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Acquisition of Antibiotic Resistance in *Escherichia coli* Exposed To a Locally Produced Herbal Drug

Monsi, Tombari Pius[1*], Amala, Smart Enoch[1] and Ugoaru, Nkechinyere Francisca[1]

[1]Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nkpolu – Oroworukwo, P.M.B. 5080, Rivers State, Nigeria.

Authors' contributions

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

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ABSTRACT

Background: Antimicrobial resistance poses great threats to the treatment and eradication of pathogenic microbes. An increasing number of infectious diseases are now becoming difficult to treat in the developing world, but the root of the problems is vaguely known.
Aims: This study aimed at determining the phenotypic properties of *Escherichia coli* isolates exposed to a locally prepared drug, usually called Goko Alcoholic Bitters (GAB), a commonly consumed herbal medication in Nigeria.
Methods: In order to determine GAB-induced resistance the antimicrobial efficacy of GAB against *E. coli* isolates was first determined using the disc diffusion method and was compared to a control (Ampicillin). A growth response assay was performed to monitor the response of *E. coli* exposed to GAB and analyzed by bacterial count on nutrient agar as well as using a spectrophotometer to measure optical density. Finally, GAB-treated *E. coli* was investigated for drug resistance acquisition using antibiotic discs.

**Corresponding author: E-mail: monsitp@live.com, tombari.monsi@ust.edu.ng;*

Results: Different concentrations of GAB did not show considerable zones of inhibition compared to the Ampicillin (30 µg) control. The effects of GAB on growth of *E. coli* showed that the highest concentration (neat concentration = 0.036 g/ml) of GAB inhibited growth of *E. coli*, but did not completely eliminate bacteria (bacteriostatic). Antimicrobial susceptibility testing of surviving *E. coli* isolates previously exposed to different concentrations of GAB showed significantly reduced sensitivity (*P*-value=0.01) to Ampicillin compared to untreated *E. coli*. This implies that resistance has been induced in bacteria by GAB.
Conclusion: This study suggests the possibility of GAB conferring resistance of some Gram-negative such as *E. coli* against some antibiotics like Ampicillin.

Keywords: Antimicrobial resistance; herbal medication; Escherichia coli; antimicrobial susceptibility; drug-induced virulence.

1. INTRODUCTION

The ability of bacteria to circumvent the action of a standard antibiotic that previously destroyed or inhibited its growth is referred to as antimicrobial resistance. There has been an increase in antimicrobial usage in developing countries such as Nigeria [1]. The motivation of this study arose from the understanding that antimicrobial agents produced locally without previous knowledge of their mode of action and ability to induce resistance might have public health implications. These antimicrobial agents may or may not have been tested for their ability to inhibit target microbes of interest without understanding further untoward effects such as antimicrobial resistance of the normal flora within the gastrointestinal tract when administered orally.

The continuous rise in antibiotic resistance in developing nations could be the result of natural selection of resistant strains of bacteria exposed to conditions, which may form a new dominant population of resistant strains. In other words, consumption of yet to be verified and approved antimicrobials (herbal medications) could make bacteria previously sensitive to some antibiotics resistant. Resistance mechanisms which could be employed by these microbes include the acquisition of antibiotic resistance plasmids from other bacteria or phages (horizontal gene transfer or transduction), mutations in specific genes, and alteration of the bacterial surface [2, 3]. It is common knowledge that the continuous use of particular antimicrobial agents has resulted in increased antibiotic resistance of bacteria globally.

Some possible causes of this rise in antimicrobial resistance have previously been described [4]. The first is overuse and inappropriate prescription of antibiotics. Sub-inhibitory and sub-therapeutic doses of antibiotics can cause resistance through genetic alterations [5]. The extensive use of agricultural animal growth supplements could serve as a source for antimicrobial resistance through natural selection. By this process of natural selection, antimicrobial agents targeting particular microbes could confer resistance on the microbes via selection. The consumption of agricultural products containing antibiotic-resistant pathogens could then serve as a means of generating antimicrobial resistant microbes in humans. Hence, antimicrobial resistance development can occur via three key pathways: antibiotics in agricultural supplements kill sensitive bacteria, but retain the resistant bacteria; resistant bacteria ingested through food consumption cause infection(s) and spontaneous mutation of bacterial resistance genes.

