

ANTIBACTERIAL PROPERTY OF SOME LIQUID HERBAL ANTI-INFECTIVES MANUFACTURED AND MARKETING IN SOUTH-EASTERN NIGERIA.

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Abstract

The present work strives to assess the antibacterial property of liquid herbal anti-infective manufactured and marketed in South-Eastern Nigeria. Bacteriostatic and bactericidal activities of the liquid herbal anti-infective preparations as well as antibiotic susceptibility test were conducted on the selected clinical isolates using Disc diffusion method. The presence of conventional antibiotics in these herbal preparations was assessed using High Performance Liquid Chromatography (HPLC) assay. Maximum inhibitory dilution (MID) of the most active herbal preparations was also obtained. Only 20% of these herbal products showed very significant activity across all the strains of the test organisms. Kill kinetic experiment showed that these herbal preparations had bactericidal activity. The MID result showed that at higher dilutions the herbal preparations had high (against strains of *Staphylococcus aureus*) or little antibacterial activity against other species. HPLC-DAD assay revealed absence of conventional antibiotics. The Multiple Antibiotic Resistance Index (MARI) evaluated on the species of the isolated bacteria revealed their high exposure to an antibiotic environment. Comparison of the antibacterial activity of the herbal anti-infectives with the selected conventional antibiotics yielded $F=294.961$ at $p < 0.05$, for the herbal and $F = 780.847$ at $p < 0.05$, for the conventional. Thus results revealed that herbal anti-infective preparations exhibited higher antibacterial activity than the conventional.

Keywords: Herbal Anti-infectives, Antibacterial Property, Bacteriostatic, Bactericidal, Susceptibility.

1.0 Introduction

Herbal medicine has been used extensively in disease treatment for thousands of years for their easy accessibility and restricted side effects (Padmavatti, 2013).

Traditional medicines that are utilized by 80% of the population have compounds derived from herbal plants (Arukumar and Muthuselvan, 2009). The medicinal values of these plants are usually due to the presence of phytochemical content as stated by (Essien *et al.*, 2012) and the most important of these phytochemicals include alkaloids, tannins, flavonoids and phenolic compounds. Medicinal plants are sources for varieties of natural antioxidants and are used for the treatment of diseases throughout the world (Rafieian-Kopaie and Baradarant, 2013). Medicinal plants, or their extracts, have been used in the prevention and treatment of several chronic diseases such as cardiovascular diseases, inflammatory diseases, arthritis,

diabetes, and others as reported by Juhas *et al.* (2008). The use of plant resources mainly for herbal preparations, forage, and food in Nigeria represents a long history of human interaction with the environment; there in vitro and in vivo properties to pathogenic microorganisms have been widely reported (Okafor, 2001; Iwalokun, 2004; Hashish, 2003). With this increased usage, the quality of these medicines has been a serious concern for health authorities and professionals (Oluyeye and Adelabu, 2010). In developing African countries, traditional medicine has always been viewed as an integral part of their culture as some plants possess important therapeutic properties even though this form of medicine is not well organised as, for example, in India and China (Ogunsheet *et al.*, 2006; Sami *et al.*, 2013). Nowadays, it is estimated that plant materials are present in (or have provided) the models relatively for 50 % western drugs (Rodders 1996). However, the use of herbal medicines has come under scrutiny due to their perceived long term toxicity among other considerations. The causes of the toxicities, which could be attributed to the chemical and mineral contents of various plants, are also linked to the source of the material (Lekouchet *et al.*, 2001).

2.0 Methods

The sample size was determined by cumulative distribution function; X_i , $i = 1, 2, \dots, n$ were independent observations taken from a normal distribution with unknown mean μ and known variance σ^2 . Two hypotheses were considered; namely, a null hypothesis: $H_0: \mu = 0$ and an alternate hypothesis: $H_1: \mu = \mu^*$

2.1 Determination of the Anti-bacterial Activity

2.1.1 Standardisation of Inoculums

Few colonies were taken from overnight culture of the inoculums of different strains of clinical isolates (test organisms) and the isolates from the herbal products (from nutrient agar slants), suspended in sterile saline. The turbidity of the suspension (bacterial dilution) was adjusted using spectrophotometer to conform to the 0.5 McFarland standards.

