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Antibacterial Activities of the Combined Leaf Extract of *Phyllanthus muellerianus* and Ciprofloxacin against Urogenital Isolates of *Staphylococcus aureus*

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Abstract

Infectious diseases caused by multi-resistant strains of *Staphylococcus aureus* are on the increase and fighting them with natural products may be more efficacious. The aim of this study was to investigate the *in vitro* antibacterial activity of methanol leaf extract and the solvent fractions of *Phyllanthus muellerianus* (Kuntze) Excell as well as their combinations with ciprofloxacin against various isolates of *Staphylococcus aureus*. Phytochemical screening was done following established method while the *in vitro* antimicrobial properties of the extract and fractions of the extract were investigated by the agar diffusion method. Agar diffusion Checkerboard and Overlay Inoculum susceptibility disc methods were further employed to study the interaction of the extract, fraction and ciprofloxacin against eight (8) isolates of *S. aureus*. Results showed that the extract and the fractions contain glycosides, flavonoids, saponins and alkaloids. N- Hexane and methylene chloride fractions showed no activity against the test microorganisms, while methanol fraction and methanol crude extract produced a concentration dependent antibacterial activity against the organisms. Result of interaction study revealed synergism in some of the combination ratios but the predominant effects were indifference and antagonism. The study has demonstrated that the leaf extract and the polar fraction of the plant, *P. muellerianus*, possess potent antibacterial effect against various strains of *S. aureus*. Combination of the extract or the fraction with ciprofloxacin could be beneficial in treating infections caused b *S. aureus*.

Keywords: Antibacterial; Ciprofloxacin; Methanol crude extract; *Phyllanthus muellerianus; Staphylococcus aureus;* Synergism

Introduction

In an attempt to improve the quality of life, men have used plants as source of food, shelter, clothing, medicine and cosmetics. Some plants are medicinal because they contain active substances that cause certain reactions from relenting to the cure of diseases on the human organism [1]. In Africa, traditional medicine practice dates back many centuries ago and was the sole system for healthcare before the advent of orthodox or modern medicine. Plants have been exploited for the treatment of many infections and diseases. Plants readily synthesize substances for defense against attack by insects, herbivores and microorganisms. The world is experiencing an increasing rate of resistance by pathogens to some of the synthetic drugs. This has challenged the scientific community to initiate research programmes aimed at seeking solutions from plant species [2]. One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents [3]. Many plants have therefore become sources of important drugs and the pharmaceutical industries have come to consider traditional medicine as a source of bioactive agents that can be used in the preparation of synthetic medicines. Some of these plants have systematically been investigated for various pharmacological activities [4*5]. *Phyllanthus* is a large genus which contains over 600 species of shrubs, trees and annual or biennial herbs distributed throughout the tropical and sub-tropical regions of both hemispheres [6]. The plant *Phyllanthus muellerianus* (Kuntze) Excell, is a glabrous, monocot shrub or small tree up to 12 m tall. It is widely used to treat intestinal troubles, severe dysentery, constipation, stomach ache, jaundice and urethral discharges [7]. Throughout West Africa, pastes of the leaves are applied as wound dressing [8] and various extracts of the leaf are used as vapour bath to treat veneral diseases and toothache [9]. Preliminary investigation into the ethanolic extract of the stem bark of *P. muellerianus* against some selected bacteria had shown that the extract produced a dose dependent antibacterial activity [7].

In vitro antimicrobial studies on plants used in traditional medicine have severally been evaluated on a wide range of pathogenic bacteria including *Staphylococcus aureus* which is a major resistant pathogen found on the skin of humans. Studies on the combined effect of two antimicrobial agents are very limited and few studies have been reported [10-13]. Thus in our present study, the antibacterial properties of methanol leaf extract of *P. muellerianus* and their various fractions were studied singly and in combination against various isolates of *S. aureus*.

Materials and Methods

Plant material

The leaves of the plant *P. muellerianus* were collected from Nsukka in Enugu State between the months of June and July, 2010 and were authenticated by Mr. A. O. Ozioko of Bioresource Development and Conservative Program Centre (BDCP) in Nsukka, Enugu State, Nigeria. The voucher specimen for identification was deposited at the center.

