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Assessment of microbial quality and detection of extended spectrum β-lactamase genes in Gram-negative bacterial isolates of herbal mixtures commonly hawked in Sagamu metropolis, Ogun State, Nigeria

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Abstract:

Background: The use of herbal mixtures in the treatment of diseases is on the rise. Many of these herbal drugs are not produced under hygienic conditions and safety issues associated with herbal medicines may have an exacerbated impact in immunocompromised and elderly individuals. This study aimed to determine the microbial loads of locally prepared herbal mixtures and detect extended spectrum beta-lactamase (ESBL) genes in any isolated Gram-negative bacteria pathogen.
Methodology: Fifty local herbal mixtures were purchased randomly from three locations in Sagamu town (Sagamu market, Ita-Oba Road and Isale Oko) in Ogun State, Nigeria. The mean total viable bacterial (MTVB), mean total coliform (MTC), and mean total fungal (MTF) counts were determined by the plate count method. The bacterial isolates were streaked on differential bacteriological media while the fungi isolates were grown on potato dextrose agar. The isolates were identified upon growth on culture media using conventional biochemical tests. Antibiotic susceptibility pattern of the isolates was determined using Kirby-Bauer disk diffusion technique. Phenotypic detection of ESBL was done by the modified double disc synergy test followed by amplification detection of bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes with polymerase chain reaction (PCR) assay.
Results: Bacteria and fungi were isolated from 38 (76.0%) and 25 (50.0%) of the herbal samples respectively. Ten (20.0%) and 14 (28.0%) of the samples had mean bacterial and fungal load that exceeded 10^5CFU/mL or g, respectively. Nineteen (38.0%) of the herbal samples analyzed had total coliforms. Fifty-one isolates belonging to eight bacterial genera and 28 fungi isolates belonging to four fungal genera were obtained. Thirty-two (62.7%) of the bacterial isolates were Gram-negative while 19 (37.3%) isolates were Gram-positive. *Staphylococcus aureus* was the most common bacterial isolate (33.3%) while *Aspergillus* species was the most prevalent fungus (60.7%). Sixteen (84.2%) *S. aureus* and 26 (81.3%) Gram-negative isolates were multidrug resistant, and 6 (18.8%) of 32 Gram-negative isolates were ESBL producers. ESBL-encoding genes were detected in 7 (27%) of the 26 multidrug resistant Gram-negative bacteria with TEM and SHV being the most prevalent 4 (14.8%) while CTX-M was identified in only one isolate.
Conclusion: This study reported the presence of microbial contaminants which exceeded the safety limits of 10^5 CFU/g according to World Health Organization. The use of locally prepared herbal medicines poses a major health risk due to the lack of microbial quality standards.

Keywords: Herbal medicines, Gram-negative bacteria, extended spectrum beta-lactamase, microbial contaminant

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Évaluation de la qualité microbienne et détection de gènes de β-lactamase à spectre étendu dans des isolats bactériens à Gram

négatif de mélanges d'herbes couramment vendus dans la métropole de Sagamu, dans l'État d'Ogun, au Nigeria

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Résumé:

Contexte: L'utilisation de mélanges à base de plantes dans le traitement des maladies est en augmentation. Bon nombre de ces médicaments à base de plantes ne sont pas produits dans des conditions d'hygiène et les problèmes de sécurité associés aux médicaments à base de plantes peuvent avoir un impact exacerbé chez les personnes immunodéprimées et les personnes âgées. Cette étude visait à déterminer les charges microbiennes de mélanges à base de plantes préparés localement et à détecter les gènes de bêta-lactamase à spectre étendu (BLSE) dans n'importe quel agent pathogène bactérien à Gram négatif isolé.
Méthodologie: Cinquante mélanges d'herbes locales ont été achetés au hasard dans trois endroits de la ville de Sagamu (marché de Sagamu, Ita-Oba Road et Isale Oko) dans l'État d'Ogun, au Nigeria. Les nombres moyens de bactéries viables totales (MTVB), de coliformes totaux moyens (MTC) et de champignons totaux moyens (MTF) ont été déterminés par la méthode de comptage sur plaque. Les isolats bactériens ont été striés sur des milieux bactériologiques différentiels tandis que les isolats de champignons ont été cultivés sur gélose au dextrose de pomme de terre. Les isolats ont été identifiés lors de la croissance sur des milieux de culture à l'aide de tests biochimiques conventionnels. Le profil de sensibilité aux antibiotiques des isolats a été déterminé à l'aide de la technique de diffusion sur disque de Kirby-Bauer. La détection phénotypique des BLSE a été effectuée par le test de synergie à double disque modifié suivi de la détection par amplification des gènes bla_{TEM}, bla_{CTX-M} et bla_{SHV} avec un test de réaction en chaîne par polymérase (PCR).
Résultats: Des bactéries et des champignons ont été isolés de 38 (76,0%) et 25 (50,0%) des échantillons d'herbes, respectivement. Dix (20,0%) et 14 (28,0%) des échantillons avaient une charge bactérienne et fongique moyenne supérieure à 10^5 UFC/ml ou g, respectivement. Dix-neuf (38,0%) des échantillons de plantes analysés contenaient des coliformes totaux. Cinquante et un isolats appartenant à huit genres bactériens et 28 isolats de champignons appartenant à quatre genres fongiques ont été obtenus. Trente-deux (62,7%) des isolats bactériens étaient Gram-négatifs tandis que 19 (37,3%) isolats étaient Gram-positifs. *Staphylococcus aureus* était l'isolat bactérien le plus courant (33,3%) tandis que l'espèce *Aspergillus* était le champignon le plus répandu (60,7%). Seize (84,2%) isolats de *S. aureus* et 26 (81,3%) isolats à Gram négatif étaient multirésistants, et 6 (18,8%) des 32 isolats à Gram négatif étaient producteurs de BLSE. Des gènes codant pour ESBL ont été détectés dans 7 (27,0%) des 26 bactéries Gram-négatives multirésistantes, TEM et SHV étant les 4 les plus répandues (14,8%) tandis que CTX-M n'a été identifié que dans un seul isolat.
Conclusion: Cette étude a rapporté la présence de contaminants microbiens qui dépassaient les limites de sécurité de 10^5 UFC/g selon l'Organisation mondiale de la santé. L'utilisation de médicaments à base de plantes préparés localement pose un risque majeur pour la santé en raison de l'absence de normes de qualité microbienne.