2.1.2 Agar Well Diffusion Method

Exactly 0.2 ml of the standardised suspension of different strains of clinical isolates of *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia* was aseptically seeded into labelled Petri dishes. This was followed by pouring of sterile molten nutrient agar (20 ml) that was cooled to about 45°C. It was gently swirled so as to ensure homogeneous mixture and allowed for solidification. After solidification, sterile cork borer (6mm) was aseptically used to make holes; about 0.1 ml each of the test solution (herbal preparations) of different concentrations (two-fold dilution) was introduced into the wells. They were allowed on the bench for pre-diffusion, and then incubated aerobically for 18-24 h at 37°C. Control was not left out as one of the wells contains sterile water. The plates were observed for inhibition zone diameter (IZD), which indicates the degree of susceptibility of the test organisms. They (IZD) were measured with a meter rule and compared with the control.

2.1.3 Determination of Bactericidal Activity using Kill Kinetics

A known volume of each sterile nutrient broth (according to the Maximum Inhibitory Dilution) were dispensed into six culture tubes (labelled 0hr to 5hrs); this was followed by a known volume of standardised inoculums (overnight broth culture) of the test organisms. Also an appropriate volume of each of the different herbal preparations that showed significant antibacterial activity by agar diffusion method were added to the culture tubes to serve as the reaction mixture. They were well mixed and held at room temperature (28±2°C). Nutrient agar media (double strength) were aseptically prepared to serve as recovery media of

any survival organism from the reaction mixture. Each tube was serially diluted (10-fold) up to 10^{-5} . Bactericidal activity was determined by plating the suspension (reaction mixture) at every one hour, up to five hours, using drop method (Miles and Misra). The plates were left on the bench for proper diffusion before incubation at 37°C for 18 – 24hrs. Thereafter, colony forming units were counted.

2.2 High Performance Liquid Chromatography-DIODE Array Detection (HPLC-DAD).

The samples were first freeze-dried to obtain a constant weight. Each of the samples (2mg) was then reconstituted with 2ml of HPLC grade methanol. The mixture was sonicated for 10 minutes, followed by centrifugation at 3000 rpm for 5 minutes. Then 100 μL of the dissolved samples were transferred into HPLC vials containing 500 μL of the HPLC grade methanol. The HPLC-DAD analysis was carried out on the samples with Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germany); detection was at 235, 254, 280, and 340 nm. The separation column (125mm x 4mm, length x international diameter) was pre-filled with euosphere-10C18 (Knauer, Germany) and a linear gradient of nanopure water (adjusted to pH2 by addition of formic acid) and methanol was used as eluent. The absorption peaks for each sample were analysed by comparing with those in the HPLC-Ultraviolet (UV) visible database which contains over 1600 registered compounds.

2.3 Determination of Multiple Antibiotic Resistance Index (MARI) of the Test Bacteria.

Seven antibiotics (ofloxacin, ciprofloxacin, tetracycline, gentamicin, ceftriazone, sulphamethoxazole, perfloxacin) were used. The formula, $\text{MARI} = a/b$, where 'a' represents aggregate resistance of antibiotic to all isolates, while 'b' represents the total number of antibiotics that was used.

Data Analysis

Data were analysed using Statistical Package for Social Sciences (SPSS – computer software package) and expressed as mean values \pm [Standard error of Mean] of three replicates. Analysis of variance (ANOVA) was used to determine the statistical significant difference in inhibition zone diameter (IZD) between the herbal anti-infectives and the conventional antibiotics.

3.0 RESULT & DISCUSSION

The results of the microbial load of the different herbal anti-infectives are presented in Table 1 below. The mean bacterial count of the different herbal sample products ranged from 2.5×10^4 to 1.75×10^5 CFU/ml.

Table 1: Mean Bacterial and Fungal Count Materials and Methods

Bacterial Count in CFU/ml					Fungal Count CFU/ml			
Code	Plate 1	Plate 2	Plate 3	Mean \pm SEM	Plate 1	Plate 2	Plate 3	Mean \pm SEM
1	2	2	2	$1 \times 10^4 \pm 0.00$	0	0	0	-
2	5	5	5	$2.5 \times 10^4 \pm 0.00$	0	0	0	-
3	0	0	0	-	0	0	0	-
4	0	0	0	-	0	0	0	-

