Evaluation of the Claims of Microbiological Activity and Microbiological Quality of Some Oral Herbal Medicinal Products Sold in Port-Harcourt Metropolis

ABSTRACT

The incidences of chemotherapeutic failure and high cost of orthodox medicines may have led to increased use of herbal medicinal products as alternative medicines. However, the non-standardization and improper regulation of these herbal products in countries like Nigeria may raise a lot of questions about the inherent health risk associated with the consumption of these products. This study was carried out to evaluate the claims of antimicrobial activity and microbiological quality of some herbal products sold in Port Harcourt Metropolis. Forty (40) herbal medicinal products were examined in this study. All claimed to have antibacterial effect and had National Agency for Food and Drugs Administration and Control (NAFDAC) registration number. *Escherichia coli, Klebsiella pneumoniae, Proteus mirablis, Pseudomonas aeruginosa* and *Staphylococcus aureus* were isolated from the herbal samples. The bacterial isolates were characterized and identified by standard microbiological and biochemical methods. Antibacterial susceptibility of the isolates was determined using Kirby-Bauer disk diffusion method. The claims of antibacterial activity of the 40 samples were tested against clinical isolates of *Escherichia coli, Klebsiella species, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Forty (40) percent and fifty (50) percent of the liquid and solid dosage forms respectively were found to have gross microbial contamination above the recommended limit according to the National Policy for Assessments of Herbal Products, 2007. *Staphylococcus aureus* was isolated in approximately sixty-four (64) percent and twenty-nine (29) percent of the solid and liquid dosage forms respectively. Antibiotic susceptibility testing showed that most of the herbal products contained pathogenic bacteria with single and multiple drug resistance patterns. The need for Good Manufacturing Practices (GMPs), standardization, stricter controls and education to safeguard the health of the consuming public demands urgent attention.

Keywords: Herbal medicines, microbiological quality, contaminants and pathogens.

INTRODUCTION

Herbal medicine or phytomedicine, refers to the use of plant parts such as seeds, berries, roots, leaves, barks or flowers for medicinal purposes [1]. Herbal preparations are defined as preparations obtained by subjecting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration and fermentation [2]. In Nigeria, the use of herbal medicine dates back to the earliest history of mankind as in other cultures worldwide. Before the advent of orthodox medicines, people relied wholly on herbal medicinal products or complementary and alternative medicines for their healthcare needs. This included the use of herbs, animal and mineral based herbal medicines often laced with spiritual ingredients such as incantations [3]. Back when technology was still unheard of, primitive men utilised the vast flora around them to the fullest extent, observing both plant and animal life and their components, eventually giving birth to herbal medicine'[4]. In the early 19^{th} century, when methods of chemical analysis first became available, scientists began extracting and modifying the active ingredients from plants. Later, chemists began making their own version of plant compounds, beginning the transition from raw herbs to synthetic pharmaceuticals. Over time, the use of herbal medicines declined in favor of pharmaceuticals [1]. Many conventional drugs that are available today originated from plant sources. In a study by the World Health Organisation on herbal medicinal use, about 80 percent of the World's populace still rely on herbal medicine to cure certain ailments and about 74 percent of the drugs we use today contain at least one botanical element.[4]. This may not be unconnected to the active mass media advertisement embarked upon by the producers and marketers of the herbal medicinal products who have taken the advantage of the relatively high cost of conventional pharmaceutical dosage forms, inaccessibility of the orthodox medical services to a vast majority of people particularly in the rural areas and the reservations by the public due to the prevalence of fake, substandard or counterfeit drugs in the market [5]. In a study in 2007 [6] evaluated the susceptibility and resistance pattern of bacterial and fungal isolates obtained from herbal medicine products (HMPs) marketed in Nigeria to conventional antibiotics. They screened seventy-five (75) bacteria and fifty-two (52) fungi isolated from the HMPs for susceptibility to conventional antibiotics. While most

of the bacteria isolates were susceptible to fluoroquinolones and aminoglycosides, they were significantly resistant to the penicillins.

Herbs and herbal materials normally carry a large number of bacteria and moulds, often originating in soil or derived from manure. While a large range of bacteria and fungi form the naturally occurring microflora of medicinal plants, aerobic spore-forming bacteria frequently predominate. Current practices of harvesting, production, transportation and storage may cause additional contamination and microbial growth. Proliferation of microorganisms may result from failure to control the moisture levels of herbal medicines during transportation and storage, as well as from failure to control the temperatures of liquid forms and finished herbal products. The presence of *Escherichia coli*, *Salmonella* spp. and moulds may indicate poor quality of production and harvesting practices.

Microbial contamination may also occur through handling by personnel who are infected with pathogenic bacteria during harvest/collection, post-harvest processing and the manufacturing process.

Bacteria such as *Salmonella* and *Shigella* species must not be present in herbal medicines intended for internal use, at any stage. Other microorganisms should be tested for and should comply with limits set out in regional, national or international pharmacopoeias. Different pharmacopoeias have different testing requirements and these should be consulted when making the appropriate choice for the selected herbal materials and herbal products. The Limit for microbial contamination for total aerobic count **is** 10^5 CFU/ml as recommended by the National Policy for Assessments of Herbal Products,[7]. The aim of this study was therefore to determine the antimicrobial activities and microbiological quality of herbal medicinal products commercially available in Port Harcourt, Rivers State

MATERIALS AND METHOD

Reagents and chemicals: MacConkey agar, Mannitol Salt Agar, Peptone water, Sterile distilled water, Crystal violet, Nutrient broth, Plate Count Agar, Simmons Citrate Agar, Triple sugar iron agar, Urea broth base, Oxidase reagent, Indole reagent, Catalase reagent, Lugol's iodine, McFarland standard.

Sample collection sites: The herbal samples used were obtained from Rumuokoro, Mile 3 Choba, Artillery and Mile 1 markets in Port Harcourt, Rivers state.

Sample collection: The herbal medicinal products were purchased between November 2016 and January 2017. A total of forty (40) samples of indigenous herbal medicinal products were used in this study. Twenty (20) of these samples were liquid while the other (20) were solid dosage forms. All forty (40) samples had NAFDAC registration number and claimed to have antibacterial properties.

Preparation of media: All culture media used were prepared according to manufacturer's instructions. The preparations were sterilized by autoclaving at 121^{o}C for 15 mins at 15 psi. The preparations were preserved until required.

Sample analysis: The samples were processed in the Pharmaceutical Microbiology Laboratory of the Faculty of Pharmaceutical Sciences, University of Port Harcourt, Choba in Rivers State. Before microbial analyses, the covers of the bottles containing liquid herbal medicinal products were disinfected with seventy percent alcohol before opening them. The sachets containing the herbal medicinal capsules were also disinfected with seventy percent alcohol, before ejecting the capsules while one edge of the sachets containing the herbal medicinal powder was disinfected with seventy percent alcohol and a flamed and cooled pair of scissors was used to cut the disinfected edge.