Mots-clés: Médicaments à base de plantes, bactéries Gram-négatives, bêta-lactamase à spectre étendu, contaminant microbien

Introduction:

Herbal medicine is becoming more popular around the world because of the easy availability of raw materials and low cost compared to synthetic industrial preparations (1,2). Herbal drugs are used as a primary mode of treatment by up to 80% of the population in Africa and are still used to treat 70–80% of the population in many industrialized economies (3,4). Herbal materials may contain microbial contaminants due to their origin. Microorganisms of different types are able to adhere to the leaves, stems, floral, seeds, and root systems from which herbal medicine can be prepared, and potential pathogens may be introduced during harvesting and processing (5,6). Consequently, the safety of medicinal herbs has become a serious public health problem (7), as consumers may become ill as a result of ingesting herbs contaminated with pathogenic microorganisms.

Microbial contamination of traditional medicinal herbs has long been recognized as a source of infections, which can result in gastroenteritis, sepsis, blindness, and even death (6, 8). The microbiological limit determination of herbal preparations is therefore required to ensure that the final product is free of health-related risks (9). Herbal medicines in Africa harbor bacterial contaminants which are highly resistant to antibiotics (10). Several studies on

medicinal herbs have revealed the presence of pathogenic microbial strains that are resistant to multiple antibiotics which can be transferred to consumers if present in medicinal herbs (6, 11).

The extensive use of herbal preparations or medicines necessitates the assurance of long-term availability of high-quality, safe preparations of these herbs, particularly for rural or low-income populations, without jeopardizing patients' health (7). A higher standard of hygienic practices during production is required to minimize contamination (9,12). However, most African countries are finding it difficult to implement the World Health Organization (WHO) safety policies regarding herbal medicines since herbalists continue to disregard safety concerns about herbal drug preparations (12,13). The majority of herbal producers in Nigeria lack the necessary skills to perform quality control and resolve safety concerns with the products they produce (9).

Despite the growing number of herbal products in the market in Nigeria, there is still scarcity of information on microbiological quality of herbal drugs in some regions and the genetic basis of resistance in these bacteria (9,10). Therefore, the goal of this study was to determine the level of microbiological contamination and the prevalence of extended spectrum beta-lactamase genes in Gram negative bacterial contaminants of herbal preparations sold in Sagamu, Ogun state, Nigeria.

Materials and method:

Study site, design and sampling process

This cross-sectional study of herb producers was conducted in Sagamu town, Ogun State, Nigeria, between August and September 2021. A total of 50 oral and local preparations (38 liquid and 12 solid indigenous herbal mixtures) were purchased at random from 15 voluntarily consented herb sellers at three locations (Sagamu market, Ita Oba Road, Isale Oko). About 10ml of liquid and 5g of solid herbal medicines were collected into sterile screw-capped bottles and transported to the Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu within one hour for processing.

Inclusion and exclusion criteria

The herb preparations included in the study were powder and liquid preparations meant to be taken orally and topically without further processing. Herbal medicinal products (in liquid and powder forms) that had undergone additional processing or were administered through other routes were excluded.

Informed consent

Each study participant gave informed consent. All of the information obtained during the study was kept private and confidentially.

Determination of bacterial and fungi load

The determination of bacteria and fungi counts was done using the pour plate method. Ten-fold serial dilution of the samples was performed by placing 1 ml of liquid samples (or 1 g of powdered samples) into 9 ml of physiological saline and allowed to soak for 1 hour. From the suspension, 1 ml was transferred to another tube containing 9 ml of physiological saline and thoroughly mixed. This dilution procedure was further repeated so that there were series of five tubes, giving serial dilutions of 10^{-1} to 10^{-5}. An aliquot of 0.5 ml was pipetted from 10^{-5} dilution for each sample into the sterile Petri dishes. Thereafter, 20 ml of molten Nutrient agar, MacConkey agar and Potato Dextrose agar (PDA) was introduced into each of the Petri dish plates and swirled. The plates were allowed to solidify and then incubated at 37°C for 24 hours for bacteriological analyses and for 5 days for fungal analysis. The study experiment was performed in duplicate.

After incubation, visible colonies on duplicate agar plates were enumerated and recorded as mean colony forming units/ml (CFU/ml). The mean total colony counts were calculated using the formula; Mean total colony counts (CFU/ml) = Mean number of colony formed (x dilution factor) / Volume plated.

Identification of bacterial isolates

A discrete colony of the bacteria to be identified was collected from cultured plates using sterile wire loop and sub-cultured on Mannitol salt agar, MacConkey agar, Eosin Methylene Blue agar and Salmonella-Shigella agar. The plates were incubated aerobically at 37°C for 24 hours. After incubation, the isolates were transferred to Nutrient agar slants for further test. The conventional biochemical tests used to identify different bacterial species were sugar fermentation on triple sugar iron (TSI) agar, hydrogen sulphide production as well as citrate, methyl red, Voges-Proskauer, oxidase, indole and urease tests for Gram-negative, and catalase and coagulase tests for Gram-positive bacteria (14).

