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GROWTH AND RESISTANCE PHENOTYPES OF *KLEBSIELLA PNEUMONIAE* PRE–TREATED WITH HERBAL DRUGS

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ABSTRACT

Background: Bacteria develop resistance to antimicrobial agents through prior sensitization to antibiotics or compounds possessing some antimicrobial properties. **Aim**: The aim of this study was to determine the growth phenotypes and resistance profile of *Klebsiella pneumoniae* pretreated with herbal drugs. **Methods**: The efficacies of the herbal drugs Ruzu bitters, Goko alcoholic bitters, Goko bitters, New hope herbal mixture, Danko solution, Beta cleanser, Golden seed and Evacuation solution to *K. pneumoniae* were carried out using well–diffusion method. The growth patterns of *K. pneumoniae* in the herbal drugs were studied in Tryptone Soy Broth (TSB) for 24 hrs. To determine the concentration of serum to use in the serum resistance study, different concentrations of normal human serum (NHS) was used ranging from 10–90% of normal human serum on the clinical and laboratory strains of *K. pneumoniae*. Forty percent (40%) of normal serum and heat–inactivated serum concentration was exposed to *K. pneumoniae* exposed to the different herbal drugs. **Results**: The zones of inhibition observed in Ruz, Gab, Gob and Bet are moderately sensitive while New, Dan, Gol and Eva are classified as resistant. All the herbal drugs showed significantly lowered ($P < 0.05$) zone of clearance compared to the control antibiotic, Imipenem. The continuous growth curve showed the bacteria showed peak growth after 24 hrs which was adopted in the exposure studies. There was an exponential decrease in the number of bacteria at 40% serum concentration before levelling off. The clinical strain in Bet, Gab and Gob did not show significant variation in the zone of inhibition compared to the control ($P > 0.05$). **Conclusion**: To sum up, some of the herbal drugs possess some levels of efficacies against *K. pneumoniae* but prior exposure to herbal drugs sensitizes the bacteria to become resistance when exposed to antibiotics (Meropenem, Imipenem and Gentamicin) as well as serum.

KEYWORDS: *Klebsiella pneumonia*, Meropenem, Imipenem and Gentamicin.

1. INTRODUCTION

Herbal medicines are derived from the plants or plant extracts containing therapeutic substances. The herbal medicine practice is generally called as complementary and alternative medicine (CAM) (Drew & Myers, 1997). Many essential oils are relatively easy to obtain, have low mammalian cell toxicity, and degrade quickly in water and soil, making them relatively easy to use and environment friendly antibiotic alternatives (Kavanaugh & Ribbeck, 2012).

In marine fish hatcheries, the indiscriminate use of antibiotics in prophylactic treatment has led to the development of the resistant strains and the need to switch over to other antibiotics. In case of herbal drugs no herbal resistance has been reported till now. The antibiotics also may reduce the larval growth and inhibit defense mechanisms of the fish larvae. Many of the antibiotics and other synthetic drugs have shown sensitization reaction and other undesirable side effects. At the global level, people have understood the adverse effect of antibiotics and they are now shifting over to natural products. Herbal extracts have important properties like control diseases due to their antioxidant and antimicrobial. Natural plant products have been reported to promote various activities like anti-stress, growth promotion, appetite stimulation, tonic and immune stimulation and to have aphrodisiac and antimicrobial properties in fin fish and shrimps larvae culture (Citrasu, 2010).

When herbal drugs are consumed they settle in the gut where they interact with gut microbiota. The gut microbiota is the community of commensal, beneficial, and pathogenic microorganisms that inhabit the gastrointestinal tract of humans and other animals. Forces that shape the composition of the gut microbiota, as well as other microbial communities, can include stochastic processes such as dispersal, genetic diversification, and ecological drift (Turnbaugh *et al.*,

2009). However, deterministic interactions between species, individuals, and the environment also create defined niches and thereby influence community composition. Species with overlapping fundamental niches can co-exist by adjusting to each other and segregating their realized niches in a process called niche differentiation. If niche differentiation is not possible, the competing species most well-adapted to the niche would be expected to outcompete and completely exclude the inferior competitor. Though various factors such as host genotype, immune status, and health state can affect the composition of the gut microbiota, the primary driver appears to be the composition and intake levels of host diet (Wu *et al.*, 2011; David *et al.*, 2014; Zarrinpar *et al.*, 2014; Carmody *et al.*, 2015).

Due to the selective actions of drugs, some vital gut flora could either be eliminated or acquire antimicrobial resistance. This does not only distort the microbial composition of the gut but also induce certain bacteria to become more pathogenic as observed in Monsi *et al.*, (2018). The aim of the current study are to assess the antimicrobial properties of some commonly sold herbal drugs in Nigeria and to evaluate the antibiotic sensitivity after prior sensitization with herbal drugs.

2. MATERIALS AND METHODS

2.1 Sample Location

The *Enterobacteriaceae* was collected from Rivers State University Teaching Hospital, Department of Medical Microbiology with the ethical permission from Rivers State Ministry of Health.

