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A Treatment Option or Source of Bacterial Pathogen Transmission? The Case of Herbal Mixture in Nigeria

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ABSTRACT: Herbal mixture such as agbo, has been used in recent years in Nigeria to treat various sicknesses including malaria, typhoid, dysentery and cholera. However, the bacteriological quality and safety is of paramount importance spurring the argument whether it is treatment option or sources of pathogen transmission. Hence this study was conducted to investigate the bacteriological analysis of agbo herbal preparations. Samples of herbal mixture were purchased from five different markets (Uselu, New Benin, Oba, Santana and Ogida Markets) in Benin City, Edo State, Nigeria. Bacteriological analysis was carried out using pour plate isolation method. Identification of isolated bacteria was based on their cultural, morphological, biochemical and molecular techniques. Antibiotic sensitivity pattern was carried out using disk diffusion method. The plasmid profile of multiple drug resistance bacterial genes isolated was also analyzed. Bacteriological analyses showed that the total bacterial counts (TBC) of all the test herbal samples obtained from the various markets ranged from 0.04 x 10^4 to 1.13 x 10^4cfu/ml. Eight bacterial species were identified and they include; *Bacillus cereus, Bacillus subtilis, Escherichia coli, Lactobacillus casei, Serratia marcescens, Micrococcus varians, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The least occurring bacterial isolates were *Serratia marcescens and Pseudomonas aeruginosa* (5.26%) while the highest occurring was *Bacillus cereus* (21.05%). Isolated bacteria were resistant to commonly used antibiotics. Plasmid profile revealed presence of plasmid genes in the bacterial isolates. Since applications of herbal medicines for curative purposes is on the increase, there is need to monitor and ensure its bacteriological quality before distributing to final consumers.

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The use of herbs as medicine is the oldest form of healthcare known to humanity and has been used in all cultures throughout history (Barnes *et al*., 2007). Early humans recognized their dependence on nature for a healthy life and since that time humanity has depended on the diversity of plant resources for food, clothing, shelter, and medicine to cure myriads of ailments. According to WHO, traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness. It is a holistic approach, that is, processes of the physical body, mind, emotions and spirit working together in determining good health or ill health (Mandel, 2009). The equation of good health or ill health also includes the interaction and relationship between nature, the cosmos and human beings (Mandel, 2009). Medicinal plants may be

associated with a broad variety of microbial contaminants, represented by bacteria, fungi, and viruses. Inevitably, this microbiological background depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations (Kira *et al*., 2021). Risk assessment of the microbial load of medicinal plants has therefore become an important subject in the establishment of modern Hazard Analysis and Critical Control Point (HACCP) schemes.

The commonly used herbal materials include chewing sticks, herbal pastes, powders, herbal mixtures and suspensions. Most of these herbal materials are prepared and sold under unhygienic conditions. A number of oral health care materials are hawked when not packaged and this raises the possibility of contamination. Most of these materials are used directly without further processing (for example chewing sticks) thereby increasing the risk of disease transmission (de Sousa Lima *et al*., 2020). Herbal preparations are used in different forms and may carry a large number of microbes originating from soil usually adhering to various parts of herb.

Therefore, the objective of this study was to screen commercially sold agbo herbal mixture obtain from five different markets (Uselu, New Benin, Oba, Santana and Ogida Market) in Benin City of Edo State, Nigeria for the presence of multidrug resistant bacteria pathogens.

MATERIALS AND METHODS

Collection of Sample: Commercially sold agbo herbal mixture was purchased from five different markets (Uselu, New Benin, Oba, Santana and Ogida Market) in Benin City, Edo state, Nigeria. Two samples were purchased from each market and were immediately transported to the laboratory for bacteriological analysis. The media used were prepared according to the manufacturer's instructions. They were nutrient agar (NA) and Muella Hilton agar.