Identification of fungal isolates

The fungal morphology was studied macroscopically by observing the colony features such as size, shape, color and hyphae. Microscopic examination of fungi was done by mounting a small portion of the colonies from

culture plate on a slide and staining with Lactophenol-in-cotton blue. The slides were observed under the compound light microscope for characteristic conidia, conidiophores and arrangement of spores (15,16).

Antimicrobial susceptibility testing of isolates

The antibiotic susceptibility patterns of the bacterial isolates were determined using Kirby-Bauer disk diffusion technique (17). Standard conventional antibiotic discs such as levofloxacin (5µg), imipenem (10µg), azithromycin (15µg), gentamicin (10µg), cefuroxime (30µg), carbenicillin (100µg), cefotaxime (30 µg), amoxicillin/clavulanic acid (30µg), cefepime (30µg), cephalexin (30µg) and ceftazidime (30µg) were used. All the isolates were first sub-cultured on nutrient agar and incubated overnight. Then, 3-4 colonies of each isolate were picked and suspended in sterile distilled water, to give turbidity equivalent to 0.5 McFarland standards. The isolate suspension was inoculated on sterile Mueller-Hinton agar plates using a sterile swab stick. Antibiotic discs were placed on the surface of the inoculated agar plates and gently pressed down onto the agar with the aid of a sterile pair of forceps to ensure complete contact with the agar surface.

The plates were left on the bench for 30 minutes to give time for the antibiotics to diffuse into the agar, and then incubated aerobically at 37°C for 24 hours. The diameters of zone of growth inhibition were measured in millimeters, reported and interpreted as sensitive, intermediate or resistant using the Clinical and Laboratory Standards Institute performance standards for antimicrobial susceptibility testing (17).

Phenotypic detection of ESBL

ESBL detection was done by using the modified double-disc synergy test (MDDST). The bacterial colonies obtained were sub-cultured into Nutrient broth, and following incubation for 24 hours at 37°C, the broth cultures were further streaked on Nutrient agar and incubated overnight, 3-4 colonies of each isolate were picked and suspended in sterile distilled water and serially diluted to give turbidity equivalent to McFarland standards ($1x10^8$ cells/ml). The isolate suspension was inoculated on Mueller-Hinton agar plates using sterile swab stick. Amoxicillin-clavulanic acid (30µg) disc was placed at center of the plate, and discs containing cefotaxime (30µg), cefepime (30µg), ceftazidime (30µg**)** and cefixime (30µg**)** were placed 2 cm (center to center) from amoxicillin-clavulanic acid disc using a pair of sterile forceps. The plates were then incubated at 37°C for 24 hours. A clear extension of the edge of the zone of growth inhibition of the cephalosporin discs towards amoxicillin-clavulanic acid disc was interpreted as positive for ESβL production (17).

Extraction of DNA from Gram-negative isolates

The bacterial chromosomal DNA was extracted by a boiling method (18). Briefly, 18-24 hours culture from tryptic soy agar (TSA) was inoculated in 2 ml Luria Bertani broth (LB) and incubated for 18-24 hours. The LB broth was centrifuged (10 000 rpm/min for 10 min) and bacterial cells were suspended in 500 µl of phosphate buffer (100 mM, pH 7) to weaken the membranes and immersed in a boiling water-bath at 100°C for 15 min to release the genetic materials. The DNA was precipitated with 250 µl of absolute alcohol, washed twice in 1000 µl of 70% alcohol, and re-suspended in 100 µl of sterile water (18).

ESBL genes detection in Gram-negative isolates

The ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$) were detected by PCR in a thermal cycler (Applied Biosystems, USA). The sequences of the different primers are presented in Table 1. The PCR mix consisted of 1 µl of DNA, 12.5 µl of WizPure™ PCR 2x Master (Wizbiosolutions, South Korea), 1 µl of each primer (0.2 pmol/µl) (Inqaba Biotechnical Industries, South Africa) and molecular grade water to make 25 µl final volume. Two PCR types were performed; a duplex PCR for *bla*$_{TEM}$ and *bla*$_{SHV}$, and a simplex PCR for *bla*$_{CTX-M}$ (19,20). The amplification condition for *bla*$_{TEM}$ and *bla*$_{SHV}$ consists of initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 40s, annealing at 50°C for 40s, and elongation 72°C for 1 min, with a final elongation step at 72°C for 7 min. The amplification condition for *bla*$_{CTX-M}$ consists of initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 40s, annealing at 60°C for 40s and elongation 72°C for 1 min with a final elongation step at 72°C for 7 min.

Table 1: Primer sequences for amplification of ESBL genes

ESBL genes	Sequence (5'-3')	Amplicon size (bp)	References
TEM	F-GAGTATTCAACATTTTCGT R-ACCAATGCTTAATCAGTGA	857	Maynard et al., (20)
SHV	F-TCGCCTGTGTATTATCTCCC R-CGCAGATAAATCACCACAATG	768	Maynard et al., (20)
CTX-M	F-TTTGCGATGTGCAGTACCAGT AA R-CGATACGTTGGTGGTGCCATA	544	Edelstein et al., (19)

Separation of the amplicons in an electrophoretic tank with migration at 100 volts for 1 hour was performed on 2% agarose gel stained with ethidium bromide (0.5µg/ml) to visualize the PCR products. A 100bp DNA marker was used as reference to determine the molecular size of each amplicon. After migration, the various bands were observed under UV transillumination and photographed using a gel documentation and analysis system (Geno-Sens 1560).