5	0	0	0	-	0	0	0	-
6	11	11	12	$5.65 \times 10^4 \pm 0.33$	0	0	0	-
7	0	0	0	-	0	0	0	-
8	0	0	0	-	0	0	0	-
9	28	28	27	$1.38 \times 10^5 \pm 0.33$	0	0	0	-
10	0	0	0	-	0	0	0	-
11	17	18	18	$8.8 \times 10^4 \pm 0.33$	0	0	0	-
12	29	30	46	$1.75 \times 10^5 \pm 5.51$	0	0	0	-
13	0	0	0	-	0	0	0	-
14	25	29	20	$1.2 \times 10^5 \pm 2.60$	0	0	0	-
15	0	0	0	-	0	0	0	-
16	0	0	0	-	0	0	0	-
17	0	0	0	-	0	0	0	-
18	0	0	0	-	0	0	0	-
19	5	8	12	$4.16 \times 10^4 \pm 2.03$	0	3	4	$1.16 \times 10^4 \pm 1.2$
20	11	18	26	$9.16 \times 10^4 \pm 4.33$	0	0	0	-

Table 2: Microorganisms Isolated From the Herbal Preparations

The result of identified isolates which consists of eight (8) bacterial genera and one (1) fungal genera from Herbal Preparations shown below in table 2

Sample Code	Identity of Bacteria Isolated	Identity of Fungi Isolated
1	<i>Bacillus popilliae</i>	Nil
2	a) <i>Proteus vulgaris</i> b) <i>Proteus vulgaris</i>	Nil
3	Nil	Nil
4	Nil	Nil
5	Nil	Nil
6	a) <i>Pseudomonas aeruginosa</i> b) <i>Vibrio orientalis</i> c) <i>Lactobacillus debruecki</i>	Nil
7	Nil	Nil
8	Nil	Nil
9	a) <i>Staphylococcus aureus</i> b) <i>Micrococcus varians</i>	Nil

10	Nil	Nil
11	a) <i>Vibrio orientalis</i> b) <i>Proteus vulgaris</i> c) <i>Pseudomonas fluorescense</i>	Nil
12	a) <i>Pseudomonas aeruginosa</i> b) <i>Pseudomonas fluorescense</i> c) <i>Bacillus populliae</i> d) <i>Micrococcus luteus</i>	Nil
13	Nil	Nil
14	a) <i>Bacillus populliae</i> b) <i>Pseudomonas aeruginosa</i>	Nil
15	Nil	Nil
16	Nil	Nil
17	Nil	Nil
18	Nil	Nil
19	a) <i>Corynebacterium xerosis</i> b) <i>Corynebacterium xerosi</i>	<i>Microsporum canis</i>
20	a) <i>Bacillus sphaericus</i> b) <i>Corynebacterium kutscheri</i>	Nil

