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Antibacterial Susceptibility of *Klebsiella pneumoniae* Isolates from Respiratory Tract Infections to Honey and Lemon

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Authors' contributions

This work was carried out in collaboration between all authors. Author GOA designed the study, supervised the laboratory work and worked on the manuscript. Author BMM carried out the laboratory work, wrote the first draft of the manuscript and performed the statistical analysis and author JAO supervised the laboratory work on the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To assess the effectiveness of lemon and/or honey on some causative agents of Respiratory Tract Infections, Isolation and identification of some infectious bacteria of respiratory tract infections, Collection of pure honey and lemon fruits, Determination of the inhibitory activities of honey, lemon and honey/lemon mixture at varied concentrations on the bacterial isolates by agar diffusion and broth dilution techniques, Evaluation of the rate of kill of the bacterial isolates by the agents (honey and lemon) and Comparative analysis of the susceptibility pattern of the bacterial isolates to the honey and lemon separately and in combination.
Study Design: Isolation, identification and antibiotic susceptibility determination of the test *Klebsiella pneumoniae* isolates, Zones of inhibition, Minimum Inhibitory and Bactericidal Concentrations and Rate of kill determination.
Place and Duration of Study: Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University Teaching Hospital (A. B. U. T. H.), Zaria, University Hospital Services (U. H. S.) Samaru Campus Ahmadu Bello University (A. B. U.), Zaria, between March 2012 and April 2013.
Methodology: Agar well diffusion and broth dilution methods were employed to ascertain

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degree of susceptibility of the isolates to honey and/or lemon, and the standard antibiotics. Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations were carried out. Rate of Kill was also carried out to know the death/survival rate of the bacterial isolates after exposure to the agents.

Results: Mean zones of inhibition (mm) of 14-32 (Ceftriaxone), 7-27 (Gentamicin), 8-35 (Amoxicillin-Clavulanic acid), 12-27 (Levofloxacin), 7-21 (Azithromycin), 10-23 (100% v/v Honey), 10-24 (100% v/v Lemon), and 19-26 (Honey/Lemon mixture) were obtained. However, the Minimum Inhibitory Concentrations range between 1.95-125 µg/ml (Ceftriaxone), 1.56-100 µg/ml (Gentamicin), 3.91-125 µg/ml (Amoxicillin-Clavulanic acid), 0.98-15.6 µg/ml (Levofloxacin), 3.13-100 µg/ml (Azithromycin), 20-65 µg/ml v/v (Honey), 15-35 µg/ml v/v (Lemon), 15-35 µg/ml (Honey/Lemon mixture). Furthermore, for the rate of kill; Lemon, Honey and Lemon mixture (20 µg/ml) effected complete killing at 120 minutes and 240 minutes respectively. Therefore, it was observed that lemon, honey and lemon mixture, Ceftriaxone, Levofloxacin and Gentamicin showed higher antibacterial activity. While Amoxicillin-Clavulanic acid and Azithromycin had less antibacterial activity. At *P*-value $P \geq 0.05$ there is significant difference between Honey and Lemon mixture and Honey, but not with lemon.

Conclusion: It was observed that lemon, honey and lemon mixture, Ceftriaxone, Levofloxacin and Gentamicin showed higher antibacterial activity. While Amoxicillin-Clavulanic acid and Azithromycin had less antibacterial activity. Better bactericidal activity was observed with Lemon and the mixture of Honey and Lemon than the Honey alone. This research therefore scientifically approves the use of Honey and Lemon as an alternative medicine by the populace in the treatment of respiratory tract infections.

Keywords: Honey; lemon; antibacterial activity; standard antibiotics; susceptibility.

1. INTRODUCTION

The respiratory tract extends from the larynx to the nostrils and comprises of oropharynx and the nasopharynx together with the communicating cavities, the sinuses, the middle ear and extends to the lungs. Infections involving this tract are referred to as respiratory tract infections [1]. Several parts of the respiratory tract may be affected by the infection giving rise to several conditions including pharyngitis, sometimes involving tonsillitis, and giving rise to a “sore throat”, nasopharyngitis, otitis media, sinusitis, epiglottitis, etc. Infection of the respiratory tract is one of the commonest illness in the general population and results in significant morbidity, which may account for missed days of work and school, it also contribute to mortality.