2.2 Collection of Organisms

The laboratory strain also known as control strain of *K. pneumoniae* WCDM 0097 (ATCC 13889) was purchased from Sigma United Kingdom. Molecularly identified clinical isolate of *K. pneumoniae* was provided by Monsi, Tombari Pius (Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria) which has been identified using molecular techniques at Lahor Research Laboratory, Agbor, Edo State.

2.3 Media Preparations

2.3.1 Tryptone Soya Agar (TSA), Tryptone Soya Broth (TSB)

The microbial media used were TSA and TSB. These were prepared according to the manufacturer's instructions and autoclaved for 15 minutes at 121°C. TSA was aseptically poured into sterile Petri dishes.

2.4 Determination of the Efficacy of Locally-Made Drugs on *K. pneumoniae*

An overnight bacterial culture at an optical density (OD) of 0.5 was diluted by a ten-fold serial dilution from the overnight culture concentration to 10^9 in TSB medium. This final dilution is equivalent to 10^2 CFU/ml when enumerated on TSA. Five different concentrations of locally-made drugs were obtained by serial dilution of locally-made drugs in TSB medium containing the overnight bacteria broth as diluents. The final concentrations of the antibacterial agent from the first to fifth Bijou bottle was 100%, 50%, 25%, 12.5% and 6.25%, respectively. These different concentrations were then plated in a 24-well plate. All cultures were incubated at 37°C and their optical densities were measured after 24 hrs of incubation.

2.5 Antibiotic Susceptibility of Locally-Made Drug Sensitized *K. pneumoniae*

Overnight cultures of *K. pneumoniae* in TSB were serially diluted to 100 CFU/ml in fresh TSB. Using this initial inoculum concentration, 2 ml of the bacteria cultures were aliquoted into five different sterile universal bottles. Different concentrations of herbal preparations were made by serial dilution in TSB. The various final concentrations of herbal drug used to treat the bacteria were 100%, 50%, 25%, 12.5% and 6.25%. A control that was not treated with herbal drug was also carried out. Both the herbal drug-treated and untreated conditions were incubated at 37°C and their OD was measured after 24 hrs to determine the response to herbal drug. After 24 hrs incubation, 200 µl of the culture was spread on the surface of TSA using a glass rod. The Ceftriaxone, Gentamycin, and Ciprofloxacin-impregnated discs were placed on the culture plates. Sensitivity plates were incubated at 37°C for 24 hrs and the zones of inhibition were measured.

2.6 Serum Bactericidal Assay

The serum bactericidal studies were modified from the work of Posdschun *et al.*, 1993. To determine serum sensitivity of herbal drug-pretreated *K. pneumoniae* isolates were grown to the mid-log phase (OD = 0.5) and incubated with 30% (in normal saline) either pooled normal human serum (NHS) or heat-inactivated human serum (HIS) for 3 hrs. The heat inactivation was achieved by heating the serum in a sterile test tube up to boiling for 3 minutes. Several concentrations of serum from 10% to 90% were tested, and 40% was determined as the most efficient concentration. Bacteria were serially diluted and plated immediately following inoculation on nutrient agar, and at 3 hrs intervals for 12 hrs. The serum bactericidal effect was calculated as a percentage survival, using the bacterial counts when incubated with HIS values as 100%. All experiments were performed in triplicate and results were expressed as percent survival.

2.7 Data Analyses

All experiments were performed at least in duplicate and on at least two independent occasions. Results were presented as mean ± SD where necessary. Where appropriate, statistical analyses were performed using an unpaired t test in which a two-tailed *P*-value was calculated (GraphPad Prism Software Version 5.03, San Diego, CA). Statistical significance was defined as a *P*-value of less than 0.05 at 95% confidence interval.

2.8 Ethical Consideration

Collection of serum samples were performed in strict accordance with the ethical recommendations of the Ethical Committee under the jurisdiction of the Rivers State Ministry of Health in Port Harcourt of Nigeria.

3. RESULTS

3.1 Antimicrobial Efficacy of Herbal Drugs on *K. pneumoniae* Isolates

Figure 3.1 depicts the efficacy of the herbal drugs on *K. pneumoniae*. Ruz, Gab, Gob and Bet showed significantly lower zones of clearance ($P < 0.05$) when compared with the control (Imipenem). These herbal drugs also showed higher efficacies than New, Dan, Gol and Eva. The latter group of herbal drugs showed significantly lower efficacies compared to the control (Imipenem). According to the NCCL 2010, the zones of inhibition observed in Ruz, Gab, Gob and Bet are moderately sensitive while New, Dan, Gol and Eva are classified as resistant. All the herbal drugs showed significantly lowered ($P < 0.05$, Table 3.1) zone of clearance compared to the control antibiotic, Imipenem.

