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Development of Antibiotic Resistance in Herbal Drug-sensitized *Staphylococcus aureus* Isolate

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Authors' contributions

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

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ABSTRACT

Background: Herbal remedies are locally produced drugs usually made from plant sources. Due to their inexpensive nature, low-income individuals prefer them over modern antibiotics in the treatment of infections.
Aims: The purpose of the current study was to determine the ability of a herbal drug called Goko Alcoholic Bitters (GAB) to induce resistance in opportunistic pathogens such as *Staphylococcus aureus*.
Methods: To evaluate the antimicrobial efficacy of GAB on *S. aureus,* Disc diffusion method was employed to observe the zones of clearance by GAB. Overnight cultures of *S. aureus* grown in Tryptone Soy Broth (TSB) at an optical density of 0.5 were serially diluted to 10^9. Five (5) different concentrations of GAB (0.018 g/ml, 0.006 g/ml, 0.002 g/ml, 0.0007 g/ml and 0.0002 g/ml) were added to the serially diluted culture. The growth responses of these different dilutions were checked against *S. aureus* isolate *in vitro*. The experiment was monitored for 24 and 48 hrs conditions using two different growth determination methods; turbidity and colony forming unit counting methods. The five (5) different conditions were further analyzed by plating 200 µl of

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bacterial broth on Tryptic Soy Agar (TSA) and antibiotic sensitivity discs were embedded onto the agar. 200 µl of control bacterial Tryptone Soy broth containing *S. aureus* not exposed to the herbal drug was also performed alongside for comparison. This was repeated for both 24 and 48 hrs exposure to GAB.

Results: The efficacy of GAB showed some levels of antimicrobial activity, however, were significantly lower compared to Ampicillin (10 µg). The growth response analysis showed gradual reduction but not complete elimination with the highest concentration of GAB. GAB-sensitized *S. aureus* showed resistance seen in the reduced zones of clearance to some Septrin 10 µg (SEP), Ampicillin 10 µg (AMP) and Nalidixic acid 10 µg (NA) which were effective against untreated *S. aureus* (control).

Conclusion: GAB has some levels of antimicrobial efficacy but antibiotic resistance gets induced in the case of surviving GAB-pretreated *S. aureus*.

Keywords: Staphylococcus aureus; herbal drugs; antibiotic resistance; opportunistic pathogens.

1. INTRODUCTION

Infectious diseases are leading cause of mortality in developing countries. An increasing number of infectious diseases are now becoming difficult to treat due to the development of resistance to antibiotics by pathogens. The acquisition of antimicrobial resistance is a natural phenomenon [1] but can also be accelerated by human activities such as agricultural practices and overuse of antibiotics [2]. Antimicrobial resistance hinders the prevention and control of infectious diseases which makes it a serious health problem.

Treatment of infections using herbs is an age-long tradition. In recent years, the use of herbs as drugs has gained huge popularity and is being used in many places across the globe [3]. In some countries such as India, it has been reported that herbs constitute about 70% of the modern drugs produced [4]. The understanding and perception of the general population about herbal medicine action are that they are devoid of side effects. Hence, individuals with a low standard of living especially in developing nations with little access to modern medications frequently use herbal drugs as their first-line of treatment [5,6,7].

In recent times, the Nigerian markets have been flooded with many brands of herbal drugs which are patronized by a lot of consumers. Some of them include Yoyo, Alomo, Goko cleanser, Orijin, Agbo, Action, Washing and Setting, Baby-Oku, Skelewu, Man-Power, Swedish, Goko Alcoholic Bitters (GAB), Ruzu, kola nuts, and bitter kolas. These drugs are sold in different places which include markets, supermarkets, highways, roadsides, and buses / vehicles. The manufacturers of these herbal drugs do not provide scientific evidence of safety and efficacies to the drug enforcement agency and public prior to marketing hence the untoward effects remain unknown.

The herbal preparation investigated in the current study is GAB. The active constituents of GAB from the manufacturer's label are; ethanol, *Calamus rhizome*, *Azadirachta indica,* and Caramel. Ethanol kills bacteria by denaturing their proteins and dissolving the lipid layer. It is used as an antiseptic against bacteria, fungi, and many viruses but usually ineffective against bacteria spores [8]. The *Calamus rhizome* is a tall perennial wetland monocot of the Acoraceae family in the genus *Acorus*. The leaves are used as sedative, laxative, a diuretic with counter side-effects of hallucinogens [9].

The aim of this study was to determine whether clinical isolate of *Staphylococcus aureus* develops resistance to modern drugs when treated with the traditional drug *in vitro*. The hypothesis of the research was that on exposure to GAB, *S. aureus* isolate will develop resistance.

