PRESENCE OF MULTIDRUG-RESISTANT GRAM-NEGATIVE BACTERIA IN SOME ORALLY-ADMINISTERED HERBAL PREPARATIONS SOLD IN SOUTH-EASTERN NIGERIA

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ABSTRACT

Background: The presence of infectious microorganisms in orally-administered herbal medicines is of great clinical importance and may portend grave danger to unsuspecting users of the products.

Objectives: This study, therefore, investigated the presence of multidrug-resistant (MDR) Gram-negative bacteria in orally-administered herbal preparations sold in South-Eastern Nigeria.

Methods: Using standard microbiological techniques, strains of *Escherichia coli* and *Pseudomonas aeruginosa* were isolated from some orally-administered liquid herbal preparations purchased from retail outlets located within the five states (Abia, Anambra, Ebonyi, Enugu and Imo) that make up South-Eastern Nigeria. Antibiotic susceptibility testing was performed on the isolates using the Kirby-Bauer disk diffusion method. The isolates were further screened for extended spectrum β – lactamase (ESBL) and metallo-β-lactamase production (MBL) using the double disk synergy test (DDST) and carbapenem - ethylene diamine tetra-acetic acid (EDTA) DDST respectively.

Results: *In vitro* antibiotic susceptibility studies revealed that all isolated *E. coli* and *P. aeruginosa* strains demonstrated high degree of resistance to most of the antibiotics tested including the carbapenems - meropenem and imipenem. ESBL production was recorded only among the *E. coli* strains and MBL production was observed only in *P. aeruginosa*.

Conclusion: It may be concluded from this study that some orally-consumed herbal medications in South-Eastern Nigeria are contaminated with pathogenic MDR Gram-negative bacteria which pose great threat to the health of the consumers. Efforts should therefore be geared at standardizing the quality of herbal products through strict adherence to good manufacturing practices.

Keywords: Multidrug-resistant bacteria, extended spectrum β – lactamase, metallo-β-lactamase, herbal medicine, South-Eastern Nigeria.

INTRODUCTION

Botanicals are indisputably used to promote good health and disease prevention in many parts of Africa. Moreover, medicinal plants are the most easily accessible health resource available to the community, and because of cultural beliefs, they are frequently the preferred option for patients. However, in several cases, the alleged health benefits of

natural health products and herbal medicines are based on common myths, as well as preliminary findings from pre-clinical studies and early-stage clinical trials that are not confirmed by regulatory-approved evidence of safety and efficacy (Mahomoodally, 2013; Chugh *et al.*, 2018).

There are some challenges in the advancement of herbal medicine, as the numerous adverse drug reactions of herbal remedies represent specific risk factors that can lead to increased vulnerability to human health difficulties (Kamsu-Foguem and Foguem, 2014). While some of the reported side effects and adverse drug reactions may be due to the inherent bioactive secondary metabolites present in the herbal formulations, many are due to the poor quality of the products that can be attributed to such factors as contamination (with chemicals, pesticides, microorganisms and/or heavy metals), adulteration with pure drug compounds, poor quality control and unhygienic measures (Onyegbule *et al.*, 2017).

According to a study conducted by Onyegbule *et al*. (2017), some herbal medicinal products sold in open markets across Nigeria do not meet regulatory specifications as they contain microbial loads above recommended levels for herbal products for oral administration. Another study found that some herbal anti-infective products produced and marketed in South-Eastern Nigeria did not meet microbiological safety standards and contained pathogenic microbial contaminants that could endanger the health of the products' consumers (Ujam *et al*., 2013). The presence of high levels of pathogenic microbial contaminants in the anti-infective herbal products that are supposed to treat infections caused by microorganisms may reflect non-compliance to good manufacturing practices (GMP) in the production of these medicines, leading to the proliferation of drug-resistant microbial strains in the products.