However, the fact that antimicrobials are being misused for treatment of cold, lemon and honey are considered natural soothers which have been utilized in some of these mild illnesses.

Honey is a sweet food made by bees using nectar from flowers. Hydrogen peroxide (H_2O_2), methylglyoxal (MGO), bee defensin, pH and osmotic effect of honey are known to be responsible for the antimicrobial effects [2].

Lemon was mainly used as ornament and medicine. In 1747, [3] experiments on Seamen suffering from Scurvy involved adding Vitamin C to their diets with lemon juice. Moreover, Vitamin C content of the lemon can actively aid in suppressing the onset of the cold. There

are different varieties of lemon. However, the species used in this work was the Eureka species as identified in the Herbarium section of Biological Science, Ahmadu Bello University Zaria. Eureka fruit has a markedly ribbed surface. The fruit colour is yellow at maturity. It is a sour lemon variety and usually has fewer seeds.

1.1 Significance and Health Implications of the Test Bacteria

Klebsiella pneumoniae though not the common cause of respiratory tract infections, but a bacterial complication after viral infection sets in has been considered a respiratory pathogen of Pneumonia patients. The symptoms include: toxic presentation with sudden onset, high fever, and hemoptysis. Many hospital cases around the world have been linked to *K. pneumoniae*. *K. pneumoniae* is commonly found in the gastrointestinal tract and hands of hospital personnel [4]. Although found in the normal flora of the mouth, skin, and intestines, it can cause destructive changes to human lungs if aspirated [5]. Treatment is done by antibiotics such as clinafloxacin [6]. But, there are an increasing amount of antibiotic-resistance strains. Ciprofloxacin is an antibiotic that is becoming less effective [7].

2. MATERIALS AND METHODS

2.1 Methodology

2.1.1 Study area

The study areas were Ahmadu Bello University Teaching Hospital, (A. B. U. T. H.) Zaria and University Hospital Services (U. H. S.), Ahmadu Bello University (A. B. U.), Samaru Campus, Zaria, Kaduna State, Nigeria. However, the research was conducted in the Faculty of Pharmaceutical Sciences, Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University (A.B.U.), Zaria, Nigeria.

2.1.2 Collection of materials and samples

Pure honey was collected from Taraba State, Nigeria. However, the lemon (*Citrus limon,* Eureka variety) was obtained from the Staff quarters in Area-A, A.B.U., Zaria. Samples of sputum, throat, ear swab and nasal secretions were collected by a laboratory scientist from admitted patients after seeking their consent. These were then transported immediately to the Pharmaceutical Microbiology Laboratory A. B. U. Zaria and processed.

2.1.3 Isolation and identification of bacteria from respiratory tract infections

Samples of sputum throat, ear swab and nasal secretions were collected from patients in A. B. U. T. H., Zaria and U. H. S., A.B.U. Samaru Campus, Zaria. The samples were then inoculated on Blood Agar, Chocolate agar, MacConkey agar and cetrimide agar, and the plates incubated at 37 ^{0}C for 24-48 hours. Identification of the growing microorganisms was done by colony morphology and Gram-Staining method. Pure colonies were sub-cultured on Blood agar, Nutrient agar and Chocolate agar media. Further identification or confirmation was carried out using Biochemical tests as recommended by [8].