2. MATERIALS AND METHODS

2.1 Collection of Drugs

GAB and Septrin, Ampicillin and Nalidixic acid-impregnated discs (Maxicare Medical Laboratory, Nigeria) were purchased from Mile 3 market, Port Harcourt, Rivers State, Nigeria.

2.2 Isolation and Identification of the Test Organism

Staphylococcus aureus was isolated and identified at the University of Port Harcourt Teaching Hospital (UPTH), Nigeria. The

organism was morphologically and biochemically identified according to Chesbrough [10]. Isolated *S. aureus* was stored on Tryptic Soya Agar (TSA) slant at 4°C for subsequent use.

2.3 Media Preparations

2.3.1 Tryptic soya agar (TSA) and tryptic 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 GAB on *S. aureus*

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 GAB were obtained by serial dilution of GAB 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 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 densities were measured after 24 and 48 hrs of incubation.

2.5 Determination of Growth Response of GAB-sensitized *S. aureus*

Growth response was investigated by spectrophotometry and bacterial count as used in Freestone et al. [11]. For the spectrophotometric method, overnight culture (OD of 0.5 at 600 nm) was serially diluted to 10^2 CFU/ml of the test organism and exposed to various concentrations of GAB using TSB as the diluent. The culture was incubated for 18 hrs at 37°C. The OD of the various culture concentrations was measured at 600 nm. The bacterial count method used the above-described procedure of drug exposure but growth was determined by enumerating bacterial colonies on TSA.

2.6 Drug Impregnation of Discs

Disc of 6 mm of Whatman filter paper No. 540 were made and sterilized in an autoclave. These were placed in the final concentrations (0.018 g/ml, 0.006 g/ml, 0.002 g/ml, 0.0007 g/ml and 0.0002 g/ml) of the herbal preparation. These were allowed to impregnate for 5 minutes. The concentration of the herbal preparation used in this study was determined by drying up 10 ml of GAB herbal solution in a test tube. The weight of the dried preparation was 3.6 g hence; making the neat (initial) concentration of the herbal drug 0.36 g/ml. Different concentrations were obtained by serial dilutions. Commercially purchased antibiotic discs were used as a control.

2.7 Antibiotic Susceptibility of GAB-Treated *Staphylococcus aureus*

Overnight cultures were prepared following the directions as previously described in section 2.5. Using this initial concentration, 2 ml of bacteria aliquots were put in five different sterile universal bottles. Different concentrations of herbal preparations were made by serial dilution in TSB. The various final concentrations of GAB used to treat the bacteria were 0.018 g/ml, 0.006 g/ml, 0.002 g/ml, 0.0007 g/ml and 0.0002 g/ml. A control that was not treated with GAB was also carried out. Both the GAB-treated and untreated conditions were incubated at 37°C and their OD was measured after 24 hrs to determine the response to GAB. After 24 hrs incubation, 200 µl of the culture was spread on the surface of TSA using a glass rod. The Septrin, Ampicillin and Nalidixic acid-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.

2.8 Data Analysis

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

3. RESULTS

3.1 GAB Efficacy

The efficacy of GAB was determined using discs diffusion method to check the sensitivity of *S. aureus*. This experiment showed that GAB possesses some efficacy (Fig. 1). However, this was significantly lower ($P<0.05$) when compared to the control (Ampicillin 10 µg). Concentrations 0.018 g/ml, 0.006 g/ml, 0.002 g/ml, 0.0007 g/ml and 0.0002 g/ml show 5 mm zone of clearance while 0.36 g/ml showed 12 mm and the control drug showed 27 mm. The control showed an

approximately 6-fold increase in the zone of clearance compared to the concentrations with 5 mm zone of clearance and approximately 2-fold increase than the 0.36 g/ml concentration.

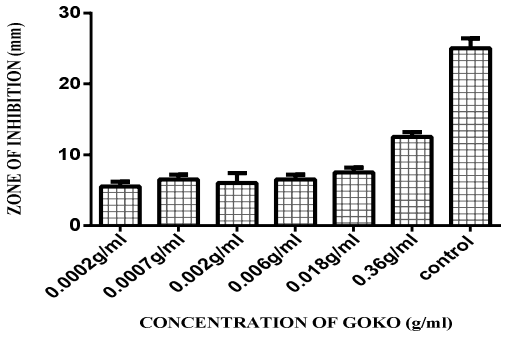

Fig. 1. Efficacy of GAB on *S. aureus*

S. aureus were treated with different concentrations of GAB incubated at 37°C for 24 hrs were spread evenly across TSA plates. Discs impregnated with various concentrations of GAB and control (Ampicillin 10 µg) was incubated with the bacteria overnight