This research was therefore designed to investigate the presence of multidrug-resistant (MDR) Gram-negative bacteria, such as the extended spectrum β – lactamase (ESBL) and metallo-β-lactamase (MBL) – producing bacteria, in orally-administered herbal preparations sold in South-Eastern Nigeria.

MATERIAL AND METHODS

Sample Collection and microbial isolation

A total of 20 brands of commercially available orally-administered liquid herbal preparations were randomly purchased from retail outlets located within the five states (Abia, Anambra, Ebonyi, Enugu and Imo) that make up South-Eastern Nigeria. Details on the collection of these herbal preparations, as well as the isolation and identification of *Escherichia coli* and *Pseudomonas aeruginosa* strains from the samples, are documented by Ujam *et al*. (2013).

Antibiotic Susceptibility Test (AST) of the Isolates

Susceptibility of isolates to different antibiotics was tested following the Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute, 2015). Pure cultures of the isolated bacteria were emulsified in sterile normal saline to obtain a homogeneous suspension of the bacterial cells. Standardized concentration (McFarland 0.5) of each isolate was swabbed on the surface of a Mueller-Hinton agar (MHA) (Oxoid, UK) plate. Commercially available single antibiotic disks (Oxoid, UK) – sulfamethoxazole-trimethoprim (1.25/23.75 µg), ciprofloxacin (5 µg),

ofloxacin (5 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg) amoxicillin/clavulanic (amoxi/clav) acid (20/10 µg), gentamicin (10 µg) and ampicillin (10 µg) were then placed on the inoculated agar plates. The plates were inverted within 30 min of applying the disks and incubated aerobically at 37 ^{0}C for 18 – 24 h. The inhibition zone diameters (IZDs) around the disks were measured in millimeter (mm) using a meter rule. This process was carried out in triplicate for every antibiotic disk and the mean IZDs were calculated and rounded to the nearest whole number. Following the Clinical and Laboratory Standards Institute's (CLSI) guidelines on antibiotic breakpoints for *Enterobacteriaceae* and *P. aeruginosa*, the isolates were described as either resistant, intermediate or sensitive (Clinical and Laboratory Standards Institute, 2015).

Phenotypic detection of ESBL production

The strains showing resistance to the 3rd generation cephalosporins: ceftazidime, ceftriaxone, and cefotaxime in the AST were further subjected to double disks synergy test (DDST) for confirmation of ESBL production following the method described by Chukwunwejim *et al.* (2018). Standardized bacterial suspension (0.5 McFarland) was aseptically swabbed on MH agar plates. Amoxi/clav acid (20/10 µg) disk was placed at the centre of the MH agar plate, while cefotaxime (30 µg) and ceftazidime (30 µg) disks were each placed at a distance of 15 mm (centre to centre) from the central disk. The plates were afterwards incubated for 18 – 24 h at 37 ^{0}C. A 5 mm increase in the IZD for either of the cephalosporins tested in combination with amoxi/clav acid, versus its IZD when tested singly confirms ESBL production (Ejikeugwu *et al.,* 2013; Chukwunwejim *et al.,* 2018).

Phenotypic detection of MBL production

E. coli and *P. aeruginosa* strains that showed resistance to any of the two carbapenem antibiotics: meropenem and imipenem in the AST were screened for MBL production using the imipenem/meropenem-EDTA DDST as described by Ejikeugwu *et al.* (2017). Meropenem (10 µg) and imipenem (10 µg) disks were placed 25 mm apart on MHA plates inoculated with standardized concentration of the test bacteria. A volume of 1 µL of sterilized 0.5M EDTA solution was added to each of the imipenem and meropenem disks respectively. Also, supplementary imipenem (10 µg) and meropenem (10 µg) disks without EDTA were placed adjacent to the carbapenem disks impregnated with EDTA. The plates were incubated at 37 ^{0}C for 18 – 24 h. A difference of $\geq$7 mm between the zones of inhibition of any of the carbapenem disks with and without the chelating agent (EDTA) infers MBL production (Ejikeugwu *et al.*, 2017).