2.1.4 Antibacterial activity testing

2.1.4.1 Susceptibility testing of the bacterial isolates to honey, lemon and the standard antibiotics

The honey was diluted with sterile distilled water to concentrations of between 25% (v/v) to 100% (v/v). The lemon was washed with water to remove sand and other particles and rinsed with sterile distilled water. It was cut with sterile knife before the juice was squeezed out and sieved. The sieving was done to remove the seeds and other particles. The juice was diluted with sterile distilled water to concentrations of between 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). Agar well diffusion technique as described by [9] and [10] was used to determine the antibacterial activities of the Honey, Lemon and the combinations of the two agents. 20 mls of Mueller Hinton agar were prepared and poured into sterile petri-dishes, and then allowed to set. Overnight culture of the test organism which was diluted in sterile normal saline to match 0.5 McFarland turbidity [11] was then spread thinly with sterile swab stick on the surface of the agar. Thereafter, holes were bored using sterile cork-borer (number 4) to make uniform wells on the inoculated agar. The bottom of the hole was then sealed with 2 drops of molten sterile Mueller Hinton agar and then filled with the test antibacterial agent (honey, lemon, honey/lemon). The standard antibiotic discs were placed at some points in the same Petri dishes with the test antibacterial agents (Honey and/or Lemon) for them to undergo the same conditions. Pre-incubation diffusion time (45 minutes to 1 hour) was allowed, after which the petri-dishes were incubated at 37ºC for 18-24 hrs. After the incubation period, the diameters of the zones of inhibition were measured in millimetres. Interpretation of zones sizes in terms of sensitivity or susceptibility, and resistance was based on the values provided by [12].

2.1.4.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the agents

The MIC was carried out using the broth dilution method [13]. Stock solutions of 125 µg/ml were prepared for CRO, AMC, and LEV; 100 µg/ml was also prepared for CN and AZM respectively 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 (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). The overnight cultures of the test bacterial isolates were diluted to match 0.5 Mc Farland turbidity. At this point, the organisms should be at a concentrations of approximately 10^5 cfu/ml. 100 µl of the standardized inoculum was then inoculated to the different dilutions of the agents and the antibiotics in the 8 test tubes plus the organism-control test tube. 15 minutes pre-diffusion of the organisms was allowed prior to incubation at 37ºC for 24 hours. The lowest concentration (highest dilution) of the

honey and/or lemon or the antibiotics which showed clear solution or no visible bacterial growth (i.e. no turbidity) when compared with the control tubes, was regarded as the M.I.C. However, M.B.C. was determined from the broth dilution tests, by sub-culturing to antibiotic-free Mueller Hinton agar (i.e. Mueller Hinton Agar+5% v/v tween 80) from tubes showing no visible growth after 24 hours incubation at 37ºC. The lowest concentration of an antibacterial agent that kills more than 99.9% of the initial inoculation after the 24 hours incubation represents the M.B.C. [14].

2.1.4.3 Rate of kill

0.1 ml of standardized overnight culture of the test organisms that were susceptible and those that were resistant to the standard antibiotics, honey, lemon and mixture of both (approximately 10^6 cfu/ml) was added to 9.9 ml each of test antibacterial agents (honey and/or lemon) formulated with sterile distilled water (using the concentrations of Sub-MIC, Around-MIC, and Above-MIC). This was mixed thoroughly and kept inside Digital shaker bath (Mc Donald Scientific International) at 37ºC. At different time interval (0, 30, 60, 120, 240, 360 and 1440 minutes). 1 ml test organism/extract admixture was taken and ten-fold dilution protocol performed with sterile inactivated normal saline (i.e. normal saline with 5% (v/v) Tween 80). These dilutions were then plated out in duplicates on sterile molten Mueller – Hinton agar supplemented with 5% (v/v) Tween 80. The agar plates were then incubated at 37^{o}C for 24 hours. After incubation, colonies observed were counted with the aid of a Colony Counter (NAPCO Model 630 Porland, Oregon, U. S. A.) [15,11]. These procedures were repeated for Sub-MIC, Around-MIC, and Above-MIC values of levofloxacin and ceftriaxone; as the standard antibacterial agents.

2.1.5 Statistical analysis

The zones of inhibitions obtained from the susceptibility tests carried out were expressed as Mean±Standard Error of Mean (SEM). The mean zone of inhibition of honey and lemon were also compared with that of the mixture and with that of the various antibiotics using Analysis of Variance (ANOVA) to determine the significant differences. Differences were considered significant if P≤0.05 and not significant if P≥0.05.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Sample collection

A total of 126 samples were collected from Sputum (83), Throat swab (26), Ear swab (14) and Nasal secretion (3). The isolates identified and confirmed from these samples include 15 *Klebsiella pneumoniae* (26.8%), 14 *Staphylococcus aureus* (25.0%), 2 *Haemophilus influenzae* (3.57%), 12 *Pseudomonas aeruginosa* (21.4%), 7 *Streptococcus pneumoniae* (12.5%), 6 *Streptococcus pyogenes* (10.7%). However, for the purpose of this research, more focus will be on *Klebsiella pneumoniae*.

