Antimicrobial Resistance Evaluation of Organisms Isolated from Liquid Herbal Products Manufactured and Marketed in South Eastern Nigeria.

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ABSTRACT

Objective: To determine the susceptibility and resistance pattern of bacteria and fungi isolates obtained from herbal anti-infective liquid preparations manufactured and marketed in South-East Nigeria to conventional antibiotics.
Study Design: Experimental
Place and Duration of the study: Pharmaceutical Microbiology and biotechnology Laboratory, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu Campus between October 2011 and March 2012
Methodology: Isolation and characterization of contaminating microorganisms were carried out using standard procedures. A total of forty-nine (49) bacteria and forty (40) fungi isolated from the herbal products were examined for susceptibility to conventional antibiotics using the disc diffusion method. The bacterial isolates were tested against ciprofloxacin, ofloxacin, amoxicillin-clavulanic acid, gentamicin, cefotaxime, ceftazidime, ceftriazone, sulphamethoxazole, tetracycline and ampicillin were employed while fungi isolates were tested against five common antifungal-griseofulvin, nystatin, ketoconazole, fluconazole and clotrimazole. The Multiple Antibiotic Resistance Index (MARI) of each of the isolated bacteria was obtained following the standard method.
Result: The antimicrobial susceptibility-resistance profile of the bacteria isolates revealed that most of the bacteria were sensitive to ciprofloxacin, ofloxacin, gentamicin, and ceftriaxone, On the other hand, a good number of the isolates demonstrated high level of resistance to common antibiotics like Ampicillin, amoxycillin-clavulanic acid, trimethoprim-sulphamethoxazole, and moderate level of resistance to Tetracycline, and some of the third generation cephalosporins - ceftazidime and cefotazime. Multiple Antibiotic Resistance Index (MARI) evaluation revealed that most of the isolates were resistance to more than fifty percent (50%) of the number of antibiotics used. The fungal isolates were susceptible to nystatin, ketoconazole and clotrimazole, resistance to fluconazole and high resistance recorded against griseofulvin.
Conclusion: The results of this study revealed that the herbal medications can serve as a trail of spread of antibiotic-resistance genes.

Keywords: {Susceptibility, antibiotic resistance, herbal anti-infectives}

1. INTRODUCTION

The use of herbal medicine has always been part of human culture, as some plants possess important therapeutic properties, which can be used to cure human and other animal diseases [1]. Herbal medicine is becoming increasingly

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popular in both developing and developed countries [2]. A World Health Organization survey indicates that about 70–80% of the world population, particularly in developing nations; rely on non-conventional medicines mainly of herbal sources in their primary health care [3]. Medicinal plant materials normally carry a large number of microbes originating from the soil. Microorganisms of various kinds are normally adhered to leaves, stems, flowers, roots and seeds. Additional contaminants may also be introduced during harvesting, handling and production of various herbal remedies since no conscious efforts are made to decontaminate the herbs other than by washing them. [4]. Herbal medicines are therefore vulnerable to attack by microorganisms and as such are disposed to spoilage. Accordingly, gross microbial contamination of herbal medicinal products commonly consumed in Nigeria has been severally demonstrated [5, 6,7]. The presence of antibiotic resistant microbial isolates in the Herbal Medicinal Products (HMPs) could lead to transfer of antibiotic resistance traits to hitherto sensitive gut or oral micro flora of consumers [8].

The emergence of multiple drug resistant bacteria (MDR) has become a major cause of failure of the treatment of infectious disease [9]. As a result, society is facing one of the most serious public health dilemmas over the emergence of infectious bacteria displaying resistance to many and in some cases, effective antibiotic [10] much like the situation in human medicine. Bacteria and fungi resistance to antimicrobial drugs has continued to grow in the last decades [11]. The increased prevalence of their resistant is due to extensive use and misuse of antimicrobials. This has rendered the current available antimicrobial agents insufficient to control microbial infections and create major public health problem.