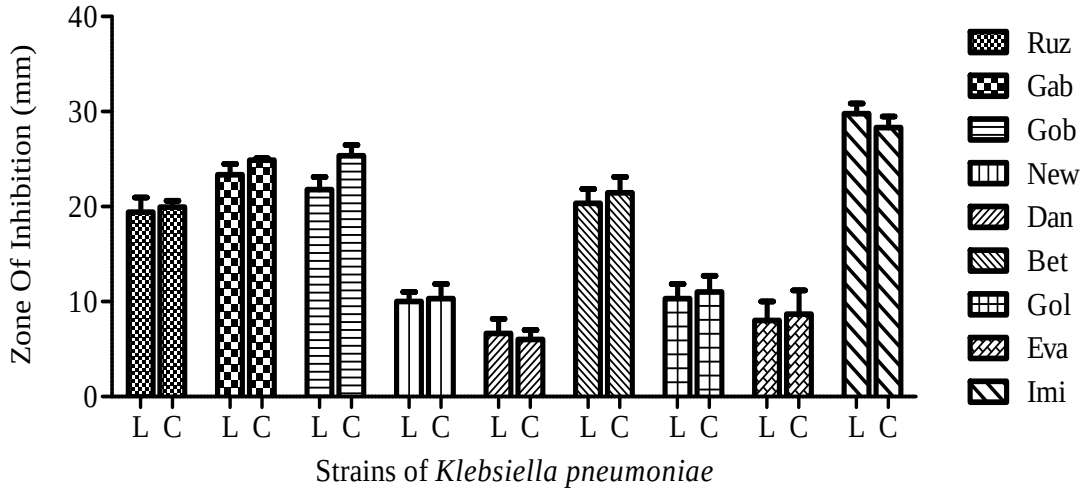


Figure 3.1: Efficacy of herbal drugs on *K. pneumoniae*. Experiment was performed in triplicates on three independent occasions. The bars represent Mean ± SD. Zone of inhibition is expressed in mm. Key: Ruz = Ruzu bitters, Gab = Goko alcoholic bitters; Gob = Goko bitters; New = New hope herbal mixture; Dan = Danko solution; Bet = Beta cleanser; Gol = Golden seed; Eva = Evacuation solution; Imi = Imipenem. L= Laboratory strains; C = Clinical strains.

Table 3.1: Comparisons of the Herbal Drugs and Imipenem Efficacies on of *K. pneumonia.*

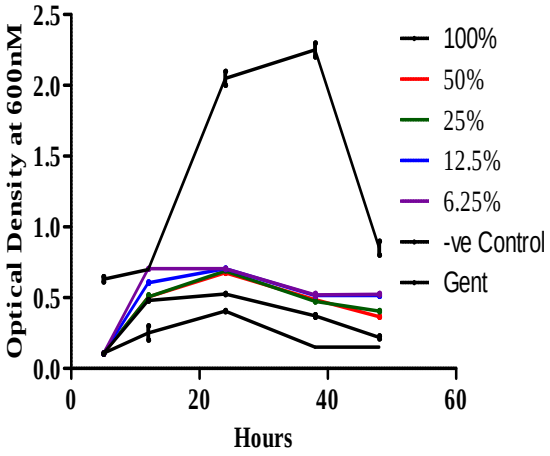
				Clinical Strain				
	Ruz	Gab	Gob	New	Dan	Bet	Gol	Eva
P-value	0.0004	0.0078	0.0357	0.0001	0.0001	0.0045	0.0001	0.0003
				Laboratory Strain				
P-value	0.0006	0.0021	0.0013	0.0001	0.0001	0.0009	0.0001	0.0001

The statistical analysis performed was a two-tailed unpaired t test at confidence interval of 95%. Hence, results were considered statistically significant at $P < 0.05$. Key: Ruz = Ruzu bitters, Gab = Goko alcoholic bitters; Gob = Goko bitters; New = New hope herbal mixture; Dan = Danko solution; Bet = Beta cleanser; Gol =Golden seed; Eva = Evacuation solution; Imi = Imipenem. L= Laboratory strains; C = Clinical strains.

3.2 Growth Curves in Different Locally–Made Drug

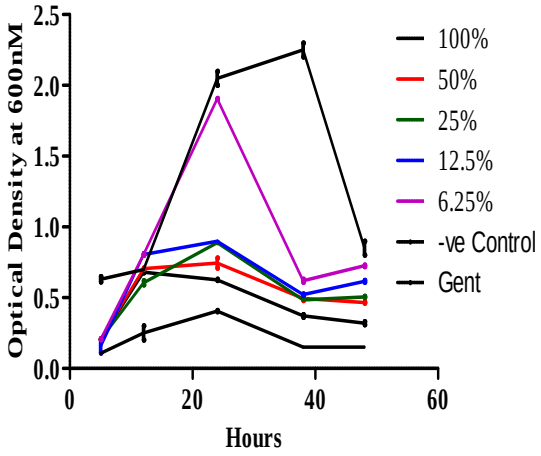
Figures 3.2 A–H show the time course of growth of *K. pneumoniae* exposed to herbal drugs as well as the positive (Gentamicin supplemented cultures) and negative (unsupplemented culture) controls. For both strains, the time courses of growth show initial increases in the optical density all treatment conditions. However, the herbal drug–supplemented cultures and the negative control showed higher optical densities than the positive control after 24 hrs of exposure. Again, while the negative control (with no drug) shows the highest level of growth, the positive control (Gentamicin) showed a much the lowest peak.