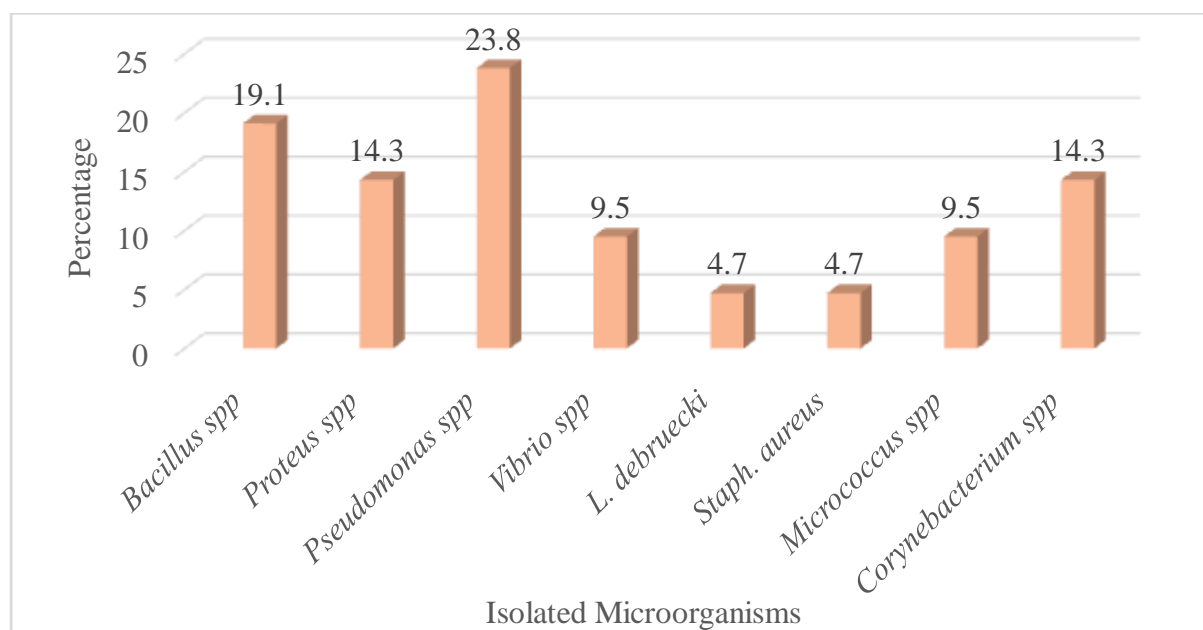


Figure 1: Percentage of Bacteria Species Isolated from the Herbal Anti-Infective

Table 3: Antibiotic Susceptibility Profile of the Isolated Organisms from the Herbal Anti-infective Products

Isolates	Inhibition Zone Diameter of Drugs (mm)									
	OFX	CIP	CN	CRO	SXT	PEF	AMC	S	SP	E
Ba1	20±0.33	23±0.58	10±0.00	6±0.33	4±0.00	22±0.33	0±0.00	18±0.33	18±0.33	10±0.00
Ba12	16±0.00	0±0.00	12±0.33	14±0.33	0±0.00	14±0.67	0±0.00	0±0.00	20±0.00	0±0.00
Ba14a	18±0.00	20±0.33	14±0.33	9±0.33	0±0.00	22±0.33	8±0.00	20±0.00	18±0.33	15±0.33

Ba20	18±0.33	26±0.33	12±0.33	7±0.33	0±0.00	24±0.00	0±0.00	18±0.33	18±0.00	10±0.33
P2a	22±0.00	24±0.00	22±0.67	0±0.00	0±0.00	22±0.00	15±0.00	15±0.33	23±0.00	11±0.33
P2b	31±0.67	25±0.58	26±0.00	22±0.33	27±0.67	30±0.58	4±0.33	24±0.00	24±0.33	18±0.33
P11	22±0.33	34±0.33	28±0.00	22±0.00	31±0.67	15±0.33	6±0.33	25±0.33	32±0.00	26±0.33
Ps6	16±0.33	20±0.00	16±0.33	26±0.58	24±0.33	14±0.00	0±0.00	21±0.58	14±0.33	10±0.33
Ps12a	27±0.58	34±0.00	19±0.33	15±0.58	16±0.00	31±0.67	0±0.00	30±0.00	31±0.33	0±0.00
Ps12b	26±0.33	32±0.33	15±0.58	10±0.00	8±0.33	28±0.00	0±0.00	21±0.33	26±0.00	0±0.00
Ps14b	19±0.33	16±0.58	12±0.00	20±0.00	0±0.00	14±0.00	0±0.00	32±0.33	14±0.33	0±0.00
V6	17±0.00	12±0.33	18±0.00	0±0.00	26±0.00	12±0.00	0±0.00	22±0.33	0±0.00	0±0.00
V11	22±0.33	22±0.00	10±0.00	26±0.33	12±0.33	11±0.67	0±0.00	26±0.00	18±0.33	16±0.00
L6	18±0.33	22±0.00	12±0.33	14±0.33	0±0.00	12±0.00	0±0.00	0±0.00	20±0.00	0±0.00
Sa9	24±0.67	29±0.33	20±0.33	10±0.33	0±0.00	23±0.00	16±0.67	20±0.33	21±0.33	11±0.33
M9	22±0.00	30±0.33	4±0.00	20±0.33	0±0.00	15±0.58	11±0.00	18±0.33	20±0.00	10±0.33
M12	25±0.67	32±0.33	20±0.00	32±0.33	20±0.00	28±0.00	18±0.33	32±0.33	20±0.00	5±0.33
C19a	17±0.67	20±0.00	10±0.00	9±0.33	15±0.33	22±0.33	0±0.00	12±0.00	10±0.00	10±0.00
C19b	20±0.00	24±0.33	11±0.67	6±0.00	0±0.00	24±0.33	0±0.00	22±0.33	15±0.00	12±0.00
C20	19±0.00	20±0.00	11±0.67	6±0.33	0±0.00	20±0.00	0±0.00	14±0.33	19±0.33	11±0.58

Ba= *Bacillus* spp, **P** = *Proteus* spp, **Ps** = *Pseudomonas* spp, **V** = *Vibriospp*, **L**= *Lactobacillus* *debrucki*, **Sa** = *Staphylococcus aureus*, **M** = *Micrococcus* spp, **C** = *Corynebacterium*spp. Gentamicin (CN, 10µg), ofloxacin (OFX, 5µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75µg), amoxicillin-clavulanic acid (AMC, 20/10µg), perfloxacin (10µg), ceftriazone (CRO, 30µg), erythromycin (10µg), streptomycin (30µg), sparfloxacin (5µg), and ciprofloxacin (CIP, 5µg).