Total viable count: 5ml of the liquid herbal samples was introduced into 5ml of nutrient broth while 1g of the solid samples were introduced into 10ml of nutrient broth and incubated for 24 hours at 37^{o}C. Ten-fold serial dilutions of the incubated herbal sample mixed with nutrient broth were carried out with normal saline in universal bottles. A 0.1ml aliquot of each of the final dilutions was inoculated into 20ml of cooled sterile molten Plate Count Agar (PCA) in a universal bottle, mixed thoroughly and poured into Petri dishes. They were allowed to solidify and then incubated at 37^{o}C for 24hrs. The tests were performed in duplicates. The mean total count was determined and expressed in CFU/ml for aerobic bacteria.

Isolation of bacteria: 5ml of each of the liquid samples and 1g of each of the solid herbal medicinal samples were introduced into 5ml and 10ml of nutrient broth respectively. These were incubated at room temperature and 37^{o}C for 24 hours. After incubation, a loopful of each of the broth culture was streaked on MacConkey Agar (MCA) and Mannitol Salt Agar (MSA) plates and incubated at 37^{o}C for 24 hours for the isolation of viable bacteria.

Identification and Characterization of Isolated Organisms: The isolated organisms were identified by their cultural, microscopic and biochemical characteristics using standard methods.

Gram Staining: A colony of the bacterial isolate was collected using a wire loop and emulsified on a clean grease-free slide with a loopful of sterile distilled water. The smear was air-dried and heat fixed by passing it over a Bunsen flame intermittently for a few seconds. The slide was flooded with crystal violet for 30 seconds and rinsed with water. The slide was then covered with Lugol's iodine for 1 minute. This was rinsed with water, decolourized with acetone for 30 seconds, rinsed with water again and counterstained with Safranin for 30 seconds. The Safranin was also rinsed off with water and the slide was air-dried. A drop of immersion oil was placed on the slide and examined at 100 x objective. The Gram reactions were recorded.

Biochemical Tests

Catalase test: A drop of 3 percent hydrogen peroxide was placed on a clean grease-free glass slide. A colony of the test organism was emulsified in the drop of the reagent. Evolution of gas bubbles was an indication of a positive result while the absence of gas bubbles was an indication of a negative result.

Coagulase test: A volume of 0.5ml of a 1 in 10 dilution of plasma was placed into each of two small test tubes. A 0.5ml aliquot of a 24 hour broth culture was added into one tube and both tubes were incubated at 37^{o}C. The tubes were examined after one hour and at intervals of up to 24 hours. The presence of clumping of the cells was an indication of a positive result while the absence of clumping of the cells was an indication of a negative result.

Staphylococcus aureus (ATCC 25923) and *Staphylococcus epidermidis* were used as positive and negative controls.

Oxidase test: A piece of Whatman (No. 1) filter paper was moistened with freshly prepared one percent aqueous tetra methyl-p-phenylenediamine hydrochloride solution. A speck of the isolate was smeared on the moistened filter paper with the flamed and cooled edge of a clean grease-free glass slide. A positive result was indicated by the appearance of a purple color within ten seconds along the smeared portion while a negative result was indicated by the absence of a purple color.

Indole test: Sterile peptone water measuring 5ml in a McCartney bottle was inoculated with a loopful of 24 hours broth culture of bacteria isolate and incubated at 44^{o}C for 48 hours. After the incubation, three drops of Kovac's reagent were added into the mixture with a sterile Pasteur pipette. The color reaction was recorded. Formation of a red ring colour was an indication of a positive reaction while the absence of a red ring colour was an indication of a negative reaction.

Citrate utilisation test: A sterile straight wire was used to inoculate a 24 hour peptone water broth culture of bacterial isolate into Simmon citrate agar slant in a McCartney bottle. Inoculation was performed by streaking the agar slant and then stabbing the butt. The cap of the McCartney bottle was screwed slightly and incubated at 37^{o}C for 48 hours. A change in colour of the medium from green to blue was an indication of citrate utilisation which is a positive result, while a negative result was indicated by an absence of change of the green colour of the medium.

Triple Sugar Iron (TSI) agar test: A sterile straight wire was used to inoculate the bacterial isolates into Triple Sugar Iron Agar prepared in a long screw-capped test tube. This was done by first stabbing the butt and then streaking the slope in a zig-zag pattern. The tube was incubated at 37^{o}C for 24hours with their caps loosely closed to allow aeration. A sterile Triple Sugar Iron Agar was also incubated without any inoculum to serve as a control.

Urease test: A sterile straight wire was used to inoculate bacterial isolate into the urea agar in a McCartney bottle. This was done by streaking the slant first and followed by stabbing the butt. The butt was incubated at 37^{o}C for 24 hours. A positive urease production was indicated by a change in color of the urea agar slant to pink while the absence of the pink color was an indication of a negative reaction.

Antimicrobial Susceptibility Test for the Bacterial Isolates: This was carried out using the modified Kirby-Bauer method. A 0.1ml aliquot of a standardized isolated bacteria suspension (1.5×10^{6} CFU/ml) was pipetted into 20ml of cooled sterile molten Mueller Hinton agar in a universal bottle. The content of the bottle was mixed thoroughly by rotating the bottle on the palm and poured into a sterile Petri dish. It was allowed to solidify and a sterile forceps was used to gently place commercially prepared antibiotics discs on the surface of the agar. The tests were performed in duplicates and allowed to stand for some time at room temperature to allow for diffusion of the antibiotics. The plates were then incubated at 37^{o}C for 24 hours after which the zones of inhibition were measured and the mean calculated for each antibiotic. Using Clinical Laboratory Standards Institute (CLSI) standard zones of inhibition, the zone size of each antimicrobial agent was interpreted and the isolate was reported as being "resistant", "intermediate", or "susceptible".

In-vitro evaluation of antimicrobial activities of the herbal medicinal products: A 0.1ml aliquot of standardized bacterial suspension (1.5×10^{8} CFU/ml) was pipetted into 20ml of cooled sterile molten Mueller Hinton agar in a universal bottle. This was mixed thoroughly by rotating the bottle on the palm. The inoculated medium was poured into a sterile Petri dish and allowed to set. A flamed and cooled cork borer (6mm in diameter) was used to bore six wells in the plate. About 2-3 drops of each of the herbal medicinal products were used to fill each of the wells. This was done in duplicates. The plates were left at room temperature for one hour and then incubated in an upright position at 37^{o}C for 24 hours after which the diameter of each zone of inhibition was measured in millimeters and the mean calculated for each of the herbal products.

Statistical analysis: Statistical analysis of the results was done using Statistical Package for Social Sciences (SPSS) version 16. Antibacterial efficacies of the herbal medicinal products, prevalence of bacterial and fungal isolates, antibacterial resistance and multiple drug resistance were expressed in frequency. Also, comparative rate of resistance against the antimicrobial agents was determined by Analysis of Variance (ANOVA).

RESULT

Table 1 displays the mean by CFU/ml of total counts of microbial isolates from herbal medicinal products in comparison with the CFU/ml recommended by National Policy for Assessments of Herbal Products, 2007. Sixty percent (60%) of the liquid samples were acceptable while 40% were not acceptable. For the solid samples, 50% were acceptable and the other 50% were not acceptable. This shows the level of microbial contamination. Table 2 shows the bacterial isolates identified.