3.2 Growth Response Curve

The growth response experiment performed to monitor the growth of *S. aureus* to GAB is shown in Fig. 2. This was done using bacteria plate count and spectrophotometer. The actual number of viable bacteria cells that are present in the different concentrations of the GAB is shown in Fig. 2a. There is a gradual decrease in the number of viable bacteria Fig. 2a. The growth rate of the bacteria by their optical densities after 24 and 48 hrs incubations is demonstrated in Fig. 2b. As the concentration of the GAB increases, the number of *S. aureus* cells that survived GAB exposure decreased (Fig. 2).

3.3 Effect of Antibiotics on GAB-treated *S. aureus*

The zone of inhibition of GAB-treated *S. aureus* with Septrin-SEP, Ampicillin-AMP, and Nalidixic acid-NA discs are shown in Fig. 3a to 3c. *S. aureus* pre-exposed to Goko drug demonstrated a varying degree of susceptibility to the different commercial antibiotics.

Significantly lower zones of clearance were seen in the bacteria exposed to SEP across all the GAB concentrations (Fig. 3a). AMP also showed a reduction in the zone of inhibition of the bacteria but the 0.006 g/ml showed the same zone of clearance with the untreated control (Fig. 3b). The all other concentrations showed a significant reduction in their zones of inhibition compared to control except 0.002 g/ml. The NA showed a significantly reduced zone of clearance (Fig. 3c).

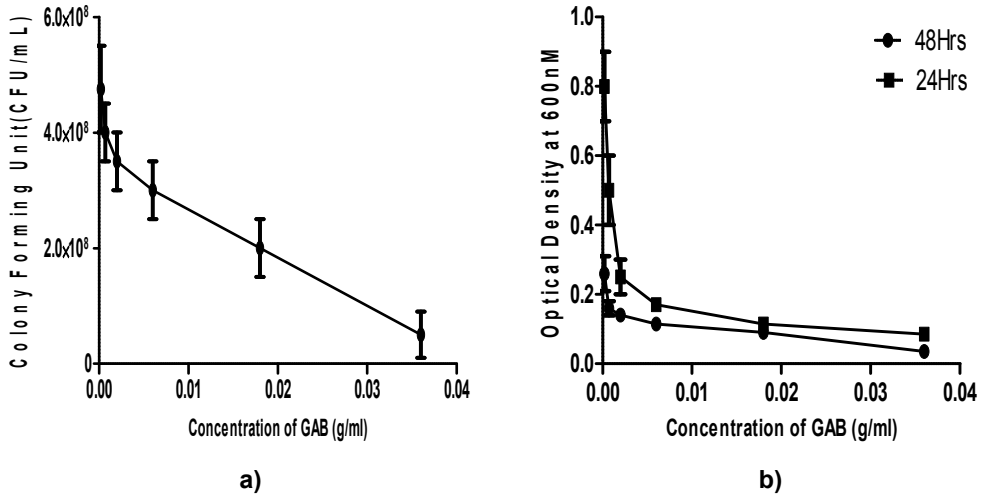

Fig. 2. Growth responses of *S. aureus* on exposure to GAB

Overnight inoculum was diluted to 10^{-9} (approximately $1.4x10^9$ CFU/ml) initial concentration. a) The bacteria growth was enumerated using TSA after 24 hrs. b) This was treated with Goko drug and incubated at 37°C for 24 and 48 hrs using a spectrophotometer. The experiment was performed on two independent occasions