Resistant bacteria strains may develop almost anywhere particularly in a pressurized environment containing previously non-resistant bacteria strains as contaminants. One of such environments can be created by widespread use of HMP. HMPs have been previously implicated as a pool for such contaminations [12,13]. It is of utmost importance to both monitor and ascertain the microbial purity of HMPs given the huge medical and economic implications of any such microbial contamination especially with multiple drug resistant strains. Such surveillance will both help to identify microbial contamination of herbal products and slow down and prevent the emergence of drug-resistant strains. The present study evaluated the presence of contaminating organisms and the susceptibility-resistance pattern of the isolated organisms.

2. EXPERIMENTAL DETAILS

2.1 MATERIALS: HERBAL SAMPLES

A total of twenty liquid herbal anti-infectives were purchased randomly from different shops and herbal outlets located within the five states that make up the south-east, Nigeria and were used in this study. The samples which were within their shelf lives and were kept at room temperature (as indicated by their manufacturers) were used within two weeks of collection.

2.2 METHODS

2.2.1 Isolation and Identification of Microbial Contaminants in the Herbal

The herbal anti-infectives were serially diluted and plated on nutrient agar and sabouroud dextrose agar plates in triplicate and incubated at 37^{o}C for 18-24 hours and 20^{o}C- 27 ^{o}C for 72-168hours for bacteria and fungi respectively. The resultant colonies were further purified, isolated and characterized using standard methods [14].

2.2.2 Characterization of Microorganisms Isolated From the Herbal Preparations

The bacteria isolates were characterized using the morphological appearance (macroscopy) of their colonies, their Gram stain reaction and confirmatory biochemical tests. The fungi isolates were also identified on the basis the morphological characteristics (macroscopy) of their colonies, microscopy, staining with ordinary stain and the appearance of their mycelia [15].

2.2.3 Antibiotics Susceptibility Testing

The susceptibility tests were performed following the method M2-A6 disc diffusion method recommended by the National Committee for Clinical Laboratory Standards [16] using Mueller Hinton and Sabouraud Dextrose Agar. The bacterial isolates from the samples were reactivated by sub-culturing from agar slant onto nutrient agar plate and was incubated for 18-24 hours. The inoculum was standardized by transferring three distinct and separate colonies of the pure culture of the test organism using sterile wire loop into 3mls of sterile nutrient broth. The suspension was incubated for 3 hours at 37°C to allow for the growth of test organism till the density was equivalent to the turbidity of 0.5 McFarland. The standardized inocula were swabbed onto Mueller-Hinton agar and Sabouraud Dextrose Agar plate and the discs were placed on the inoculated plates and pressed firmly onto the agar plate for complete contact. The bacterial strains were tested against the following discs:, ofloxacin (OFX, 5µg) ; ciprofloxacin (CIP,5µg) ; amoxicillin/clavulanic acid (AMC,20/10µg) ; gentamicin,(GN ,10µg) ; ceftazidime (CAZ,30µg) ; cefotaxime (CTX,30µg) ; trimethoprim-sulfamethoxazole (SXT,1.25/23.75µg); Ampicillin (AMP,10µg) ; tetracycline (TE, 30µg); ceftriaxone (CRO, 30µg).The fungal strains were tested against the following discs: nystatin (N,20µg) ; clotrimazole (C,20µg) ; griseofulvin (G,20µg) ; ketoconazole (K,20µg) and fluconazole (F,20µg). The Plates were inverted and left on the work table for 30 minutes to allow for pre-diffusion of antibiotics into the agar. The plates were incubated at 37°C for 18-24 hours and at 25^{o}C 24-48hours for bacteria and fungi respectively. The susceptibility of each isolate to each antibiotic was shown by a clear zone of growth inhibition and this was measured using a meter rule in millimeters and the diameter of the zones of inhibition was then interpreted using standard chart [17].

2.2.4 Determination of Multiple Antibiotics Resistance Index (MARI)

The Multiple Antibiotics Resistance Index (MARI) of ten antibiotics (ofloxacin, ciprofloxacin, gentamicin, amoxycillin-clavulanic acid, sulphamethoxazole-trimethoprim, ceftriazone, ceftazidime, cefotazime, tetracycline and ampicillin) were determined using the formula, MARI = a/b

Where; a = the aggregate resistance of antibiotics to all isolates and b = the total number of antibiotics that was used

3. RESULTS AND DISCUSSION

3.1 RESULTS

Table 1: Microorganisms Isolated from the Herbal anti-infective Products.