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Extraction of plant material

The pulverized dried leaves (1 kg) were weighed with a triple beam balance. A soxhlet extractor was used for the extraction of the pulverized leaves and the recovered extract was evaporated to dryness under room temperature to get the methanol crude extract (ME; 180 g). Forty grams (40 g) of the crude extract was dissolved in about 400 ml of methanol and adsorbed onto silica gel (50-200, Lab Tech chemicals) and eluted in succession with n-hexane, methylene chloride and methanol in order of increasing polarity and the fractions were labeled as NF, MCF and MF respectively. The solvent was removed by evaporation to obtain solvent free fractions.

Phytochemical tests

Phytochemical analysis of the crude methanol extract together with n-hexane, methylene chloride and methanolic fractions of the extract was carried out based on procedure described by Harborne [14] and Evans [15].

Test microorganism and evaluation

A total of eight isolates of *S. aureus* isolated from urogenital specimens (urine, high vaginal swab (HVS), urethral swab (US) and semen) of patients who visited Adonai Diagnostic and Research Laboratory in Nsukka, Enugu State were used. Clinical isolates of *S. aureus* were isolated from patients manifesting symptoms of urogenital tract infections [16]. Isolation and characterization of the micro-organisms were done according to the method described by Cheesbrough [17], Cowna and Steet [18]. The stock culture of each clinical isolate was stored in nutrient agar slants at 4°C. Prior to use, the cultures were activated by successive daily sub-culturing first to blood agar; then into nutrient agar slant to ensure there was no contamination [16].

Antimicrobial screening test

Antimicrobial screening of the crude extract (ME) and fractions of the plant and standard antibiotic (ciprofloxacin) against the bacteria was done by cup-plate agar diffusion method [19,20]. A known concentration (200 mg/ml) of each of the extract and each of the fractions in DMSO was further diluted serially to produce 100, 50, 25 and 12.5 mg/ml. Similarly, two-fold serial dilutions of 2 mg/ml stock solution of ciprofloxacin were carried out to obtain 1, 0.5, 0.25 and 0.125 mg/ml concentration levels. Molten nutrient agar was seeded with 0.1 ml of standardized broth cultures of *S. aureus*. A total of six wells, 8 mm in diameter were made on the solid agar using a sterile cork borer. As a procedural control, DMSO was put in the center well. The various concentrations of the test agents and standard antibiotic were added into the respective labeled wells on the seeded agar plates. The plates were left for 1 h at room temperature for proper pre-diffusion after which they were incubated at 37°C for 24 h. Mean of triplicate determinations was taken in each case.

Evaluation of the minimum inhibitory concentration (MIC)

The MIC of the antimicrobial agents was determined using agar diffusion method [20]. The MIC was determined by plotting the graph of the IZD^2 (mm^2) against the log of concentration and the point of intersection on the x-axis was used to calculate the MIC.

In vitro evaluation of the extracts and the standard antibiotic in combination

The effects of the extracts in combination with ciprofloxacin against the *S. aureus* were evaluated using agar diffusion Checkerboard method and the Overlay Inoculum Susceptibility Disc method as described by Okore [21]. In the agar diffusion, various combination ratios (1:9, 2:8… to 9:1) of ciprofloxacin and the test agents (MF or ME) were prepared and evaluated. For each of the combinations, six 2-fold serial dilutions were done and the MIC determined. The fractional inhibitory concentration (FIC) of each of the combination ratios for the various isolates was determined using Eqn.1:

$$\mathrm{FIC}(\text{extract A}) = \frac{\text{MIC of the extract A in combination}}{\text{MIC of A alone}} \quad (1)$$

The sum of FIC (A) and FIC (B) gives the FIC (index) (Eqn. 2) from where an inference can be drawn, thus:

$$\mathrm{FIC}(\text{Index}) = \mathrm{FIC}(\text{A}) + \mathrm{FIC}(\text{B}) \quad (2)$$