Table 4: Multiple Antibiotic Resistance Index (MARI) of the Isolated Bacteria

S/N0	Isolates	Multiple Antibiotic Resistance Index (MARI)
1	Ba1	0.8
2	Ba12	0.7
3	Ba14	0.5
4	Ba20	0.6
5	P2a	0.4
6	P2b	0.1
7	P11	0.2

8	Ps6	0.4
9	Ps12a	0.2
10	Ps12b	0.4
11	Ps14	0.6
12	V6	0.6
13	V11	0.4
14	L6	0.6
15	Sa9	0.5
16	M9	0.5
17	M12	0.3
18	C19a	0.6
19	C19b	0.6
20	C20	0.6

Ba= *Bacillus* spp, P = *Proteus* spp, Ps = *Pseudomonas* spp, V = *Vibriospp*, L= *Lactobacillus debrucki*, Sa = *Staphylococcus aureus*, M = *Micrococcus* spp, C = *Corynebacterium* spp.

Multiple Antibiotic Resistance Index (MARI) evaluation of the isolated bacteria (Table 4) revealed that four species of *Bacillus* (Ba1, Ba12, Ba20, Ba14) showed high level of multiple antibiotic resistance to the panel ranged from 0.5 – 0.8 of antibiotics: eight (CN,CRO, SXT AMC, E,S,SP,CIP); seven (CIP, CN, SXT, AMC, S, SP, CRO); six (CN, CRO, SXT, AMC, E, S) and five (CN, CRO, SXT, AMC,E) antibiotics respectively out of the ten used. Three *Proteus* species have MARI values ranging from 0.1 – 0.4, each resistant to four CRO, SXT, AMC, E, one (AMC) and two AMC and PEF antibiotics respectively.

The MARI results of four *Pseudomonas* species ranged from 0.2 – 0.6, with Ps6 and Ps12b being resistant to four antibiotics; CRO, SXT, AMC, E while Ps12a and Ps14b were resistant to (AMC, E) and (SXT, AMC, E, CN, PEF, SP) antibiotics respectively. Two *Vibrio* species (V6 and V11) had their MARI ranged from 0.4 – 0.6, each being resistant to (CRO, PEF, AMC, SP, E, CIP) and (CN, SXT, PEF, AMC) antibiotics respectively. *Micrococcus* species (M9 and M12) had their MARI ranged from 0.3 – 0.5, each being resistant to (CN, SXT, AMC, PEF, E) and (E, AMC, CN) antibiotics respectively. We had three isolates of *Corynebacterium* species and the MARI values are 0.6 each were resistance to (CN, CRO, SXT, AMC, SP, E) antibiotics out of the ten tested.

However, *Lactobacillus* specie and *Staphylococcus aureus* were resistance to (CN, SXT, PEF, AMC, S, E) and (CRO, SXT, AMC, E, S) antibiotics respectively out of the ten antibiotics used in this study. This MARI entails that all the bacterial isolates in one way or the other originated from the environment where these antibiotics were overused (Paul *et al.*, 1997).

Conclusion

The high rate of resistance to antibiotics exhibited by the isolated bacteria may be a very serious indication of widespread antibiotic resistance among bacteria from different sources. Therefore, indiscriminate intake of herbal medicines should be discouraged; also, herbal practitioners should strictly observe the current good manufacturing practices (cGMPs) in their production processes (Esimone *et al.*, 2007a).

In all, the comparison of the antibacterial activity of the herbal products with that of the conventional antibiotics showed that the herbal products recorded higher zones of inhibition than the conventional antibiotics at 95% level of significance.

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Authors Contribution

OCF, UNT, UJ, OAN and ECO developed the manuscript with the statistical analysis involved. OCF and ECO conceived and designed the experiment. OCF performed the experiment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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