Table 1: Enumeration of microbial load of herbal samples

Liquid sample			Solid sample		
Sample code	Mean total cell count (CFU/ml)	Remarks	Sample code	Mean total cell count (CFU/ml)	Remarks
H1	0	Acceptable	H21	1.6×10^{8}	Not Acceptable
H2	4.0×10^{2}	Acceptable	H22	0	Acceptable
H3	1.9×10^{3}	Acceptable	H23	1.2×10^{8}	Not Acceptable
H4	2.1×10^{2}	Acceptable	H24	3.5×10^{5}	Not Acceptable
H5	4.1×10^{6}	Not Acceptable	H25	8.2×10^{8}	Not Acceptable
H6	9.0×10^{2}	Acceptable	H26	1.2×10^{3}	Acceptable
H7	0	Acceptable	H27	1.7×10^{5}	Not Acceptable
H8	0	Acceptable	H28	6×10^{3}	Acceptable
H9	1.3×10^{5}	Not Acceptable	H29	5.1×10^{7}	Not Acceptable

H10	7.3×10^3	Acceptable	H30	0	Acceptable
H11	2.6×10^3	Acceptable	H31	0	Acceptable
H12	4.0×10^6	Not Acceptable	H32	5.8×10^8	Not Acceptable
H13	3.7×10^4	Acceptable	H33	3.1×10^4	Acceptable
H14	3.5×10^5	Not Acceptable	H34	0	Acceptable
H15	4.0×10^6	Not Acceptable	H35	4.0×10^{12}	Not Acceptable
H16	1.0×10^6	Not Acceptable	H36	0	Acceptable
H17	3.8×10^{12}	Not Acceptable	H37	0	Acceptable
H18	8.0×10^{10}	Not Acceptable	H38	2.2×10^8	Not Acceptable
H19	1.3×10^{11}	Not Acceptable	H39	5.2×10^{12}	Not Acceptable
H20	5.0×10^2	Acceptable	H40	0	Acceptable
Percentage acceptable 45%;		**60%**			
Percentage Not acceptable 55%		**40%**			

Table 2: Incidence of Bacterial Isolates in the various herbal medicinal products

Liquid sample no	Isolated organisms	Solid sample no	Isolated organisms
1	*No growth*	21	*S. aureus, Pseudomonas*
2	*No growth*	22	*No growth*
3	*Klebsiella pneumoniae*	23	*S. aureus*
4	*Pseudomonas aeruginosa, E. coli, Proteus*	24	*S. aureus*
5	*Klebsiella pneumoniae*	25	*S. aureus*

6	*Klebsiella pneumoniae*	26	*Pseudomonas*
7	*No growth*	27	*S. aureus*
8	*No growth*	28	*S. aureus*
9	*S. aureus*	29	*E. coli*
10	*S. aureus*	30	*No growth*
11	*S. aureus, Klebsiella*	31	*No growth*
12	*S. aureus, Escherichia coli*	32	*S. aureus*
13	*S. aureus*	33	*S. aureus*
14	*S. aureus, Klebsiella, E.coli, Proteus*	34	*No growth*
15	*S. aureus, Proteus mirabilis*	35	*Klebsiella*
16	*S. aureus*	36	*No growth*
17	*E. coli*	37	*No growth*
18	*S. aureus, E. coli*	38	*S. aureus*
19	*E. coli, Proteus*	39	*Klebsiella*
20	*Klebsiella, Pseudomonas, Proteus*	40	*No growth*

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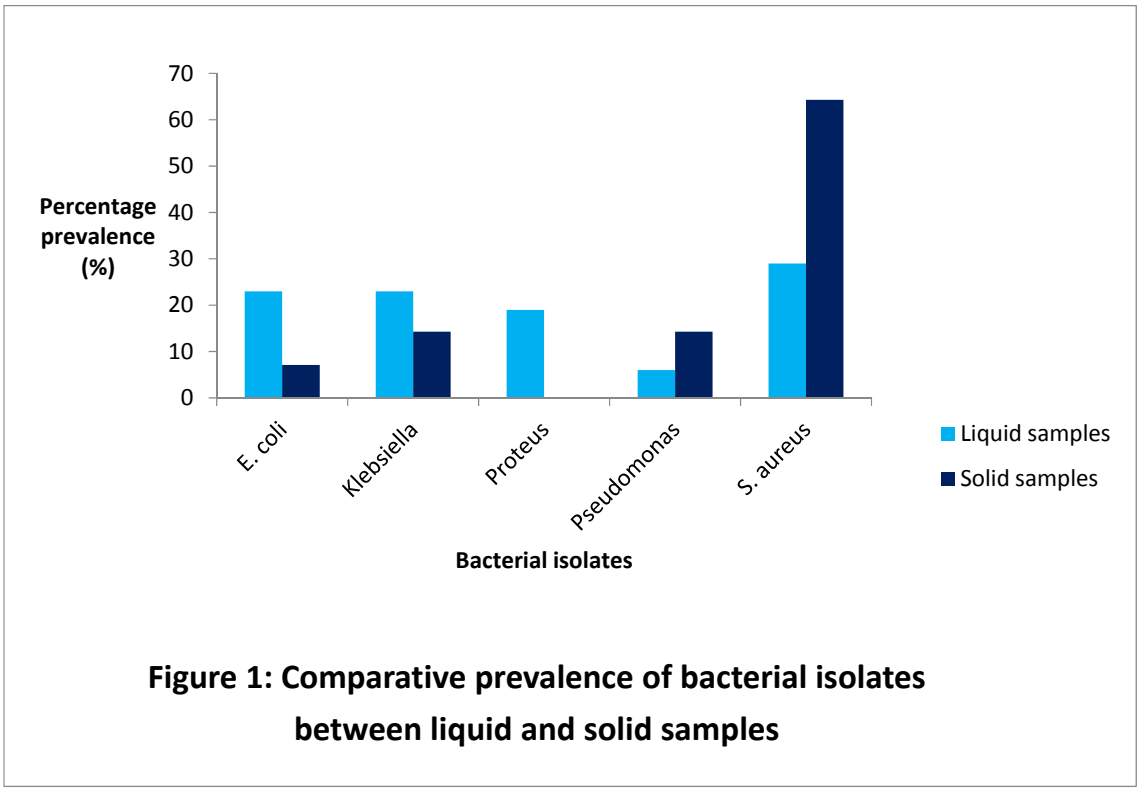

Figure 1: Comparative prevalence of bacterial isolates between liquid and solid samples

Table 3: Characterization and Identification of Bacterial Isolates from Herbal Samples

Table 3a: For *S. aureus*

Culture morphology on Mannitol Salt Agar	Gram stain and microscopic morphology	Coagulase	Catalase	Organism identified
Small yellow colonies	Gram +ve cocci	+ve	+ve	*Staphylococcus aureus*

Table 3b: For Enterobacteria

Culture morphology on MacConkey agar	Gram stain and Microscopic morphology	Indole	Oxidase	Citrate	Urease	TSI	Organism identified
Mucoid pink colonies, lactose fermenting	Gram negative rods	+ve	-ve	-ve	-ve	A/A, Gas	*Escherichia coli*
Mucoid pink colonies, lactose fermenting	Gram negative rods	-ve	-ve	+ve	+ve	A/A, Gas	*Klebsiella*
Large circular gray	Gram	-ve	-ve	+ve	+ve	K/A, Gas	*Proteus*

smooth colonies, non-lactose fermenting; swarming occurs; fishy odor	negative rods					and H2S +ve	*mirabilis*
Pale colored colonies;	Gram negative rods	-ve	+ve	+ve	+ve	K/K	*Pseudomonas aeruginosa*

TSI interpretation: A/A, Gas = Glucose and lactose and /or sucrose fermentation; Gas fermentation. K/A, Gas, H2S = Glucose fermentation only; Gas and H2S produced K/K = No fermentation; peptone catabolized.