The effects of the combinations were classified as synergistic, additive, indifference and antagonistic, if the FIC (Index) <1, = 1, >1 ≤ 2, and >2 respectively [22]. For the overlay inoculum susceptibility disc technique, a sub-inhibitory concentration of one of the test agents (ME) for the combination was prepared in molten nutrient agar. The antibiotic agar was poured into sterile clean petri dish and allowed to solidify to form the base antibiotic agar. About 2 ml of molten nutrient agar was inoculated with the test organism and shaken gently to ensure uniformity of the cells in the medium. The inoculated medium was then poured on the surface of the base antibiotic agar to form a thin uniform layer, the overlay inoculum agar. When the inoculum agar has solidified; the second antibiotic in the form of an antibiotic disc was placed on the agar medium (ciprofloxacin). Two control plates were prepared. Control A contained only the base antibiotic and the overlay inoculum agar, while control B contained the base agar without antibiotic, the overlay inoculum and the antibiotic disc. The three plates in the set were kept at room temperature for 1 h to allow for pre-diffusion, and then incubated at 37°C for 24 h. The zone of inhibition formed on the test plates was used to determine the combined effect of the two antibiotics, by comparing it with the result in control B. Synergism is obtained when the diameter of the zone of inhibition in the test plate is greater than that in control B by at least 19%; lower than 19% indicates additivity, equal to control B indicates indifference; when it is less than control B, there is antagonism.

Statistical data analysis

The SPSS (version 18.0) was used for statistical analysis. The values obtained were expressed as Mean ± SD and differences between the MIC and FIC (index) values were considered significant at $p<0.05$ using a one way analysis of variance (ANOVA).

Results

The results of phytochemical tests show the presence of glycosides, alkaloids, terpenoids, steroids and the absence of proteins in the crude extract (ME) and the various fractions of the extract. There were variations in the presence of the other phytoconstituents in the extract and fractions (Table 1). The analysis revealed the absence of reducing sugar, saponins, tannins and acidic compounds in the n-hexane (NF) and methylene chloride (MCF) fractions and the presence of these phytochemicals in the MF and ME. The results of sensitivity test of the extract and fractions (methylene chloride, n-hexane and methanol fractions) and the standard drugs are presented in table 2. Methylene chloride and n-hexane fractions of the extract of the leaves were not presented because they did not show any activity against the test microorganism. ME and MF produced clear zones of inhibition on

Phytoconstituents	Phyllanthus muellerianus			
	ME	NF	MCF	MF
Reducing Sugar	+	-	-	+
Glycosides	+	+	+	+
Alkaloids	+	+	+	+
Flavonoids	+	+	-	+
Terpeniods	+	+	+	+
Steroids	+	+	+	+
Protein	-	-	-	-
Saponin	+	-	-	+
Tannin	+	-	-	+
Lipids	+	+	-	-
Carbohydrates	+	-	+	+
Resins	+	+	-	+
Acidic compounds	+	-	-	+

Key–ME: Methanol Crude Extract; NF: N- hexane Fraction;
MCF: Methylene Chloride Fraction
MF: Methanol Fraction; +: Present; -: Absent

Table 1: Phytochemical constituents of the methanol leaf extract and the fractions of Phyllanthus muellerianus.

Isolates	MF (mg/ml)					ME(mg/ml)					Cipro (mg/ml)				
	200	100	50	25	12.5	200	100	50	25	12.5	2	1	0.5	0.25	0.125
	IZD (mm)					IZD (mm)					IZD (mm)				
1	13	10	9	8	6	11	9	8	-	-	33	30	27	22	18
2	13	11	9	9	7	16	14	10	8	-	31	30	28	22	20
3	13	10	9	8	7	12	8	7	-	-	28	22	16	8	-
4	10	8	6	-	-	12	10	8	-	-	27	20	19	7	-
5	11	9	7	7	-	12	10	7	6	-	32	30	26	23	12
6	11	9	7	-	-	11	11	7	-	-	28	30	26	20	11
7	14	12	11	9	-	14	12	9	7	-	33	30	26	20	11
8	9	7	6	-	-	12	11	8	-	-	30	24	22	14	7

