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Research Article

Comparative Study of Antibacterial Efficacy of Honey, Lemon and Standard Antibiotics against *Staphylococcus aureus* Isolates from Upper Respiratory Tract Infections

Mshelia BM*, Adeshina GO and Onaolapo JA

Department of Microbiology, Faculty of Sciences, Federal University Birnin Kebbi, Kebbi State, Nigeria

***Corresponding Author:** Mshelia BM, Department of Microbiology, Faculty of Sciences, Federal University Birnin Kebbi, Kebbi State, Nigeria.

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Abstract

The antibacterial effect of honey and lemon were investigated. Their use separately and in combination were compared with that of standard antibiotics against *Staphylococcus aureus* isolates from sputum, ear swab, nasal secretion and throat swab samples of patients from Ahmadu Bello University Teaching Hospital (ABUTH) and University Health Services (UHS) A.B.U., Zaria, Nigeria. Honey and/or lemon, and the standard antibiotics were used against the isolates using agar diffusion and broth dilution techniques in order to detect their effects. Minimum Inhibitory Concentrations (MIC) was also conducted to also check the efficacy of the agents used. Test for synergy was also carried out to know if there is synergistic, additive or antagonistic effect between the honey and the lemon. Excellent antibacterial activity was observed with lemon, honey and lemon mixture (with 50:30 to 50:10 v/v lemon/honey mixture having the best activity), Levofloxacin, Ceftriaxone and Gentamicin against the *Staphylococcus aureus* isolates. While less antibacterial activity was observed with Azithromycin and Amoxicillin-Clavulanic acid. There is no synergistic, nor antagonistic activity between the mixture of honey and lemon. Their activity is within the additive level (> 0.5 to ≤ 4) of Fractional Inhibitory Concentration index. This research therefore scientifically justifies the use of Honey and Lemon as an alternative medicine by the populace in the treatment of upper respiratory tract infections.

Keywords: Honey; Lemon; Standard Antibiotics; Synergistic Effect; Antibacterial Activity; Zone of Inhibition

Introduction

The upper respiratory tract extends from the larynx to the nostrils and comprises the oropharynx and the nasopharynx together with the communicating cavities, the sinuses and the middle ear. Upper respiratory tract infections (URTIs) are the illnesses caused by an acute infection which involves the upper respiratory tract: nose, sinuses, pharynx or larynx [1]. Upper respiratory tract infection (URI) causes at least of one-half of all symptomatic illness in a community, exacting huge tolls that can be measured as morbidity, absenteeism from school and work, direct health care costs, and overuse of antibiotics leading to the emergence of drug-resistant bacteria [2]. This disease burden is largely explained by anatomy. The nose, mouth, and pharynx are exposed to circulating viruses and are normally colonized by large numbers of bacteria including potential pathogens such as *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae* and group *A streptococci.* Mucosal injury caused by viral infection, allergy, or other factors compromises the mucociliary barriers designed to maintain sterility of the middle ears, paranasal sinuses, and lungs. Most URIs are self-limited but progression to life-threatening acute illness occurs and progression to chronic disease is common [2]. The upper respiratory tract hosts a vast range of commensals and potential pathogenic bacteria, which form a complex microbial community [3]. The upper respiratory tract can be the site of several types of infection. Which are mostly; sinusitis, epiglottitis, pharyngitis, nasopharyngitis, otitis media, etc. Infections then seems to be the commonest and serious complications of URT. There are currently no medications or herbal remedies which have been conclusively demonstrated to shorten the duration of illness completely [4]. Treatment comprises symptomatic support usually via analgesics for headache, sore throat and muscle aches [1].

Honey is highly nutritious, it has traces of minerals and vitamins not to mention the antioxidants which destroy free radicals and delay ageing; although not an herb, honey is a plant by-product and used medicinally around the world [5]. Honey was found to possess bactericidal activity against wide variety of microorganisms [6]. The antibacterial mechanisms of honey known to date are Hydrogen peroxide (H_2O_2), methylglyoxal (MGO), bee defensin-1, the osmotic effect and the pH [7]. Historically, honey has been used by humans to treat a variety of ailments, from gastric disturbances to ulcers, wounds and burns, through ingestion or topical application [8].

