Evaluation Of Antmicrobial Properties, Acute Toxicity and Immunostimulatory Potential of *Phyllanthus amarus*

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ABSTRACT

The proposed incorporation of medicinal plants into mainstream primary healthcare programmes in Nigeria will require rigorous scientific scrutiny of both their therapeutic potentials and safety. Phyllanthus amarus is one of the medicinal plants widely used traditionally for the remedy of upper respiratory tract infections in Nigeria. The methanolic extract of the plant was fractionated and assessed for antimicrobial activity against Bacillus subtilis, E. coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus and Candida albicans by agar diffusion method. All the fractions were active against the test organisms with the exception of the chloroform fraction. Ethyl acetate fraction exhibited the highest activity with zones of inhibition of 23, 20, 20, 18.5, 23 and 18mm against the test organisms respectively. The minimum inhibitory concentration of the extract against the test organisms were 3.125, 6.25, 25, 25, 3.125, and 1.56 mg/ml respectively while the minimum bactericidal concentrations were 6.25, 100, 50, 100, 6.25 6. 25 mg/ml. The extract was found to be practically non toxic with LD_{50} = 774.6mg/kg body weight. The plant extract also showed a good immunostimulatory activity by increasing white blood cell proliferation when administered into Wister rats at doses of 100 and 1000mg/ml. The result of this investigation supports the popular use of this plant for the traditional remedy of infectious diseases possibly caused by these test organisms.

INTRODUCTION

Traditional herbal medicines still remain the basic health care means for a large majority of rural and urban dwellers in Nigeria. The full integration of herbal medicine into the mainstream primary health care programmes requires rigorous scientific scrutiny of both their therapeutic potentials as well as the assessment of safety issues. *Phyllanthus amarus* is one of the important medicinal plants that has been reported to be used for treating diarrhea and dysentery[1], its use for treating jaundice was also reported[2]. It is also used to treat stomach disorder and as blood tonic[3]. The increasing incidence of antibiotic resistance among human

pathogens has compelled the scientific community to look for alternative sources of medicines for the treatment of infectious diseases. The scientific scrutiny of *Phyllanthus amarus* is as a result of the need to look for other sources of novel antimicrobial agents particularly from medicinal plants based on ethno-pharmacological information. Various workers[4,5,6] have reported on the antimicrobial properties of this plant. However, significant phytochemical diversity among this plant when collected from different geographical conditions has been reported[7]. The inconsistencies of the reports on the plant's antimicrobial properties as well as lack of information on its acute toxicity and immunostimulatory potentials prompted this investigation. The present study, was, therefore, conducted to evaluate antimicrobial activity of fractions from the methanolic extract of Phyllanthus *amarus* and to assess its acute toxicity as well as its immunostimulatory potential.

MATERIALS AND METHODS

Collection, Identification and Extraction of Plant Materials

Phyllanthus amarus was collected from in and around Federal College of Education, Otite-Okene. The plant was earlier identified and authenticated at the herbarium of the Dept of Biological Sciences, A.B.U., Zaria with voucher number 555. The leaves were air-dried under shade at ambient temperature, pulverized into fine powder, packed into soxhlet extractor, defatted with n-hexane, and subsequently extracted with methanol. The extract was concentrated using rotary evaporator at $40^{O}C$ and transferred into a clean container. This crude extract was fractionated using liquid-liquid extraction into chloroform, ethyl acetate and n- butanol and distilled water.

Test Microorganisms

Strains of the following microorganisms were sourced from the stock of Dept. of Pharmaceutics and Pharm. Microbiology, A.B.U., Zaria. These include *Bacillus subtilis, Candida albicans, E. coli, Pseudomonas aeruginosa, Salmonella typhi, and Staphylococcus aureus.*

Standardization of Inocula

The bacteria strains were tested for sterility and then grown in Nutrient broth at 37 ^{o}C for 24hr. The overnight cultures were subsequently diluted to give 0.5 McFarland standard (approximately 1.5×10^{8} cfu/ml.).

Antimicrobial Susceptibility Test

The agar cup diffusion method according to[8] was adopted. Nutrient Agar plates were flooded with overnight culture of the standardized organisms and the excess drained off. After drying, wells measuring 7mm were bored aseptically into each inoculated plate using sterile cork borer. The wells were filled with the extract (100 µl of 100mg/ml) with the aid of Pasteur pipette. Standard antibiotic disc (Ofloxacin 5µg/disc) was used as positive control while sterile distilled water served as

negative control. The plates were allowed to stand at room temperature for 1 h for the extracts to diffuse into the agar. Diameters of zones of inhibition were determined after incubating plates at 37^{o}C for 24 hr. The experiments were replicated and the zones of inhibition (mm) expressed as the mean and standard errors on means.