3.1.2 Susceptibility pattern of the bacterial isolates to the tested standard antibiotics

(Table 1.1) Shows the mean zones of inhibition for the standard antibiotics. Whereas, (Fig.1) shows the percentage susceptibility pattern of the test organisms to the test standard antibiotics.

3.1.3 Susceptibility pattern of the bacterial isolates to honey and lemon juice

(Table 1) Shows the mean zones of inhibition of honey, lemon and honey/lemon mixture. However, (Fig. 1.1) shows the percentage susceptibility pattern of all the test bacterial isolates to Honey, Lemon and the mixture of Honey and Lemon. There is significant difference between the mean zone of inhibition of 100% v/v Honey and 30:50, 40:50, 50:50% v/v concentrations of Honey/Lemon mixtures against *Klebsiella pneumoniae* (Fig. 1.2).

3.1.4 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 (Fig. 1.3).

3.1.5 Comparing the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results between honey, lemon and honey/lemon

Generally, there's reduction in MIC and MBC in the mixture of Honey and Lemon compared to the Honey alone (Table 1.2 to 1.4). There's increase in MIC and MBC in the mixture of Honey and Lemon compared to the Lemon alone (Table 1.2 to 1.4).

3.1.6 Rate of kill

Lemon effected complete killing at 240 minutes which is depicted by gradual decrease in cell population from 30 minutes to 120 minutes, and a sharp decrease at 240 minutes; Honey and Lemon however effected complete killing at 360 minutes, as also depicted by gradual decrease in cell population from 30 minutes to 240 minutes and a sharp decrease at 360 minutes, while CEF, LEV, HONEY could not produce complete killing even at 1440 minutes for the resistant *Klebsiella pneumoniae* (Fig. 1.4).

Lemon effected complete killing at 120 minutes, as depicted by gradual decrease in cell population from 30 minutes to 90 minutes and a sharp decrease down at 120 minutes; Honey and Lemon mixture effected complete killing at 240 minutes, as shown by a steady decrease in cell population from 30 minutes to 120 minutes, and gradual decrease down at 240 minutes; while CEF, LEV and Honey produced complete killing at 1440 minutes, as observed by a gradual decrease from 30 minutes to 360 minutes and a steady decrease down to 1440 minutes for the susceptible *Klebsiella pneumoniae* (Fig. 1.5).

Table 1. Mean zone of inhibition (mm) ± SEM of the bacterial isolates to honey, lemon and honey/lemon mixture.

Bacterial Isolates	HA	HB	HC	LA	LB	LC	H/L
K. pneumoniae	19.3±0.4	16.0±0.4	11.7±0.4	21.3±0.4	18.3±0.4	15.1±0.4	22.8±0.6

Key: *HA, HB, HC=100%, 50%, 25% v/v of Honey respectively, LA, LB, LC=100%, 50%, 25% v/v of Lemon respectively, H/L=Honey/Lemon.*

Table 1.1 Mean zone of inhibition (mm) ± SEM of the bacterial isolates to the standard antibiotics

Bacterial Isolates	CRO	CN	AMC	LEV	AZM
K. pneumonia	20.7±1.5	19.3±1.5	17.7±1.7	19.8±1.2	15.5±1.1

Key: *CRO=Ceftriaxone, CN=Gentamicin, AMC=Amoxicillin-clavulanic acid, LEV=Levofloxacin and AZM=Azithromycin.*

Table 1.2 Average minimum inhibitory concentration (MIC) (µg/ml v/v) values for the test agents (Honey and Lemon) against the bacterial isolates

Bacterial isolates	Honey	Lemon
Klebsiella pneumonia	43.0	21.3

Key:*10:50, 20:50, 30:50, 40:50, 50:50=The various concentrations of Honey/Lemon mixture.*

Table 1.3 Average Minimum Inhibitory Concentration (MIC) (µg/ml v/v) values for the test agents (Honey/Lemon mixture) against the bacterial isolates

Bacterial isolate	10:50	20:50	30:50	40:50	50:50
Klebsiella pneumonia	33.7	32.0	29.3	28.8	28.3