Lemon belongs to the family of Rutaceae. It is used to treat infections, and it also has antioxidant and astringent properties [9]. The juice of lemons is excellent and effective remedy to treat disorders of the throat and persistent catarrh. Lemon contains high level of Vitamin C and this effectively aid in reducing the onset of the cold or catarrh. Michelle reported that lemons are rich in vitamin C and flavonoids that work in conjunction for a serious punch against infection. Therefore, drinking lemon juice is very good for treating common colds and flu; Lemon juice used as a gargle or oral wash also helps bring relief from sore throats, tonsillitis, and can help alleviate gingivitis, as well as canker sores in a person [10]. Citric acid makes up about five to six percent of the juice and tissues of lemons and limes. At this percentage with its low pH, breaks down the cell membrane of bacteria, similar to the effects of heating [11]. In a research by Hakim and Harris, it was found that the peel showed strong potential for significantly reducing risk of non-melanoma skin cancers [12]. Different varieties of lemon exist. They are: Lisbon, Ponderosa, Eureka, Bush lemon tree, Variegated pink and Meyer. Eureka species was used in this research. This was identified in the Herbarium section of Biological Science in Ahmadu Bello University Zaria, Nigeria.

Significance and health implications of the test bacteria

It is estimated that 20% of the human population are long-term carriers of *S. aureus* [13], which can be found as part of the normal skin flora and in anterior nares of the nasal passages [13,14]. Each year, about 500,000 patients in American hospitals contract a Staphylococcal infection [15]. *Staphylococcus aureus* is responsible for many infections but it may also occur as a commensal. So, isolating *S. aureus* does not always indicate infection. The treatment of choice for *S. aureus* infection is penicillin; in most countries, though, penicillin resistance is extremely common, and first-line therapy is most commonly a penicillinase-resistant β-lactam antibiotic (for example, oxacillin or flucloxacillin). Combination therapy with gentamicin may be used to treat serious infections, such as endocarditis [16], but its use is controversial because of the high risk of damage to the kidneys [16]. The duration of treatment depends on the site of infection and on severity.

Materials and Methods

Study area

The study area was Zaria, Kaduna State, Nigeria. The research was carried out in the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, ABU, Zaria, Nigeria.

Collection of the agents

The pure honey used was obtained from some parts of Taraba, Nigeria. While the collection of lemon used was done at the Staff quarters in Area-A of Ahmadu Bello University, Zaria.

Isolation and identification of bacteria

Different samples were used for this research. These include: sputum, throat, ear swab, and nasal obtained from patients in ABUTH, Zaria and UHS, ABU Samaru Campus, Zaria. Samples obtained were inoculated onto Mannitol Salt agar, Chocolate agar and Blood agar after which the plates incubated at 370C for a period of 24 - 48 hours. Colony morphology and Gram-Staining technique were used in the identification of the growing cultures. Nutrient agar, Mannitol salt agar and Blood agar media were used to subculture the pure colonies. Biochemical tests such as catalase, coagulase, etc were used to confirm the presence of this organisms as recommended by Cheesbrough [17].

Culture media preparation

The media were prepared according to the manufacturer's direction, and sterilized in autoclave (Adelphi MFG Co. Ltd., Portland) at 1210C for 15 minutes. The media were allowed to cool to about 470C and poured into sterile plates.

Gram staining

The Gram staining was carried out in accordance to the standard method [17].

Biochemical tests

The following Biochemical tests were carried out to confirm the bacteria isolated: Catalase test and Coagulase [17].

Testing for antibacterial activity

Standardization of innocula: The pure cultures were incubated for 18 - 24 hours and diluted in sterile normal saline to match 0.5 Mc Farland turbidity (Mc Farland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number

of bacteria will be within a given range to standardize microbial testing. The standard most commonly used in clinical microbiology laboratory is the 0.5 Mc Farland standard, which is prescribed for antimicrobial susceptibility testing and culture media performance testing) [18].

Testing the susceptibility of the bacterial isolates to honey, lemon and the standard antibiotics