Furthermore, availability of relatively few antibiotics due to the low cost of drugs compared to production costs of cancer drugs has been linked to the persistence of antimicrobial resistance. This makes drug manufacturing companies reluctant in producing more antimicrobial agents as the profit margin is low [6]. Moreover, the challenges involved in obtaining approval due to regulatory issues and difficulties in drug approval present serious problems. This occurs as a result of bureaucratic delays, differences in regulatory licensing rules and difficulty in channels of communication. These factors have resulted in boycotts of the regulatory process by local drug manufacturers and in-house preparation of their products as is currently the case in most developing countries such as Nigeria.

The local source of antimicrobial agent used in this study was Goko Alcoholic Bitters (GAB). The contents of this drug consist of some plants extracts, whose phytochemical properties are not yet known [7]. The bacteria used in this study

were clinical isolates of *E. coli*, which are usually an essential component of the gut flora and an etiological agent for both hospital and community-acquired infections in humans [8]. As with other bacterial pathogens, this bacterium can develop multiple drug resistance to several antimicrobials, which has made antimicrobial chemotherapy of invasive *E. coli* infections very difficult.

The ability of microbes to acquire resistance to herbal antibacterial agents is poorly understood. Some studies have suggested that microbes cannot develop resistance to herbal drugs [9, 10]. However, *Escherichia coli* ATCC 252922 and environmental *E. coli* isolates exposed to aqueous extracts of banana, lemon grass and turmeric showed resistance [11]. Also, some herbal drugs have been shown to harbor drug resistant microbes. High colony forming units of enteric microbiota were discovered in dried herbal products [12]. Ogunshe et al. [11] demonstrated that herbal medications in Nigeria contain bacterial flora, which showed multiple resistance to modern antibiotics.

The aim of the study was to investigate whether a locally prepared herbal antibacterial agent, GAB, confer resistance to opportunistic bacteria such as *E. coli.* The hypothesis was that exposure of *E. coli* to GAB will induce resistance in *E. coli*.

2. MATERIALS AND METHODS

2.1 Collection of the Herbal Drug GAB

The herbal drug, Dr. Iguedo's GAB was purchased from Mile 3 market, Port Harcourt, Rivers State, Nigeria. GAB contains ethanol, *Calamus rhizome*, *Azadirachta indica* and Caramel.

2.2 Isolation and Identification of the Test Organism

Isolation and identification of *E. coli* isolates were carried out at the University of Port Harcourt Teaching Hospital (UPTH), Nigeria. The organism was obtained by culturing urine samples on Cysteine Lactose Deficiency Agar (CLED). The bacterium was identified according to Cheesbough [13]. A pure culture of the isolate was obtained and stored on Tryptic Soya Agar (TSA) at 4°C for subsequent use. Ethical approval was obtained from the University of Port Harcourt Teaching Hospital before the collection procedure.

2.3 Media Preparations

2.3.1 Tryptic soya agar (TSA) and tryptic soya broth (TSB)

TSA and TSB were prepared according to the manufacturer's instructions and autoclaved for 15 minutes at 121°C. The medium was allowed to cool and 20 ml of TSA was aseptically poured into sterile Petri dishes. Plates were allowed to solidify at room temperature and stored at 4°C for subsequent use.

2.4 The Efficacy of GAB on *Escherichia coli* Isolates

An overnight culture was first diluted 10^9 (equivalent to 10^2 CFU when enumerated on TSA) in TSB medium. Five different concentrations of GAB were obtained by serial dilution of GAB in TSB medium containing the overnight bacteria broth as diluents. The concentration of the antibacterial agent from the first to fifth Bijou bottle was 0.018 g/ml, 0.006 g/ml, 0.002 g/ml, 0.0007 g/ml and 0.0002 g/ml, respectively. All cultures were incubated at 37°C and their optical density was measured after 24 and 48 hrs of incubation.

2.5 Determination of Growth Response

Growth response was determined using two methods; spectrophotometry and bacterial count according to Freestone et al. [14]. In the spectrophotometer method, an overnight culture with an optical density (OD) of 0.5 at 600 nm was diluted to contain 10^9 colony forming units (CFU) of the test organism and treated with different concentrations of GAB in TSB for 18 hrs at 37°C. The OD of the various culture concentrations was measured at 600 nm. In the bacterial count method, the above described procedure was repeated and enumeration of bacterial colonies was carried out by bacterial plate count on TSA.