Key: MF: Methanol Fractioion, MF (mg/ml) IZD (mm); ME: Methanol Crude Extract, ME (mg/ml) IZD (mm); Cipro: Ciprofloxacin, Cipro (mg/ml) IZD (mm)

Table 2: IZD produced by the extract of the plant, fraction and ciprofloxacin against Staphylococcus aureus

Isolate	MIC (mg/ml)		
	MF	ME	Cipro
1	6.18	11.11	0.04
2	4.08	14.10	0.02
3	4.08	27.85	0.22
4	22.94	16.36	0.20
5	85.44	15.10	0.60
6	19.39	3.20	0.11
7	5.30	13.71	0.80
8	17.57	14.52	0.12

Table 3: MIC values of the extracts, fraction and ciprofloxacin agains *S. aureus*.

all the eight isolates used. The IZD produced by ME and MF by the different isolates of *S. aureus* varied, ranging from 6 mm to 16 mm. ME produced the highest inhibitory activity at a concentration of 200 mg/ml with an IZD of 16 mm while the lowest inhibition was produced on isolate 5 (6 mm) at a concentration of 25 mg/ml. IZD of 6 mm was also produced by MF against the isolates (1, 4 and 8). The IZD produced by the standard antibiotic, ciprofloxacin, ranged from 7 mm at 0.125 mg/ml to 33 mm at 2 mg/ml. The MIC values of the crude leaf extract (ME) and fraction (MF) and the standard reference antibiotic against *S. aureus* are presented in table 3. Results showed that the MIC values ranged from 3.20 to 27.85 mg/ml for ME while MF produced MIC values ranging from 4.08 to 22.94 mg/ml. Ciprofloxacin was found to produce MIC ranging from 0.02 to 0.22 mg/ml against the various isolates. The most sensitive isolates were isolate 6 (ME, MIC=3.20 mg/ml), isolate 2 and 3 (MF, MIC=4.08 mg/ml). *In vitro* evaluation of the MF and ciprofloxacin against *S. aureus* as determined by Checkerboard method is presented in table 4. The combinations predominantly showed indifference and antagonism at the various ratios against the various isolates. The interaction between the methanol crude extract (ME) and ciprofloxacin against *S. aureus* as determined by Checkerboard method is present in table 5. Synergism was observed in the combination ratio of 8:2 (Cipro:ME). Other combinations yielded predominantly indifference effect. The antimicrobial activity of ciprofloxacin disc and ME using the overlay inoculum susceptibility disc method is shown in table 6. In the calculation, it was assumed that the amount of antibiotic remaining in the disc at the time of inhibition zone formation must at least tend to zero. It was further assumed that the amount of antibiotic diffusing beyond inhibition zone margin must be infinitesimally small. Under such conditions, the effective concentration of disc antibiotic can be calculated as the quotient of the disc potency and the volume of the inhibition zone. From table 6, it can be seen that the combination of ciprofloxacin and ME produced synergistic effect on isolate 4 of *S. aureus* (% increase=21.43 over control). Isolate 6 showed additive effect (% increase=7.40), isolate 9 showed indifference while all the other isolates of the test organism showed antagonism.

Discussion

Results of the phytochemical analysis of *P. muellerianus* leave extract using the solvents showed that their phytoconstituents differed depending on the nature of solvent used. Successful prediction of chemical compounds from plants materials is largely dependent on the type of solvent used in the extraction procedure. The traditional healers