RESULTS

The 20 herbal preparations purchased from retail outlets in different parts of South-Eastern Nigeria yielded a total of ten (10) *Escherichia coli* and four (4) *Pseudomonas aeruginosa* strains. Distribution of strains isolated from the various herbal anti-infective products is shown in Table 1. Eleven (11) of the 20 samples screened were positive for *E. coli* and/or *P. aeruginosa* strains.

Ujam *et al.* (2013) first reported the antibiotic susceptibility/resistance profile of the isolates against ten antibiotics namely: ofloxacin, ciprofloxacin, amoxicillin-clavulanic acid, gentamicin, ceftazidime, cefotaxime, trimethoprim-sulfamethoxazole, ampicillin, tetracycline and ceftriaxone. However in our study, the susceptibility/resistance of isolates to the aforementioned antibiotics, as well as to

imipenem and meropenem, was re-determined and results are shown in Table 2. The mean IZDs produced by the test isolates in the AST, as well as their resistance profile are presented in Table 2. It can be observed that these organisms were generally resistant to the different classes of antibiotics tested. Percentage resistance to the antibiotics used in the AST ranged from 58 – 75% and 0 – 100% for the *E. coli* and *P. aeruginosa* strains respectively.

In general, all *E. coli* strains showed high degree of resistance (100%) to cefotaxime, ampicillin, sulfamethoxazole-trimethoprim, amoxicillin/clavullanic acid and imipenem, followed by meropenem (90%), tetracycline (90%), and ceftazidime (80%). They were however readily inhibited by ciprofloxacin, ofloxacin, gentamicin, and ceftriaxone with percentage resistance of 0, 10, 10, and 30% respectively. The *P. aeruginosa* strains were tested only against ceftazidime, ofloxacin, ciprofloxacin, gentamicin, imipenem and meropenem according to the CLSI performance standards for antimicrobial susceptibility testing (Clinical and Laboratory Standards Institute, 2015). These *P. aeruginosa* strains showed 75% resistance to imipenem and meropenem, 50 % resistance to ceftazidime and 25% resistance to ofloxacin, ciprofloxacin and gentamicin (Table 2).

ESBL and MBL - production was investigated in all the isolates. In the DDST, an increase of $\geq$5 mm in the cephalosporin-produced IZDs when tested in combination with amoxi/clav acid confirmed ESBL production (Figure 1). In the test for MBL production, an increase of $\geq$7 mm in IZDs produced by the EDTA-containing carbapenem disks compared to individual EDTA-free disks confirmed the production of MBL (Figure 2). Out of the 10 *E. coli* strains, 2 were ESBL – positive strains (strains E4 and E14), and 1 out of the 4 *P. aeruginosa* strains tested positive for MBL production (strain P16). None of the *E. coli* strains tested positive for MBL production, and none of *P. aeruginosa* strains was ESBL – positive) (Table 2, Figure 3).

Table 1 Distribution of *E. coli* and *P. aeruginosa* strains isolated from the herbal anti-infective products

Strains	Sample codes																				
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**Total**
E. coli	-	E2	-	E4	-	E6	E7	-	-	-	E11	-	E13	E14	E15	E16	-	E18	-	-	10
P. aeruginosa	-	P2	-	-	-	-	P7	-	-	P10	-	-	-	-	-	P16	-	-	-	-	4