2.6 Preparation of Herbal Discs

Discs of the herbal preparation were prepared for a sensitivity test by punching Whatman filter paper No. 540. The size of discs used was 6 mm. The discs were sterilized in an autoclave, placed in the solution of the herbal preparation in sterile Petri dishes at final concentrations of

0.018 g/ml, 0.006 g/ml, 0.002 g/ml, 0.0007 g/ml and 0.0002 g/ml and allowed to impregnate for 5 minutes. The concentration of the herbal preparation used in this study was made by drying up 10 ml of GAB herbal solution in a test tube. The weight of the dried preparation was 0.36 g hence; making the neat (initial) concentration of the herbal drug 0.036 g/ml. Different concentrations were obtained by serial dilutions. Commercially purchased antibiotic discs (Ampicillin 30 µg) were used as a control. The concentration of the drug was determined by evaporating 10 ml of the drug to dryness.

2.7 Antibiotic Susceptibility of GAB-Treated *Escherichia coli* Isolates

Inocula from overnight cultures were made as previously described (See 2.5). From this starting concentration 2 ml of bacteria, aliquots were placed in five different sterile Bijou bottles. Five concentrations of herbal preparations made by serial dilution using TSB at 0.018 g/ml, 0.006 g/ml, 0.002 g/ml, 0.0007 g/ml and 0.0002 g/ml, respectively were used, inoculated and incubated at 37°C and their OD was measured after 24 hrs to determine the response to GAB. After 24 hrs, 10µl of the culture was spread over the entire surface of TSA using the spreading technique and the Ampicillin disc was carefully placed on the respective culture plates. As a control, *E. coli* not previously exposed to GAB was subjected to the same conditions. Sensitivity plates were incubated at 37°C for 24 hrs and the zones of inhibition were measured.

2.8 Data Analysis

Data were statistically analyzed on Graph Pad Prism 6 using T-test.

3. RESULTS AND DISCUSSION

Resistance to antimicrobial agents is a serious public health challenge especially when it is acquired by opportunistic pathogens [9]. In the last decades, the emergence of drug resistant microbes has increased the public awareness of using antimicrobial drugs. The rationale behind our study was to determine whether a locally prepared herbal preparation (extract), GAB, could confer resistance to an opportunistic pathogen. In order to study the effect of GAB on *E. coli* we adopted the *in vitro* methodology employed by Freestone et al. [14], where they assessed the effects of inotropic agents on growth and biofilm formation in *E. coli*. This current study utilized a low starting inoculum as previously described by Freestone et al. [14] in an attempt to detect any possible changes in the number of bacteria.

Bacteria sense the presence of chemical substances within their environment and react in ways to enable their survival. A study on the effect of catecholamines conducted by Lyte et al. [15] showed that bacteria develop virulence factors when exposed to inotropic agents such as catecholamines. A similar principle was discovered here for the detection of antimicrobial resistance upon exposure to GAB. Also, an investigation carried out by Freestone et al. [14] showed that inotropic drugs tested *in vitro* resuscitated antibiotic-damaged bacteria. The concept employed in our study was that as endogenous molecules such as catecholamines can induce virulence in bacteria, possibly exogenous molecules in the form of locally manufactured drugs could also cause virulence by the induction of resistance in opportunistic pathogens.

3.1 Efficacy of Goko Alcoholic Bitters on *E. coli*

The first task in this study was to evaluate the ability of GAB to kill *E. coli* using the disc diffusion method, which has been previously used to determine antibiotic susceptibility of organisms to antibiotics [9,16,17]. To determine whether *E. coli* was sensitive to GAB, different concentrations of GAB were tested alongside the Ampicillin (30 µg) control impregnated on discs. By comparing the efficacy of the different concentrations of GAB to the control, the different concentrations of GAB did not show considerable zones of clearance. The implication of this is that GAB is less effective than Ampicillin. Since there were no considerable zones of clearance by GAB compared to the control, there could be some untoward effects on opportunistic normal flora such as *E. coli*. A crucial unfavorable effect is the possibility of inducing drug resistance in opportunistic pathogens.