Statistical analysis:

The quantitative data were analyzed statistically using SPSS (version 20) statistical software. Variables were described using standard descriptive statistics such as means, frequencies and percentages.

Results:

Microbial load of herbal mixtures

Twenty-three (46.0%) of the 50 herbal samples were alcohol based, 15 (30.0%) were water based, while 12 (24.0%) were in powdered form. Of the 50 samples, 43 (86.0%) had bacterial and fungal growth while 7 (14.0%) had no bacterial or fungi growth. The microbial loads of the herbal mixtures are presented in Table 2. Bacterial and fungal growths were observed in 38 (76.0%) and 25 (50.0%) of the herbal samples respectively. Ten (20.0%) of the samples had bacterial load that exceeded the safety limit ($\leq 10^5$ CFU/ml or g) set by the World Health Organization (WHO), while 14 (28.0%) samples exceeded the safety limits for fungal growth. Of the herbal samples analyzed, 19 (38.0%) were positive for total coliforms.

The highest total mean bacterial load was $2.6x10^7$ CFU/ml (sample O) while the least total mean bacterial load was $1.0x10^4$ CFU/ml (sample TA). The highest total coliform count and total fungal count were $9.0x10^6$ CFU/g and $4.4x10^7$ CFU/g respectively (sample F), while the least total coliform count was 1.3×10^4 CFU/ml (sample AA) and the least total fungal load was 2.0×10^4 CFU/ml (sample DA).

Bacterial isolates

Fifty-one bacterial isolates were obtained from the 38 herbal samples consisting of seven bacterial genera. Thirty-two (62.7%) of the isolates were Gram-negative while 19 (37. 3%) were Gram-positive. *Staphylococcus aureus* was the most frequent bacterial pathogen 17 (33.3%), followed by *Pseudomonas aeruginosa* 9 (17.6%), and *Acinetobacter baumannii* was the least frequent species (Table 3).

Fungal isolates

Twenty-eight fungal isolates belonging to four genera (*Aspergillus*, *Rhizopus*, *Penicillium* and *Alternaria*) were identified in 23 herbal mixtures. *Aspergillus* species was the most frequently isolated species (60.7%), followed by *Rhizopus* species (25%) (Table 4).

Antibiotic resistance profile of bacterial isolates from herbal mixtures

Fig 1 shows the antibiotic resistance profile of bacterial isolates. *Acinetobacter* species was the most resistant isolate with 100% resistance to cefuroxime, cefotaxime, cefepime, cephalexin and ceftazidime, followed by *Shigella* species with 100% resistance to cefuroxime, cefotaxime, cephalexin and ceftazidime.

Antibiotic resistance pattern of multi-drug resistant (MDR) isolates

Sixteen (94.1%) of the 17 *S. aureus* were multidrug resistant (resistant to three or more classes of antibiotics) while 26 (81.3%) of the 32 Gram-negative isolates were multidrug resistant. All *Escherichia coli* (n=6, 100%) and *Klebsiella oxytoca* (n=6, 100%) isolates were multidrug resistant, while 7 (77.7%), 6 (85.7%), 4 (80%) and (50%) of *K. pneumoniae*, *P. aeruginosa*, *Salmonella* and *Shigella* species respectively were MDR (Table 5).

Table 2: Bacterial and fungal load of herbal mixtures

S/N	Sample	Mean total viable bacterial count (CFU/ml or g)	Mean total coliform count (CFU/ml or g)	Mean total fungal count (CFU/ml or g)
1	A	2.4×10^5	-	1.4×10^6
2	B	3.0×10^5	-	4.0×10^5
3	C	1.0×10^5	2.0×10^5	1.0×10^5
4	D	3.3×10^6	-	-
5	E	-	1.2×10^6	1.4×10^7
6	F	1.52×10^7	9.0×10^6	4.4×10^7
7	G	1.24×10^7	8.0×10^5	1.2×10^6
8	H	-	2.0×10^5	1.6×10^6
9	I	1.6×10^7	4.0×10^5	1.22×10^7
10	J	6.0×10^5	2.0×10^5	2.0×10^7
11	K	1.4×10^5	1.0×10^6	1.2×10^6
12	L	8.0×10^5	-	6.0×10^5
13	M	2.0×10^5	-	4.0×10^5
14	N	8.0×10^5	-	1.48×10^7
15	O	2.6×10^7	2.2×10^6	3.32×10^7
16	P	-	-	2.0×10^7
17	Q	-	-	1.76×10^7
18	R	-	-	2.4×10^6
19	S	3.6×10^5	-	-
20	T	1.7×10^5	-	-
21	U	4.4×10^5	1.8×10^5	-
22	V	2.7×10^5	-	-
23	W	2.3×10^5	-	-
24	X	5.2×10^5	-	-
25	Y	-	-	2.0×10^4
26	AA	3.9×10^5	1.3×10^4	-
27	BA	-	-	-
28	CA	2.6×10^5	-	-
29	DA	1.6×10^5	-	2.0×10^4
30	EA	1.4×10^5	1.6×10^5	7.0×10^4
31	FA	1.3×10^4	-	-
32	GA	-	-	-
33	HA	9.0×10^4	-	-
34	IA	2.1×10^5	1.7×10^5	-
35	JA	1.5×10^6	1.1×10^6	-
36	KA	2.4×10^5	1.3×10^5	1.5×10^7
37	LA	-	-	-
38	MA	2.0×10^4	-	-
39	NA	1.1×10^5	0.9×10^5	-
40	OA	1.7×10^5	0.4×10^5	-
41	PA	1.2×10^7	-	1.2×10^5
42	QA	1.8×10^6	-	6.0×10^4
43	RA	1.0×10^5	-	-
44	SA	-	-	-
45	TA	1.0×10^4	-	1.4×10^5
46	UA	-	-	-
47	VA	-	-	2.7×10^5
48	WA	3.2×10^7	2.8×10^6	-
49	XA	2.2×10^4	-	-
50	YA	1.9×10^6	2.5×10^4	-