Bet [L_c]



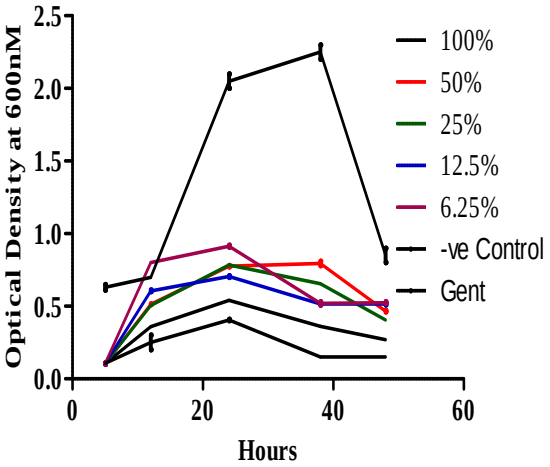
A

Bet [C_c]



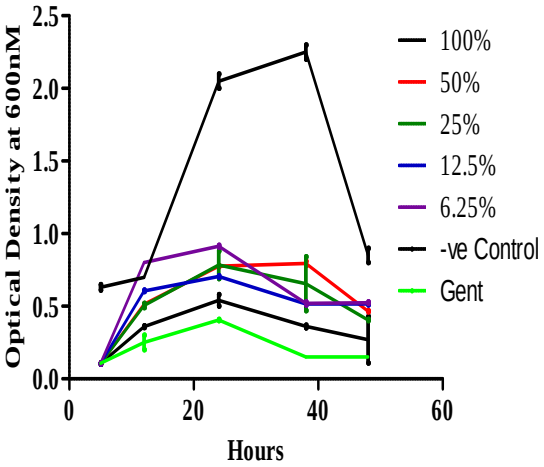
B

Gab [L_c]



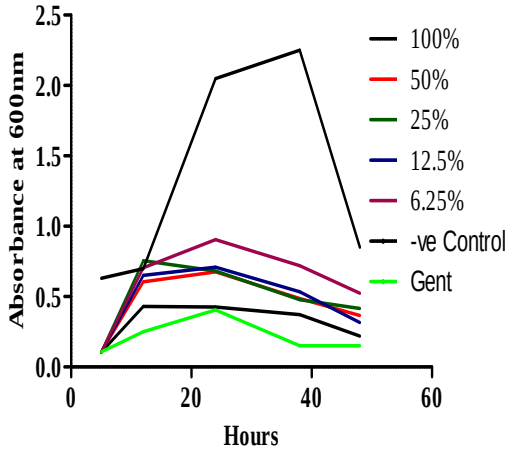
C

Gab [C_c]



D

Gob [L_c]



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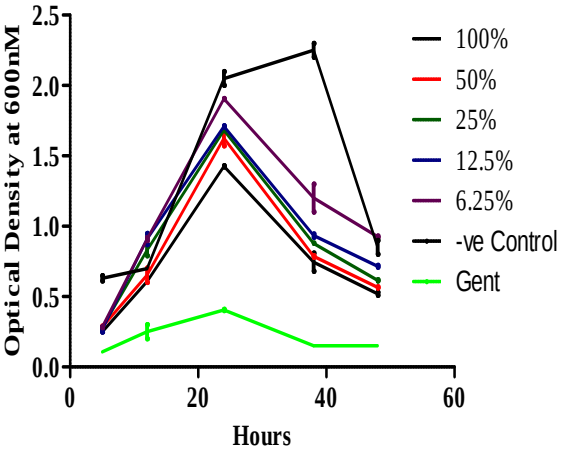
Gob [C_c]