Samples Code	Shelf life	Contents	Therapeutic Claims	Identity Of Bacteria Isolated	Identity Of Fungi Isolated
1	(Man.: Feb. 2010 Exp.: Feb 2014)	*Carica papya, Magnifera indica, Newbouldlia, leavis, Azadricha indica, Jaminum officionili, Aloe, barbedensis, Ginseng, Treated water 60cl.*	Antibacterial, Antimalarial, Ant rheumatic, infertility, Antiviral.	*a)Staphylococcus aureus b)Proteus spp*	*a) Microsporuim spp. b) Aspergillus spp c) Nigrospora spp*
2	(Man.: May 2011 Exp.: May 2014)	38 African Roots, Herbs, Fruits, Barks plus ginseng, Aloevera and Garlic.	Antibacterial, Antirheumatic, Antifungal and Antiviral.	*a)Escherichia coli b)Staphylococcus aureus c)Staphylococcus epidermidis d)Pseudomonas aeruginosa e)Bacillus spp f)Proteus spp*	*a) Candida tropicalis b) Microsporum canis*
3	(Man.: July 2011 Exp.: Jan 2014)	60% herbs, 25%flower, 10% leaves, 5% roots.	Antibacterial, Antirheumatic, Antifungal, Earlier Menopause, Painful and irregular menstruation.	*a)Staphylococcus aureus b)Bacillus subtilis c)Bacillus cereus d)2 Salmonella spps*	*a) Candida albicans b) Candida tropicalis c) Trichosporon spp*
4	(Man.: April 2011 Exp.: April)	Aloe vera plus 31 roots and herbs ,fruits and barks	Antibacterial, Antifungal.	*a)Escherichia coli b)Staphylococcus areus c)Streptococcus spp d)Bacillus spp*	*a) Coccidioides immitis b) Microsporuim audounii*
5	(Man.: May 2009 Exp.: May 2012)	Water, herbs, root and fruits.	Antibacterials, Antimalarial, Antiparasitic, Internal heat, pile, and reduces sugar.	*a)Staphylococcus epidemidis b)Streptococcus spp c)Yersinia spp*	-
6	(Man.: May 2011 Exp.: 2013)	-	Antibacterial, Treatment of all form of eye infections.	*a)Escherichia.coli b)Bacillus spp c)Proteus spp*	*a)C.topicalis b) Microsporuim audounii c)Aspergillus niger*
7	(Man.: Oct. 2011 Exp.: Oct 2012)	Nauclea diiderchi 10%,Hippocrates pallens 20%,Alluim sativum12.5%,Cochios permum planchoni 5.5%,Uvaria chame 5%,Punica granatum	Antibacterial, Antimalarial.	*a)Escherichia coli b)Staphyloccus spp c)Pseudomonas d)Bacillus spp*	*a) Blastomyces b) Microporuim canis*