The honey was diluted with sterile distilled water to different concentrations ranging from 25% (v/v) to 100% (v/v). Water was used in washing the lemon in order to remove sand and other particles. After which it was rinsed with sterile distilled water. The knife was sterilized before cutting the lemon. The juice was then squeezed out and sieved. This was done in order to remove the seeds and other particles therein. Sterile distilled water was used to dilute the juice to different concentrations ranging from 25% (v/v) to 100% (v/v). However, for the combination, Lemon:Honey was diluted at 10:50, 20:50, 30:50, 40:50, (using sterile distilled water 40%, 30%, 20%, 10% respectively to make it up to 100% (v/v) concentrations, 50:50 v/v concentration) and Honey:Lemon at 10:50, 20:50, 30:50, 40:50, (using sterile distilled water 40%, 30%, 20%, 10% respectively to make it up to 100% (v/v) concentrations, and 50:50 v/v concentration). The method that was used in determining the antibacterial activities of the Honey, Lemon and their combinations was agar well diffusion technique as described by Adeniyi., *et al.* [19]; Adeshina., *et al* [20]. After the media was sterilized, 20 mls each of Mueller Hinton agar were dispensed into sterile petri-dishes, and then allowed to solidify. The inocula were then applied to the surface of the solidified media by thinly spreading it using sterile swab stick. Cork-borer (number 4) was used to bore uniform holes on the inoculated agar. Two (2) drops of molten sterile Mueller Hinton agar was used to seal the bottom of the hole. After which the hole was filled with the test antibacterial agents at different concentrations and combinations. Care was taken to ensure that the concentration of the agent was correctly measured and added into each of the holes, and to avoid spillage. The same condition was used for the test antibacterial agents and the standard antibiotic discs by placing the discs at some points in the same petri dishes with the test agents. This was allowed to undergo a pre-incubation diffusion of 45 minutes to 1 hour before the petri-dishes were incubated for 18 - 24 hrs at 37°C. Clinical and Laboratory Standard Institute [21] was used in interpreting the zones in terms of sensitivity and resistance.

Determining the minimum inhibitory concentration (MIC) of the agents

The broth dilution technique as described by Kabir., *et al.* [22] was used in determining the MIC. 125 µg/ml was prepared for CRO, AMC, and LEV; 100 µg/ml was also prepared for CN and AZM respectively as stock solutions. This was prepared based on their different MIC break point values. Two fold serial dilution of the stock solutions were made in eight (8) test tubes (plus three control test tubes; one containing Mueller Hinton broth and the test bacteria, another containing Mueller Hinton broth and the standard antibiotics and the other containing Mueller Hinton broth and Sterile distilled water) of Mueller Hinton broth, with the first test tube being a double strength and the others single strength to obtain concentrations between 125 - 0.98 µg/ml, 100 - 0.78 µg/ml and 50.0 - 0.39 µg/ml. However, for the Honey and Lemon, 100 µg/ml (v/v), 90 µg/ml (v/v), 80 µg/ml (v/v), 70 µg/ml (v/v) and 60 µg/ml (v/v) of stock solutions were prepared. Two fold serial dilution of the stock solutions were also made in eight (8) test tubes, plus three control test tubes (same procedure as above) to obtain concentrations between 100 - 0.78 µg/ml (v/v), 90.0 - 0.71 µg/ml (v/v), 80.0 - 0.63 µg/ml (v/v), 70.0 - 0.55 µg/ml (v/v) and 60.0 - 0.47 µg/ml (v/v). 0.5 McFarland turbidity was made by diluting 18 - 24 hrs cultures of the test bacterial isolates to match the McFarland standard. Once this is achieved, the organisms are at a concentration of 10^5 cfu/ml approximately. After which, 100 µl of the standardized inoculums (10^5 to 10^6 cfu/ml) was added to the different concentrations of the agents, and the antibiotics in 8 test tubes plus the control test tube. Pre-diffusion period of 15 minutes was allowed before the incubation was done at 37°C for 24 hours. The highest dilution (lowest concentration) of the agents or the antibiotics which did not show any form of turbidity (indicating no visible bacterial growth) when compared with the control tubes was regarded as the MIC.

Test for synergism

Synergism is more likely to be expressed when the ratio of each antibiotic to the MIC of that antibiotic was same for all the components of the mixture. A Fractional Inhibitory Concentration Index (FICI) was used to interpret the results [23]. The ∑FICs were calculated as follows: ∑FIC=FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone [24]. The combination is considered synergistic when the Fractional Inhibitory Concentration (∑FIC) index is ≤ 0.5. Indifference or additive effect was indicated by a FIC index > 0.5 to ≤ 4, while antagonism when the ∑FIC is > 4 [25].

Statistical Tests

The susceptibility tests results produced different values. The values obtained from the zones of inhibitions were expressed as Mean ± Standard Error of Mean (SEM). To determine the significant difference, average zone of inhibition of the agents used separately was compared with their use in combination and with that of the different antibiotics using Analysis of Variance (ANOVA). If $P \leq 0.05$ differences were considered to be significant. However, if $P \geq 0.05$, the difference was not significant.