F

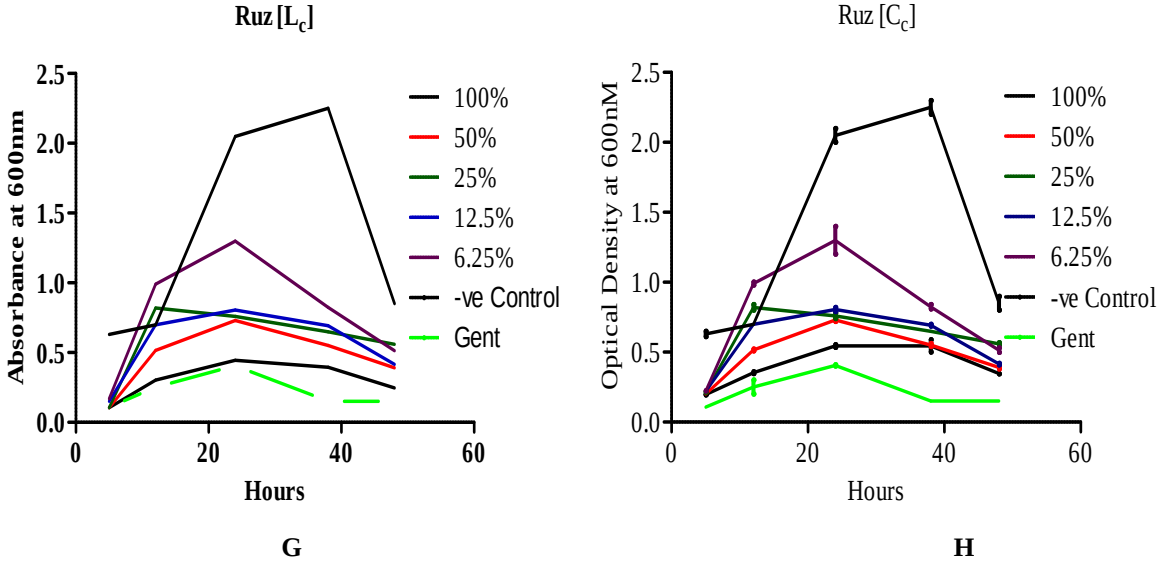
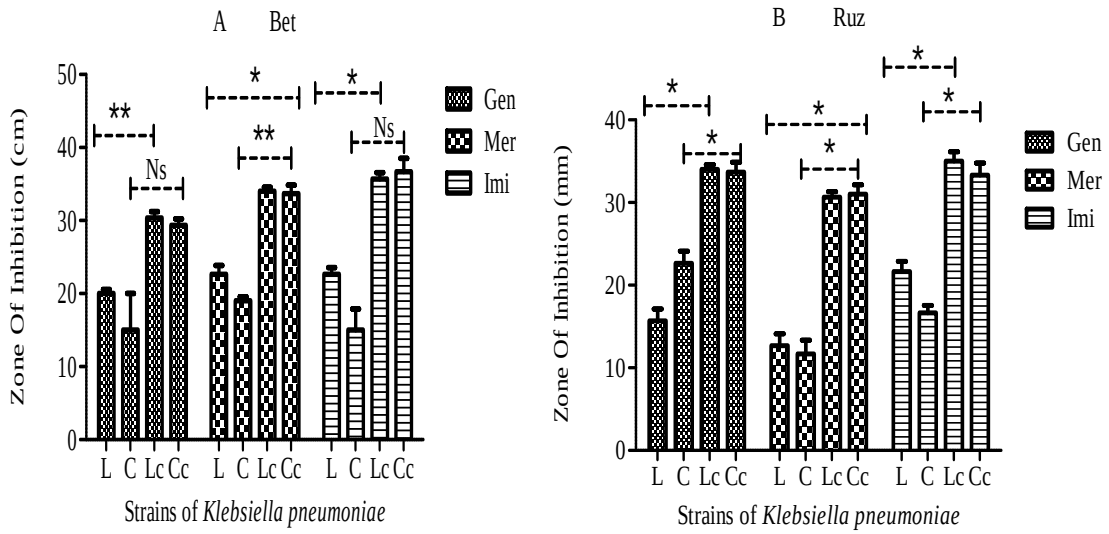


Figure 3.2 A–H: Time courses of growth of *K. pneumoniae* in herbal drug – supplemented TSB medium. Keys: Beta cleanser [Bet], Goko alcoholic bitters [Gab], Goko bitters [Gob], and Ruzu bitters [Ruz], L_c: non–sensitized Laboratory strain, C_c: non–sensitized clinical strain, -ve Control: untreated *K. pneumoniae*, and Gent: Gentamicin.

3.3 Effect of Antibiotics on Herbal Drug-Treated *K. pneumoniae*

Figure 3.3 represents the effect of modern antibiotics on *K. pneumoniae* previously exposed and unexposed to herbal drugs. The 50% concentration of all the herbal drugs was chosen for antibiotic sensitivity studies as this showed the significant variation in the biofilm production across all the drugs in previous studies (Monsi *et al.*, 2018). Hence, *K. pneumoniae* isolates treated with 50% concentration of the herbal drugs for 24 hrs at 37°C were further exposed to Meropenem, Imipenem and Gentamicin antibiotic discs from Oxoid, UK. The zones of inhibition were measured and experiment repeated on two independent occasions. Ruz showed significantly lower levels ($P < 0.05$) of zones of clearance of herbal drugs exposed *K. pneumoniae* isolates. The clinical strain in Bet, Gab and Gob did not show significant variation in the zone of inhibition compared to the control ($P > 0.05$).



