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Evaluation of the antibacterial properties of *Picralima nitida* stembark extracts

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Abstract: The antibacterial activity of the alcohol extract of the stembark of *Picralima nitida* was tested against *Staphylococcus aureus* ATCC12600, *Pseudomonas aeruginosa* ATCC 10145, *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 11775, *Salmonella kintambo* Human 1,13,23 mt: and three clinical isolates. The antibacterial assay was by both the agar-well diffusion and macro-broth dilution methods. Separation of the crude extract by column chromatography yielded six fractions of which, all contained alkaloid except F_1. The spectra of activity for the crude extract and active fractions was 50% each, with no apparent activity recorded against the clinical isolates. Fractions F_1 and F_5 exhibited no antibacterial activity. The control drug, Gentamicin had 62.5% spectrum of activity. There was significant difference ($p<0.05$) in antibacterial activity between the crude extract and its fractions, with F_2 exhibiting the highest antibacterial activity. The MIC range for the crude was 25-50 $mgmL^{-1}$; F_2, 3.125-50 $mgmL^{-1}$; F_3, 1.563-50 $mgmL^{-1}$ and F_6, 3.125-50 $mgmL^{-1}$. The combined activity of the fractions showed that the active components might be antagonistic to one another. Both the MBC and kill-rate study show that the partially purified fractions had higher bactericidal effect as compared to the crude extract. These results provide scientific justification for the use of locally distilled alcohol, as solvent for most herbal preparations.

Key words: Picralima nitida, stembark extract, antibacterial activity, ethnomedicine, Nigeria

INTRODUCTION

The earlier preliminary study with *Picralima nitida*, family *Apocynaceae*, (common name: Akuamma plant; Ibo: Osi-Igwe) revealed some antibacterial activity. This result is in line with the plant's widely varied applications in Nigeria folk medicine and claims of some herbalist to have used the leaves, seeds or stembark as treatment for various disorders and diseases.[1-3] The seed, stem and roots have been reported to be effective as a cough suppressant anodyne, as an aphrodisiac and hypoglycaemic agent in treatment of diabetes.[4,5] However, there is apparently no scientific report on the antibacterial properties of the plant especially in the form in which it is administered. The present study was solely designed to authenticate folklore medicinal use of the plant using the traditional solvent (locally distilled alcohol, kai-kai). Thus, the locally distilled alcohol (kai-kai) extracts of the stembark and it's partially purified column fractions were quantitatively evaluated for activity against eight bacterial strains, including four from American Type Culture Collection (ATCC), one stereotype local salmonella strain, and three clinical isolates. This study part of a comprehensive project to evaluate the therapeutic potentials of Nigerian herbs with a view to conserving and developing them, and exploring their potential to application in orthodox medicinal practice.

MATERIALS AND METHODS

Collection and identification of plant material: Fresh stembarks of *P. nitida* were obtained from a local herbalist at Egbelubi-Eziama in Imo State, southeastern Nigeria. A. O. Ozioko of the Department of Botany, University of Nigeria, Nsukka, taxonomically authenticated the plant and a voucher specimen was deposited in the departmental herbarium.

Preparation of extracts: A 100-g amount of the pulverized dried stembark was continuously extracted with local alcohol (the solvent mostly used in traditional herbal preparations in eastern Nigeria) in a soxhlet extractor for 2½ h and the solvent distilled off in the rotatory evaporator. The extract was then poured into a weighed flask and further dried in a desiccating chamber to a constant weight. The dried extract was exposed to UV. rays for 24 hours and checked for sterility by streaking on nutrient agar plate.

Separation and partial purification of extract: Two fractionation techniques were used, namely: Thin Layer Chromatography (TLC) and column chromatography. The alcohol stembark extract (crude) of *P.nitida* was first separated by Thin Layer Chromatography

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(Analytical TLC) on silica gel G_{60} using different solvent systems; local alcohol alone, alcohol/chloroform (1:2), alcohol/acetic acid (1:1), n-hexane/alcohol/acetone (1:1:1), and alcohol/acetone (5:1). This was to determine the best solvent for the column chromatography[6] . After each separation, the TLC plates were exposed to iodine fumes in an iodine chamber to visualize the spots. The solvent system that gave the best resolution was used for the column chromatography.[23]

The column separation of the extracts was carried out with a glass column of internal diameter 40 mm and length of 1.0 m. A 250-g of silica gel 60, particle size 0.063 0.200mm (EM SCIENCE) was wet-packed using the alcohol/acetone (5:1) solvent system determined from the TLC. After settling, the alcohol extract (6.25 g) was redissolved in the alcohol and gradually poured down the side of the glass containing the column. The extract was allowed to drain completely into the gel before the eluent alcohol/acetone (5:1) was continuously used to elute the stationary phase. Then 10 mL fractions (aliquots) were collected; each subjected to thin layer chromatography. The fractions showing similar TLC mobility were pooled in a beaker and the solvent evaporated under a steady air current in a fume cupboard.[6] The yield and retention factors (R_f) were determined.