Isolation and Identification of Microorganisms: One milliliter (1ml) of each sample was measured into sterile test tube containing 9ml of sterilized distilled water. The 10^{-1} suspension was serially diluted using tenfold serial dilution up to 10^{-3}. Aliquot of 1ml of the appropriate dilution was plated in nutrient agar for isolation of bacteria. The inoculated nutrient agar plates were incubated at 37ºC for 24-48 hours. After incubation, the number of discrete colonies was counted in terms of colony forming units. The viable counts were obtained by reference to the serial dilution used. The colonies were collected with a sterile wire loop and were streaked on already solidified medium to obtain pure culture. Each pure culture was then sub-cultured into agar slants in bijou bottles and kept as stock culture. The bacterial isolates were initially identified using their cultural, morphological and biochemical characteristics before molecular identification was carried out.

The identity of the bacterial isolates was confirmed using polymerase chain reaction and gel electrophoresis to get DNA bands which were further sequenced.

Antibiotic susceptibility test was carried out using commercially available antibiotic disk, while Plasmid profile of multi drug resistant bacterial isolates was carried out using PCR method. Plasmid curing was done using ethidium bromide.

RESULTS AND DISCUSSION

Total bacterial counts ranged from 0.04±0.002 x10^4 cfu/ml in agbo samples purchased from Santana market to 1.13±0.7 x10^4 cfu/ml in samples from Uselu market (Table 1). Plate 1 reveals the different bands of DNA of the bacteria in agarose gel. Bacterial isolates recovered from agbo herbal mixture included *Bacillus* cereus, *Escherichia coli, Serratia marcescens, Lactobacillus casei, Bacillus subtilis, Micrococcus varians, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The most occurring bacterial isolate was *Bacillus cereus* with percentage occurrence of 21.05% while the least occurring bacteria were *Serratia marcescens* and *Pseudomonas aeruginosa* (5.26%) (Figure 1). Table 2 shows antibiotics sensitivity pattern of bacterial isolates. *Escherichia coli* and *Pseudomonas aeruginosa* showed resistance to all but two (pefloxacin and ofloxacin) of the antibiotics tested. *Serratia marcescens* was resistant to septrin (SXT), sparfloxacin (SP), ciprofloxacin (CPX), and gentamicin (CN) but was sensitive to augmentin (AU), pefloxacin (PEF) and ofloxacin (OFX). *Bacillus subtilis* was sensitive to almost all antibiotics tested except Ampicillin (APX). *Bacillus cereus* was also sensitive to most antibiotics tested but showed resistance to ampicillin and amoxicillin. Plasmid profile revealed presence of plasmid genes in the bacterial isolates.

Table 1: Total bacterial counts in agbo herbal mixture

Abgo	Mean counts (x10^4 cfu/ml)
	Bacteria
Ogida	0.17±0.01
Uselu	1.13±0.7
New Benin	0.3±0.2
Oba	0.17±0.11
Santana	0.04±0.002

Result of the microbial load in agbo mixture is in consonance with the observation by Agbulu *et al.* (2016) who reported the microbiological quality of cough syrups and herbal solutions in Markudi, Benue State. The result is slightly different from that of Oluyege and Adelabu (2010) who reported a higher bacterial count of 4.0 x 10^4 to 1.7 x 10^6cfu/ml in hawked herbal products in Ado-Ekiti. The difference could be attributed to the fact that the Ado-Ekiti samples were hawked around in the streets, exposing the products to different microorganisms. Also, the source of water and level of hygiene of the producers and vendors could impact on microbial load. This is also true for the differences in microbial counts observed from samples obtained from the different markets in Benin City. Herbal medicines harbour various pathogenic microorganisms. This is because herbs are made from trees and these plants have microorganisms adhered to their stems, barks, leaves, flowers, fruits and roots. Though these microorganisms exist in their natural environment, and are normal flora of the tree, they could be sources of infection, when in contact with human body.