Table 2 Antibiotic susceptibility profile of the isolates

Isolates		CTX	CRO	CAZ	TE	AMP	OFX	CIP	SXT	AMC	CN	IPM	MEM	Resistance (%)	ESBL	MBL
		Inhibition zone diameters in millimeters [resistance (R), susceptibility (S) or intermediate(I)]														
E. coli strains	E2	16(R)	22(I)	19(I)	8(R)	8(R)	21(S)	29(I)	0(R)	0(R)	20(S)	0(R)	0(R)	58	neg	neg
	E4	20(R)	17(R)	0(R)	0(R)	0(R)	34(S)	34(S)	0(R)	10(R)	25(S)	13(R)	0(R)	75	pos	neg
	E6	12(R)	23(S)	0(R)	9(R)	10(R)	14(I)	23(I)	0(R)	0(R)	20(S)	9(R)	13(R)	67	neg	neg

	E7	12(R)	23(S)	0(R)	8(R)	0(R)	19(S)	22(I)	0(R)	6(R)	21(S)	0(R)	12(R)	67	neg	neg
	E11	20(R)	36(S)	0(R)	8(R)	10(R)	10(R)	23(I)	0(R)	0(R)	21(S)	8(R)	0(R)	75	neg	neg
	E13	20(R)	20 (I)	0(R)	0(R)	0(R)	20(S)	23(I)	0(R)	6(R)	21(S)	11(R)	0(R)	67	neg	neg
	E14	0(R)	14(R)	0(R)	0(R)	0(R)	20(S)	27(I)	0(R)	10(R)	22(S)	18(R)	21(I)	67	pos	neg
	E15	11(R)	34(S)	0(R)	23(S)	0(R)	19(S)	24(I)	0(R)	0 (R)	10(R)	8 (R)	0(R)	67	neg	neg
	E16	15(R)	12(R)	24(S)	0(R)	0(R)	19(S)	26(I)	0(R)	7(R)	20(S)	10(R)	0(R)	67	neg	neg
	E18	20(R)	30(S)	0 (R)	6 (R)	10(R)	20(S)	25(I)	0(R)	0 (R)	21(S)	12(R)	0(R)	67	neg	neg
Resistance (%)		100	30	80	90	100	10	0	100	100	10	100	90			
P. aeruginosa strains	P2	NA	NA	24(S)	NA	NA	24(S)	32(S)	NA	NA	20(S)	8(R)	0(R)	33	neg	neg
	P7	NA	NA	24(S)	NA	NA	19(S)	29(I)	NA	NA	23(S)	24(S)	18(I)	0	neg	neg
	P10	NA	NA	0(R)	NA	NA	21(S)	29(I)	NA	NA	18(S)	14(R)	0(R)	50	neg	neg
	P16	NA	NA	12(R)	NA	NA	0(R)	0(R)	NA	NA	0(R)	0(R)	0(R)	100	neg	pos
Resistance (%)		NA	NA	50	NA	NA	25	25	NA	NA	25	75	75			

CTX: cefotaxime; CAZ: ceftazidime; CRO: ceftriaxone; TE: tetracycline; AMP: ampicillin; OFX: ofloxacin; CIP: ciprofloxacin; SXT: sulfamethoxazole-trimethoprim; AMC: amoxicillin/clavullanic acid; CN: gentamicin; IPM: imipenem; MEM: meropenem. R: resistant; I: intermediate; NA: not applicable; S: susceptible; pos: positive; neg: negative