Table 1.4 Average minimum inhibitory concentration (MIC) (µg/ml v/v) values for the test agents (Lemon/Honey mixture) against the bacterial isolates

Bacterial isolate	10:50	20:50	30:50	40:50	50:50
Klebsiella pneumoniae	19.3	21.3	25.3	27.6	28.3

Key*: 10:50, 20:50, 30:50, 40:50, 50:50= The various concentrations of Lemon/Honey mixture.*

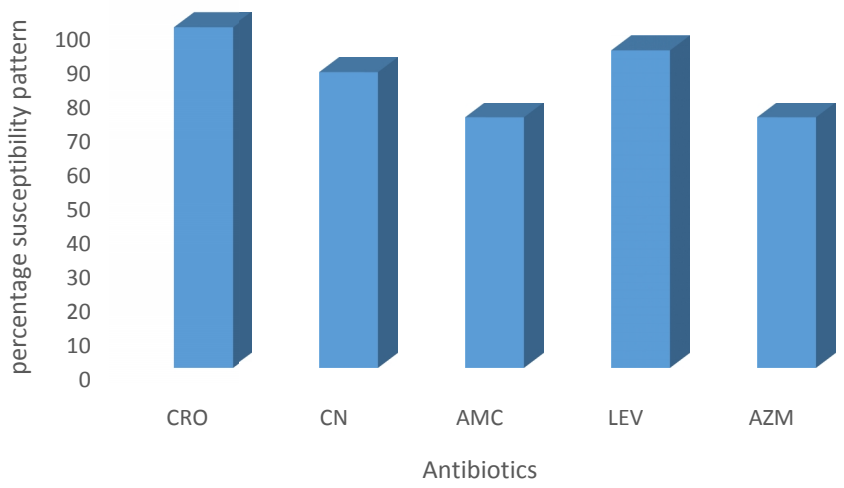

Fig. 1. The percentage susceptibility pattern of *Klebsiella pneumonia* to the standard antibiotics

Key: CRO=Ceftriaxone, CN=Gentamicin, AMC=Amoxicillin-clavulanic acid, LEV=Levofloxacin and AZM=Azithromycin

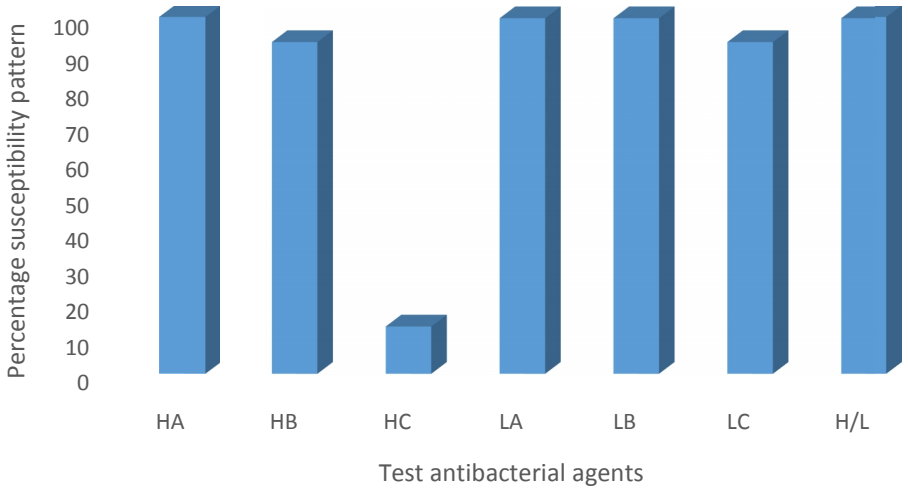

Fig. 1.1 The percentage susceptibility pattern of *Klebsiella pneumonia* to honey and lemon

Key: HA, HB, HC (LA, LB, LC) and H/L=100%, 50%, 25% v/v Honey (Lemon) concentrations and Honey/Lemon mixture