		47%.			
8	(Man.: June 2011 Exp.: June 2014)	Aloe vera 40%, Olong tea 20%, Flower and roots 40%, Saracin.	Antibacterial.	*a)Staphylococcus spp b)Salmonella spp*	*a) Blastomyces*
9	(Man.: April 2009 Exp.:April 2012)	Aloe Vera	Antibacterial Anti-malarial, HBP, Cough Antirheumatism, e.t.c.	*a)Staphylococcus areus*	*a) Blastomyces spp b) Cryptococcus neoformans c) Histoplasma*
10	(Man.: June 2011 Exp.: Dec. 2014	Aloe Vera, Flowers, Fruits seed barks.	Antibacterial, Hypertension, Antiviral, fibroid, stroke.	*a)Pseudomonas aeruginosa*	*a)Penicillum spp b)Aspergillus spp*
11	(Man.: June 2011 Exp.: June 2013)	Honey (natural), Lime juice, Zingiber officillinar, Herbal seeds and roots.	Antibacterial and Asthmatic cough.	*a)Escherichia coli b)Staphylococcus*	*a)Candida spp*
12	(Man.: March 2010 Exp.: Dec. 2012)	-	Anti-bacterial Antiviral, Diabetes, Reduces cholesterol.	*a)Staphylococcus areus b)Streptocoloccus spp*	*a) C.albicans b)Blastomyces*
13	(Manu: July 2011 Exp.: July 2013)	25 different types of roots, herbs, seeds and flowers.	Anti-bacterial, Anti-malaria, Antirheumatic. Antifungal	*a)Escherichia coli b)Corynebacteruim diphtheria*	*a)Aspergillus spp b) Nigrospora spp c) C.tropicalis*
14	(Man: July 2011 Exp.: July 2016)	Herbs, water, root and fruits.	Antibacterial, Antiviral, Antirheumatic, Antifungal, Antiparatic, internal heat, pile.	*a)Escherichia coli b)Streptococcus spps c)Yersinia spp*	*a)C.albicans b) Aspergillus spp*
15	(Man.: July 2011 Exp.: Dec. 2013)	Magnifera indica, Carica papaya leaves, Psiduim guajava, Breadfruit bark, Masularia acuminate roots, Citrus lemon leaves, Zingiber Officinale roots, Cymbopogon spp.	Antibacterial, Antimalarial, Antirheumatic Antiviral.	*a)Escherichia coli*	*a)Microsporum spp b) Coccidioides spp c)Aspergillus spp.*
16	(Man.: June 2011 Exp.: May 2015)	Awapa bark, white lotus, Golden seal, Mahogany, Ukor root, Aloe barbaders, Mistletoe, Osisika Aguru, Uda roots, Uvuru ilu, Lemon grass.	Antibacterial, Antirheumatic and Arthritis, Veneral diseases.	*a)Escherichia coli b)Pseudomonas aeruginosa*	*a)C.albicans b) Mucor spp*
17	(Man.: Sept. 2011 Exp.: Dec. 2012)	Aloe Vera, Cadeperi salt, Lime	Antibacterial, Treatment and prevention of toothache.	*a)Proteus spp*	*a) Aspergillus spp*
18	(Man.2010: Exp.: Sept. 2012)	Lymbopogon citrates, Carica papaya leaves, Magnifera indica, bark, Treculia Africana, Citrus, Limonia, Psiduim guajava, Zingibar officinale root,	Antibacterial, Antirheumatism reduces sugar and cholesterol.	*a)Escherichia coli*	*a)Mucor spp*

		Alluim sativum.			
19	(Man.: Sept. 2010 Exp.: Aug. 2012)	Natural roots and barks.	Antibacterial, Antiviral, Purifies blood, Detoxifies toxins, Builds immune system, Stops dizziness, weakness.	*a) Bacillus spps*	*a) Yeast/Blastomyces b)Aspergillus spp c) Microsporum spp*
20	(Man.: Nov. 2010 Exp.: Dec. 2013)	Nuclealatifolia, Allium sativum, Aloe Vera bitter, Chick weed, Preclina nitida, Hibiscus sabdrrifa, Aqua, Ethanol.	Antibacterial, Antiparasitic, ulcer, constipation, fibroid, internal heat heart burn and diabetes	*a) Staphylococcus epidermidis*	*a)Aspergillus spp*

Table 2: Percentage of microbial isolates from the Herbal anti-infective Products

BACTERIA ISOLATES	% Occurrence	FUNGI ISOLATES	% Occurrence
E.coli	20.4	*Aspergillus spp*	22.5
S.aureus	24.5	*Microsporuim spp*	17.5
P.aeruginosa	8.2	*Candida spp*	22.5
Strep.spp	10.2	*Trichosporon spp*	2.5
Bacillus	16.3	*Coccidiodes spp*	5.0
Salmonella	6.1	*Blastomyces spp*	12.5
Proteus spp	8.2	*Cryptococcus spp*	2.5
Yersinia spp	4.1	*Histoplasma spp*	2.5
C.diphtheria	2.0	*Penicilluim spp*	2.5
		Nigrospora spp	5.0
		Mucor spp	5.0