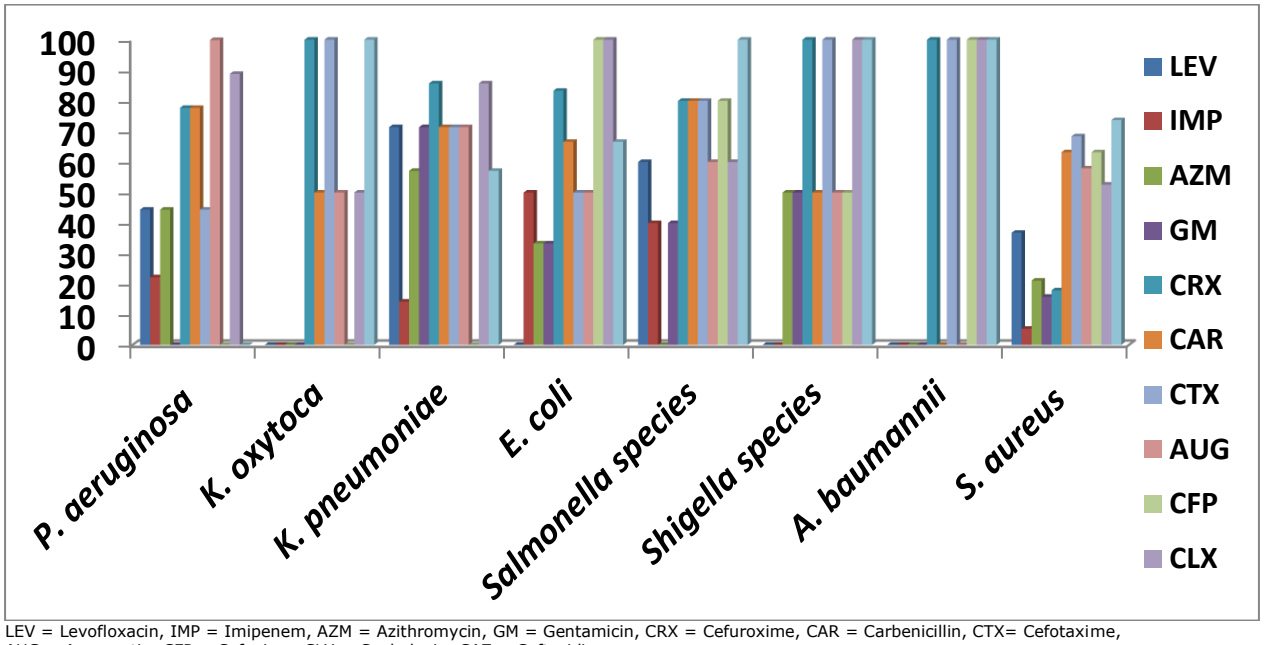
CFU/ml or g = Colony forming unit per ml or gram; - = No Growth

Table 3: Bacteria isolates recovered from the herbal mixtures

S/N	Isolated bacterial species	No of isolated bacteria	Percentage of isolated bacteria
1	*Pseudomonas aeruginosa*	9	17.6
2	*Klebsiella oxytoca*	2	3.9
3	*Klebsiella pneumoniae*	7	13.7
4	*Escherichia coli*	6	11.7
5	*Salmonella* species	5	9.8
6	*Shigella* species	2	3.9
7	*Acinetobacter baumannii*	1	1.9
8	*Staphylococcus aureus*	17	33.3
9	*Staphylococcus saprophyticus*	2	3.9
	Total	**51**	**100**

Table 4: Fungi isolates recovered from the herbal mixtures

S/N	Isolated fungal species	No of isolated fungi	Percentage of isolated fungi
1	*Aspergillus* species	17	60.7
2	*Rhizopus* species	7	25.0
3	*Penicillium* species	3	10.7
4	*Alternaria* species	1	3.6
	Total	**28**	**100**



LEV = Levofloxacin, IMP = Imipenem, AZM = Azithromycin, GM = Gentamicin, CRX = Cefuroxime, CAR = Carbenicillin, CTX= Cefotaxime, AUG = Augmentin, CFP = Cefepime, CLX = Cephalexin, CAZ = Ceftazidime

Fig 1: Percentage antibiotic resistance profile of isolates from herbal mixture

Prevalence of ESBL in Gram-negative bacterial isolates

Six (18.8%) of the 32 Gram-negative isolates were positive for ESBL production by the phenotypic detection method. The isolates were *Pseudomonas aeruginosa* (n=1), *A. baumannii* (n=1), *Salmonella* species (n=1), *K. pneumoniae* (n=1), and *E. coli* (n=2) as shown in Table 6. Figs 2 and 3 showed PCR amplification of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. ESBL-encoding genes were detected in 7 (27.0%) of the 32 multidrug resistant Gram-negative bacteria, with bla_{TEM} and bla_{SHV} being the most frequent 4 (14.8%), while bla_{CTX-M} was identified in only one isolate. The bla_{TEM} gene was detected in *P. aeruginosa*, *K. pneumoniae* and *E. coli*, bla_{SHV} was detected in *P. aeruginosa, A. baumannii*, *Salmonella* species

and *E. coli*, while *bla*$_{CTX-M}$ was detected only in *K. pneumoniae.* Co-occurrence of *bla*$_{TEM}$ and *bla*$_{SHV}$ was detected in one *E. coli* isolate while co-occurrence of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ was detected in one *K. pneumoniae.* All the 6 isolates positive for phenotypic ESBL production carried ESBL-encoding genes while one isolate that was negative for phenotypic ESBL production carried *bla*$_{SHV}$ gene as shown in Table 6.