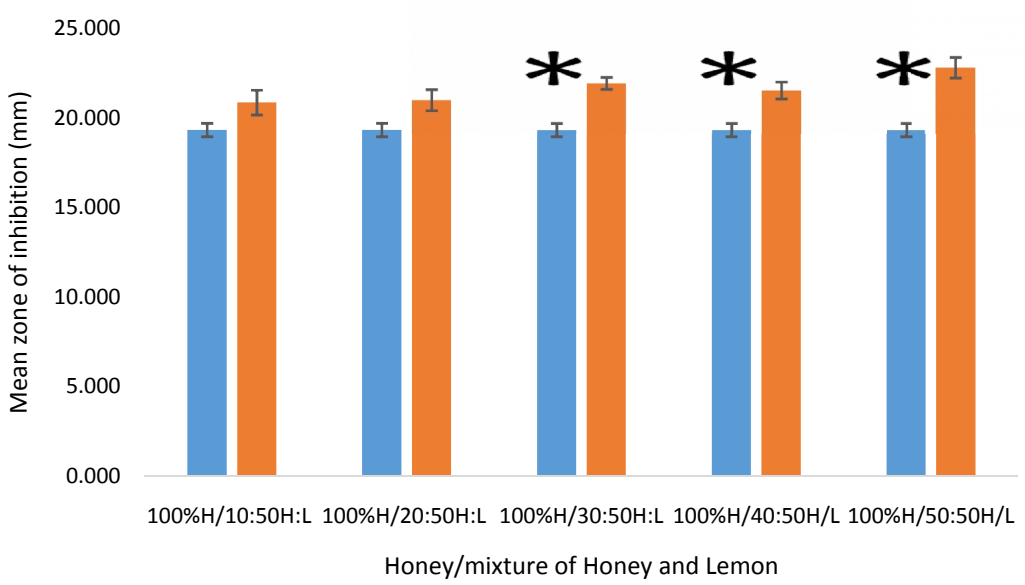

Fig. 1.2 Comparing the mean zones of inhibition of honey and honey/lemon mixture (v/v concentrations) against *Klebsiella pneumoniae*