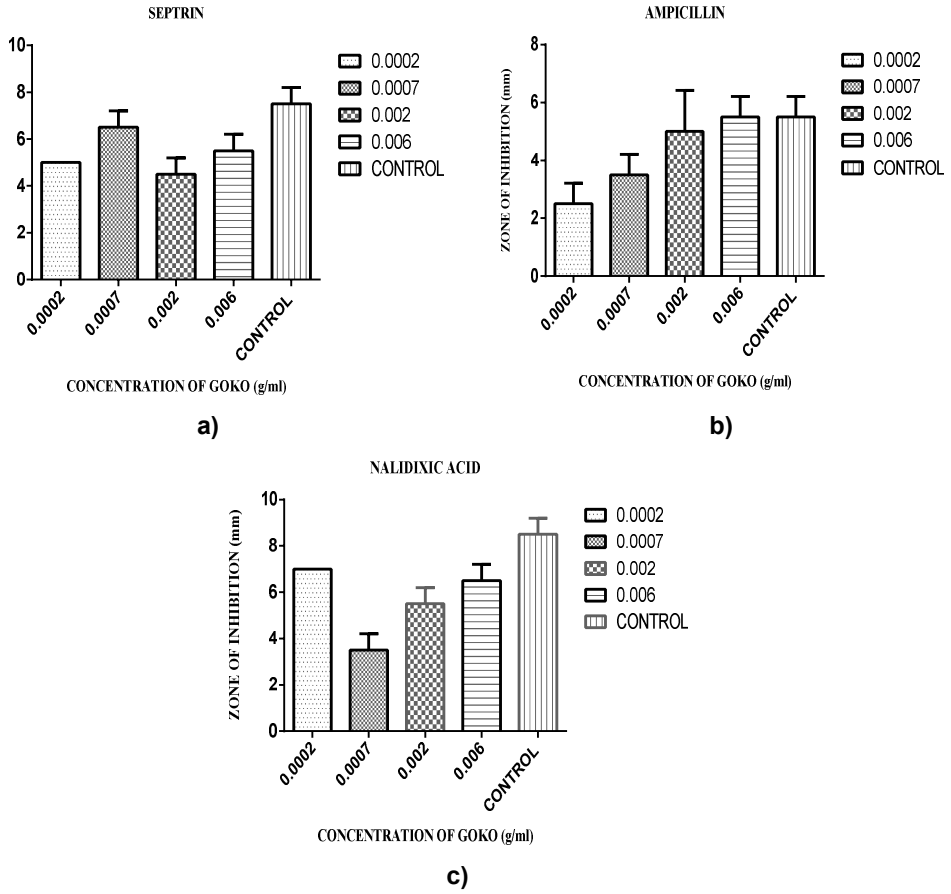

Fig. 3. Effect of commercial antibiotics on Goko treated *S. aureus* isolates

S. aureus isolates treated with various concentrations of GAB for 24 hrs at 37°C were exposed to a) Septrin, b) Ampicillin c) Nalidixic acid. The zones of inhibition were measured and experiment repeated on two independent occasions

4. DISCUSSION

The widespread antibiotic resistance has reduced the significance of antibiotics usage. This study was done to determine the antimicrobial effects of the GAB against *S. aureus* as well as drug resistance that could result from exposure of *S. aureus* to GAB. Antimicrobial effect of ethanol which is an active ingredient in the traditional drug has been shown to kill bacteria [8]. *Calamus rhizome* which is an active ingredient in GAB widely used as traditional herbs and has been found to have antimicrobial and insecticidal activities [12].

In the drug efficacy experiment, there were some levels of the zone of inhibitions in the different concentrations of GAB. Significant differences were observed when compared to the control ($P<0.05$). The control (Ampicillin 10 µg) showed significantly higher level of inhibition of *S. aureus* growth compared to all the concentrations of GAB used. Clinical and Laboratory Standard Institute (CLSI) [13] has shown that Ampicillin (10 µg) is an effective drug for killing *S. aureus* with a zone of clearance >28 mm. The zone of clearance of 27 mm was demonstrated by Ampicillin (10 µg) (Fig. 1). This means the recommended zone of clearance by CLSI is 1 mm higher than observed in our study. Even with this difference, the current study has demonstrated a moderate level of clearance zone which means the control drug is an effective antimicrobial agent that kills *S. aureus*. The difference in the zones of inhibition could arise from strain variation which has been reported by Li et al. [14] that strains difference can lead to a difference in bacteria response to external agents. Therefore since GAB is not

eliminating the bacteria like the control, a further study into its growth pattern was necessary.

In order to study the growth response patterns of *S. aureus* exposed to GAB, two methods of bacterial growth determination, turbidity and viable colony count techniques were used. This is similar to the experiment employed by Sandrini et al. [15] to show *Staphylococcal epidermidis* growth response to catecholamines. The growth response of *S. aureus* exposed to GAB after 24 and 48 hours showed a reduction in the total number of bacteria as shown in their optical densities. As the concentration of the herbal drug reduces the total number of bacteria increases (Fig. 2). This shows there is an inverse relationship between the drug and the bacteria. This is similar to others publications that studied drug efficacy on different microorganisms [13, 14,16]. The determination of viable count after 24 hours exposure also showed similar characteristics seen in the turbidity method described above as the concentrations of the drug was inversely proportional to the number of bacteria. One important observation in this experiment is that there was no complete elimination of bacteria even with the highest concentration of the herbal preparation. This was seen in both the growth experiment with a spectrophotometer and viable bacterial count. The inability of GAB to eliminate *S. aureus* led to further investigation into the possible resistance phenotypic characteristics of the surviving GAB-pretreated *S. aureus* when exposed to modern antibiotics such as SEP, AMP, and NA.