Results and Discussion

Sample Collection

A total of 126 samples of Throat swab, nasal secretion, Ear swab and Sputum were collected.

The bacterial isolates in the retrospective study were *Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Haemophilus influenzae.*

However, in the prospective study, the highest percentage of Staphylococcus aureus as predominant organisms next to Klebsiella pneumoniae isolated is in close proximity with several other findings such as that of Shopsin [26] and Zetola [27] who reported that Staphylococcus aureus strains colonizes up to 35% of young children and are associated with a wide range of diseases including soft tissue infections, sepsis, and pneumonia.

Susceptibility pattern of the bacterial isolates to the standard antibiotics, honey, lemon juice and honey/lemon mixture

Table 1 compares the average zones of inhibition of the standard antibiotics, honey, lemon and honey/lemon mixture against the bacterial isolates. Highest zone of inhibition was observed with Levofloxacin, while least zones of inhibition was observed with honey and Azithromycin. Honey/Lemon mixture had higher zone of inhibition than CN, CRO, AMC and AZM.

Organism	CRO	LEV	AMC	AZM	CN	Honey	Lemon	Honey/lemon
Staphylococcus aureus	20	25.9	20.3	19.6	21.6	19.4	20.1	24.5

Table 1: Comparing the average mean zones of inhibition (mm) of the standard antibiotics, Honey, Lemon and Honey/Lemon mixture against *Staphylococcus aureus* isolates.

Minimum Inhibitory Concentration (MIC)

The percentage resistance profile for the MIC test using the standard antibiotics

The percentage resistance profile to the standard antibiotics for all the bacterial isolates are as shown in figure 1, with highest percentage of resistance shown against Amoxicillin-Clavulanic acid, followed by Azithromycin and the least percentage of resistance shown against Ceftriaxone.

The percentage sensitivity profile for the MIC test using the honey, lemon and honey/lemon mixture

The percentage sensitivity profile of the *Staphylococcus aureus* to the honey, lemon and honey/lemon mixture are as shown in figure 2, with highest percentage of sensitivity shown to honey/lemon mixture, 100% v/v honey, 100% v/v lemon and least percentage sensitivity profile observed with 25% v/v honey.

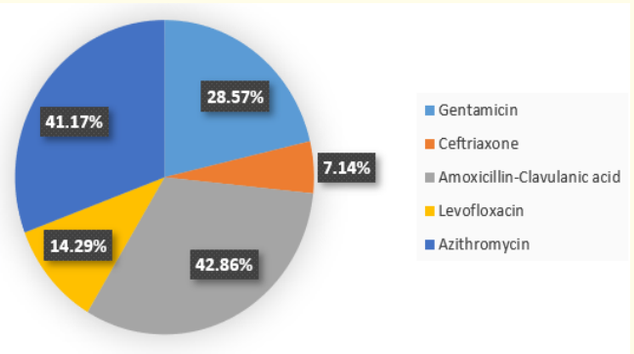

Figure 1: Resistance profile of *Staphylococcus aureus* to the standard antibiotics.

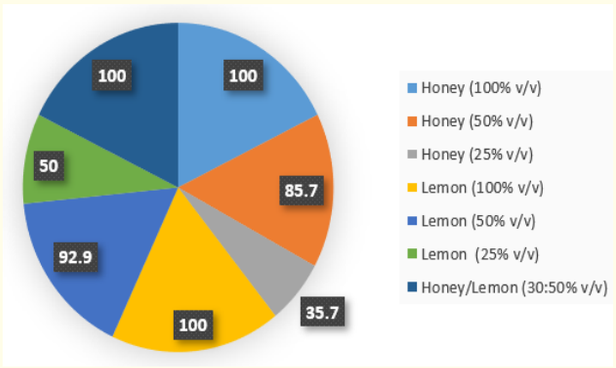

Figure 2: Sensitivity profile of *Staphylococcus aureus* to honey and lemon.

Comparison of the minimum inhibitory concentration (MIC) results among the agents

There is a decrease in the MIC values of the Honey and Lemon combination compared to the use of Honey alone (Table 2 to 4).

Bacterial isolates	Honey	Lemon
Staphylococcus aureus	47.1	17.9

Table 2: Average Minimum Inhibitory Concentration (MIC) (µg/ml v/v) values for the test agents (Honey and Lemon) against the bacterial isolates.