Combination ratio (Cipro:MF)	Isolate								Predominant Effect
	1	2	3	4	5	6	7	8	
	FIC Index of isolates								
9:1	3.70[d]	1.85[c]	1.48[c]	1.85[c]	1.83[c]	2.20[d]	1.84[c]	1.99[c]	Indifference
8:2	6.41[d]	3.40[d]	3.38[d]	1.70[c]	1.79[c]	0.60[a]	0.40[a]	1.99[c]	Antagonism/ Indifference
7:3	11.35[d]	3.10[d]	3.11[d]	3.10[d]	1.70[c]	1.30[c]	0.19[a]	4.00[d]	Antagonism
6:4	9.87[d]	2.80[d]	2.82[d]	2.80[d]	1.60[c]	1.40[c]	0.64[a]	2.88[d]	Antagonism
5:5	4.18[d]	2.50[d]	2.50[d]	2.51[d]	1.50[c]	1.50[c]	1.24[c]	4.00[d]	Antagonism
4:6	6.44[d]	2.20[d]	2.21[d]	2.20[d]	1.40[c]	1.80[c]	1.00[b]	4.01[d]	Antagonism
3:7	5.03[d]	1.90[c]	1.87[c]	1.89[c]	1.30[c]	2.00[c]	0.49[a]	4.41[d]	Indifference
2:8	6.32[d]	1.60[c]	1.62[c]	1.59[c]	0.80[a]	2.00[c]	0.19[a]	2.00[c]	Indifference
1:9	2.47[d]	1.30[c]	1.30[c]	1.10[c]	1.10[c]	1.90[c]	0.27[a]	2.00[c]	Indifference

[a](FIC Index<1)=synergy; [b](FIC Index=1)=additivity; [c](FIC Index >1 ≤ 2)=indifference; [d](FIC Index >2)=antagonism

Table 4: Combined effects of ciprofloxacin and MF against various strains of *S. aureus* as determined by the Checkerboard method.

Combination ratio (Cipro:ME)	Isolate								Predominant Effect
	1	2	3	4	5	6	7	8	
	FIC Index of isolates								
9:1	0.91[a]	1.88[c]	0.97[a]	1.88c	1.68[c]	1.29[c]	0.87[a]	1.00[b]	Indifference
8:2	0.84[a]	13.40[d]	3.38[d]	1.77[c]	1.52[c]	1.59[c]	0.88[a]	0.99[a]	Synergy/Indifference
7:3	1.46[c]	11.81[d]	2.99[d]	0.83[a]	1.36[c]	0.93[a]	1.64[c]	1.00[b]	Indifference
6:4	2.75[d]	10.20[d]	2.74[d]	1.54[c]	1.21[c]	0.54[a]	2.77[d]	0.99[a]	Antagonism
5:5	4.94[d]	4.29[d]	2.33[d]	1.50[c]	1.95[c]	0.63[a]	1.19[c]	0.99[a]	Indifference/Antagonism
4:6	1.08[c]	3.48[d]	1.94[c]	1.34[c]	3.30[d]	2.80[d]	1.00[b]	0.99[a]	Indifference/Antagonism
3:7	0.93[a]	1.30[c]	3.24[d]	0.61[a]	2.60[d]	3.09[d]	1.63[c]	1.01[c]	Indifference/Antagonism
1:9	1.34[c]	2.11[d]	0.44[a]	0.53[a]	2.40[d]	1.13[c]	1.73[c]	0.99[a]	Synergy/Indifference

[a](FIC Index <1)=synergy; [b](FIC Index =1)=additivity; [c](FIC Index >1 ≤ 2)=indifference; [d](FIC Index >2)=antagonism

Table 5: Combined effects of ciprofloxacin and ME against various strains of *S. aureus* as determined by the Checkerboard method

Strain	IZD (mm)			
	Test plate	Cipro	%Increase	Effect
1	-	13.00	-	-
2	30.00	35.00	-	Antagonism
3	34.00	28.00	21.43	Synergism
4	29.00	27.00	7.40	Additivity
5	-	21.00	-	-
6	10.00	25.00	-	Antagonism
7	28.00	28.00	0	Indifference
8	-	30.00	-	-

Cipro=Ciprofloxacin; - =no growth; Test plate=methanol leaf extract of *P. Muellerianus* (ME)

Table 6: Antibacterial activity of Ciproflo acin disc and ME against *S. aureus* as determined by Overlay Innoculum susceptibility disc method.