*Key: * =significant difference*

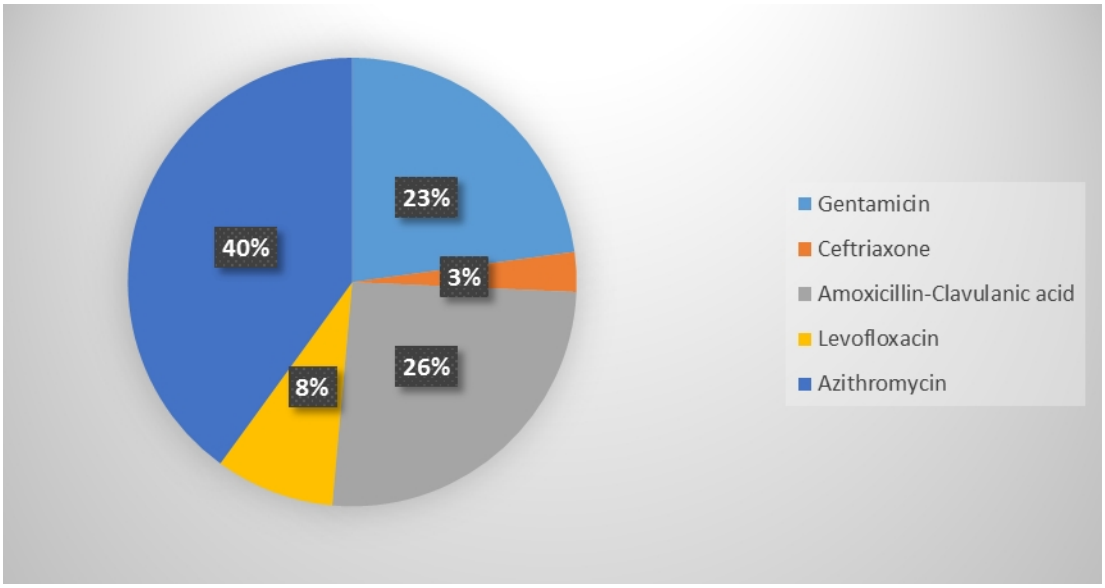

Fig. 1.3 Resistance profile of *Klebsiella pneumoniae* to the test antibiotics

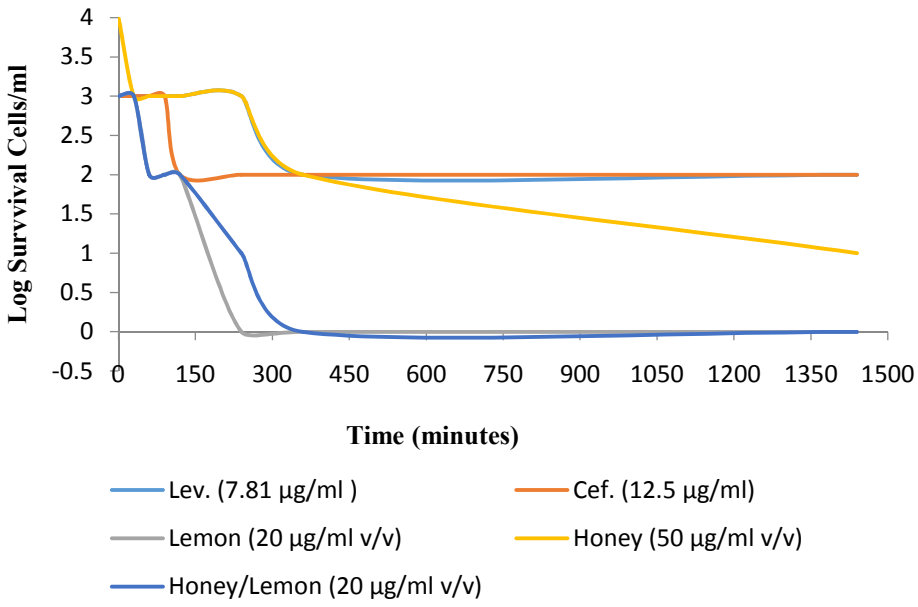

Fig. 1.4 The log of survival cells/ml of *Klebsiella pneumoniae* (Resistant) on exposure to standard antibiotics and honey, lemon and honey/lemon mixture

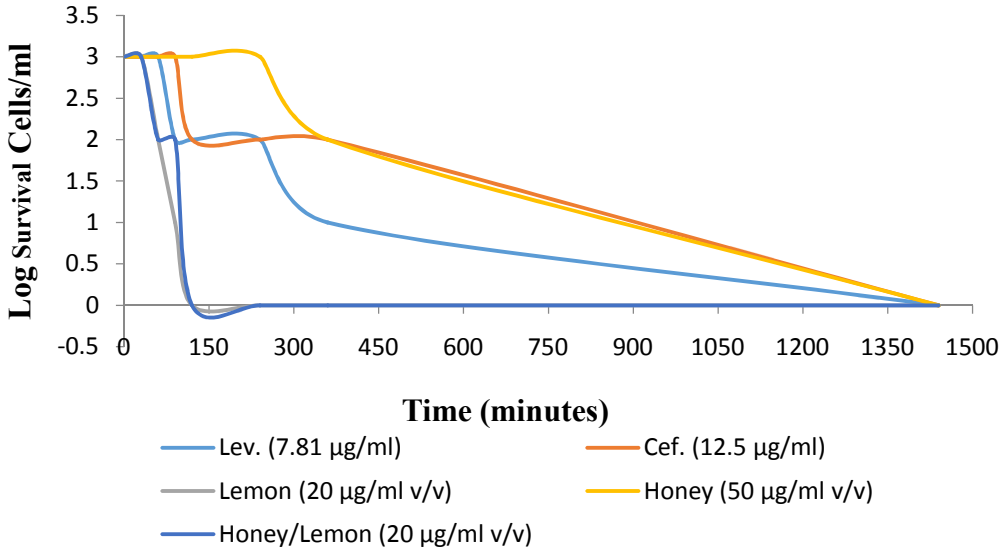

Fig. 1.5 Log of Survival cells/ml of *Klebsiella pneumoniae* (Sensitive) on exposure to standard antibiotics and honey, lemon and honey/lemon mixture

3.2 Discussion

In this study, the highest percentage of *Klebsiella pneumoniae*, as predominant organisms isolated is in close proximity with several other findings such as a study conducted by Achana and Harsh [16], who found that out of the fifty nine clinical isolates they used, twenty were *Klebsiella pneumoniae* which had the highest percentage of the isolates; and a study conducted in Benin City by Ophori and Wemabu, [17], the identified bacterial isolates included *H. influenzae, K. pneumoniae, S. pneumoniae, Moraxella catarrhalis* and *S. pyogenes*. However, *H. influenzae* had the highest percentage prevalence of 20.8%, followed by *K. pneumoniae* (19.2 %).