Table 5: Antibiotic resistance profile of multidrug resistant bacterial isolates

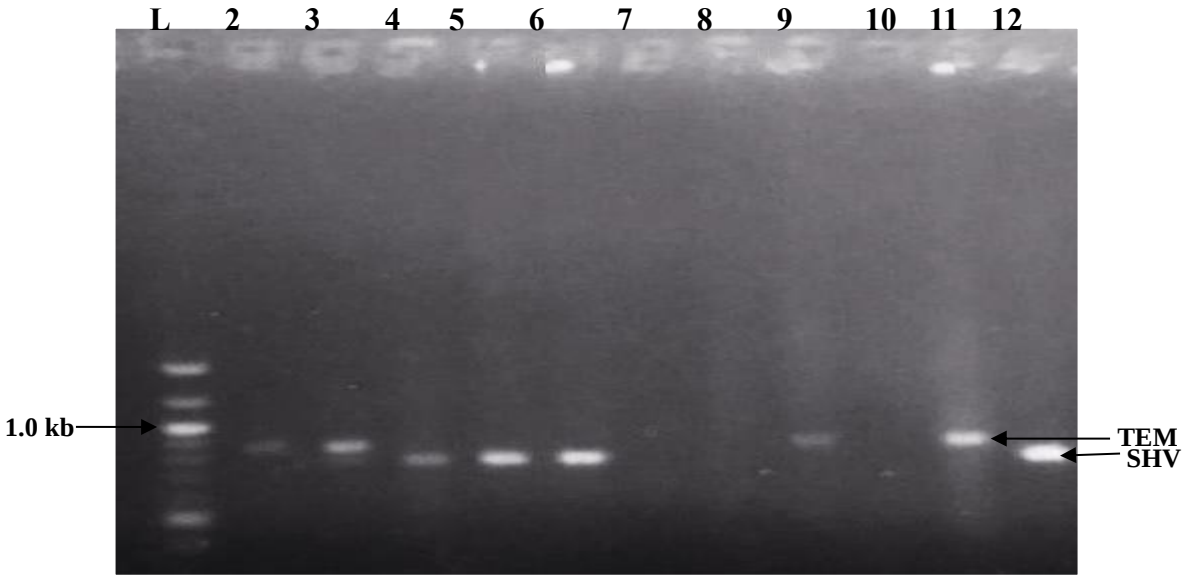
Isolate code	Isolates	Resistant profile
A1	*Staphylococcus aureus*	CRX, AUG, CLX
D1	*Staphylococcus aureus*	LEV, AZM GM, CRX, CAR, CTX, CAZ
F1	*Staphylococcus aureus*	IMP, CRX, CAR, AUG, CLX, CAZ
G1	*Staphylococcus aureus*	LEV, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
H1	*Staphylococcus aureus*	AZM, CRX, CTX, CLX, CFP, CAZ
S	*Staphylococcus aureus*	LEV, AZM, CRX, CTX, AUG, CFP, CLX, CAZ
T	*Staphylococcus aureus*	LEV, CRX, CTX, AUG, CFP, CLX
X	*Staphylococcus aureus*	LEV, CRX, CAR, CTX, CFP, CLX, CAZ
CA2	*Staphylococcus aureus*	CAR, AUG, CLX
FA	*Staphylococcus aureus*	LEV, CRX, CAR, CFP, CAZ
XA	*Staphylococcus aureus*	CRX, CAR, CTX, AUG, CFP, CLX, CAZ
K2	*Staphylococcus aureus*	CRX, CAR, CTX, AUG, CFP, CAZ
M1	*Staphylococcus aureus*	AZM, GM, CRX, CAR, CTX, AUG, CLX, CFP, CAZ
MA	*Staphylococcus aureus*	CRX, CAR, CTX, AUG, CLX
QA	*Staphylococcus aureus*	CRX, CAR, CTX, AUG, CFP
TA	*Staphylococcus aureus*	LEV, CRX, CAR, CTX, CFP, CAZ
B2	*Pseudomonas aeruginosa*	CRX, CAR, AUG, CFP, CLX
E3	*Pseudomonas aeruginosa*	CRX, CAR, AUG, CLX
DA	*Pseudomonas aeruginosa*	LEV, IMP, AZM, GM, CRX, CAR, CTX, AUG, CLX
U2	*Pseudomonas aeruginosa*	AZM, CRX, AUG, CLX
AA1	*Pseudomonas aeruginosa*	LEV, AZM, CRX, CAR, CTX, AUG, CLX
CA1	*Pseudomonas aeruginosa*	LEV, AZM, GM, CRX, CAR, CTX, AUG, CLX
HA	*Pseudomonas aeruginosa*	LEV, IMP, GM, CRX, CAR, CTX, AUG, CLX
AA2	*Klebsiella oxytoca*	CRX, CAR, CTX, AUG, CLX, CFP, CAZ
L	*Klebsiella oxytoca*	CRX, CTX, CLX, CFP, CAR
O2	*Klebsiella pneumoniae*	AZM, GM, CRX, CTX, AUG, CFP, CLX, CAZ
JA	*Klebsiella pneumoniae*	IMP, GM, CRX, CAR, CTX, AUG, CLX, CFP
KA	*Klebsiella pneumoniae*	AZM, GM, CRX, CAR, CTX, AUG, CLX, CFP, CAZ
W	*Klebsiella pneumoniae*	LEV, AZM, GM, CRX, CAR, CTX, AUG, CLX, CAZ
WA	*Klebsiella pneumoniae*	LEV, CRX, CAR, CLX
NA	*Klebsiella pneumoniae*	LEV, AZM, GM, CRX, CAR, CTX, AUG, CFP, CLX
A2	*Escherichia coli*	IMP, GM, CRX, CAR, AUG, CLX, CFP
M2	*Escherichia coli*	LEV, IMP, AZM, GM, CRX, CAR, CTX, AUG, CLX, CFP, CAZ
EA	*Escherichia coli*	CRX, CTX, CLX, CAZ, CFP
U1	*Escherichia coli*	CAR, CTX, CLX, CAZ, CFP
OA	*Escherichia coli*	AZM, CRX, CTX, AUG, CLX, CFP, CAZ
YA	*Escherichia coli*	LEV, CRX, CAR, AUG, CFP, CLX, CAZ
I3	*Salmonella* species	CRX, CAR, CTX, AUG, CFP, CAZ
IA	*Salmonella* species	CRX, CAR, CFP, CLX, CAZ
V	*Salmonella* species	LEV, IMP, GM, CRX, CAR, CTX, AUG, CLX, CAZ
RA	*Salmonella* species	LEV, IMP, GM, CRX, CAR, CTX, AUG, CLX, CAZ
J	*Shigella* species	AZM, GM, CRX, CAR, CTX, AUG, CLX, CFP, CAZ