Phytochemical screening: The dried extracts (crude and it's column fractions) were first reconstituted in the respective solvents used for their extraction and then tested by standard phytochemical method for the presence of alkaloid, flavonoid, tannin, saponin, glycosides and protein.[6,8]

Test bacterial strains: Clinical isolates of *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa* and locally isolated typed strain of *Salmonella kintambo* (Human, 1,13,23: mt:-) were collected from the Department of Veterinary Microbiology and Pathology, University of Nigeria, Nsukka. Standard typed cultures of *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), and *Staphylococcus aureus* (ATCC 12600) were obtained from Bioresources Development and Conservation Project (BDCP), Nsukka. Re-isolating three times in Mueller Hinton agar (Oxoid) purified each test bacterial strain and identity confirmed by standard bacteriological methods.[9,10].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The MIC for the crude and fractions of the alcohol extract were determined by two test methods, a modified agar-well diffusion method[11]and a modified macro-broth dilution technique.[12] In the agar-well diffusion technique, A two-fold serial dilution of the extracts were prepared by first reconstituting in 20% DMSO then diluting in sterile distilled water to achieve a decreasing concentration range of 50 $mgmL^{-1}$ to 0.781 $mgmL^{-1}$. A 100-il volume of each dilution was introduced in triplicate wells into MHA plates already seeded with the standardized inoculum ($5x10^5$) of the test bacterial cells. All test plates were incubated at 37°C for 24 h. The least concentration of each extract showing a clear zone of inhibition was taken as the MIC. In the macro broth dilution technique, a two fold serial dilution of the reconstituted extract was prepared in Mueller Hinton Broth (MHB). Each dilution was seeded in triplicates with 100- µl of the standardized suspension of the test bacterial strain to achieve a final concentration of $5x10^5$ cfu/mL. All cultures tubes were incubated at 37°C for 18 24 h. Then each tube was plated on solid MHA plates and incubated for 24 h at 37°C. The least concentration showing no growth on the MHA plates was taken as the MIC.

The MBC was taken as the least concentration showing no growth after 48 h plating on MHA, as in the macro-broth MIC determination above. From the MICs obtained by the two techniques, MIC indices were determined. Similar determinations of the MIC, MBC and MIC-MBC indices were done for the column fractions and the control antibiotic (Gentamicin).

Test for combined activity of fractions: A total of five different combinations of the fractions were tested against the susceptible bacterial strains at a 50 $mgmL^{-1}$ concentration. The combinations were, 1 F_2; F_3; F_4 and F_6, 2;F_2; F_3 and F_6, 3;F_2 and F_6, 4;F_2 and F_3, and 5;F_3 and F_6. The agar well diffusion method was used and the inhibition zone diameter measured to the nearest mm.

Effect of autoclaving and chilling on antibacterial activity of extract: The crude and fractions F^2 and F^3 were used for this study. The extracts were autoclave at 121°C for 15 minutes; refrigerated at 4°C for 18 hours or incubated at room temperature for 24 h. All extracts were allowed to return to ambient (room) temperature before testing for antibacterial activity using the agar well diffusion technique.

Determination of rate of bacterial cell killing by the extracts: The assay of cell death time of crude and its column fractions F^2 and F^3 against *Staphylococcus aureus* ATCC 12600 were by the macro-broth dilution method[13]. The dried extracts were aseptically reconstituted in 20% DMSO and diluted in sterile water

for each experiment. The extracts were sufficiently diluted such that aliquots of 1.0 mL each, added to 9 mL of MHB culture with a concentration of 10^8 cfu per mL, achieved a concentration of 50 mgmL^{-1} for the crude and 20 mgmL^{-1} each for fractions F_2 and F_3. Two sets of control tubes were included for each experiment. One set was seeded with the test bacterium but contained no extract. The other contained 80-µg/mL^{-1} of Gentamicin (Gentalek^R). All cultures were incubated in shaker water bath at 37°C and samples were withdrawn at 30 minute interval up to 120 minutes. Each sample was diluted in ten-fold series of sterile normal saline and 0.1 mL (100 µl) of each dilution spread plated in triplicates in nutrient agar. After incubation for 24 h at 37°C, the bacterial colonies were counted and compared with the control. The results were expressed as negative or positive $\log_{10}$ values[14].