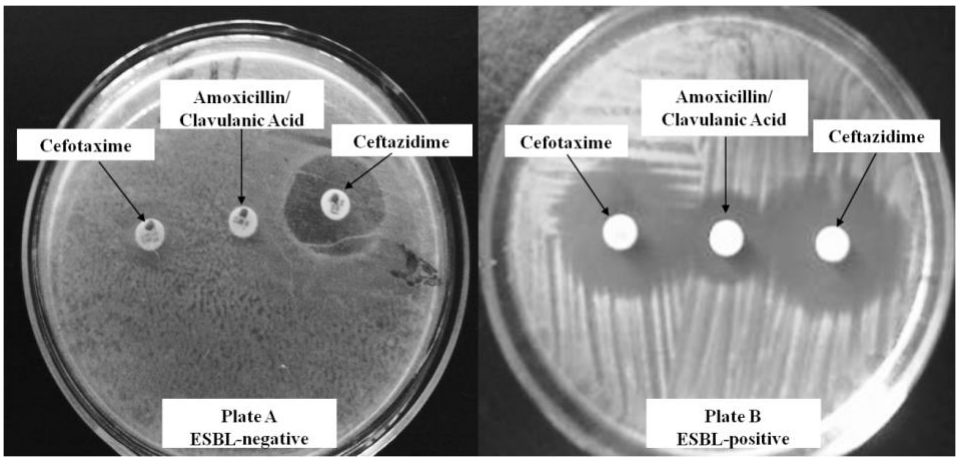


Figure 1 Agar plates showing ESBL – negative (Plate A) and ESBL – positive (Plate B) strains in the DDST testing. In Plate A, the lack of synergy between amoxi/clav acid (20/10 µg) disk at the center and the corresponding cefotaxime 30 µg and ceftazidime 30 µg disks can be observed. Synergism between the disks can be observed in Plate B.

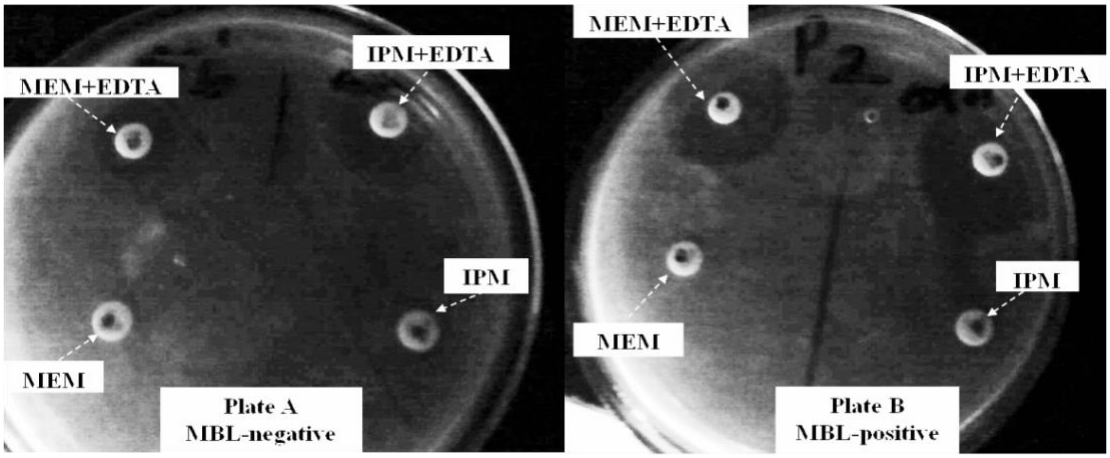


Figure 2 Agar plates showing MBL – negative (Plate A) and MBL – positive (Plate B) strains in the phenotypic testing for confirmation of MBL production. In Plate A, IZDs produced by both carbapenem disks are larger than (≥7 mm) the individual disks not containing EDTA indicating MBL production. MEM=meropenem; IPM=imipenem; EDTA=ethylene diamine tetra-acetic acid.