Several antibiotics which cannot treat infections are classified as antimicrobial agents due to results obtained in antimicrobial susceptibility testing. The current study investigated whether *S. aureus* previously exposed to GAB can develop resistance to modern antibiotics using disc diffusion method. The GAB-treated *S. aureus* showed reduced zones of inhibition upon exposure to SEP, AMP, and NA (Fig. 3). Freestone et al. [11] have shown that even antibiotic-damaged *S. aureus* shows the ability to resuscitate when exposed to a drug such as a catecholamine. These observations were similar to our findings that all herbal drug-treated *S. aureus* survived the different concentrations of GAB used in the study. According to Li et al. [14], growing of *Pseudomonas putida* and *Pseudomonas aeruginosa* previously exposed to Ciprofloxacin and Tetracycline showed increase growth and lag phase of the bacteria which depicts resistance. From this study, GAB-pretreated *S. aureus* showed reduced sensitivity to modern antibiotics. This reduced sensitivity could translate to development of resistance. Hence, sub-culturing of these GAB-treated *S. aureus* could lead to increase in growth.

Further investigations are necessary for the aspect of drug resistance studies using kinetic models that study the lag phase of herbal drug pretreated bacteria. Using minimum inhibitory concentration and lag phase time could provide very valuable information on bactericidal and resistance phenotypes of bacteria exposed to herbal drugs.

5. CONCLUSION

GAB commonly used as a source of antimicrobial agents in developing countries & could induce resistance to an opportunistic pathogen such as *S. aureus*. Hence, herbal preparations should be evaluated scientifically and disseminated effectively to reduce cases of resistance that may arise as a result of the drug abuse.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Fleming A. Penicillin. Nobel Lecture; 1945.
2. Vadhana P, Singh BR, Bharadwaj M, Singh SV. Emergence of herbal antimicrobial drug resistance in clinical bacterial isolates. Pharmaceutica Analytica Acta. 2015;6:434.
3. Nasri H. Herbal drugs and new concepts on its use. Journal of Preventive Epidemiology. 2016;1(1):1.
4. Rabe T, Van-Staden J. Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethno-pharmacology. 1997;56:81-87.
5. Jain R, Rao B. Critical analysis of India's National Mission on Medicinal Plants (NMMP) in providing access to quality botanical drugs to improve public health. Journal of Ayurveda and Integrative Medicine. 2015;6:198-207.
6. McEwen BJ. The influence of herbal medicine on platelet function and coagulation: A narrative review. Seminars in Thrombosis and Hemostasis. 2015;41: 300-314.

7. Damnjanovic I, Kitic D, Stefanovic N, Zlatkovic GS, Catic-Djordjevic A, Velickovic RR. Herbal self-medication use in patients with diabetes mellitus type 2. Turkish Journal of Medical Sciences. 2015; 45:964-971.
8. Becker CE. The clinical pharmacology of alcohol. California Journal of Oriental Medicine. 2013;113(3):37-45.
9. Balakumbahan RK, Rajamani, KK. *Acorus calamus*, an overview. Journal of Medicinal Plants Research. 2010;4(25):2740–2745.
10. Cheesbrough M. District laboratory practice in tropical countries, part 2. Cambridge University Press. Madrid, Spain; 2002.
11. Freestone PPE, Haigh RD, Lyte M. Catecholamine inotrope resuscitation of antibiotic-damaged *Staphylococci* and Its blockade by specific receptor antagonists. Journal of Infectious Disease. 2008; 197(7):1044-1052.
12. Kumar SS, Akram AS, Ahmed TSF, Jaabir MSM. Phytochemical analysis and antimicrobial activity of the ethanolic extract of *Acorus calamus* Rhizome. Oriental Journal of Chemistry. 2010;26(1).
13. CLSI. M31-A. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 1999;19.
14. Li B, Qiu Y, Shi H, Yin H. The importance of lag time extension in determining bacterial resistance to antibiotics. Analyst. 2016;141:3059-3067.
15. Sandrini SM, Shergill R, Woodward J, Muralikuttan R, Haigh RD, Lyte M, Freestone PP. Elucidation of the mechanism by which catecholamine stress hormones liberate iron from the innate immune defense proteins transferrin and lactoferrin. Journal of Bacteriology. 2010; 192(2):587-594.
16. Monsi TP, Amala SE, Ugoaru NF. Acquisition of antibiotic resistance in *Escherichia coli* exposed to a locally produced herbal drug. Microbiology Research Journal International. 2017; 22(2):1-7.

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