RESULTS

Table 1 shows the yield of the crude extract as a proportion of the pulverized plant material, and its column fractions as a proportion of the dried crude extract used in the column chromatography. The column chromatography of the crude extract of the stem-bark gave six fractions F_1 to F_6. F_2 gave the highest yield (46.4%), followed by F_1 (22.4%) and F3 (9.6%). The least yield was recorded with F_5 (1.6%). The retardation factor (R) of the fractions showed a range of 0.20-0.96, with F_1 having the highest value and F_6 the least value. F_2 and F_4 contained two components each with R_f values as 0.47, 0.0.34 and 0.54, 0.38, respectively.

The constituents compounds detected by the phytochemical analysis of the crude extract and its column fractions (Table 2). All fractions, except F contained alkaloid. Glycolipids were detected in the crude, F_1, F_2 and F_6 fractions. Saponin and protein were present in the crude, F_1, F_2 and F_3. Steroids, carbohydrate, tannins and terpenoids were detected in the crude, F_1 and F_2. Flavonoids were only present in the crude and F_1. All the fractions, including the crude contained no cyanogenic or cardiac glycosides.

Table 1: Yield of crude extract and fractions

Extract	Percentage yield*	R_f value†
Crude	7.25	-
F_1	22.4	0.96
F_2	46.4	0.47, 0.34
F_3	9.6	0.48
F_4	3.2	0.54, 0.38
F_5	1.6	0.34
F_6	9.44	0.20

*Yield of fractions are expressed as a proportion of the dried extract used in the column chromatograph, while yield of crude extract as a proportion of the pulverized stembark.† R_f =distance of compound from origin over the distance of solvent front from origin

Table 2: Results of phytochemical analysis of crude extract and fractions

Secondary metabolites	Crude	F_1	F_2	F_3	F_4	F_5	F_6
Alkaloids	+	-	+	+	+	+	+
Cyanogenic glycoside	-	-	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-	-	-
Glycolipid	+	+	+	-	-	-	+
Saponin	+	+	+	+	-	-	-
Steroids	+	+	+	-	-	-	-
Carbohydrate	+	+	+	-	-	-	-
Tannins	+	+	+	-	-	-	-
Terpenoids	+	+	+	-	-	-	-
Protein	+	+	+	+	-	-	-
Flavonoids	+	+	-	-	-	-	-

+ Present
- Not detected

The highest spectrum of activity against the test bacterial strains were recorded for the crude F_2, F_3, F_4 and F_6, with 50% each of the test bacterial strains susceptible to the extracts. The control drug Gentamicin (Gentalek^R) was active against 62.5% of the test bacterial strains (Table 3). The crude extract as well as the fractions, F_2, F_3, F_4 and F_6 showed activity against all the standard type cultures with the exception of *Escherichia coli* ATCC 11775. Fractions F_1 and F_5 showed no activity against the test bacterial strains. Both the crude extract and its fractions exhibited no antibacterial activity against the local clinical isolates. The crude extract and its fractions exhibited significant difference ($p<0.05$) in activity against the test bacterial strains. F_2 and F_4 generally gave the best activity. The activity of the crude extract was compared to the pure analytical ethanol (BDH) extract. The result showed no significant difference ($p>0.05$) in antibacterial activity between the extracts (Table 3).

Ciproxin had the highest spectrum of activity (87.8%), while Rifampin and Amoxil were not sensitive to the bacteria strains (Table 4).

The corresponding MIC values determined by the two test methods (agar-well diffusion and macro-broth dilution methods) were not significant for the crude as compared to its fractions (Table 5).

Combinations 1,4 and 5 had activity to at least one of the test bacterial strains while 2 and 3 exhibited no antibacterial activity (Table 6).

Storage at room temperature did not affect the activity of the crude, F_2 and F_3 likewise autoclaving, with the exception *Ps. aeruginosa* ATCC 10145 while the chilling affected the activity with the exception if *B.subtilis* ATCC 6051 (Table 7).