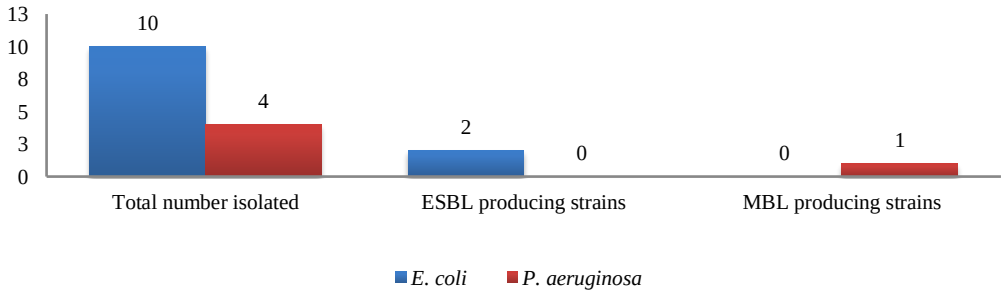


Figure 3 Total numbers of ESBL- and MBL-producing isolates

DISCUSSION

Our study was a continuation of the work of Ujam *et al.* (2013), who described the isolation, identification, and antibiotic resistance patterns of numerous microbial strains isolated from herbal samples. The microbial strains included 49 bacterial strains from the genera *Staphylococcus, Escherichia, Bacillus, Streptococcus, Pseudomonas, Proteus, Salmonella, Yersinia,* and *Corynebacterium*, as well as 40 fungal strains from the genera *Aspergillus, Candida, Microsporum, Trichosporon, Coccidioides, Blastomyces, Cryptococcus, Histoplasma, Penicillium, Nigrospora* and *Mucor*. Among all of these potentially pathogenic microbial strains, our study focused on Gram-negative bacterial species from the genera *Escherichia* and *Pseudomonas* because they are more likely to harbor MDR-genes such as those responsible for ESBL and MBL production (Yong *et al*., 2002; Chukwunwejim *et al*., 2018).

Production of beta (β)-lactamases is the most common mechanism of resistance to β-lactam antibiotics, including the third-generation cephalosporins, especially among *Enterobacteriaceae*. ESBLs are Class A and

D β-lactamases that hydrolyze expanded-spectrum cephalosporins and monobactams and are inhibited by clavulanate. Infections caused by ESBL-producing organisms are difficult to manage as empiric therapy consisting of β-lactam antimicrobials is often ineffective, and these organisms tend to also be resistant to other classes of antimicrobials including fluoroquinolones and aminoglycosides (Clinical and Laboratory Standards Institute, 2015; Chukwunwejim *et al.*, 2018). ESBLs are considered one of the most important mechanisms by which pathogens exert their antibiotic resistance capabilities, and according to previous studies (Mehrgan *et al*., 2010; Freitas *et al*., 2003; Yusha'u *et al.*, 2011), the prevalence of bacteria producing these ESBLs has steadily increased. ESBLs have been reported worldwide in many different genera of *Enterobacteriaceae* and *P. aeruginosa*. However, ESBL production has been previously reported to be most common in *Klebsiella* species and *Escherichia coli* (Chukwunwejim *et al.*, 2018).

Metallo-β-lactamases (MBLs), on the other hand, are Class B β-lactamases produced by bacteria which hydrolyze the carbapenems (such as imipenem, meropenem, and ertapenem). This means that MBLs help bacteria that produce them to be resistant to the carbapenems and as such, render the antibiotics ineffective for treatment (Ugwu *et al*., 2018; Ejikeugwu *et al*., 2019). The increase in the prevalence of carbapenem resistance mediated by acquired MBLs has been reported, particularly for *P. aeruginosa* clinical isolates in several countries (Yong *et al.*, 2002). The production of MBLs by bacteria allows the organism to resist the antimicrobial onslaughts of the carbapenems. The carbapenems are often the last line of treatment option for a variety of bacterial related infections including those caused by MDR organisms such as ESBL – positive bacteria (Ejikeugwu *et al*., 2019). MBL – positive isolates leads to serious therapeutic failure because they carry MDR genes and the only treatment option available is potentially toxic polymyxin B and colistin (Livermore and Woodford, 2000).