Table 4: CLSI standards for Gram-positive and Gram-negative antibiotic discs

Table 4a: Gram positive discs:

Antimicrobial agent	Disc content	Sensitive (mm)	Intermediate (mm)	Resistance (mm)
Erythromycin	5ug	≥ 23	14-22	≤ 13
Ceftriaxone	30ug	≥ 23	20–22	≤ 19
Gentamicin	10ug	≥ 15	13-14	≤ 12
Cefuroxime	30ug	≥ 30	15–22	≤ 14
Cloxacillin	5ug	Not stated	Not stated	Not stated
Ofloxacin	5ug	≥ 18	15-17	≤ 14
Ceftazidime	30ug	≥ 21	18–20	≤ 17
Augmentin	20/10ug	≥ 18	14–17	≤ 13

Table 4b: Gram negative discs:

Antimicrobial agent	Disc content	Sensitive (mm)	Intermediate (mm)	Resistance (mm)
Nitrofurantoin	300ug	≥ 17	15–16	≤ 14
Ampicillin	10ug	≥ 17	14–16	≤ 13
Ceftazidime	30ug	≥ 21	18–20	≤ 17
Cefuroxime	30ug	≥ 30	15–22	≤ 14
Gentamicin	10ug	≥ 15	13-14	≤ 12
Ciprofloxacin	5ug	≥ 21	16-20	≤ 15
Ofloxacin	5ug	≥ 16	13-15	≤ 12
Augmentin	20/10ug	≥ 18	14–17	≤ 13

Table 5: Comparison of the Inhibition Zone Diameter of Antibiotic Discs against Gram-negative Organisms Isolated from Solid and Liquid Herbal Products.

Antibacterial Agent	Form	Zones of Inhibition Mean ± SEM	P- value
Nitrofurantoin	Solid	18.05 ± 0.51	< 0.05
	Liquid	23.80 ± 0.56	
Ampicillin	Solid	0.50 ± 2.32	>0.05
	Liquid	0.00 ± 0.00	
Ceftazidime	Solid	14.48 ± 1.29	>0.05
	Liquid	16.20 ± 2.59	
Cefuroxime	Solid	9.86 ± 1.09	>0.05
	Liquid	14.00 ± 1.94	
Gentamicin	Solid	12.74 ± 0.84	>0.05
	Liquid	13.00 ± 1.80	
Ciprofloxacin	Solid	18.56 ± 1.18	>0.05
	Liquid	19.80 ± 0.78	

Ofloxacin	Solid	16.50 ± 1.06	>0.05
	Liquid	20.73 ± 0.93	
Augmentin	Solid	4.18 ± 0.80	<0.05
	Liquid	10.40 ± 2.34	

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242 **Table 6: Comparative Activity of the Gram-negative Discs against *E. coli, Klebsiella,***
243 ***Proteus and Pseudomonas***

Gram negative disks	Isolated organisms	Mean	± Std. Error	Subset for alpha = 0.05	
				F	Sig.
Nitrofurantoin	*E. coli*	16.12	0.91	11.22	< 0.05
	Klebsiella	20.89	0.60		
	Proteus	18.00	1.04		
	Pseudomonas	22.75	0.70		
	Total	19.11	0.50		
Ampicillin	*E. coli*	0.00	0.00	2.13	> 0.05
	Klebsiella	1.22	0.70		
	Proteus	0.00	0.00		
	Pseudomonas	0.00	0.00		
	Total	0.41	0.23		
Ceftazidime	*E. coli*	9.25	2.03	5.91	< 0.05
	Klebsiella	14.33	2.15		
	Proteus	21.44	0.69		
	Pseudomonas	17.00	3.09		
	Total	14.80	1.15		
Cefuroxime	*E. coli*	4.75	1.41	11.03	< 0.05
	Klebsiella	9.67	1.77		
	Proteus	15.33	1.70		
	Pseudomonas	17.50	0.67		
	Total	10.63	0.97		
Gentamicin	*E. coli*	11.50	1.60	0.84	> 0.05
	Klebsiella	12.59	1.42		
	Proteus	13.17	1.46		
	Pseudomonas	15.25	0.73		
	Total	12.79	0.75		
Ciprofloxacin	*E. coli*	14.50	1.83	3.60	< 0.05
	Klebsiella	22.00	1.72		
	Proteus	18.72	2.09		
	Pseudomonas	20.25	0.55		

	Total	18.79	0.97		
Ofloxacin	*E. coli*	12.96	1.83	4.54	< 0.05
	Klebsiella	19.44	1.47		
	Proteus	17.00	1.89		
	Pseudomonas	21.50	0.47		
	Total	17.28	0.90		
Augmentin	*E. coli*	2.62	1.45	3.60	< 0.05
	Klebsiella	6.00	1.67		
	Proteus	9.17	1.04		
	Pseudomonas	3.50	1.84		
	Total	5.33	0.82		

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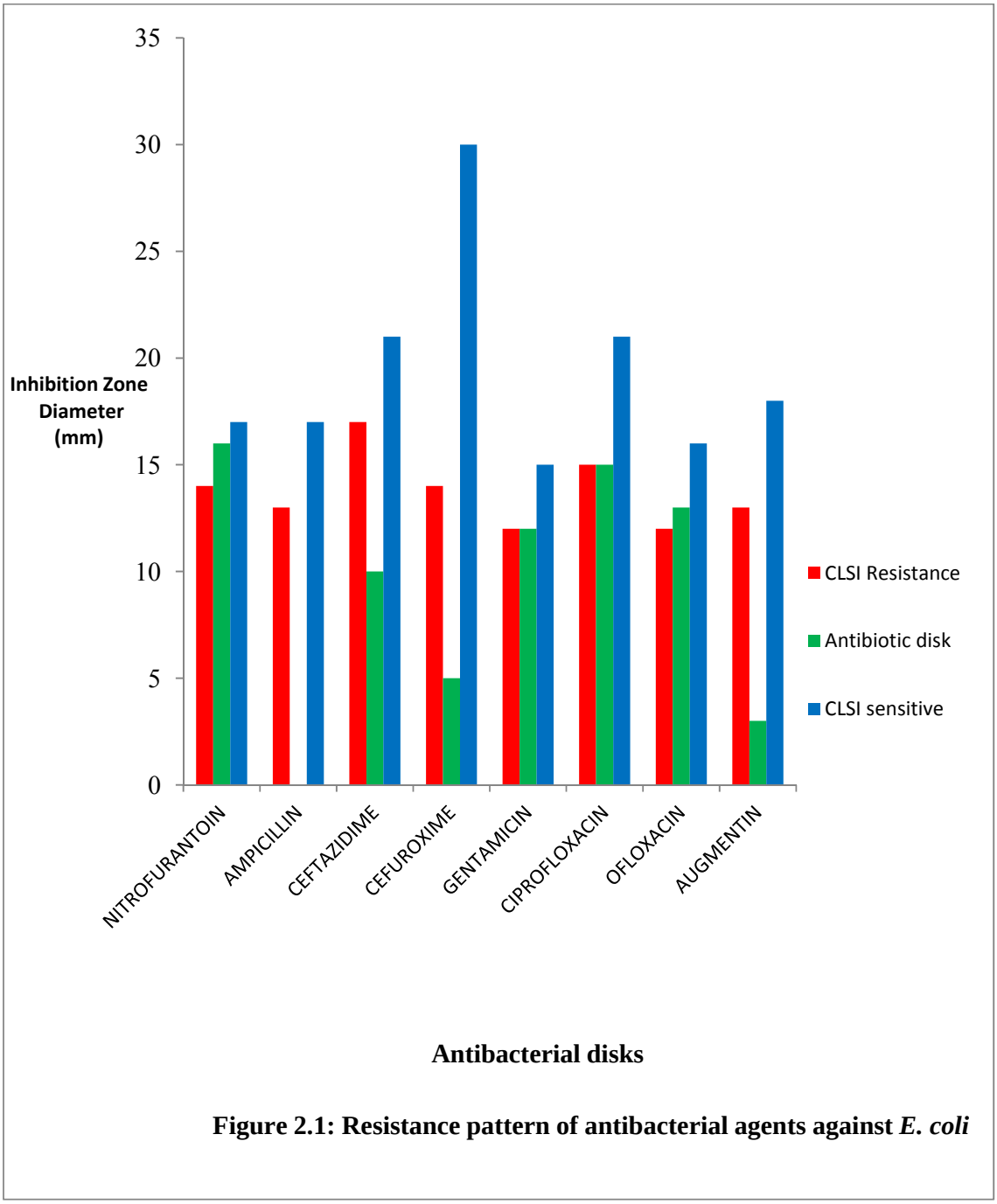