Table 8 shows the result of a constant viable cell concentration of 10^8 cfu mL^{-1} exposed to the single concentration of the Crude, F_2, F_3 and control drug, at varied time intervals. The result showed an increasing net reduction in viable cell count through out the duration of exposure time (30, 60, 90, 120 mins) with all the

Table 3: Mean zone of inhibition of crude extract and fractions against the test bacterial strains

Bacterial strains	E - extract*	Crude	F_1	F_2	F_3	F_4	F_5	F_6	Gentamicin(16 µg)
Staph.aureus ATCC 12600	20 ± 1.73	17.0± 0.71	0	22.0± 1.41	16.0± 1.41	24.0± 0.71	0	16.0± 2.83	11.0± 0.71
P. aeruginosa ATCC 10145	20 ± 1.0	15.0± 0.71	0	16.0± 1.41	12.0±1.41	18.0± 1.41	0	20.0± 0.71	0
B subtilis ATCC 6051	16 ± 2.0	14.0± 1.41	0	21.0± 1.41	22.0± 0.71	17.0± 0.71	0	17.0± 1.41	24.0± 0.0
E. coli ATCC 11775	14 ± 1.73	0	0	0	0	0	0	0	0
Sal. kintambo (Human 1323)	25 ± 2.65	21.0± 1.41	0	14.0± 0.71	16.0± 0.71	15.0± 1.41	0	16.0± 1.41	19.0± 0.71
Staph aureus (l.c.i)	0	0	0	0	0	0	0	0	0
Ps aeruginosa (l.c.i)	0	0	0	0	0	0	0	0	18.0± 0.0
E coli (l.c.i)	0	0	0	0	0	0	0	0	14.0± 0.71
% susceptibility	62.5	50.0	0	50.0	50.0	50.0	0	50.0	62.5

L.c.i – clinical isolates
*Pure analytical ethanol extract.

Table 4: Mean zone inhibition (mm) of the antibiotics on the test bacterial strains

Bacterial strains	C10 (µg)	R30 (µg)	NA30 (µg)	E10 (µg)	STX25 (µg)	AMX30 (µg)	VAN 30 (µg)
Staph.aureus ATCC 12600	24.0	8.0	0.0	13.0	0.0	0.0	24.0
Ps. aeruginosa ATCC 10145	14.0	0.0	8.0	0.0	0.0	0.0	0.0
B subtilis ATCC 6051	25.0	15.0	13.0	20.0	17.0	0.0	0.0
E. coli ATCC 1175	16.0	14.0	0.0	0.0	0.0	0.0	0.0
Sal. kintambo (Human 1323)	21.0	12.0	0.0	0.0	0.0	0.0	10.0
Staph aureus (l.c.i)	30.0	0.0	0.0	0.0	0.0	0.0	20.0
Ps aeruginosa (l.c.i)	25.0	0.0	0.0	15.0	0.0	0.0	0.0
E coli (l.c.i)	30.0	0.0	0.0	0.0	0.0	0.0	0.0
Resistance	≤15.0	≤16.0	≤13.0	≤10.0	≤13.0	≤10.0	≤10.0
% Sensitive	87.5	0	25	12.5	0	12.5	25

C – Ciproxin; R – Rifampin; NA – Nalidixic acid; E – Erythromycin; STX – Septrin; AMX – Amoxil; VAN – Vancomycin

Table 5: The MIC*, MBC and MIC-MBC ratios of the crude extract and fractions.

	Bacterial strains			
	Staph. aureus ATCC 12600	*P. aeruginosa* ATCC 10145	*B. subtilis* ATCC 6051	*Sal. kintambo* (Human 1,13,23:mt:-)
Crude Extract				
MIC(mgmL^{-1})	25	50	50	>50.0
MBC (mgmL^{-1})	25	>50.0	>50.0	-
MIC-MBC ratio	1	-	-	-
F2				
MIC (mgmL^{-1})	3.125	12.5	50	6.25
MBC (mgmL^{-1})	25	25	>50.0	25
MIC-MBC ratio	0.125	0.5	-	0.25
F3				
MIC (mgmL^{-1})	1.563	50	12.5	12.5
MBC (mgmL^{-1})	3.125	50	50	12.5
MIC-MBC ratio	0.5	1	0.25	1
F6				
MIC (µgmL 1)	25	50	3.125	>50.0
MBC (µgmL 1)	50	50	3.125	-
MIC-MBC ratio	0.5	1.0	1	-
Gentamicin				
MIC (mgmL^{-1})	40	160	20	20
MBC (mgmL^{-1})	80	>320	40	40
MIC-MBC ratio	0.5	-	0.5	0.5

*Macro-broth MIC

Table 6:Effect of the test methods on the MIC of crude extract and Gentamicin against test bacterial strains