A total of 89 microbial strains (49 bacterial and 40 fungal strains) were isolated from the herbal preparations. The identified microbial isolates consists of nine (9) bacterial genera and eleven (11) fungal genera which include *Staphylococcus, E. coli, Bacillus, Streptococcus, Pseudomonas, Proteus, Salmonella, Yersinia, Corynebacteruim diphtheria* and *Aspergillus, Candida, Microsporuim, Trichosporon , Coccidiodes, Blastomyces, Cryptococcus, Histoplasma, Penicilluim, Nigrospora*, Mucor respectively. The most frequently isolated bacteria and fungi specie were *Staphylococcus* spp (24.5%) and *Aspergillus* spp/*Candida* spp (22.5%) respectively. The least frequently isolated bacteria specie was *Corynebacteruim diphtheria* (2.0%) and that of fungi were *Trichosporon* spp, *Cryptococcus* spp, *Histoplasma* and *Penicillium* spp (2.5%). See Tables 1 and 2 above.

Table 3: The Antibiotic Susceptibility Profile of the isolated bacteria

Drugs and Strength (µg)			OFX-5	CIP-5	SXT-1.25/ 23.75	AMC-20/10	GN-10	CTX-30	CAZ-30	TE-30	AMP-10	CRO-30
			N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
BACTERIA ISOLATES	*E. coli*	S	8 (80)	10 (100)	0 (0)	0 (0)	9 (90)	0 (0)	3 (30)	1 (10)	0 (0)	5 (50)
		I	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (20)
		R	1 (10)	0 (0)	10 (100)	10 (100)	1 (10)	10 (100)	7 (70)	9 (90)	10 (100)	3(30)
	P. aeruginosa	S	3 (75)	3 (75)	0 (0)	0 (0)	3 (75)	0 (0)	2 (50)	0 (0)	0 (0)	2(50)
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (75)	0 (0)	0 (0)	0 (0)	1 (25)
		R	1 (25)	1 (25)	4 (100)	4 (100)	1 (25)	1 (25)	2 (50)	4 (100)	4 (100)	1(25)
	Staphylococcus spp	S	8 (67)	10 (83)	0 (0)	0 (0)	10 (83)	0(0)	2 (17)	2 (17)	0 (0)	7(58)
		I	4 (33)	2	0 (0)	0 (0)	0 (0)	2	1 (8)	2 (17)	0 (0)	4

			(17)				(17)				(33)
	R	0 (0)	0 (0)	12 (100)	12 (100)	2 (17)	10 (83)	9 (75)	8 (67)	12 (100)	1(8)
Salmonella spp	S	1 (33)	1 (33)	0 (0)	0 (0)	3 (100)	2(67)	2 (67)	1 (33)	0 (0)	1(33)
	I	1 (33)	2 (67)	0 (0)	0 (0)	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	1(33)
	R	1 (33)	0 (0)	3 (100)	3 (100)	0 (0)	1 (33)	1 (33)	2 (67)	3 (100)	1(33)
Streptococcus spp	S	3 (60)	4 (80)	0 (0)	0 (0)	4 (80)	2 (40)	2 (40)	1 (20)	0 (0)	1(20)
	I	1 (20)	1 (20)	0 (0)	1 (20)	1 (20)	0 (0)	1 (20)	1(20)	0 (0)	0(0)
	R	1 (20)	0 (0)	5 (100)	4 (80)	0 (0)	3 (60)	2 (40)	3 (60)	5 (100)	4 (80)
Bacillus spp.	S	3 (38)	5 (63)	0 (0)	0(0)	6 (75)	0 (0)	1 (13)	4 (50)	0 (0)	5(63)
	I	5 (63)	3 (38)	0 (0)	1(13)	0 (0)	1 (13)	1 (13)	1 (13)	1 (13)	1(13)
	R	0 (0)	0 (0)	8 (100)	7(88)	2 (25)	7 (88)	6 (75)	3 (38)	7 (88)	2(25)
Proteus spp	S	2 (50)	1 (25)	0 (0)	0 (0)	2 (50)	1 (25)	2 (50)	2 (50)	0 (0)	1 (25)
	I	2 (50)	3 (75)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	2 (50)
	R	0 (0)	0 (0)	4 (100)	4 (100)	2 (50)	3 (75)	1 (25)	2 (50)	4 (100)	1(25)
Yersinia spp	S	0 (0)	1 (50)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	I	2 (100)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)
	R	0 (0)	0 (0)	2 (100)	1 (100)	0 (0)	2 (100)	1 (50)	2 (100)	2 (100)	1 (50)
C. diphtheria	S	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
	I	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1(100)
	R	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)