In our study, the presence of MBL – positive and ESBL – positive MDR bacteria was demonstrated using phenotypic methods in several herbal medicines sold in South-Eastern Nigeria. It is planned that future research will include PCR detection and molecular characterization of the MDR genes. ESBL production was recorded only among strains of *E. coli*, and MBL production was recorded only for *P. aeruginosa* (Table 2, Figure 3). The ESBL – positive strains (E4 and E14) displayed 75 and 67% resistance to the antibiotics used in the AST. The other ESBL – negative *E. coli* strains also showed high degree of resistance (58 – 75%) to the test antibiotics, including the cephalosporins (cefotaxime, ceftazidime, and ceftriaxone) and carbapenems (imipenem and meropenem). The MBL – positive *P. aeruginosa* strain (P 16) showed 100% resistance to all antibiotics tested (Table 2). As observed in this study, and according to a previous report (Walsh *et al*., 2005), MBL production is found to be more prevalent in *P. aeruginosa* isolates than the *Enterobacteriaceae.*

The presence of these MDR bacteria in these herbal medicines could be as a result of lack of GMP in their production. The European Pharmacopeial specifications for the acceptance of herbal medicinal products indicate the absence of *E. coli* 1 ml of the preparation (European Pharmacopeia, 2010). The presence of MDR strains of *E. coli* (a particular indicator organism of fecal contamination) and *P. aeruginosa* in the orally-consumed herbal medicines investigated in this study is of great clinical

significance and may portend grave danger to unsuspecting consumers.

It is worth noting that the orally-consumed herbal medicines examined in this study are promoted and sold as anti-infectives that are supposed to cure microbe-caused diseases. These anti-infectives, however, contain harmful MDR microorganisms that can cause infections in unsuspecting consumers. This is particularly true for vulnerable or immunocompromised individuals who may succumb to bacterial toxin-induced diseases of the gastro-intestinal tract or other infections as a result of ingesting these harmful products. Such infections may be difficult to treat due to the resistance to commonly used first-line antibiotics. The implications of this situation include the risk of treatment failures for infections caused by MDR species due to limited treatment options, which could result in longer patient hospitalizations, higher health-care expenses, and possibly higher morbidity and death.

Many Nigerians use herbal medicines and they regards it to be safe, effective and beneficial (Oreagba, 2011). The Nigerian society stands to gain more working towards the integration of traditional medicine into its health care delivery system (Adefolaju, 2011). However, there are many challenges that need to be overcome for its full potential to be realized. The results of this study, like other previous studies (Ujam *et al*., 2013; Builders *et al*., 2015; Onyegbule *et al* 2017), reflect the lack of quality control in the production herbal medicines in Nigeria. These data point to the need for deployment of effective systems of supervision of the manufacture, storage, distribution and marketing of these products, as well as insightful programmes of quality control on the part of National regulatory agencies (Onyegbule *et al* 2017).

The unregulated use of traditional and herbal medicines adversely affects the health and wellbeing of the population. If herbal medicines are to be respected in modern health care, the quality of the data and the quality of the herbal products themselves, as well as regulatory control of herbal medicines, must significantly improve (Erah, 2002). Appropriate legislation should be put in place by the national authorities to regulate the trado-medical industry. These laws will expectedly take care of issues such as training, documentation, quality control, coordination, standardization and ethical issues. These steps will go a long way in reinforcing the desire of traditional medical practitioners to modernize their practice and put them on the same pedestal with their western counterparts. This will eventually lead to an improvement in the health sector of the economy and end the rivalry between the two medical practices that has become a veritable threat to health care delivery in the country. More importantly, such integration would boost primary health care delivery, reduce the high maternal and child mortality, reduce cost of medical care, provide employment and eventually improve the economy (Adefolaju, 2011).

CONCLUSION

It can be concluded from this study that some orally-consumed herbal preparations commercially marketed in South-Eastern Nigeria are contaminated with potentially harmful Gram-negative bacteria. The presence of hazardous MDR microorganisms in these products is of great clinical importance, and may portend grave danger to unsuspecting consumers. Concerted effort by the Nigerian government, manufacturers, health practitioners, marketers, and users of herbal medicines should be geared towards the standardization of the quality and safety of the products.

CONFLICT OF INTEREST

The authors declare no conflict of Interest.

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