	Agar well MIC†					Macro-broth MIC†					MIC Index (MICi)					Effect of Test methods‡				
Bacterial strains	crude	F_2	F_3	F_4	Gen	crude	F_2	F_3	F_4	Gen	crude	F_2	F_3	F_4	Gen	crude	F	$_2$F	$_3F_4$	Gen
Staph.aureus ATCC 12600	50	50	12.5	12.5	8	25	3.125	1.563	25	40	2	16	8	0.5	0.2	NS	S	S	NS	NS
Ps. aeruginosa ATCC 10145	50	50	50	25	128	50	12.5	50	50	160	1	4	1	0.5	1.25	NS	NS	NS	NS	NS
B subtilis ATCC 6051	25	50	25	25	1	50	50	12.5	3.125	20	0.5	1	2	8	0.05	NS	NS	NS	S	S
E. coli ATCC 1175	ND	ND	ND	ND	64	ND	ND	ND	ND	80	ND	ND	ND	ND	0.8	-	-	-	-	NS
Sal. kintambo (Human 1323)	25	50	50	25	2	ND	6.25	12.5	ND	20	ND	8	4	ND	0.1	-	S	NS	-	S
Staph aureus (l.c.i)*	ND	ND	ND	ND	64	ND	ND	ND	ND	80	ND	ND	ND	ND	0.8	-	-	-	-	NS
Ps aeruginosa (l.c.i)	ND	ND	ND	ND	16	ND	ND	ND	ND	80	ND	ND	ND	ND	0.2	-	-	-	-	NS
E coli (l.c.i)	ND	ND	ND	ND	2	ND	ND	ND	ND	40	ND	ND	ND	ND	0.05	-	-	-	-	S

*Local clinical isolates†Mean of three replicates of the experiment, ND= Not determined.MIC index = Agar well MIC/macro-broth MIC; S= significant (MIC index>4.0 or ≤ 0.125); NS= Non significant (MIC index of > 0.125 to 4.0).‡The interpretation of MIC indices for significance or non-significance is adapted from the modified definition of fractional inhibitory concentration index (FICI).[22] By this definition, MIC indices which lie outside the inherent two-fold error of MIC (MICi = > 4.0 or ≤ 0.125) is considered significant. That is, the test method has an effect on the MIC value determined. Conversely, MIC indices which lie within the two-fold error (MICi = >0.125 – 4.0) imply that the test method have no significant effect on the MIC value. Two-fold error of MIC means that the true MIC may lie between 1xMIC and 2xMIC[24].

Table 7: Mean zone of inhibition (mm) of the different combinations of the column fraction on the test bacterial strains

	Combinations*				
Bacterial strains	1	2	3	4	5
Staph.aureus ATCC 12600	8.0	0.0	0.0	0.0	8.0
P. aeruginosa ATCC 10145	0.0	0.0	0.0	15.0	18.0
B subtilis ATCC 6051	0.0	0.0	0.0	0.0	0.0
E. coli ATCC 11775	0.0	0.0	0.0	0.0	15.0

*Combinations 1 ; F_2, F_3, F_4 and F_5, 2;F_2, F_3 and F_6; 3;F_2 and F_6; 4; F_2 and F_3; 5;F_3 and F_6.

Table 8: Effect of autoclaving and chilling on the Inhibition zone diameter (IZD)* in mm of the crude and fractions

Bacterial strains	Treatment	Crude	F_2	F_3
Staph.aureus	Autoclaved	21.0 ± 1.41	16.5 ± 0.71	21.0 ± 1.41
ATCC 12600	Chilled	0.0 ± 0.0	11.0 ± 1.41	0.0 ± 0.0
	Room	17.5 ± 0.71	21.5 ± 0.71	16.5 ± 0.71
Ps. aeruginosa	Autoclave	0.0 ± 0.0	0.0 ± 0.0	11.5 ± 0.71
ATCC 10145	Chilled	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Room	14.5 ± 0.71	16.5 ± 0.71	12.5 ± 0.71
B subtilis	Autoclave	31.0 ± 0.71	25.0 ± 1.41	32.5 ± 0.71
ATCC 6051	Chilled	31.0 ± 1.41	31.0 ± 1.41	33.5 ± 0.71
	Room	14.0 ± 1.41	20.5 ± 0.71	21.5 ± 0.71
Sal. kintambo	Autoclave	17.5 ± 0.71	10.5 ± 0.71	21.0 ± 1.41
(Human 1,13,23)	Chilled	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Room	21.0 ± 1.41	14.5 ± 0.71	15.5 ± 0.71

*Each value is a mean of triplicates, test concentration of extracts - 50mg mL^{-1}.