To determine the efficacy of GAB, the disc diffusion method was used, previously applied for the determination of the sensitivity of microbes to drugs [9]. The effect of the GAB on *E. coli* tested at different concentrations (neat' or highest concentration of 0.036 g/ml) did not show any significant variation in zones of clearance compared to the Ampicillin control (Fig. 1). However, when each of the concentrations was

compared with the control a clear difference was observed in the zones of clearance. This difference in the zones of clearance of Ampicillin and the different GAB concentrations was at least three-fold. However, the differences were not significant compared to the control.

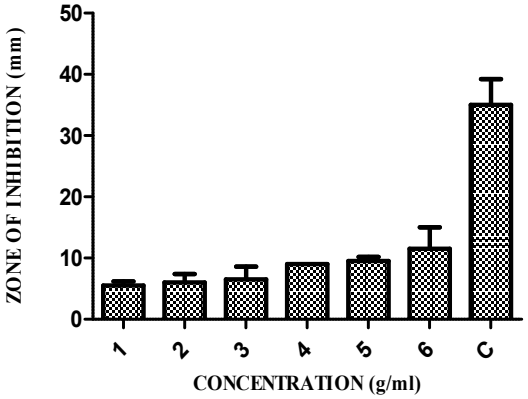

Fig. 1. Efficacy of GAB on *E. coli* isolates
The zones of inhibition were measured using a meter rule. Lanes 1-6 represents 0.0002 g/ml, 0.0007 g/ml, 0.002 g/ml, 0.006 g/ml, 0.018 g/ml, and 0.036 g/ml, respectively, and C represents the Ampicillin control (30 µg) (comparison of 1-6 with control showed P>0.05)

3.2 Growth Response Assay

The study on the efficacy of GAB showed there were no considerable zones of clearance, which prompted us to study the growth response of *E. coli* to GAB. There is no published findings yet on the growth response of bacteria to local herbal preparations. Here, GAB could not completely eliminate *E. coli* (Fig. 2). This is in contrast to the finding by Gullberg et al. [18], which showed complete clearance of *E. coli* exposed to tetracycline. Hence, we can postulate that surviving *E. coli* could acquire some resistance, which can pose a serious challenge to eradication with modern antibiotics. This is similar to the findings from other investigations [14,15] that bacteria can recognize and utilize inotropic drugs to grow and produce virulence factors. The findings that GAB could not completely eliminate *E. coli* led to further investigations of modern antibiotic efficacy on GAB-treated *E. coli*.

Growth response assays were further performed to determine the patterns of growth of *E. coli* isolates after exposure to GAB. Two types of methods employed in the investigation of growth response were adopted from Lyte et al. [15] and Freestone et al. [14]. Both the bacterial count method and the spectrophotometric method showed that the response of GAB to *E. coli* was dose-dependent (Fig. 2). For the bacteria count method, the lowest concentration showed the highest CFU/ml value, while the highest concentration was associated with the lowest CFU/ml value. The neat (highest) concentration (0.036 g/ml) demonstrated a marked reduction in the number *E. coli* colonies (Fig. 2a). There was no complete elimination of bacteria at the neat concentration compared to Ampicillin. This was demonstrated by re-plating bacteria grown in broth on nutrient agar and visible growth was observed. The growth response of *E. coli* varied according to the concentration of GAB. The same observation was made on growth response measured by spectrophotometry (Fig. 2b).

3.3 Effect of Ampicillin on GAB-Treated *E. coli* Isolates

The work performed by Fagbemi et al. [19] showed that herbal medications in Nigeria contain bacterial flora and exhibited multiple resistance to modern antibiotics. This formed the basis of resistance inducement by locally prepared drugs on pathogens. Similar observations were made in our study as *E. coli* showed different levels of resistance to Ampicillin (Fig. 3). While other concentrations of GAB showed significantly reduced zones of inhibition (*P*-value=0.01), no zones of clearance were detected at 0.0007 g/ml. This could be interpreted that this particular concentration of GAB may cause resistance to modern antibiotics. The concentration 0.0002 g/ml which is lower than 0.0007 g/ml did show zone of clearance. The implication of this experiment is when people consume GAB to treat *E. coli* infection, *E. coli* becomes sensitized by the herbal drug and acquire resistance. The resistance could be total as observed at a single concentration or reduced sensitivity at other concentrations in this study. The results of this current study contradict the suggestions and observations made by some studies that herbal drugs cannot induce resistance in microbes [11,20].