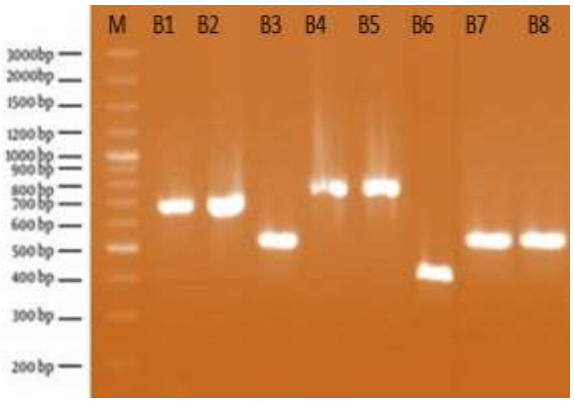

Plate 1: PCR product of 16SrRNA on 1% Agarose Gel
Lane M = molecular size marker, B1=Bacillus cereus, B2=*Escherichia coli*, B3= *Serratia marcescens*, B4=*Lactobacillus casei*, B5=*Bacillus subtilis*, B6=*Micrococcus varians*, B7=*Pseudomonas aeruginosa*, B8= *Staphylococcus aureus*

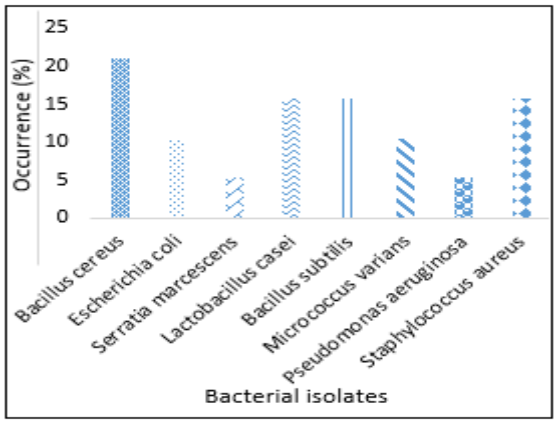

Fig 1: Distribution of bacterial isolates among different samples

The result of bacterial identification is in agreement with reports by Agbulu *et al.* 2016; Abdulahi *et al.* 2015; Oluyege and Adelabu 2010. Interestingly, Yaaba *et al* (2020) bacterial pathogens including *Pseudomonas aeruginosa*, *Micrococcus* sp., *Bacillus* sp., *Enterococcus* and coliform bacteria from liquid herbal medicine. This is in agreement with this study which also reported the presence of these bacterial pathogens in herbal medicines. In this study, *Staphylococcus aureus* was isolated which is a normal commensal of the mammalian skin, hands and mucous membranes. Upon the consideration of the extent of human contact involved in the preparation of herbal medicinal samples, it is most likely that sources of the contaminating *Staphylococcus* spp. are the producers of the Agbo. This suggests that the level of hygiene of persons involved in the preparation of the tested samples may be low. Similar studies carried out on herbal samples include work by Odedera and Memuletiwon (2014); Oluyege *et al.* (2010) and Yaaba et al. (2020) have all reported that the pathogens frequently isolated in herbal products were *S. aureus*, *E. coli* and *Pseudomonas aeruginosa*. Similar finding was reported by Kira *et al*. (2021) who reported *E. coli* and *S. aureus* from herbal drugs. This work varies by reporting a higher count of *Bacillus cereus*. Contamination by *Bacillus cereus* (21.05%) could have arisen during growth of the herbs as the bacterium is commonly found in soils. This finding is in contrast with the report of Odedera and Mumuletiwon (2014) who found more of *Escherichia coli* and *Penicillium notatum* as herbal contaminants in Abeokuta, Nigeria and this could be as a result of differences in the hygiene level of the producers. *Escherichia coli*, a major faecal coliform may have been introduced from the water used during processing of the herbs.

Microbial contamination of agbo herbal mixture as shown in this study, may also be as a result of the plant materials, utensils used during preparation, poor hygiene of the manufacturer or even the packaging vessel after processing. Considering the packaging materials, it is worthy of note that this contributes greatly to microbial contamination as the final stage of the processing is packaging. Most of the packaging cans used are not sterilized and are usually picked up where they are found littered along the road or in public places and barely washed before being used to package finished agbo products. Antibiotic resistance of microorganisms is an area of growing concern because antibiotic resistant strains can be detrimental as they are capable of transferring the resistance gene to non-resistant bacteria (some of which are part of the human microflora).