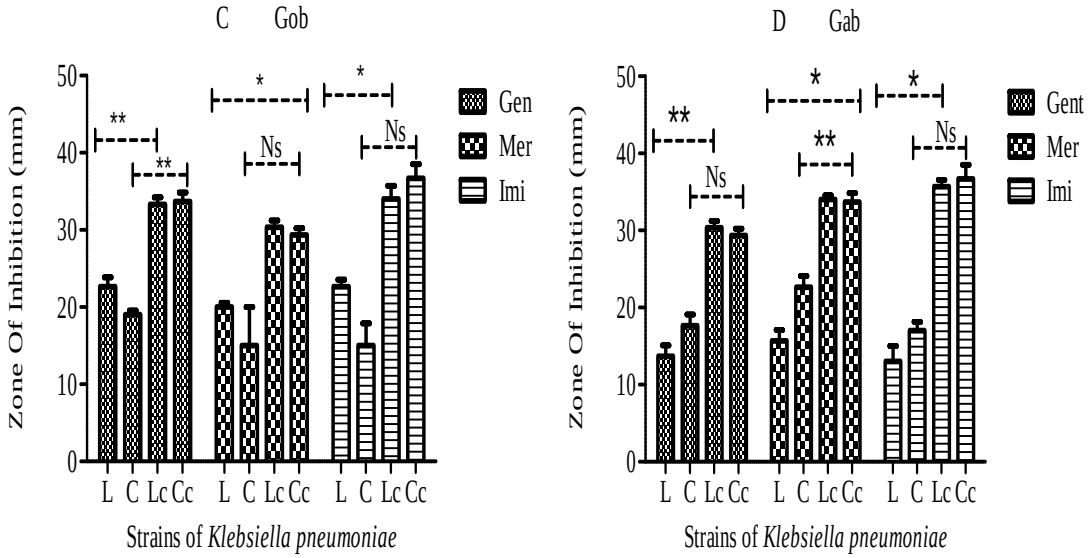


Figure 3.3 A–D: Antimicrobial sensitivity of herbal drug–pretreated *K. pneumoniae* isolates. The different pretreatment conditions are: a) Bet b) Ruz c) Gob and d) Gab. *Statistical significance at *P*<0.05. Beta cleanser [Bet], Goko alcoholic bitters [Gab], Goko bitters [Gob], and Ruzu bitters [Ruz], L: Laboratory strain, C: Clinical strain.

3.4 Serum Test of *K. pneumoniae* Isolates Exposed to Herbal Drugs

To determine the concentration of serum to use in the serum resistance study, different concentrations of normal human serum (NHS) was used ranging from 10–90% of normal human serum on the control strains as they are have not been previously exposed to the herbal drugs. This is revealed in Figure 3.4. As serum has bactericidal activities due to the presence of mainly complements as well as enzymes, we expect a decrease in the number of bacteria in the presence of the NHS. There was an exponential decrease in the number of bacteria at 40% serum concentration before levelling off. Hence, 40% serum was used as the concentration for serum resistance investigation in herbal drug–induced *K. pneumoniae* isolates. Figure 3.5 demonstrates the bactericidal actions of NHS on the *K. pneumoniae* isolates compared to their respective controls treated in heat–inactivated human serum (HIS). Ruz and Gab-treated *K. pneumoniae* isolates showed significantly higher levels ($P < 0.05$) in the number of bacteria isolated from the NHS compared to HIS. The reverse results were obtained for Gob and Bet-treated *K. pneumoniae* isolates as they were significantly lower ($P < 0.05$) in the NHS than HIS.

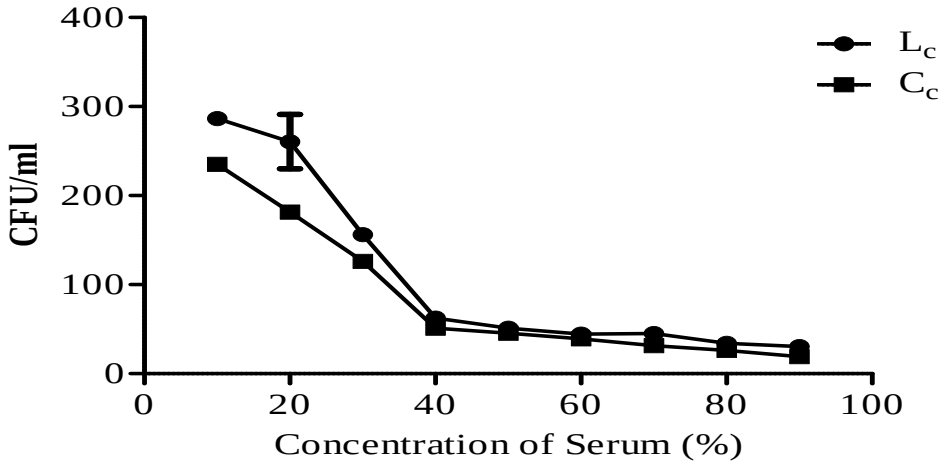


Figure 3.4: Serum concentration dependent survival of unexposed *Klebsiella pneumoniae*. *Klebsiella pneumoniae* ATCC 1388 (L_c), clinical *Klebsiella pneumoniae* (C_c) were exposed to 40% NHS and enumerated on nutrient agar. The data was obtained from two independent experiment in duplicate.