Key: S = Sensitive, **I** = intermediate, **R** - Resistance, **N** = number of organisms, OFX= ofloxacin, CIP = ciprofloxacin, CAZ = ceftazidime, TE = tetracycline, AMP = ampicillin, SXT = trimethoprim-sulfamethoxazole, GN = gentamicin, CTX = cefotaxime, CRO = ceftriaxone, AMC = amoxycillin-clavulanic acid.

Table 3 above shows the antibiogram, of all the bacterial strains isolated from the Herbal products - a representation of the bacteria that are Susceptible, Intermediate or Resistant to the different antibiotics using the NCCLS break points [17].

Table 4: Antibiogram of Fungi Isolated from the Herbal Anti-Infectives

Samples Code	Isolates	Inhibition Zone Diameter (IZD) in millimeter (mm)				
		Griseofulvin	Nystatin	Ketoconazole	Clotrimazole	Fluconazole
1	*Microsporuim spp.*	0	25	0	0	0
	Aspergillus spp	0	32	10	12	0
	Nigrospora spp	0	30	20	14	12
2	*Candida tropicalis*	0	26	23	7	15
	Microsporum canis	0	25	0	0	0
3	*Candida albicans*	0	22	12	10	7
	Candida tropicalis	0	23	11	10	8
	Trichosporon spp	7	30	32	18	8
4	*Coccidioides immitis*	0	30	0	0	0
	Microsporuim audounii	0	29	0	0	0
6	*C.topicalis*	0	27	12	11	8
	Microsporuim audounii	0	8	0	0	0

	Aspergillus niger	0	29	0	7	0
7	*Blastomyces*	0	32	17	18	0
	Microporuim canis	0	30	0	0	0
8	*Blastomyces*	0	31	18	18	0
9	*Blastomyces spp*	0	29	16	19	0
	Cryptococcus neoformans	0	0	0	0	0
	Histoplasma	0	30	29	12	15
10	*Penicillum spp*	0	31	15	7	0
	Aspergillus spp	0	23	0	12	0
11	*Candida spp*	0	22	12	10	7
12	*Candida albicans*	0	20	14	12	9
	Blastomyces	0	30	15	19	0
13	*Aspergillus spp*	0	32	17	8	0
	Nigrospora spp	0	28	14	15	0
	Candida tropicalis	0	35	25	20	0
14	*Candida albicans*	0	22	13	10	10
	Aspergillus spp	0	26	0	7	0
15	*Microsporum spp*	0	25	0	0	0
	Coccidioides spp	0	30	0	0	0
	Aspergillus spp.	0	31	10	0	0
16	*C.albicans*	0	24	23	8	0
	Mucor spp	0	26	0	0	0
17	*Aspergillus spp*	0	31	10	0	0
18	*Mucor spp*	0	25	0	0	0
19	*Yeast/Blastomyces*	0	28	18	16	0
	Aspergillus spp	0	29	0	9	0
	Microsporum spp	0	0	0	0	0
20	*Aspergillus spp*	0	36	0	0	0

Table 5: Multiple Antibiotics Resistance Index (MARI) of the Isolated Bacteria.