Table 9: Rate of kill of *Staphylococcus aureus* ATCC 12600 by the Crude, F_2 and F_3 extracts

		Reduction in viable bacterial count (log_{10} cfummL^{-1})* with time			
Extract	Concentration (mg mL^{-1})	30 (min)	60 min	90 min	120 min
Crude	50.0	-2.57	-2.74	-2.86	-3.0
F_2	20.0	-2.83	-2.89	-3.05	-3.1
F_3	20.0	-2.51	-2.77	-2.92	-3.1
Negative control	0.00	+0.21	+0.40	+0.51	+0.56
Gentamicin	80.0 µg mL^{-1}	-2.62	-3.0	-3.22	-3.41

*Bacterial cell count following treatment with extract minus initial innoculum counts (approx. 10^8 cfu)
- Net reduction in count (killing of cells by extract)
+ Net growth (no killing of cells)

extracts/drug, while the negative control gave a steady net increase in viable bacterial cell count. The crude extract achieved its highest net reduction in count by 3.0log_{10} after 120 minutes, while a similar result was recorded with F_2 in 90 minutes and control drug in 60 minutes. F_3 achieved a similar result in about 120 minutes. The highest net reduction in count by 3.41log_{10} was recorded for the control drug after 120 minutes.

DISCUSSION

Some of the phytochemical compounds detected e.g. glycoside, saponin, tannin, flavonoids, terpenoid, alkaloids, have variously been reported to have anti-microbial activity[11,15]. It is significant to note that alkaloids were detected in crude extract and the chromatographic fractions except F_1. Strikingly all the active fraction, F_2, F_3, F_4, F_6, contained alkaloids but F_1, which contained no alkaloid, appeared to have no activity. Thus, alkaloid may be a necessary ingredient in antibacterial activity of the extract except that F_5, which also contains alkaloid, showed no activity. The association of alkaloid with antibacterial activity is even stronger considering that an active fraction, F_4, like F_5 contains only alkaloid. Thus, that F_5 had no antibacterial activity inspite of the presence of alkaloid suggest that the type of alkaloid it contained was not bioactive. This further suggests that antibacterial activity may not be a characteristic of all member of a particular class of phytochemical compound but may rather depend on the specific compound in question. Therefore, assignment of antibacterial activity to any constituent phytochemical compound requires obtaining and testing the compound in pure form. Extract obtained with analytical grade ethanol gave a relatively wide spectrum of activity (62.5%) against the test strains as compared with the crude extract (50%). This could result from inhibition of active components by impurities extracted with locally distilled alcohol. Thus, purity of solvent may be a factor in potency of extract obtained. However, there was no significant difference ($p>0.05$) in antibacterial activity between the extracts of pure ethanol and that of locally distilled alcohol. In other words, the potency of extracts prepared with alcohol (i.e. kai-kai, the traditional solvent) compares favorably with extracts obtained with pure ethanol. Except in event of alcohol (kai-kai) containing impurities that are injurious to health on its own, use of this traditional solvent remain effective and economical. This justifies the decision to use the traditional solvent for most of the extractions done in this work since in a study of this nature solely designed to authenticate folklore medicinal use of the plant, traditional preparation methods and solvents would be most appropriate.

The crude extract and its chromatographic fractions gave the same spectrum of activity (50%) while Gentamicin had 62.5%. Crude plant preparations have generally been reported to exhibit lower antimicrobial activity than pure antibiotic substances such as Gentamicin[12,16,17]. The MBC showed that the crude extract was only bactericidal to *Staph. aureus* ATCC 12600, while its partially purified chromatographic fractions, F_2, F_3, F_6, were bactericidal to at least three of the test bacterial strains. This variation in degree of activity exhibited between the crude extract and its fractions further suggests that certain components of the crude extract inhibited the active principles to some extent. Thus purification of the constituents may greatly improve both the spectrum and intensity of activity. However, it has been suggested that certain components of crude plant

extract, though not bioactive in themselves may serve to protect the active ingredients against degradation until it reaches the target site[18].

The clinical isolates tested were not susceptible to both the crude and its partially purified fractions while the type strains were. It is uncertain whether this lack of susceptibility could be interpreted to mean outright resistance. There is not much report on development of resistance to natural plant products such as these extracts. However, these strains were also resistant to microorganism derived antibiotics with the exception of Ciproxin. If both results are taken as they are, this may signal the advent to resistance problem in ethnomedicine as there is in orthodox practice. The resistance of the local clinical isolates to the microorganisms-derived antibiotics may be attributable to abuse of these antibiotics since a new and expensive drug such as ciproxin, which has not been much abused, remained singularly active. It is not even known whether the resistance would account for the high MIC and MBC values recorded for the control drug (Gentamicin). It could also be that at the test concentration (50 $mgmL^{-1}$), the local clinical isolates were not susceptible to the extracts. If so, this may be another explanation for the large volumes of herbal remedies administered in traditional treatment of diseases. The exclusive susceptibility of the typed strains to the extracts could signal change in genetics and physiology of the laboratory adapted strains. They may have lost the necessary enzymes to breakdown the antibacterial plant products whereas the local clinical strains, considered wild type, still have the capacity.