LEV = Levofloxacin, IMP = Imipenem, AZM = Azithromycin, GM = Gentamicin, CRX = Cefuroxime, CAR = Carbenicillin, CTX= Cefotaxime, AUG = Augmentin, CFP = Cefepime, CLX = Cephalexin, CAZ = Ceftazidime

Table 6: Detection of extended spectrum beta-lactamase (ESBL) genes in Gram negative isolates

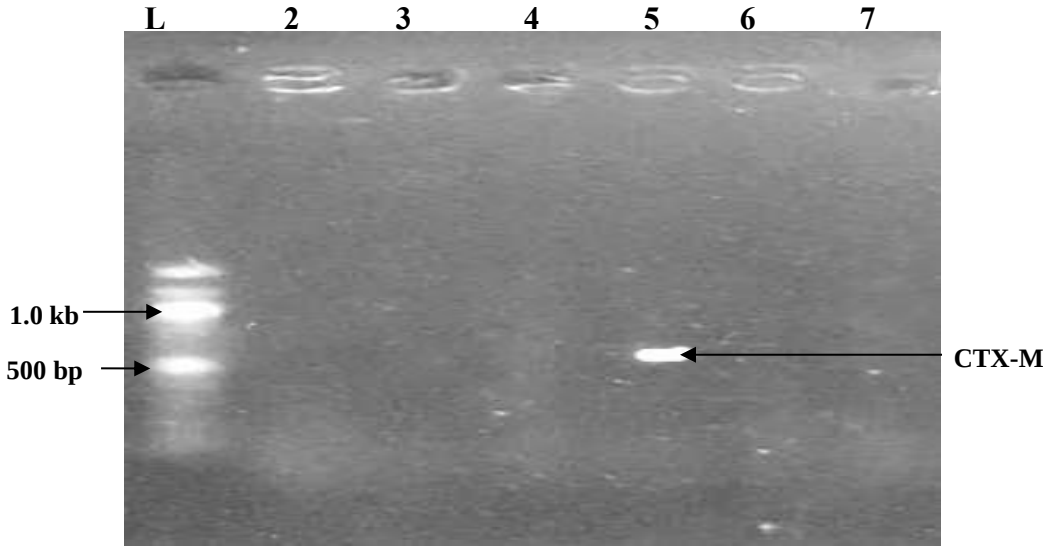
S/N	Isolate code	Isolates	ESBL genes			MDDST
			CTX-M	SHV	TEM	
1.	B2	*Pseudomonas aeruginosa*	-	-	+	+
2.	G2	*Acinetobacter* species	-	+	-	+
3.	IA	*Salmonella* species	-	+	-	+
4.	JA	*Klebsiella pneumoniae*	+	-	+	+
5.	OA	*Escherichia coli*	-	-	+	+
6.	YA	*Escherichia coli*	-	+	+	+
7.	CA1	*Pseudomonas aeruginosa*	-	+	-	-

MDDST- Modified double-disc synergy test



L = Molecular weight marker; 2 = *Acinetobacter* species, 3 = *K. pneumoniae*, 4 = *Acinetobacter* species, 5 = *Salmonella* species, 6 = *E. coli*, 7 = *P. aeruginosa*, 8 = *K. oxytoca*, 9 = *E. coli*, 10 = *K. pneumoniae*, 11 = *E. coli*, 12 = *P. aeruginosa*

Fig 2: PCR amplification of *bla*$_{SHV}$ (768 bp) and *bla*$_{TEM}$ (857 bp) genes



L = Molecular weight marker; 2-7 = 2 = *P. aeruginosa*, 3 = *Acinetobacter* species, 4 = *Acinetobacter* species, 5 = *K. pneumoniae*, 6 = *E. coli*, 7 = *E. coli*

Fig 3: PCR amplification of *bla*$_{CTX-M}$ gene (543 bp)

Discussion:

Both traditional and modern medicine use medicinal plants as a source of raw materials. Plant materials have been in use as home remedies, over-the-counter drugs, and biopharmaceutical ingredients in both the developed and developing worlds (4). Medicinal herbs are the major source of primary health care for most rural populations, particularly in developing countries (3,4). Plant-based medicines are increasingly being integrated into primary health care systems in developing countries but safety concerns are being overlooked (21,22). The production of herbal medicines by unlicensed vendors with no or limited educational backgrounds, as well as a lack of food hygiene knowledge, have contributed significantly to

the high rate of bacterial contamination of herbal drugs (23). Furthermore, most medicinal plants are prepared in open environment under unsanitary conditions, which leads to contamination with pathogens that are harmful to the public (24,25). The risk of microorganisms such as coliform and others being present in pharmaceutical products, including herbal medicines, is determined by the nature of the product, the intended use, and the possibilities for end user harm (26,27). The inability to prevent moisture levels in herbal medicines during transportation and storage, as well as the temperatures of liquid forms and finished herbal products, may have resulted in the proliferation of microorganisms (22).

Several studies have reported bacterial counts in herbal materials and herbal medicines (28,29). Bacterial and fungal contaminations are common, particularly in locally produced herbal medicines, with CFU/g levels exceeding the recommended WHO standards of $\leq 10^5$ CFU/ml (22), demonstrating increased risks in the intake of these products (22). In this study, 20.0% and 28.0% of herbal medicine preparations exceeded the safety limits for bacterial and fungal growths respectively, indicating that consuming these products poses a risk. Herbal medicines in liquid pharmaceutical form for oral use had the highest microbial contamination and were the most commonly consumed products among the elderly (25).

The locally sold herbal mixtures in our study showed a wide variety of potential pathogens such as *S. aureus, P. aeruginosa, K. pneumoniae* and *E. coli* that have been implicated in many diseases. The presence of *E. coli, Salmonella* and *Shigella* species in liquid herbal preparations makes them unfit for human consumption according to the WHO guidelines (12). Previous studies from Nigeria also identified the presence of *S. aureus, P. aeruginosa*, *Salmonella* species and other coliforms in herbal mixtures from Abuja, Nigeria (9,30). The mean total viable bacterial counts (range of $1.0x10^4$-$3.2x10^7$ CFU/ml) and mean total fungi count (range of $2.0x10^4$-$4.4x10^7$ CFU/ml or g) obtained in this study are higher than the study of Ya'aba et al., (9) who reported a range of $2.0x10^1$-$6.7x10^3$ CFU/ml for bacteria and $1.0x10^1$-$3.0x10^1$ CFU/ml for fungi. However, in a study from Jos, Nigeria, Dashen et al., (30) reported a higher mean total viable bacterial count of $1.0x10^6$–$1.4x10^7$ CFU/ml. *Aspergillus* species was the most predominant fungal isolate from this study. Previous studies have reported the presence of fungi in herbal medicines (29,31,32). The contamination of herbal preparations by fungi has been attributed to contamination by dust following storage in moist conditions (33).

According to a meta-analysis on antibiotic-resistance in medically important bacteria isolated from commercial herbal medicines in Africa from 2000 to 2021, *E. coli* was the most frequently reported MDR species, followed by *S. aureus* (10). All the bacterial isolates from herbal mixtures by Ayansina and Akinsola (29) were multidrug resistant. In our study however, 84.2% of Gram-positive isolates and 81.3% of Gram-negative isolates were multidrug resistant. Yesuf et al., (28) reported that all *E. coli* from herbal preparations were sensitive to gentamicin and ciprofloxacin, whereas our study found that all *E. coli* were sensitive to levofloxacin but only 66.7% were sensitive to gentamicin. Resistance to ceftazidime was found in all *K. oxytoca, A. baumannii* and *Salmonella* species in our study, which agrees with the findings of Ayansina and Akinsola (29), who reported all *P. aeruginosa, E. coli, Klebsiella*, and *Salmonella* isolates in their study to be ceftazidime resistant (29). Resistance to cefuroxime was seen in all *P. aeruginosa, E. coli, Klebsiella,* and *Salmonella* species (29) in our study, with resistance ranging from 78 to 86%.

The detection of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} among bacterial isolates recovered from herbal mixtures in our study agrees with the findings of Ayansina and Akinsola (29), who also detected bla_{TEM} and bla_{CTX-M} genes in bacterial isolates recovered from Nigerian herbal mixtures. The presence of multidrug-resistant Gram-negative bacteria isolates carrying ESBL-genes in herbal mixtures is a major source of concern, because of the potential risk of more difficult-to-treat ESBL infections in consumers of these herbal preparations, as well as the risk of transfer of ESBL genes, that can be carried on mobile genetic elements such as plasmids, transposons and integrons, from one isolate to another (34). Furthermore, high proportion of elderly people in Nigeria who use herbal medicines to treat a variety of ailments may be at increased risk of infections by ESBL-producing and multi-drug resistant bacterial pathogens, with dire consequences.

Conclusion:

From the finding of our study, local herbal preparations are not sufficiently safe to consume, because of high degree of contamination with pathogenic microorganisms that are resistant to most commonly used antibiotics in our environment. As a result, herbal mixtures prepared locally in an unsanitary env-

ironment or sold without regulatory approval are not safe for human consumption. It is recommended that proper hygienic conditions be maintained throughout the entire preparation process.

Authors' contributions:

OBO* conceived and designed the study; OBO* and OEA coordinated the experiment; OBO*, SEA and OBO carried out the study experiments; OBO* drafted the manuscript and analyzed the data; and OEA did necessary editing of the manuscript. All authors read and approved the manuscript.

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No conflict of interest is declared

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