Grouping	Isolates and Samples Code	Multiple Antibiotics Resistance Index (MARI)
Group A	Pa16	1
Group B	S3b	0.8
Group C	E4,11,14,15,Sa1,P2	0.7
Group D	E7,15,16,13,18,Sa2,Sa5,Sa7,Sa20,St12,St4,Ba7,Ba19b,Ba3a,Ba6,Ba3b,P17,Y5	0.6
Group E	E2,6,Sa3,Sa4,Sa8,Sa11,Sa9,Sa2,Pa10,St5,St14,Ba9a,P6,Y14,C13	0.5
Group F	Sa12,Pa2,Pa7,St14,Ba2,S8,P1	0.4

Notes for Table 5: E = *E.coli*, Sa = *Staphylococcus spp*, Pa = *Pseudomonas aeruginosa*, St = *Streptococcus spp*, Ba = *Bacillus spp*, S = *Salmonella*, P = *Proteus spp*, Y = *Yesinia spp*, C = *Corynebacteruim spp*. The numbers attached represent the product numbers

3.2 DISCUSSION

Antimicrobial susceptibility testing of the isolated microorganisms was carried out to evaluate the activity of conventional antibiotics against the isolated bacteria and fungi strains. The bacteria contaminants isolated from these herbal preparations showed wide resistance to penicillins, especially ampicillin, augmentin (amoxycillin-clavulanic acid combination) and cloxacillin, suggesting that they could be producers of penicillinases. The resistance to trimethoprim-sulphamethoxazole (co-trimoxazole) by all the isolates especially the Gram-negative isolates calls for attention. The findings of this study agree with an earlier work [12]. *Staphylococci* strains were the most frequently isolated bacteria species and it probably originated from handlers, as its habitat is human skin. *Staphylococcus* showed wide resistance to penicillins suggesting possibly that they are producers of penicillinase enzymes. Resistance to trimethoprim by *S. aureus* and *S. epidermidis* has been reported with increasing frequency [18,19, 20]. It seems probable that *S. epidermidis* serves as a reservoir for resistance, which can be transferred to *S. aureus*. Also, inter-generic transfer of resistance among different genera of Gram-positive cocci and between Bacillus species and Staphylococci and Streptococci has been reported [20, 21]. *Escherichia coli* were the second most frequently isolated species in these medications which is an intestinal bacterium and an indicator of faecal contaminant. Presence of *Escherichia coli* in the sample indicates poor hygiene practices and lack of adequate handling of the products. According to the European pharmacopoeia 2007 [22], no

Salmonella spp or Escherichia coli strain should be present in oral medicines. The presence of *E. coli* in herbal drugs had been reported by another researcher [23]. The *Escherichia coli* isolates showed a wide resistance to ampicillin, ceftazidime, sulphamethoxazole-trimethoprim, amoxycillin-clavulanic acid, and tetracycline. *Bacillus spp*. were the third most frequently found in these herbal medicaments because they are widely distributed in the soil, dust, air and because they are resistant to environmental destructive factors [20, 24]. A number of reports have described serious human infections caused by members of the genus Bacillus even though they have been regarded as non-pathogenic [25,26,27]. All the strains of *Pseudomonas* isolated were resistant to β-lactam antibiotics; Inducible β-lactamase activity is a general property of *Pseudomonas cepacia* [28]. Gram negative rods usually have wide resistance against antimicrobial agents [20] (Esimone *et al*., 2007a). *Streptococcus spp* showed high resistance to sulphamethoxazole-trimethoprim and ampicillin. *Salmonella spp*, were resistant to sulphamethoxazole-trimethoprim, amoxycillin clavulanic acid, and ampicillin. *Proteus spp*, *Yersinia spp* and *Corynebacteruim diphtheria* showed wide resistance to sulphamethoxazole-trimethoprim, amoxycillin clavulanic acid, ceftazidime and ampicillin (Table 3). On the other hand, the bacterial isolates were susceptible to some groups of the antibiotics (ofloxacin, ciprofloxacin, gentamicin and ceftriaxone).