Different combinations of the chromatographic fractions showed no activity where the fractions singly exhibited antibacterial activity. This shows that constituents of the crude extract may be antagonistic to one another as has earlier been reported on *Landolphia owerrience*[16].

The agar-well determined MIC was relatively higher (indicating less activity) than the macro-broth MIC in almost all the extracts except for the crude against *B. subtilis* ATCC 6051, and F_6 against *Staph. aureus* ATCC 12600, *Ps. aeruginosa* ATCC 10145. For Gentamicin, the reverse was the case, that is, the macro-broth MIC was higher (indicating less activity) than the agar-well MIC. A similar result has been reported[11]. The variation observed in susceptibility pattern with the two test methods may be due to differences in solubility of the active principles in the solvent used (20%DMSO). Dimethylsuphoxide (DMSO) has been widely reported to affect the activity of extracts when used as a solvent[11,19,20]. DMSO is an aprotic solvent in which certain reactions would proceed at a much lower temperature and at rates many times higher than in a protic solvent such as water. Hill *et al.*,[19] and co-workers had suggested that the reduced activity of cinnamon oil in DMSO against *Saccharomyces cerevisae* could be due to partitioning of the oil between the aqueous phase and DMSO, thus, preventing solubilization of the oil in the liquid membrane of the organism where it could have effect on cell metabolism.[10] The choice of DMSO as a solvent especially in acute toxicity studies is due to its solvency for a wide range of chemicals combined with its low acute toxicity to mammals[21].

The disparity in value of MIC obtained by the two methods may also be as a result of the differences in diffusibility of the test materials in agar and solubility in the broth. The active components of the extracts probably diffuse slowly through the agar than the pure antibiotic, Gentamicin. Also disparity in MIC values could have been influenced by strain differences.

The apparent inactivity of crude and its chromatographic fractions when chilled at +4°C relative to the activity of samples stored at room temperature (27°C) or autoclaved samples cannot easily be explained. Chemical compounds particularly of plant or animal origin would be expected to keep satisfactory when chilled. However, as has been mentioned earlier, DMSO, the solvent in which the extract was reconstituted and stored is an aprotic solvent, which could drive certain reactions at much lower temperature including +4°C[22]. Autoclaving seemed to reduce the spectrum of activity of the crude extracts and fractions but apparently enhanced the intensity of reaction (i.e. better Inhibition zone diameter, IZD). For example, autoclaving abrogated activity of the crude extract and F_2 against *Pseudomonas aeruginosa* ATCC 10145 but IZD of these samples were higher after autoclaving (31.0 and 25.0 mm, respectively) then un-autoclaved samples at room temperature (14.0 and 21.0 mm, respectively) against *B.subtilis* ATCC 6051. These observations suggest that different constituents inactivate different organisms. In other words, the mechanisms of antibacterial activity and the active principles vary with bacterial species; hence autoclaving inactivated the principle active against *Ps. aeruginosa* ATCC 10145 but not that against *B. subtilis* ATCC 6051. Secondly, it goes further to show that as has been mentioned earlier, certain constituents antagonize the others, hence where the principles inactivated by heat were active, the heat stable components tended to have higher activity in terms of intensity. Thirdly, the extracts contained heat-stable constituents, which resisted a temperature as high as 121°C for 15 min and still remained active against some of the test bacterial strains.

This is desirable since the traditional healers have no cold storage facilities and are constrained to store herbal preparations at room temperature.

The rate of killing of *Staphylococcus aureus* ATCC 12600 by the extracts and Gentamicin was time-dependent. That is, the higher the time of exposure, the more the number of bacterial cells inactivated. Though a single concentration of the extract and drug was tested, earlier report with vancomycin on some strains of *Staphylococcus* have shown no concentration-dependent killing[25]. The killing of cells was not total, that is less than 100% which is in agreement with earlier report that crude plant extract generally spare some viable cells even at the highest concentration.[11] Thus, the increased reduction in viable cell count with time suggests that these extracts are bactericidal at the test concentration.

The superior performance of the partially purified fractions may not be taken to mean that purified samples would make better drugs unless both have been investigated *in vivo* for bioavailability.

CONCLUSIONS

The results obtained in the study justify the folklore use of traditional solvent (locally distilled alcohol, kai-kai) in most of the herbal preparations of the plant and large volume administered in treatment of diseases. Further research probably involving *in vivo* assays, especially with the isolation and identification of the active principle in pure form, would be needed to establish the relationship between the MICs obtained in the study and the effective doses at which the herbs are applied in traditional practice.