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
Staphylococcus aureus	18.0	21.4	20.2	21.6	20.0

Table 3: Average Minimum Inhibitory Concentration (MIC) (µg/ml v/v) values for the test agents (Honey/Lemon mixture) against the bacterial isolates.

Key: 10:50, 20:50, 30:50, 40:50, 50:50=Honey:Lemon concentrations (µg/ml v/v).

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
Staphylococcus aureus	32.1	28.2	22.1	21.3	20.0

Table 4: Average Minimum Inhibitory Concentration (MIC) (µg/ml v/v) values for the test agents (Lemon/Honey mixture) against the bacterial isolates

Key: 10:50, 20:50, 30:50, 40:50, 50:50=Lemon:Honey concentrations (µg/ml v/v).

There is a rise in the MIC values of the Honey and Lemon combination compared to the use of Lemon alone (Table 2 to 4).

Test for Synergy

The activities of the two agents Honey and Lemon are not synergistic, and also not antagonistic, but falls within the Indifference range of the FIC Index, interpretation (> 0.5 to ≤4) (see Table 5 and 6).

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
Staphylococcus aureus	1.39	1.65	1.56	1.67	1.54

Table 5: ∑FIC values for the test agents (Honey/Lemon mixture) against the bacterial isolates.

Key: 10:50, 20:50, 30:50, 40:50, 50:50=Honey:Lemon concentrations (µg/ml v/v).

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
Staphylococcus aureus	2.47	2.18	1.70	1.64	1.54

Table 6: ∑FIC values for the test agents (Lemon/Honey mixture) against the bacterial isolates.

Key: 10:50, 20:50, 30:50, 40:50, 50:50=Lemon:Honey concentrations (µg/ml v/v).

Statistical Analysis

There is also significant difference between the mean zone of inhibition of 100% v/v Honey and 10:50, 20:50, 30:50, 40:50, 50:50% v/v concentrations of Honey and Lemon mixtures for the Staphylococcus aureus isolates (Figure 3).

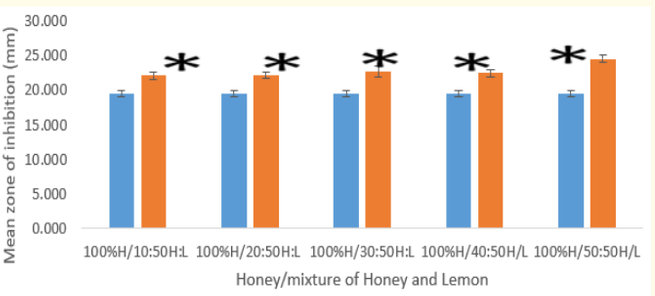

Figure 3: Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon for *Staphylococcus aureus.*

Key: *=Significant difference.

In addition, there is significant difference between the mean zones of inhibition of 100% v/v Lemon and 50:50% v/v concentrations of Honey and Lemon mixtures (Figure 4).

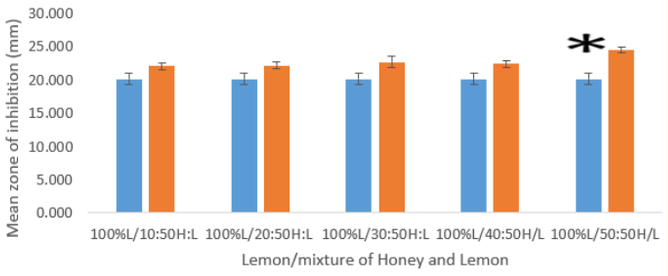

Figure 4: Comparing the mean zones of inhibition of Lemon and the mixture of Honey and Lemon for *Staphylococcus aureus.*

However, for the other concentrations, there is no significant difference between 100% v/v Honey, 100% v/v Lemon and the Honey/Lemon mixture.

Furthermore, the mean zone of inhibition between Honey and lemon and the standard antibiotics has been compared. There is no significant difference between most of them except for AMC, and AZM in some, 25% v/v Honey, and 50:50% v/v Honey/Lemon mixture.