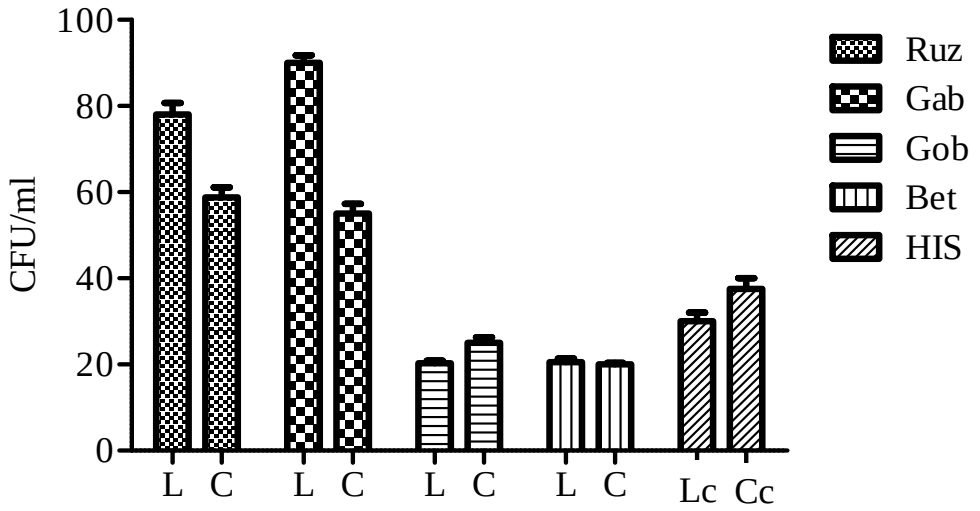


Figure 3.5: Serum resistance of *K. pneumoniae* isolates. CFU/ml of each isolates determined for each isolate following 6–hrs incubation in 40% normal human serum (NHS) and compared to the control in heat-inactivated serum (HIS). The data were obtained from a minimum of two independent experiments in which each isolate was tested in duplicate. Bars are the SD of the mean. L_C and C_C were the controls. Key: L; *K. pneumoniae* ATCC 13889, C; clinical *K. pneumoniae*, L_C; *K. pneumoniae* ATCC 13889 in HIS treated condition, C_C; clinical *K. pneumoniae* in HIS treated condition.

4. DISCUSSION

4.1 Antimicrobial Sensitivity of *K. pneumoniae* Isolates to Herbal Drugs

In the developing countries, there is an increase in the rate of consumption of herbal drugs due to the difficulty associated in managing infectious diseases and the inability of the populace to afford adequate medical treatment. In Nigeria, there are several herbal drugs peddled most of which are not duly registered with the appropriate agencies. Hence, there efficacies have not yet been scientifically proven. The current study tested the antimicrobial efficacy of eight herbal drugs namely; Ruz, Gab, Gob, Bet, New, Dan, Gol and Eva. There is no evidence to show the efficacy of these herbal drugs on *K. pneumoniae* isolates. From the results, *K. pneumoniae* isolates showed significant levels of sensitivity, when compared to the control (Imipenem), in the first four herbal drugs. Although the last four drugs showed some levels of efficacies but are resistant according to NCCLS, (2003) classification of antimicrobial efficacy.

Therefore, the results showed that *K. pneu*moniae isolates were not sensitive to 50% of the tested herbal drugs. Previous studies have demonstrated antimicrobial effectiveness of herbal alternatives such as Triphala (Prabhakar *et al.,* 2017); *Syzygium aromaticum* and *Citrus limon* (Sharmeen *et al.,* 2012) and *Mentha pulegium* essential oil (Jazani *et al.,* 2009). These drugs showed some level of inhibition of *Klebsiella* sp growth which is in accordance with the results in this current study. Our study used the Imipenem as control for the comparison as it is very effective against beta–lactamase–producing bacteria.

4.2 Continuous Growth Curve

The continuous growth study was performed to ensure the experiment adopted duration that the *K. pneumoniae* still remain active physiologically during exposure to the herbal drugs. The Imipenem showed a much reduced levels of optical density at 595 nM due to its bacteriolytic effect. This drug as the ability to lyse bacteria, hence, the lyses of *K. pneuomoniae* lead to reduced optical density when measured in the spectrophotometer. Similar findings were noted by Freestone *et al.,* (2012b). The current study results also showed that the peak of growth was observed after 24 hrs. Hence the time of exposure to herbal drug adopted in this research was 24 hrs.

4.3 Antibiotic Resistance Induction in Herbal Drug–Pretreated *K. pneumoniae* Isolate

When drugs are administered via oral mean, they move from the mouth and settle in the stomach by means of peristalsis action. The gastrointestinal tract is characterized by the finger–like projections called villi of the stomach and folding nature of the intestines. Some microorganisms have been reported to reside in some hidden crevices due to these characteristics of the gut (Hawkey, 2008). This makes antimicrobials taken orally to be available below the bactericidal concentrations for these bacteria in seclusion. Hence it has been discovered that these bacteria develop resistance. Instead of being eliminated they are only become sensitize to the drugs and develop resistance (Webber *et al.,* 2015). One of the objectives of the current study was to investigate whether *K. pneumoniae* pretreated with herbal drugs could develop resistance to modern antimicrobials. The basis for this objective is that *K. pneumoniae*, an opportunistic

pathogen in the gut, could be sensitized by the herbal drugs and acquires resistance to modern drugs.