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REFERENCES

1. Dalziel, J.M., 1961. The Useful Plants of West Tropical Africa. The Crown Agents, London.
2. Iwu, M.M., 1993. HandBook of African Medicinal Plants. CRC Press Inc. USA., pp: 219-221.
3. Nkere, C.K. and C.U. Iroegbu, 2005. Antibacterial screening of the root, seed and stembark extracts of *Picralima nitida*. Afr. J. Biotechnol., 4 : 522-526.
4. Ayensu, E.S., 1978. Medical Plants in West Africa. Reference Publications Inc. Algonac, Michigan, pp: 330.
5. Oliver, B., 1960. Encyclopedia of Medicinal Plants. College of Arts, Science and Technology, Ibadan.
6. Touchstone, J.C., 1992. Practice of Thin Layer Chromatography. 3rd (Edn.) A Wiley-Interscience Publication, New York, pp: 147-181, 338-340.
7. Bobbit, J.M., 1964. Thin Layer Chromatography. Reinhold Publishing Corporation, New York. pp: 128-182.
8. Harborne, J.B., 1984. Phytochemical Methods. Chapman and Hall, London, pp: 166-226.
9. Cheesbrough, M., 1984. Medical Laboratory Manual for Tropical Countries. Vol. II. Microbiology. Linacre House, Jordan Hill, Oxford.
10. Collins, C.H. and P.M. Lyne., 1970. Microbiological Methods. Butterwort, London.
11. Okeke, M.I., C.U. Iroegbu, E.N. Eze, A.S. Okoli and C.O. Esimone, 2001. Evaluation of extracts of the root of *Landolphia owerrience* for antibacterial activity. J. Ethnopharmacol., 78 : 119-127.
12. Ibrahim, M.B., M. O. Owonubi and J.A. Onaolapo, 1997. Antimicrobial effects of extracts of leaf, stem, and root-bark of Anogiessus leicarpus on Staphylococcus aureus NCTC 8190, *Escherichia coli* NCTC 10418 and *Proteus vulgaris* NCTC 4636. J. Pharm. Res. Develo., 2: 20-26.
13. Okli, A.S., M.I. Okeke, C.U. Iroeg and P.U.Ebo., 2002. Antibacterial activity of durgs permits declinalix of Synergy and antagonism. Anti microbial Agents and chemotherapy, 37: 260-264.
14. Leven, M., D.A. VandenBerghe, F. Mertens, A. Vlictinck and E. Lammens, 1979. Screening of higher plants for biological activities/antimicrobial activity. Planta medica, 36: 311-321.
15. Ebi, G.C. and S.I. Ofoefule, 1997. Investigating into folkloric antimicrobial activities of *Landolphia owerrience*. Phytotherapy Research, 11: 149-151.
16. Navarro, V., M.L. Villerreal, G. Rojsa and X. Lozoya. 1996. Antimicrobial evaluation of some plants used in Mexican traditional medicine for treatment of infectious diseases. J. Ethnopharmacol., 53: 143-147.
17. Kafaru, E., 1994. Immense help from nature's workshop. Elikat Health Services, Lagos, pp: 31-210.
18. Hill, P., C.S. Evans and R.G. Veness., 1997. Antimicrobial action of essential oils: The effect of dimethylsulphoxide on the activity of cinnamon oil. Letters in Applied Microbiology, 24: 269-275.
19. Weiss, L.R. and R.A. Orzel, 1967. Some comparative toxicologic and pharmacologic effects of dimethylsulphoxide as a pesticide solvent. Toxicology and Applied Pharmacology, 11: 546-557.

20. Brown, V.K., V. Robinson and D.E. Stevenson, 1963. A note on the toxicity and solvent properties of dimethylsulphoxide. J. Pharm. Pharmacol., 15: 688-692.
21. Morrison, R.T. and R.N Boyde, 1983. Organic Chemistry 4th (Edn.) Allyn and Bacon, Boston, M.A., pp: 33-35.
22. Lowdin, E., I. Odenholt and O. Cars., 1998. *In vitro* studies of pharmacodynamic properties of vancomycin against Staphylococcus aureus and Staphylococcus epidermidis. Antimicrobial Agents and Chemotherapy, 42: 2739-2744.
23. Sanders, C.C., W.E. Jr. Sanders and E.S. Moland, 1993. Decimal assay for additivity of drugs permits declination of synergy and antagonism. Antimicrobial Agents and Chemotherapy., 37: 260-264.