Figure 2.1: Resistance pattern of antibacterial agents against *E. coli*

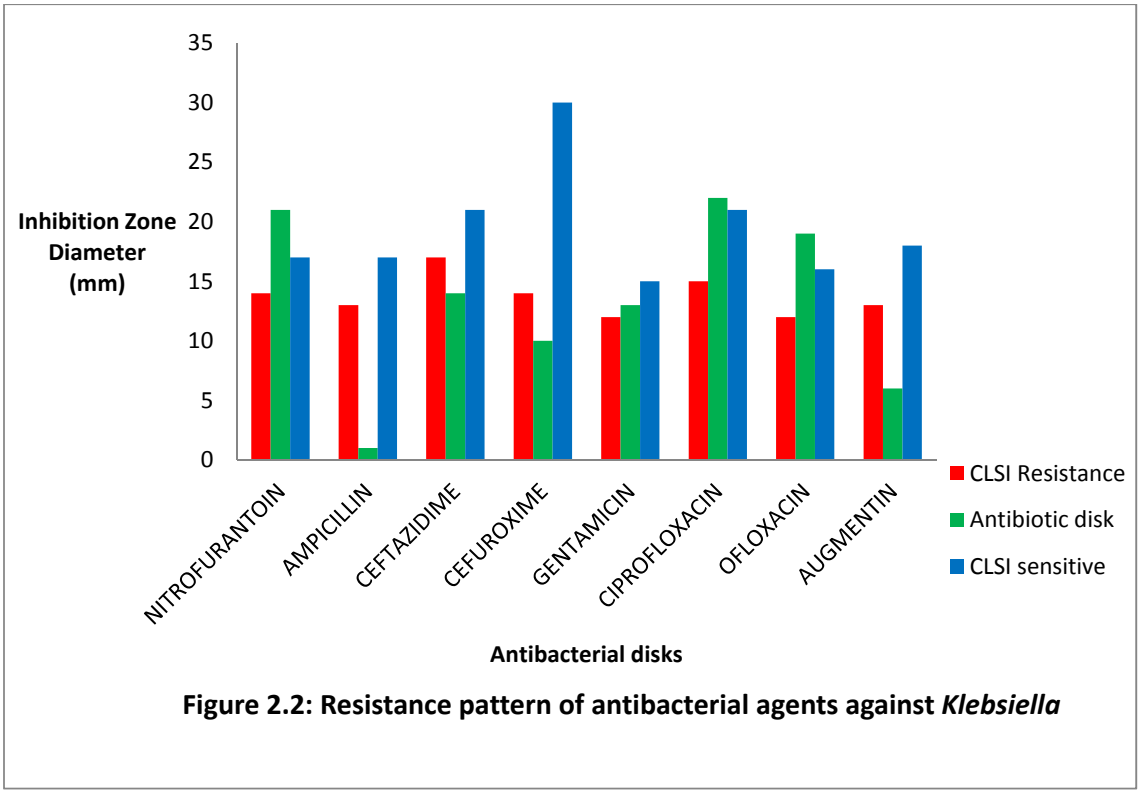

Figure 2.2: Resistance pattern of antibacterial agents against *Klebsiella*

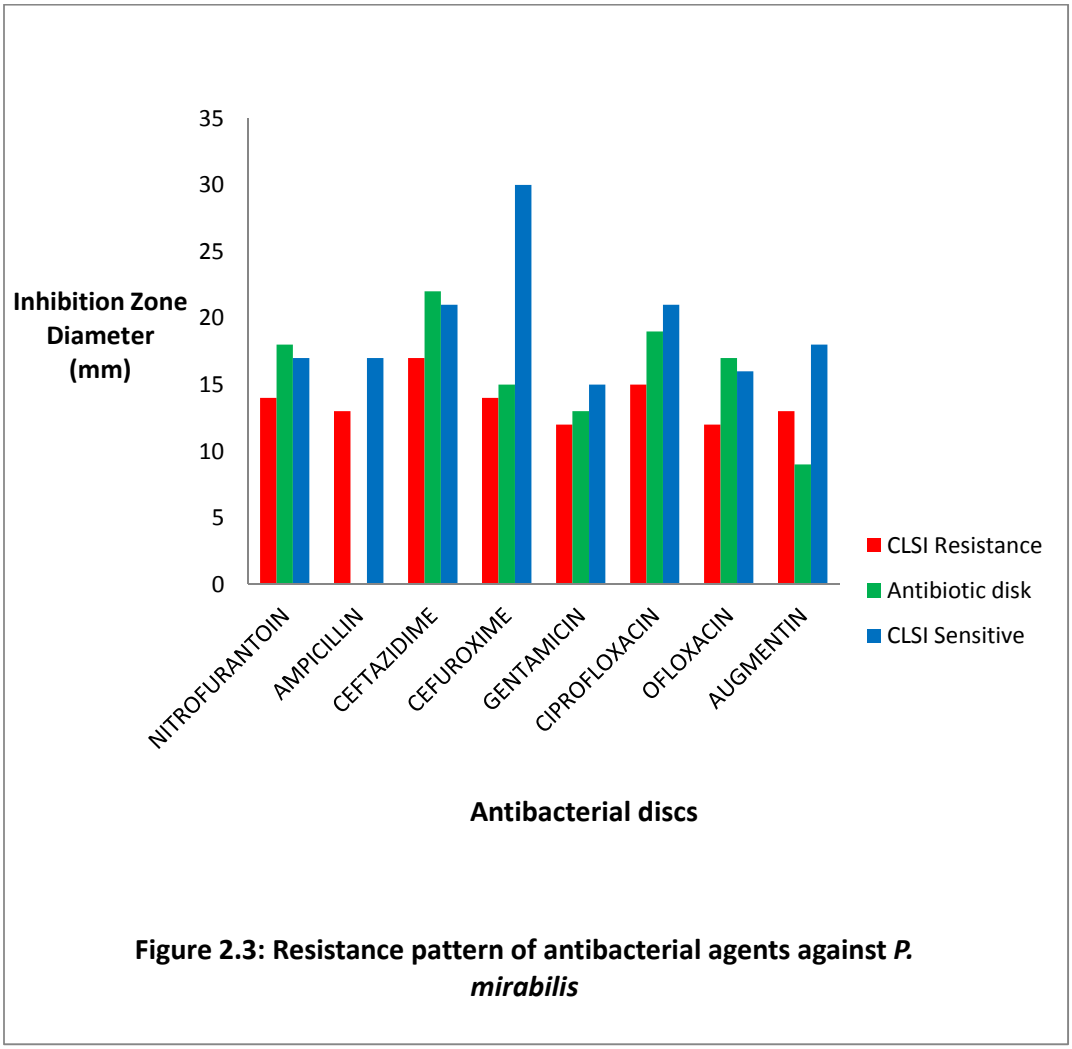

Figure 2.3: Resistance pattern of antibacterial agents against *P. mirabilis*

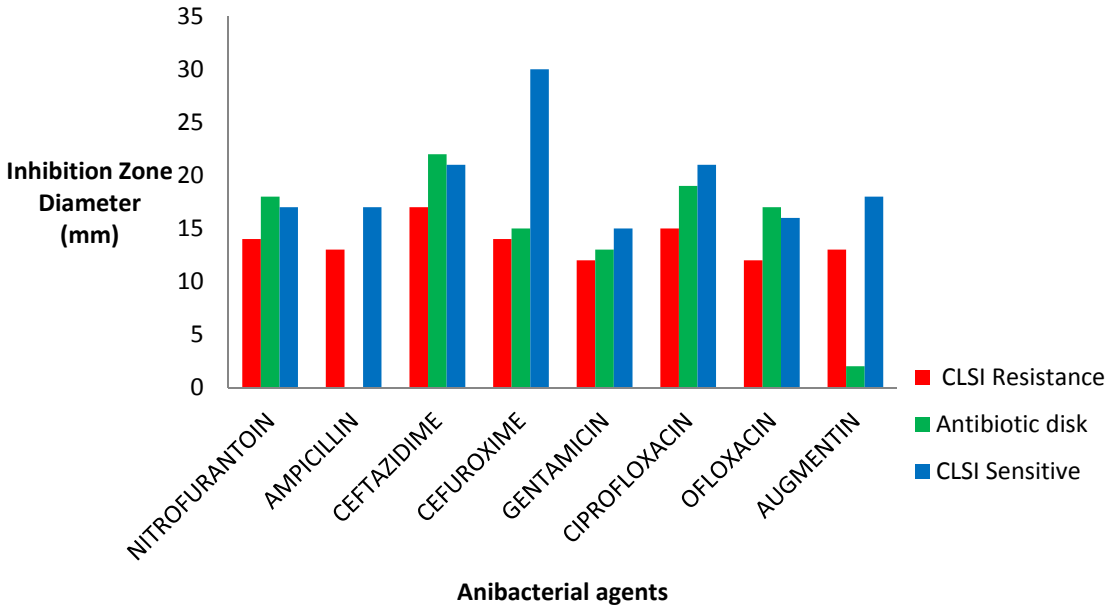

Figure 2.4: Resistance Pattern Of Antibacterial Agents Against *P. aeruginosa*

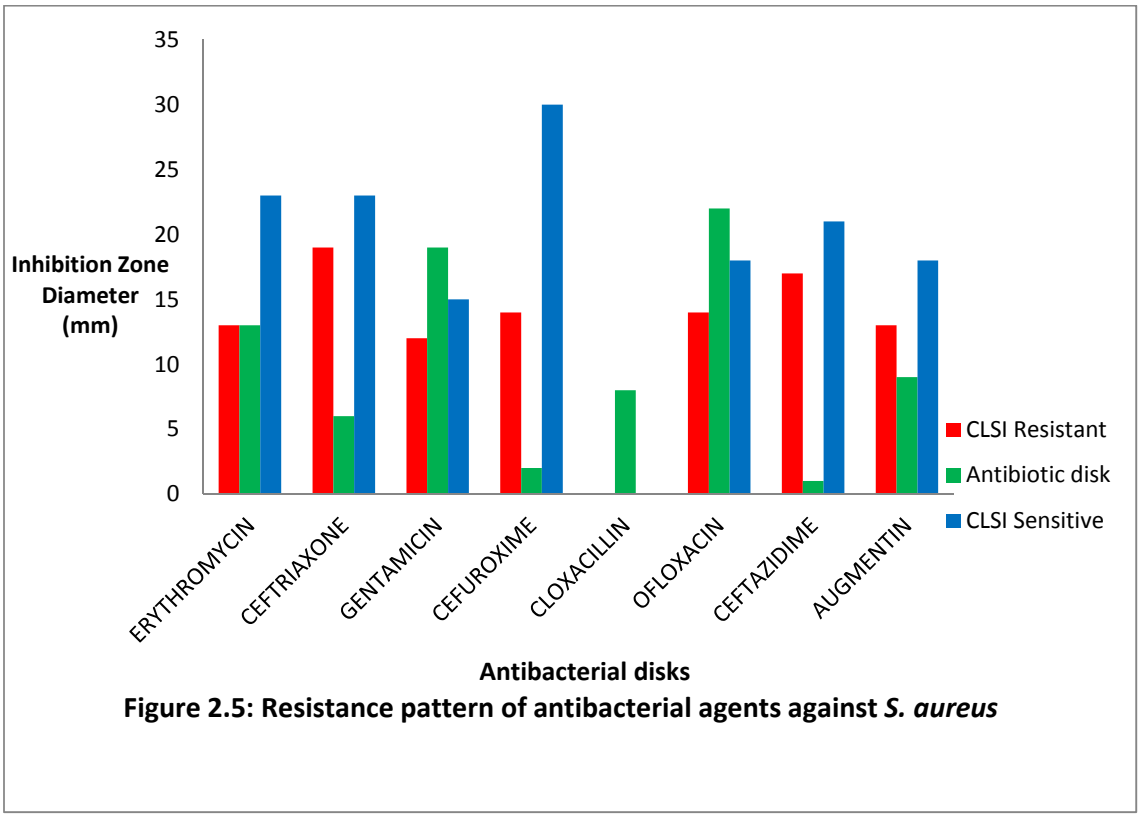

Figure 2.5: Resistance pattern of antibacterial agents against *S. aureus*

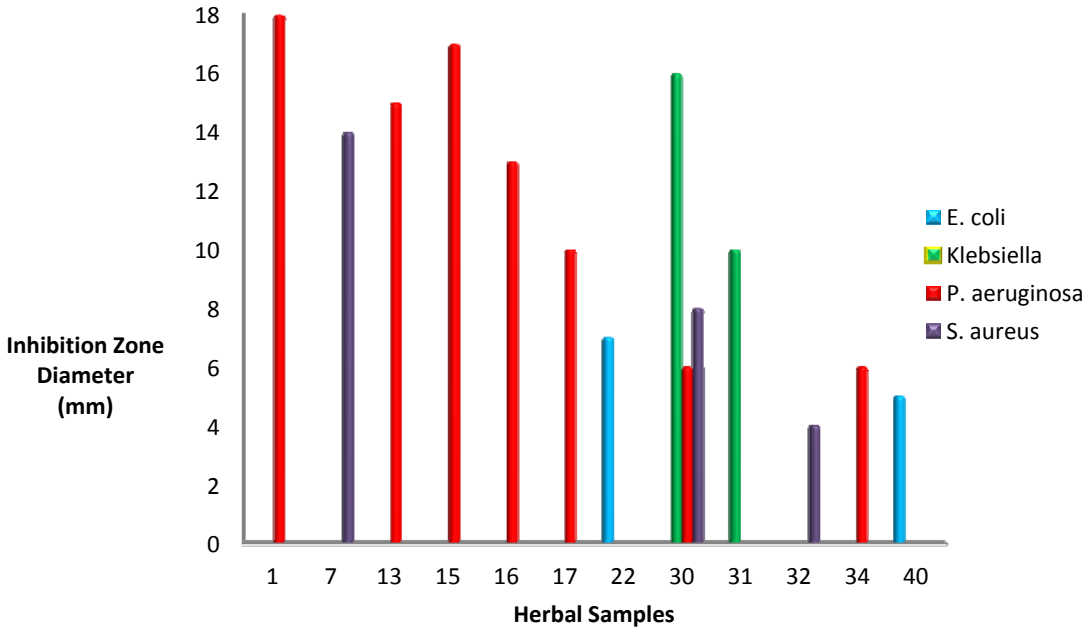

Figure 2.6: In Vitro Inhibitory Qualities Of The Herbal Products Against Selected Clinical Isolates at concentration of 100mg/ml

340 **Table 7: Phytochemical screening of the herbal medicinal product samples.**

Test/sample code	Specific tests	1	2	3	4	5	6	7	8	9	10
Alkaloids	Dragendorff	-	+	+	+	+	-	+	+	+	+
	Meyers	-	+	+	+	+	-	-	+	-	+
	Hagers	-	+	-	+	+	-	-	-	+	+
Tannins	Ferric chloride	-	+	+	+	+	-	+	+	+	+
Phlobatannins	1% hydrochloric acid	-	+	+	+	+	-	+	+	+	+
Anthraquinones	Free Anthraquinones	-	-	-	-	-	-	-	-	-	-
	Combined Anthraquinones	-	-	-	-	-	-	-	-	-	-
Flavonoids	Shinoda	+	+	+	+	+	+	+	+	+	+