Table 2: Antibiotic susceptibility pattern of bacterial isolates before curing

G-ve	**SXT**	**CH**	**SP**	**CPX**	**AM**	**AU**	**CN**	**PEF**	**OFX**	**St**
Escherichia coli	R	R	R	R	R	R	R	R	S	R
Serratia marcescens	10.0 (R)	0.0 (R)	20.0 (S)	10.0 (R)	15.0 (I)	25.0 (S)	10.0 (R)	20.0 (S)	10.0 (S)	11.0 (I)
Pseudomonas aeruginosa	0.0 (R)	9.0 (R)	0.0 (R)	10.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	11.0 (I)	15.0 (I)	10.0 (R)
G+ve	**PEF**	**CN**	**APX**	**Z**	**AM**	**Ro**	**CPX**	**St**	**SXT**	**E**
Bacillus subtilis	20.0 (S)	20.0 (S)	10.0 (R)	20.0 (S)	0.0 (R)	0.0 (R)	22.0 (S)	20.0 (S)	20.0 (S)	20.0 (S)
Micrococcus varians	15.0 (I)	18.0 (S)	0.0 (R)	11.0 (I)	0.0 (R)	10.0 (R)	21.0 (S)	14.0 (I)	10.0 (R)	16.0 (I)
Lactobacillus casei	20.0 (S)	20.0 (S)	0.0 (R)	20.0 (S)	0.0 (R)	15.0 (I)	20.0 (S)	20.0 (S)	10.0 (R)	20.0 (S)
Bacillus cereus	20.0 (S)	17.0 (I)	0.0 (R)	29.0 (S)	0.0 (R)	20.0 (S)	20.0 (S)	20.0 (S)	20.0 (S)	20.0 (S)
Staphylococcus aureus	0.0 (R)	10.0 (R)	17.0 (S)	15.0 (I)	10.0 (R)	0.0 (R)	15.0 (I)	0.0 (R)	15.0 (I)	10.0 (S)

Note: SXT = septrin, SP = sparfloxacin, CPX = ciprofloxacin, AM = amoxicillin, AU = augmentin, PEF = pefloxacin, OFX = ofloxacin, S = streptomycin, CN = gentamicin, R = rocephin, Z = zinnacef, E = erythromycin, APX = ampicillin; I= Intermediate R= Resistant S= Sensitive; Resistance (R) = ≤10 mm. Intermediate (I) = 11-17 mm. Sensitivity (S) ≥ 18mm

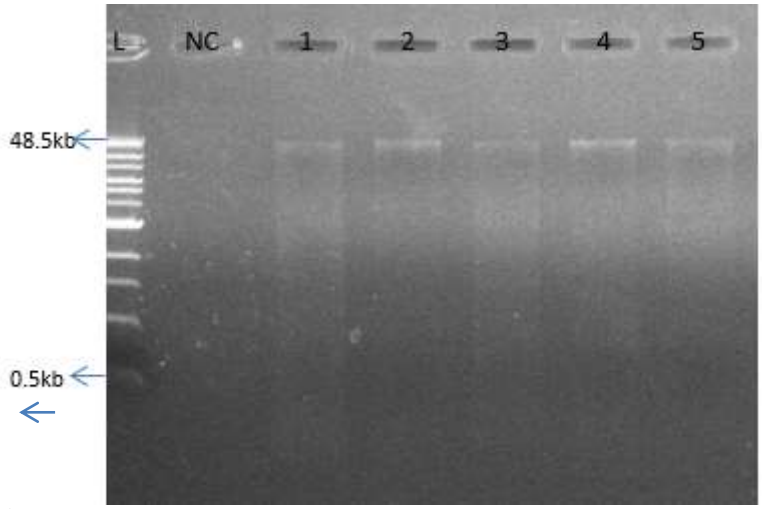

Plate 2: Plasmid profile of multiple drug resistance bacterial isolates analyzed with 0.8% agarose gel electrophoresis, stained with ethidium bromide. L is 0.5kb-48.5kb ladder (molecular marker). Samples 1, 2, 3, 4 and 5 (*E. coli, P. aeruginosa, Micrococcus virians, Staphylococcus aureus* and *Serratia marcescens*) are positive for plasmid genes. NC is a no plasmid DNA template control.