All the tested bacterial isolates were completely susceptible to the crude concentrations of Honey, Lemon and the Honey/Lemon mixture. This activity is tight to the acidic nature of the honey and lemon, beside other antimicrobial properties they were known to possess. They were also moderately susceptible to Honey at 50% v/v concentration, Lemon juice at 25% v/v concentration; but showed resistance to Honey at 25% v/v concentration. This is in agreement to works done by Ifra and Ahmad, [18] and Kawaii *et al*., [19] who reported that the stock solution of the honey samples inhibited the growth of all the bacterial isolates, but when the dilutions were made the efficacy reduced.

The resistance profile for the M.I.C. showed that the *Klebsiella pneumoniae* isolates were more resistant to Amoxicillin-Clavulanic Acid and Azithromycin. The bacterial isolates were more sensitive to Ceftriaxone and Levofloxacin; and moderately sensitive to Gentamicin. Generally, there is reduction in M.I.C. and M.B.C. in the mixture of Honey and Lemon compared to the Honey alone. However, there is increase in the M.I.C. and the M.B.C. in the mixture of Honey and Lemon compared to the Lemon alone.

Better activity was observed with the Ceftriaxone (a third generation Cephalosporin), Levofloxacin (a fluoroquinolone), and Gentamicin (an Aminoglycoside) against the *Klebsiella pneumoniae*. [16], also reported that *Klebsiella pneumoniae* were found to be highly susceptible to the Quinolones and Gentamicin. Less susceptibility pattern against Amoxicillin-Clavulanic Acid and Azithromycin was observed across the bacterial isolates. This is in agreement to a work done by Abd-El aal et al., [20]; who that the mean zone of inhibition produced by honey against the isolated Gram negative organisms were significantly higher than that of Amoxicillin-Clavulanic acid. However, Ceftriaxone, Levofloxacin and Gentamicin were observed to be the most active among the standard antibiotics.

Generally, inadequate antimicrobial treatment defined as ineffective treatment due to failure to complete prescribed dosage or prescription without carrying out the susceptibility testing is an important factor in emergence of antibiotic resistant bacteria [21]. The pressure of prolonged usage and regular abuse in our society has led to general resistance to Azithromycin and Amoxicillin-Clavulanic Acid as was observed in this research. This could be due to abuse of Azithromycin and Amoxicillin-Clavulanic Acid by the populace since in Nigeria, different antibiotics are readily available from patent medicine shops, street drug vendors, and in open markets; sources where adequate storage conditions are not adhered to.

However, resistance to Ceftriaxone and Levofloxacin was low. This is because they are relatively costly, not within the reach of the poor sector of the populace, hence less abused. Thus, high sensitivity profile shown by the organisms to the standard antibiotics used in this study (especially, Ceftriaxone, Gentamicin and Levofloxacin) is an indication that these drugs are still effective in this region. The aminoglycosides (Gentamicin), though relatively cheap, are only available as injectables and hence not easily abused.

Furthermore, the rate of kill against the resistant and even the susceptible *Klebsiella pneumoniae* on exposure to the test agents showed that Lemon effected a better killing (evidenced by the sharp decrease in the bacterial cell populations) than the Honey, Honey and Lemon mixture, and the standard antibiotics.

Therefore, as vividly demonstrated in this research, Honey, Lemon and Honey/Lemon mixture were found to possess antibacterial activity; but to varying degrees. The mixture gives a better activity compared to Honey alone, Lemon alone (in some of the organisms) and relative activity to Lemon in other organisms.

4. CONCLUSION

The bacterial isolates were found to be more susceptible to Ceftriaxone, Levofloxacin, and Gentamicin; and less susceptible to Azithromycin and Amoxicillin-Clavulanic acid.

Honey and Lemon had more inhibitory effect to all the test bacterial isolates than the commonly used antibiotics especially Azithromycin and Amoxicillin-Clavulanic acid. The bacterial isolates were found to be generally susceptible to the test agents (Honey and Lemon).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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