	NaOH	+	+	+	+	+	+	+	+	+	+
Carbohydrate	Molisch	+	+	+	+	+	+	+	+	+	+
	Fehling solution	+	+	+	+	+	+	+	+	+	+
Saponin	Emulsion	-	+	+	+	+	+	-	+	+	+
	Frothing	-	+	+	+	-	+	-	+	+	+
	Sodium bicarbonate	+	+	+	-	-	+	+	+	+	+
Cardiac glycoside	Kedde	+	+	+	-	+	-	-	-	-	-
	Lieberman-Burchard	-	-	+	-	+	-	+	-	-	+
	Salkowski	+	+	+	+	-	+	+	+	+	+
	Keller-kiliani	+	+	+	+	-	+	+	+	+	+
Cyanogenic glycoside	Sodium picrate	-	-	-	-	-	-	-	-	-	-

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Test/sample code	Specific tests	11	12	13	14	15	16	17	18	19	20
Alkaloids	Dragendorff	+	+	+	-	+	+	+	+	-	+
	Meyers	+	+	+	-	+	+	+	+	-	+
	Hagers	+	+	-	-	-	+	+	+	-	+
Tannins	Ferric chloride	+	+	+	-	+	+	+	+	-	+
Phlobatannins	1% hydrochloric acid	+	+	+	-	+	+	+	+	-	+
Anthraquinones	Free Anthraquinones	-	-	-	-	-	-	-	-	-	-
	Combined Anthraquinones	-	-	-	-	-	-	-	-	-	-
Flavonoids	Shinoda	+	-	-	-	-	+	+	+	-	+
	NaOH	+	-	-	-	-	+	+	+	-	+
Carbohydrate	Molisch	+	+	+	+	+	+	+	+	+	+
	Fehling solution	+	+	+	+	+	+	+	+	+	+
Saponin	Emulsion	-	-	-	-	+	+	+	+	+	+
	Frothing	-	-	-	+	+	+	+	+	+	+
	Sodium	-	-	-	+	+	+	+	+	+	+

	bicarbonate										
Cardiac glycoside	Kedde	-	-	-	+	+	+	+	+	+	+
	Lieberman-Burchard	+	+	-	+	+	+	+	+	+	+
	Salkowski	+	+	+	+	+	+	+	+	+	+