Table 3: Antibiotic susceptibility pattern of bacterial isolates after curing

G-ve	**SXT**	**CH**	**SP**	**CPX**	**AM**	**AU**	**CN**	**PEF**	**OFX**	**St**
Escherichia coli	15.0 (I)	20.0 (S)	12.0 (I)	0.0 (R)	18.0 (S)	0.0 (R)	19.0 (S)	18.0 (S)	20.0 (S)	15.0 (I)
Serratia marcescens	20.0 (S)	15.0 (I)	20.0 (S)	20.0 (S)	20.0 (S)	25.0 (S)	19.0 (S)	20.0 (S)	17.0 (S)	18.0 (S)
Pseudomonas aeruginosa	15.0 (I)	10.0 (R)	15.0 (I)	18.0 (S)	10.0 (R)	13.0 (I)	11.0 (I)	13.0 (I)	15.0 (I)	18.0 (S)
G+ve	**PEF**	**CN**	**APX**	**Z**	**AM**	**Ro**	**CPX**	**St**	**SXT**	**E**
Micrococcus varians	25.0 (S)	20.0 (S)	20.0 (S)	21.0 (S)	18.0 (S)	20.0 (S)	21.0 (S)	22.0 (S)	20.0 (S)	18.0 (S)
Staphylococcus aureus	11.0 (I)	18.0 (S)	17.0 (S)	20.0 (S)	18.0 (S)	10.0 (R)	18.0 (S)	10.0 (R)	17.0 (I)	20.0 (S)

Note: SXT = septrin, SP = sparfloxacin, CPX = ciprofloxacin, AM = amoxicillin, AU = augmentin, 1PEF = pefloxacin, OFX = ofloxacin, St = streptomycin, CN = gentamicin, Ro = rocephin, Z = zinnacef, E = erythromycin, APX = ampicillin; I= Intermediate R= Resistant S= Sensitive: Resistance (R) = ≤10 mm. Intermediate (I) = 11-17 mm. Sensitivity (S) ≥ 18mm

In this study, thirteen (13) selected antibiotics were used to test antibiotic resistant pattern of bacterial isolates. *Escherichia coli* and *Pseudomonas aeruginosa* showed resistance to all but two (perfloxacin and ofloxacin) of the antibiotics tested. *Serratia marcescens* was resistant to septrin (SXT), sparfloxacin (SP), ciprofloxacin (CPX), and gentamicin (CN) but was sensitive to augmentin (AU),

pefloxacin (PEF) and ofloxacin (OFX). This is in agreement with the report of Oluyege and Adelabu (2010).

Bacillus subtilis was sensitive to almost all antibiotics tested. It however was resistant to Ampicillin (APX). *Bacillus cereus* was also sensitive to most antibiotics tested but showed resistance to ampicillin and amoxicillin. It is noteworthy that all Gram positive bacteria tested (except *Staphylococcus aureus*) were resistant to ampicillin, and amoxicillin (except *Bacillus subtilis*). This could be attributed to the widespread use of these drugs. People purchase these drugs over the counter without recourse to the doctors' prescription. In fact, they are now regular drugs in most households. The ability of some of the antibiotics applied in the sensitivity tests to resist the growth of opportunistic pathogens such as *B. cereus* and *E. coli* indicate the potency of these orthodox medicine against such bacteria and might be resorted to by herbal consumers in case of probable infections (Odedera and Memuletiwon, 2014). The plasmid profile of multiple drug resistance bacterial genes isolated was also analysed. The electropherogram was positive for the respective plasmid genes of the isolates showing the contributions of the plasmid genes to drug resistance.

Conclusion: The results of this study show contamination of Agbo by multidrug resistant pathogenic bacteria. Since applications of herbal medicines for curative purposes is on the increase, there is a need for assessing the bacteriological quality of the medicinal plants especially at critical control points during processing and during final packaging before distribution to final consumers. Nigerian government also need to introduce some standards that must be met by every herbal processor and seller and create opportunities for these processors to be trained.

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