The effect of commercially prepared antibiotics on GAB-treated *E. coli* isolates was performed using the disc diffusion method with Ampicillin as the control. GAB-treated isolates showed varying susceptibility to Ampicillin. *E. coli* isolates exposed to all concentrations showed decreased susceptibility to Ampicillin activity (Fig. 3). A

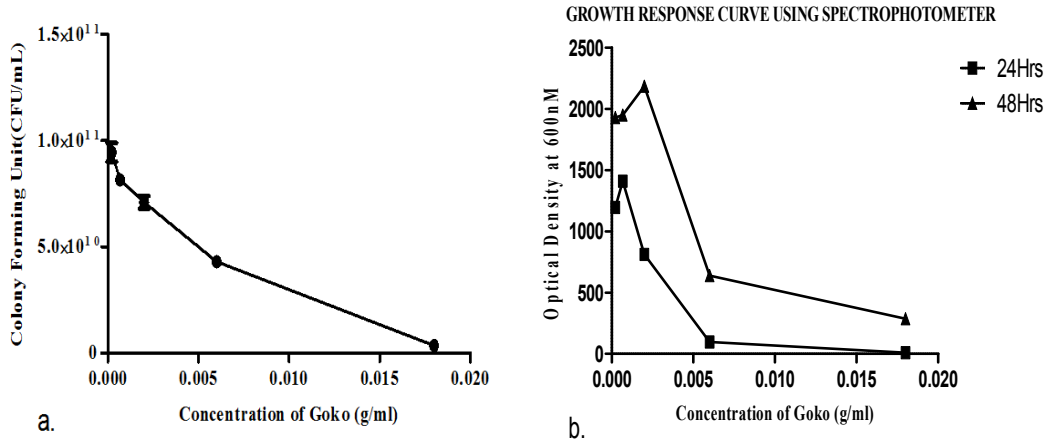

Fig. 2. Growth response of *E. coli* after exposure to GAB

The bacterial growth was measured (a) as colony forming units by enumeration on TSA and (b) by optical density spectrophotometry

striking result was observed at 0.0007 g/ml, which showed no zones of inhibition. The experiment demonstrated that *E. coli* exposed GAB had reduced potency to Ampicillin.

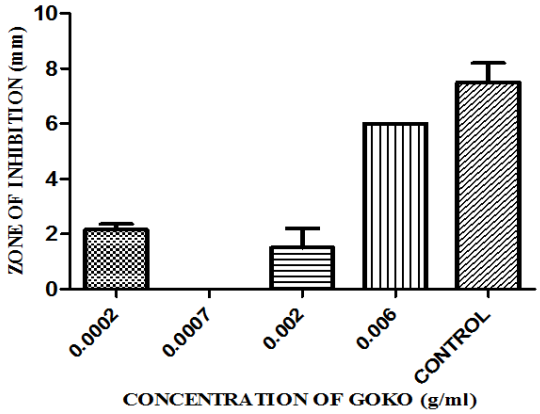

Fig. 3. Effects of modern antibiotics on GAB-treated *E. coli* isolates

Zones of inhibition were measured using a graduated meter rule. Control represents non-GAB treated E. coli. The experiment was carried out in triplicates and repeated on two independent occasions. The P-value between the control and all GAB-treated E. coli was 0.01

This antibacterial resistance study showed that GAB has the ability to induce resistance to opportunistic pathogens. GAB-treated *E. coli* showed reduced sensitivity to Ampicillin compared to non-GAB treated *E. coli*. This reduced sensitivity was significant (*P*-value=0.01) in all GAB-treated *E. coli*. It demonstrated that GAB may induce some level of resistance in *E. coli*.

4. CONCLUSION

The data obtained from this study has shown for the first time that although GAB has some potential antimicrobial properties, it can also confer resistance in *E. coli* to modern antibiotics such as Ampicillin. There was significant reduction in the zones of inhibition of *E. coli* by Ampicillin and complete resistance at a particular concentration. This implies that GAB could induce resistance in *E. coli* to Ampicillin. The use of herbal drugs as alternatives to modern antibiotics is on the increase especially in Nigeria due to the high costs of antibiotics. Drug regulatory agencies should overlook the production and sales through appropriate screening of herbal drugs to check their ability to induce resistance in opportunistic pathogens. People should patronize government recommended drugs as their side effects are usually established and indicated. This novel discovery of resistance induction in normal flora exposed to locally- produced antibacterial preparations has demonstrated a serious untoward effect of herbal drugs previously unknown.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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