The susceptibility test showed that all the tested bacterial isolates were completely sensitive to the raw Honey, Lemon and the Honey/Lemon used in combination. The low pH of the agent used could be one of the reasons for this activity apart from other antibacterial properties they possess. The bacterial isolates were also moderately susceptible to 50% v/v concentration of the Honey, 25% v/v concentration of the Lemon juice; but not sensitive to 25% v/v concentration of the Honey. The results obtained in this study is in consonant to the research of Ifra and Ahmad [28] and Kawaii., *et al.* [29] who reported that the crude concentrations of honey inhibited the growth of all the bacterial isolates, but the activity decreases when dilutions were made. However this was in disagreement to work of Mathai., *et al.* [30] who found that Honey showed the least zone of inhibition (0.5 ± 0.6 mm). Laboratory studies have revealed that honey is effective against methicillin-resistant *S. aureus* (MRSA) [31,32]. The zone diameter of inhibition of Nilgiris honeys were found to be (20 - 21) mm for *S. aureus* [33]. However, the zone diameter of inhibition of (5.3 - 11.6) mm for S. aureus was observed by Agbagwa and Frank-Peterside [34]. The bactericidal effect of the agents (honey and lemon) is reported to be dependent on concentration used and the nature of the bacteria [35,36].

The MIC results showed that the *Staphylococcus aureus* isolates were less sensitive to Azithromycin and Amoxicillin-Clavulanic Acid. Ceftriaxone and Levofloxacin showed batter activity against the isolates; while their susceptibility to Gentamicin was moderate. It has been shown that generally, there is decrease in the MIC values of the Honey and Lemon combination in comparison to the Honey used alone. The MIC values however rises for the Honey and Lemon combination than the Lemon alone.

Ceftriaxone (a third generation Cephalosporin), Levofloxacin (a fluoroquinolone), and Gentamicin (an Aminoglycoside) showed better efficacy against the *Staphylococcus aureus* isolates. This is in contrast to the work of Onah., *et al.* [37] who reported that the *Staphylococcus aureus* organisms that were suppressed by the honey/agar mixture were resistant to ceftazidime, ceftriaxone, ciprofloxacin, pefloxacin, gentamicin, ampicillin, cloxacillin, amoxicillin, and erythromycin. Less susceptibility pattern against AMC and AZM was observed across the bacterial isolates. A research by Abd-El Aal AM., *et al*. [38] is in consonant with this; Their report shows that the average of the zone of inhibition that Honey produced against the Gram negative organisms were greater in comparison to that of Amoxicillin-Clavulanic acid. Ceftriaxone, Levofloxacin and Gentamicin were showed better efficacy among the standard antibiotics used.

The act of blind treatment with broad spectrum antibiotics and not completing the prescribed dosage are contributory factors to the emergence of antibiotic resistance among many microorganisms [39]. Some are even in the habit of using drugs and keeping it for future use and many other abuses such as not maintaining the appropriate storage temperature by street vendors and open market dealers (obtainable in Nigeria) might have led to the possible resistance observed among Azithromycin and Amoxicillin-Clavulanic acid in this study. The above abuses and many others have led to resistance to most antibiotics in use. So, many tends to traditionally use the honey and lemon when the case is not that complicated, which have been found effective unto many than buying drugs from the illegal conduits for self-medication and later suffer for their actions. This has also motivated others to resort to the use of honey and lemon which can be obtained in their nearby environment, than spending their money on drugs and at the end will not be found effective due to problem of resistance.

Ceftriaxone and Levofloxacin were shown to be effective and resistance of the isolates to these drugs was so low. This could be because Ceftriaxone and Levofloxacin are not cheap, and not all can afford it, leading to less abuse of such drugs. This may be the reason why the isolates were more susceptible to Ceftriaxone, Levofloxacin and Gentamicin. The Gentamicin is cost effective but it is

administered through injection. And this could be the reason why it is still active than the other antibiotics tested. So, these antibiotics are still very active in this part of the country.

So, honey, lemon and honey/lemon combination are effective antibacterial agents which can be used at different concentrations. Combination of Honey and Lemon have better efficacy than Honey alone, Lemon alone (among some of the isolates), while additive affect was observed among the other isolates.

Conclusion

This study compared the activity of some antibiotics and Honey/Lemon against *Staphylococcus aureus* species obtained from respiratory tract infections. Of which Ceftriaxone, Levofloxacin, and Gentamicin showed better activity against the *Staphylococcus aureus* isolates than Azithromycin and Amoxicillin-Clavulanic acid. The isolates were also inhibited by Honey and Lemon better than some of the antibiotics used most especially Amoxicillin-Clavulanic acid and Azithromycin.

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