	Keller-kiliani	+	+	+	+	+	+	+	+	+	+
Cyanogenic glycoside	Sodium picrate	-	-	-	-	-	-	-	-	-	-

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Test/sample code	**Specific tests**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Alkaloids	Dragendorff	-	-	+	+	-	+	+	+	+	+
	Meyers	-	-	+	+	+	+	-	+	+	+
	Hagers	+	-	+	+	+	-	+	+	+	+
Tannins	Ferric chloride	-	-	+	+	-	-	+	+	+	+
Phlobatannins	1% hydrochloric acid	-	-	+	-	-	-	-	-	-	+
Anthraquinones	Free Anthraquinones	-	-	-	-	-	-	-	-	-	-
	Combined Anthraquinones	-	-	-	-	-	-	-	-	-	-
Flavonoids	Shinoda	+	+	+	+	+	+	+	+	+	+
	NaOH	+	+	+	+	+	+	+	+	+	+
Carbohydrate	Molisch	+	+	+	+	+	+	+	+	+	+
	Fehling solution	+	+	+	+	+	+	+	+	+	+
Saponin	Emulsion	+	+	+	-	-	+	+	+	+	+
	Frothing	+	+	+	+	+	+	+	+	+	+
	Sodium bicarbonate	+	+	+	+	+	+	+	+	+	+
Cardiac glycoside	Kedde	+	+	+	-	-	-	+	+	-	-
	Lieberman-Burchard	+	+	+	-	-	-	+	+	+	-
	Salkowski	+	+	+	+	+	+	+	+	+	+
	Keller-kiliani	+	+	+	+	+	+	+	+	+	+
Cyanogenic glycoside	Sodium picrate	-	-	-	-	-	-	-	-	-	-

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Test/sample code	**Specific tests**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
Alkaloids	Dragendorff	+	+	+	+	+	+	+	+	+	+
	Meyers	+	+	+	+	+	+	+	+	+	+