Determination of Minimum Inhibitory Concentration (M.I.C.)

M.I.C. was determined by a modification of the agar dilution method[9]. The extracts were sterilized using Corning sterile syringe filter (0.2µm pore size). 10ml of the double strength of the various extract concentrations (100, 50, 25.0, 12.5, 6.25, 3.125, 1.56, and 0.78mg/ml) were incorporated into 10ml of double strength molten agar at 50^{o}C and aseptically poured into Petri-plates. After setting, sterile paper discs (6mm) were aseptically applied to the surface of the set agar containing the various extract concentrations at equidistance in duplicates. 10µl of each standardized inoculum was then spot-inoculated onto each disc and allowed to diffuse for 20 mins before incubating at 37^{o}C for 18 hrs. The first lowest concentration that showed no visible growth of the inoculated test organism was recorded as the M.I.C. of the extract for the test organism.

Acute toxicity studies

This was done using the method of[10]. The test was conducted in two phases. In the first phase, nine (9) mice (average weight of 20 g) were divided into 3 groups of three mice each. To each group, one of the three doses of 10, 100 and 1000 mg/kg body weight was administered intraperitonially. The animals were then observed over a period of 24 hours for mortality. The response of the animals was also noted. In the second phase three (3) mice were given the extract at doses of 1600, 2900 and 5000 mg/kg each (doses higher than doses where no mortality was observed as directed in the Lorke, 1983 table). The LD_{50} was calculated using the formular:

$$LD_{50} = \sqrt{C \times D}$$

Where

C = the highest dose at which no death occurred in the second phase

D = the least dose at which death occurred in the second phase

The extract was considered as extremely toxic, highly toxic, moderately toxic, slightly toxic, practically non toxic and harmless when the LD_{50} value $\leq$ 1 mg/kg, 1-50 mg/kg, 50-500 mg/kg, 500-5000 mg/kg and >15g/kg body weight respectively.

Immuno-stimulatory effect of S. dulcis extract on Total Leucocyte Count (TLC) and Differential Leucocyte Count (DLC)

A modification of[11] method was adopted for this study. Nine (9) mice of average weight 25g were grouped into three, three in each group. The first group served as control, the second and third groups was administered with the extract at doses of 100 and 1000 mg/kg body weight intraperitonially respectively on the 1st, 5th and 9th days. On the 10th day, blood samples were collected by applying pressure on their tails and then cutting off the tip of each tail. White cell diluting pipette (capillary tube) was used to collect blood directly from the tail of each mouse for TLC. The TLC was done by making 1:20 dilution of the blood samples with white

cell diluting fluid and counting with the aid of Neubaver counting chamber under the microscope at x10 magnification. The DLC was determined by making a thin film of the blood samples on microscopic slides and staining with the Leishman's stain. The films were air dried at ambient temperature and examined microscopically under oil immersion(x100 magnification).

RESULTS AND DISCUSSION

The study showed that except the chloroform fraction all the other fractions of methanolic extract of *Phyllanthus amarus* have antibacterial activity against the test organisms (Table 1). This result agrees with those of[4] for *E. coli*, those reported by[5] for S. typhi and the report of[6] for *E.coli*, *P. aeruginosa* and *S. aureus*. This result is at variance with that of[12] who reported that the plant extract does not have activity on *S. typhi*, *P. aeruginosa* and *E. coli*. The ethyl acetate fraction showed higher antimicrobial activity on average than the remaining fractions. The zones of inhibition of the ethyl acetate fraction against the test organisms ranged fro 18- 25 mm, the activity of the n-butanol fraction ranged from 15-22 mm while that of the aqueous fraction was between 16.5-21 mm. The minimum inhibitory concentration of the ethyl acetate fraction against *B. subtilis*, *E. coli*, *P. aeruginosa*, *S.typhi*, *S. aureus* and *C. albicans* were *3.125*, *6.25*, *25*, *25 3.12* and 1.565 mg/ml respectively while the minimum bactericidal concentrations for the test organisms were found to be 6.25, 100, 50, 100, 6.25 and 6.25 mg/ml respectively. The MIC and MBC of the ethyl acetate fraction against the test organisms were far less than the control for the bacteria. This is possibly because the extract is still in the crude form has better activity than the control for the fungus