or practitioners make use of water primarily as a solvent. In this study, extraction was done using organic solvent such as n-hexane, methanol and methylene chloride. This is because these solvents are easily evaporated and permit an easier estimation of extract concentration, which is difficult to obtain with water as solvent. The analysis revealed that the most common substances found in the extract and fractions of the plant were glycosides, alkaloids, steroids, terpenoids and flavonoids. *S. aureus* isolates were sensitive to ME (methanol crude extract) and MF (methanolic fraction) which are rich in flavonoids. This is probably due to their ability to complex with bacterial cell walls; being lipophilic flavonoids may disrupt microbial membranes [23,24]. Besides, alkaloids have been demonstrated to intercalate between DNA strands [25]. The presence of sterols, tannins, glycosides and reducing sugars in ME and MF could also account for the important antimicrobial activity exhibited by the plant against the test microorganisms. Lack of antibacterial activity of NF and MCF fractions against the test organism could possibly be attributed to the absence of saponins, tannins or the acidic compounds in these chromatographic fractions of the plant extract.

The variations observed in the IZDs and MICs of MF and ME of the isolates could be related to the different sources (patients) from which the organisms were isolated. Furthermore, the isolates used were different strains of *S. aureus* which may have developed resistance to some of the active components of the plants used. Microorganisms are known to vary widely in the degree of their susceptibility depending on their sources. Though the activity of ciprofloxacin was found to be more than those of the test agents, it is possible that higher concentrations of the extract could produce antibacterial activity that is comparable with that of the standard antibiotic against the test organism. Nonetheless, the results of the present study indicate that the leaves of the studied plant have promising antimicrobial properties which when isolated and further purified can be used to combat the fast emerging resistant urogenital pathogens. Earlier workers on the other parts of the plant, *P. muellerianus,* have demonstrated similar antibacterial properties against the *S. aureus* as seen with the ME and MF in the present work [7,26].

The interaction study shows that ciprofloxacin and MF in combination have a promising antimicrobial activity against the pathogen (*S. aureus*) studied since synergism was observed at some of the combination ratios. With combination of ciprofloxacin and methanol extract (Cipro:ME), synergism was observed at some ratios. The results of this study suggest that the combination of ciprofloxacin and the plant extracts in the treatment of staphylococcal infections will be beneficial since synergistic effect was observed at some points.

However, the predominant indifference and antagonism observed in most of the combination ratios of both MF and ME calls for caution in the use of the standard antibiotic in combination with the plant extract. Such interactions could adversely affect therapeutic outcomes [27]. Similarly, from the overlay inoculum susceptibility disc method, it was observed that the combination of ciprofloxacin and ME produced a synergistic effect on an isolate of *S. aureus*. It was similarly observed that the IZDs produced by the disc antibiotic (Cipro) were larger than that produced by the test agents used singly or in combination. This suggests that the plant extract (ME) reduced the activity of ciprofloxacin against the test organisms which may be attributed to possible phytochemical incompatibility between the combined agents or a competitive inhibition at the site of action. Also, the ciprofloxacin (disc) might have encountered problem diffusing through the agar since the agar (base agar) contained the plant extract (ME). However, both interaction study models have demonstrated the possibility of combining ciprofloxacin and the plant extract at some ratios to achieve better antibacterial effect that is superior to any of the agents used alone.

Conclusion

The methanol crude extract and the methanol fraction of the leaves of *Phyllanthus muellerianus* have been shown to have promising antimicrobial activities against various isolates of *Staphylococcus aureus*. The outcome of this study could therefore justify the ethnomedicinal uses of *Phyllanthus muellerianus* for the treatment of infections caused by *S. aureus*. The combined extract or methanol fraction with the standard drug, ciprofloxacin, produced synergistic effect in many of the combination ratios against the test organism and this has a lot of therapeutic implications in the treatment of infections caused by *S. aureus*. Further purification should therefore, be done to extract the active ingredients which may serve as the lead structure for the development of new drugs that will be beneficial to man.

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