The study used three antimicrobial agents (Gentamicin, Meropenem and Imipenem) due to their high potencies and efficacies against beta–lactamase–producing bacteria (Harino *et al.*, 2013). All isolates pretreated with herbal drugs showed lower zones of inhibition compared to the non–herbal drug treated isolates. This implies that the sensitivity levels to modern drugs reduce when *K. pneumoniae* is exposed to the herbal drugs. The levels of antimicrobial sensitivity to Gentamicin, Meropenem and Imipenem were all significant in the *K. pneumoniae* isolates treated in the Ruz compared to the untreated isolate. Bet pretreated isolates did not show significant difference from the control with Gentamicin (clinical strain) and Imipenem (clinical strain). Similar, Gob pretreated isolates did not show significant difference from the control with Meropenem (clinical strain) and Imipenem (clinical strain) and Gab pretreated isolates did not show significant difference from the control with Gentamicin (clinical strain) and Imipenem (clinical strain). Bet and Gab treated isolates showed exactly the same pattern of resistance induction by the same herbal drugs. These results show that there is antimicrobial resistance acquisition in *K. pneumoniae* exposed to some herbal drugs. Hence, consumption of herbal drugs that have not been properly screened could results to resistance development in gut bacteria.

There are diverse views on the mechanisms behind resistance development in bacteria. Some previous studies have demonstrated that stress–inducing drugs such as catecholamines enhance the growth, biofilm, virulence proteins in different species of bacteria in both serum–based and non–serum based medium (Freestone *et al.*, 2003a; Freestone *et al.*, 2012b; Lyte *et al.*, 2003). This idea suggests that compounds that cause stress in bacteria could induce virulence that promotes bacterial survival. Our study used a non-serum based medium, TSB, which is rich in nutrient necessary for bacteria growth due to the nutrient rich environment *K. pneumoniae* live in the stomach. The stomach nutritional content is a reflection of the dietary composition of an individual. Hence, a healthy gut is composed of: glucose, amino acid, vitamins, iron and other essential minerals that could enhance the normal growth of anaerobic organisms. The detection of resistance acquisition in herbal drugs–pretreated *K. pneumoniae* prompted further studies into possible mechanisms of resistance such as biofilm production.

4.4 Serum Resistance

The successful invasion of the blood by a pathogen partly depends on the ability of the microorganism to evade the bactericidal effect of the serum (Podschun & Ullmann, 1998). Although main component responsible for this killing is complement, other factors that contribute to the destruction of bacteria include; phospholipase which hydrolyse the membrane of bacteria (Dennis *et al.*, 2011), circulating leucocytes that engulf pathogens (Hancock & Scott, 2000), and lysozyme (Callewaert & Michiels, 2010). The results from the optimization of the appropriate concentration of the serum for the study showed that 40% serum concentration resulted in an exponential decrease in the number of *K. pneumoniae* for both strains. This differs from the 30% concentration of normal human serum reported by King *et al.*, (2009). The lower concentration of 30% used in this previous study may arise due to contamination of the serum during the separation stage by leucocytes from the buffy coat layer. The presence of leucocytes in the serum enhances bactericidal activities through phagocytosis of complement–opsonized bacteria (Struve *et al.*, 2015).

In addition to the optimization studies we also investigated whether the killing was mainly complement–mediated by heat inactivation of the complements due to their ability to spontaneously degrade in the presence of heat. Hence, the number of isolates exposed to the normal human serum were compared those exposed to the heat–inactivated serum. Both clinical and laboratory isolates of *K. pneumoniae* pretreated with Ruz and Gab showed significantly higher number of bacteria that survived. This shows resistance induction in the bacteria. The opposite findings were seen for Gob and Bet–pretreated *K. pneumoniae* isolates. This implies that Ruz and Gab induced serum resistance to *K. pneumoniae*. Previous studies have shown that while commensal bacteria were generally prone to bactericidal effect of serum, hospital–acquired bacteria tend to be serum resistant (Podschun & Ullmann, 1998; Podschun *et al.*, 1993; Sahly *et al.*, 2004).

Serum contains iron–binding proteins called transferrin, lactoferrin and haemosiderin. These proteins are produced by human as a mechanism to regulate the amount of iron available in the blood. Hence, they bind directly to iron and sequester them from the blood. The availability of free iron in the blood during bacteremia increased the virulence of blood–borne pathogens. Sandrini *et al.*, (2014) and Freestone *et al.*, (2012b) discovered that catecholamine, a stress inducer, can bind to these iron–binding proteins allosterically and cause their release into the blood. They further showed that this resulted to the increase in the production of biofilm responsible for resistance. We are proposing that herbal drugs could induce resistance in bacteria such as *K. pneumoniae* via making iron available in the serum.

CONCLUSION

Klebsiella pneumoniae exhibited some levels of sensitivity to four herbal drugs (Ruzu bitters, Goko bitters, Goko alcoholic bitters and Beta cleanser). The continuous growth studies showed 24 hrs is the duration to obtain peak growth of the bacteria in herbal drug treated–medium. Again prior sensitization of *K. pneumoniae* strains to the herbal drugs (Ruz, Gob, Gab and Bet) lead to ressitance inducement to antibiotics

(Meropenem, Imipenem, and Gentamicin) and human serum.

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