	Hagers	+	+	+	+	+	+	+	+	+	+
Tannins	Ferric chloride	+	+	+	+	+	+	+	+	+	+
Phlobatannins	1% hydrochloric acid	+	-	-	-	-	+	+	-	-	+
Anthraquinones	Free Anthraquinones	-	-	-	-	-	-	-	-	-	-
	Combined Anthraquinones	-	-	-	-	-	-	-	-	-	-
Flavonoids	Shinoda	+	+	+	+	+	+	+	+	+	+
	NaOH	+	+	+	+	+	+	+	+	+	+
Carbohydrate	Molisch	+	+	+	+	+	+	+	+	+	+
	Fehling solution	+	+	+	+	+	+	+	+	+	+
Saponin	Emulsion	+	+	+	+	+	+	+	+	+	+
	Frothing	+	+	+	+	+	+	+	+	+	+
	Sodium bicarbonate	+	+	+	+	+	+	+	+	+	+
Cardiac glycoside	Kedde	+	-	-	+	-	-	-	+	-	-
	Lieberman-Burchard	+	-	-	+	+	-	-	+	+	-
	Salkowski	+	+	+	+	+	+	+	+	+	+
	Keller-kiliani	+	+	+	+	+	+	+	+	+	+
Cyanogenic glycoside	Sodium picrate	-	-	-	-	-	-	-	-	-	-

DISCUSSION

Although herbal preparations are classified by pharmaceutical regulatory agencies as non-sterile pharmaceuticals because of their crude method of preparation, they should not be grossly contaminated by the herbalists whose methods of concocting herbal preparations especially with regards to conditions and environment are generally regarded to be somewhat unhygienic [6].

Plate counting was used to estimate the number of viable cells that are present in a sample while pour plate method was used to analyze microaerophilic bacterial species present in the samples.

Table 1 shows the total mean count of the herbal samples expressed in CFU/ml. Nine (22.5%) of the herbal samples had no growth while 31(77.5%) had growth. When the mean number of colonies counted was compared with the acceptable mean value of finished herbal

products according to the National Policy for Assessments of Herbal Products, 2007, it was seen that 19 (47.5%) out of the 40 herbal products had total mean counts above the recommended level for finished herbal products (10^5 CFU/ml). Contamination by microorganisms is influenced by the environment, improper handling and storage of medicinal plants [8, 9].

The most predominant bacterial isolate from the herbal preparations in this study was *Staphylococcus aureus* (40%) followed by *Klebsiella* (20%), *Escherichia coli* (17.8%), *Proteus mirabilis* (13.3%) and *Pseudomonas aeruginosa* (8.9%). This finding is in agreement with that reported by Esimone *et al.*,[6] on herbal products purchased from herbalists in Edo State, Nigeria. Table 2 shows the incidence of isolated organisms in solid and liquid herbal samples. It was observed that *S. aureus* and *Pseudomonas aeruginosa* had higher incidence in solid than in liquid samples while *E.coli* and *Klebsiella* had higher occurrence in liquids and *Proteus* was isolated only from the liquid samples.

The result of the antimicrobial susceptibility test of the bacteria isolated from the herbal products showed that *E.coli* was resistant to most of the antibiotics including the cephalosporins, the penicillins, and Gentamicin. The Gram-positive contaminant (*S. aureus*) isolated from these herbal products showed relatively high resistance to the Penicillins and cephalosporins such as Amoxicillin - clavulanic acid, Ceftriaxone, Cefuroxime and Ceftazidime. The Gram-negative isolates demonstrated high level of resistance to the penicillin derivatives which suggests that they could be penicillinase producers Resistance to cephalosporins especially cefuroxime and ceftazidime was also observed and this result agrees with that reported by Esimone *et al.*,[6]. The presence of multiple resistant bacteria in the herbal preparations could result in transfer of antibiotic-resistant traits to hitherto sensitive strains [6].

All the herbal samples used in this study claimed to have antibacterial activities. The in-vitro confirmatory test in this study showed that only 13 out of 40 herbal samples (32.5%) with acclaimed antibacterial activities truly had inhibitory properties at a concentration of 100mg/ml. This poor percentage of samples demonstrating antibacterial activities could be as

a result of suboptimal concentration, improper storage and method of processing. The in vitro conditions could also contribute to the poor antibacterial activity as they may differ from in vivo conditions seen in the biological systems. It could also be that the manufacturers may have labeled their products with false claims to attract potential consumers. The presence of microbial contaminants in non-sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the product and has the potential to adversely affect patients taking the medicines [10, 5].

Statistical analysis was carried out to compare the activity of the Gram-negative discs against isolates from the liquid and solid herbal samples. Except for Nitrofurantoin and Augmentin® which exhibited greater activity against organisms from solid than those from the liquid herbal samples (p value < 0.05), there was no significant difference in the activity of the antibacterial discs against isolates from the solid and liquid samples as shown in Table 5.

Table 6 shows the antibacterial agents that had a significant difference in their activity against the Gram-negative bacterial isolates. Nitrofurantoin and Cefuroxime were most active against *Pseudomonas.* Augmentin®, Ceftazidime and Ofloxacin against *Proteus* and Ciprofloxacin against *Klebsiella.*

Biological activity in plants is attributed to the presence or concentration of various secondary metabolites. It is believed that plants which are rich in a wide variety of secondary metabolites belonging to chemical classes such as tannins, terpenoids, alkaloids and polyphenols are generally superior in their antimicrobial activities [11]. In this study, majority of the herbal samples tested positive for one or more of these secondary metabolites. This suggests that the strength of biological activities of a natural product is dependent on the diversity and quantity of such constituents. The activity of some of the plant extracts on different organisms explains their broad spectrum nature while most of the plant extracts found to have effect on one organism may be due to their narrow spectrum of activity [12]. However, this is not the case seen here. Sample 30 had the broadest spectrum of activity against 3 different organisms but had no exceptional phytochemical result from the other samples as Table 7.

CONCLUSION

The results of this study showed that approximately 50% of the herbal medicinal products on sale in Port Harcourt were grossly contaminated with pathogenic microbes resistant to commonly prescribed antibacterial agents. In both the liquid and solid herbal samples, the most prevalent bacterium isolated was *Staphylococcus aureus* an organism that has established itself as a superbug. *E.coli*, another dangerous pathogen, was also resistant to most of the commonly used antibiotics. Only 13 out of the 40 herbal samples (32.5%) with acclaimed antibacterial activities truly had inhibitory properties at a high concentration of 100mg/ml. Herbal medicine practitioners have been known to organise massive annual herbal fairs in Port Harcourt that attracted practitioners from all parts of the country as well as members of the public who come out in their numbers to patronise them. During such fairs, they advertise their products freely on air with claims of unsubstantiated efficacy in several disease conditions that attract the unsuspecting public. In Nigeria, many consumers have ignorantly come to accept the presence of any NAFDAC registration number [13] on any product as a mark of approval of its quality and therefore purchase such products with confidence. The findings of this study reveal, however, that even the presence of NAFDAC registration number on all the selected samples used did not guarantee the safety of the herbal samples as a significant number of the samples were grossly contaminated beyond acceptable limits. This state of affairs constitutes grave danger to public health with the potential to further aggravate the problem of antimicrobial resistance. All statutory regulatory authorities saddled with this responsibility and other stake holders should work together to sanitize the system and safeguard the health of the public. The need for proper training and education of the practitioners and the continuous development and standardization of herbal medicines practice in Nigeria is more urgent than ever.

Ethical approval and consent: NA

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