Multiple Antibiotic Resistance Index (MARI) evaluation (Table 5) revealed that species of *Escherichia coli* showed high level of multiple antibiotic resistances to the panel of antibiotics used in this study. The MARI value ranged from 0-5 -0.7, with three (30%) resistant to seven antibiotic out of the ten used, six (60%) resistant to six of the antibiotics used and two (20%) resistant to five. *Staphylococcus spp* have MARI values ranging from 0.4-0.7, with one (8.3%) resistance to seven antibiotic, four (33.3%) resistant to six antibiotics, six (50%) resistant to five antibiotics and one (8.3%) resistance to four antibiotics. The MARI result of *Bacillus spp* ranged from 0.4 – 0.6, with five (62.5%) resistant to six antibiotics, One (12.5%) being resistance to five, four and three antibiotics each. *Proteus spp* MARI value is from 0.4 - 0.7, with one (25%) each of the four isolates resistant to seven, six, five and four respectively. *Pseudomonas spp* had MARI value ranging from 0.4 - 1.0, one (25%) showed high resistance index, being resistance to ten of the antibiotics used in this study, one (25%) resistant to five antibiotics and two (50%) resistance to four antibiotics. The three species of *Streptococcus* isolated showed MARI values from 0.3-0.8, that is, one (33.3%) resistant to eight antibiotics, one (33.3%) to four antibiotics and one (33.3%) to three out of the ten antibiotics. We had two isolates of *Yersinia spp* and the MARI values are 0.5 and 0.6. Lastly, *Corynebacteruin diphtheria* isolate is resistance to five antibiotics out of the ten antibiotics used in this study. Bacteria with high MAR index originate from the environment where antibiotics are over used [29].

Fungal infections are becoming an increasing cause of morbidity and mortality especially among immunocompromised patients. With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory methods to guide and select antifungal therapy have gained greater attention. However, determining antifungal susceptibilities of filamentous fungi is fraught with difficulties associated with slow growth of filamentous forms and subjectivity of interpreting visual endpoints [30]. In the present study, antifungal susceptibility testing of 40 fungi isolates was observed against five common antifungal agents (griseofulvin, nystatin, ketoconazole, clotrimazole, and fluconazole) using disc diffusion method, presence of inhibition zone was considered as sensitive while absence of inhibition zone was recorded as resistance. The fungi isolates were very sensitive to nystatin, ketoconazole and clotrimazole. Most of the fungi isolates were resistance to fluconazole while all are resistant to Griseofulvin (Table 5).

The importance of surveying resistant environmental strains is that under favourable situations, they may transfer their resistance plasmids to pathogens [31,32]. If such organisms are present in medicaments, such as herbal medicinal products they could behave as opportunist pathogens and initiate an infection, particularly in immuno-compromised patients as well as lead to transfer of antibiotic resistance traits to hitherto sensitive microorganisms co-habiting within the consumers of those products. Given the increasing rate of development of resistant bacteria strains, our challenge is to slow the rate at which resistance develops and spreads. In order to decrease the spread of resistance among antibiotics, physicians, pharmacists, researchers and consumers alike need to be more aware of the selective pressures driving these bacteria to decrease their susceptibility [33]. These selective pressures include the abuse, overuse and misuse of antimicrobials in therapy, improperly manufactured and mishandled HMPs [13, 34] as well as other numerous socioeconomic factors that govern the development of multi-drug resistant bacteria strains [35].

4. CONCLUSION:

The high rate of resistance to antimicrobial agents of strains isolated from these herbal preparations may indicate a widespread antibiotic resistance among microorganisms from different sources. It is therefore mandatory that herbal medicines should not be taken indiscriminately and that current good manufacturing practices (cGMPs) must be observed by these herbal practitioners in the production of the medicines.

Authors' Contributions:

Oli Angus N - drafting of the manuscript/Corresponding author, Esimone Charles O – conceptualized and designed the work as well as revising the manuscript critically for important intellectual content, Ujam Nonye T – carried out the experiments and did analysis and interpretation of data, Adikwu Michael Umale - revised the manuscript critically for important intellectual content, Ikegbunam MN- carried out some part of the experimental work

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