Table 1: Antimicrobial Activity of the Various Fractions from Methanolic Extract of *Phyllanthus amarus*

Fraction	Test organisms					
	Bs	*Ec*	*Pa*	*St*	*Sa*	*Ca*
	Zone of Inhibition (mm/SD)					
Aqueous	21±3.0	16.5±1.5	17±1.0	19.5±0.5	18±0.0	16.5±2.5
n-Butanol	22.5±0.5	15±1.0	19.5±0.5	18.5±0.5	21±1.0	15±1.0
Ethylacetate	23±1.0	20±2.0	20±0.0	18.5±2.5	23±1.0	18±1.0
Ofloxacin(5µg/ml)	23.6 ±2.8	23± 00	25.33±2.3	30 ±00	27 ± 3.0	-
Griseofulvin(100mg/ml)	-	-	-	-	-	26 ± 00

Table 2: Minimum Inhibitory and Bactericidal Concentration of the Ethyl acetate fraction of the Methanolic Extract of *Phyllanthus amarus* (mg/ml)

Aqueous Fraction	Test Organisms					
	Bs	*Ec*	*Pa*	*St*	*Sa*	*Ca*
MIC(mg/ml)	3.125	6.25	25	25	3.125	1.56
MBC(mg/ml)	6.25	100	50	100	6.25	6.25
Ofloxacin(5µg/ml)	10.0	0.75	1.25	10.0	5.0	-
Griseofulvin(100mg/ml)	-	-	-	-	-	50

Bs-Bacillus subtilis, Ec-E. coli, Pa-Pseudomonas aeruginosa, St-Salmonella typhi, Sa-Staph aureus and *Ca-Candida albicans*

Table 3: Acute Toxicity Study of the Methanolic extract of *P. amarus.*

Fraction	Phase	Weight of mice	Doses (mg/ml)	Mortality	LD_{50}
Methanolic Extract of *P. amarus*	First	23 20 17	1000 1000 1000	1/3	$\sqrt{CxD}$ $=\sqrt{1000x600}$ =774.6 mg/kg (practically non toxic)
		23 20 17	100 100 100	0/3	
		20 18 18	10 10 10	0/3	
	Second	18 17 18	600 1000 1600	0/1 1/1 1/1	

The calculated LD_{50} (i.p.) in mice =√1000x600=774.6mg/kg. This fell into the range of practically non toxic. This result is in agreement with those of[12] who observed that the methanolic extract of this plant is not cytotoxic. This probably account for its wide spread use in the preparation of herbal medicines in Nigeria.

The immuno-stimulatory effect of the extract on the blood parameters (Table 4) showed that the methanolic extract of *P. amarus* increase the Total Leucocyte Count (WBC). The extract also increased lymphocyte proliferation significantly in mice. This indicates that the extract stimulates the cellular immunity system in the experimental mice. This result support the traditional uses of the extract for blood cleansing and as general tonic earlier reported by[13, 16].

Table 4: Effect of Methanolic Extract of *P. amarus* on Total and Differential Leucocyte Counts in mice

Treatment Dose (mg /kg)	TLC ($X10^9$/l)	Differential Leucocyte Count (%)				
		Lymphocyte	Neutrophile	Monocyte	Eiosin	Basophile
Control	6.39 ± 1.865	74.44 ± 5.96	17.56 ± 5.96	2.66 ± 3.055	2.33 ± 2.517	0.00 ± 0.00
25	6.28 ± 3.138	76.11 ± 6.23	22.11 ± 6.51	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
50	9.01 ± 5.338	76.89 ± 1.36	19.56 ± 3.61	4.33 ± 4.163	0.667 ± 0.58	0.00 ± 0.00
100	7.02 ± 1.750	77.00 ± 6.54	*22.33 ± 6.27	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Average of three readings

- Significant ($P < 0.05$)

CONCLUSION

Various fractions obtained from Methanolic extract of *Phyllanthus amarus* showed varying antimicrobial activity against *B. subtilis, E. coli, P. aeruginosa, S.typhi, S. aureus* and *C. albicans B. subtilis, E. coli, P. aeruginosa, S.typhi, S. aureus* and *C. albicans*. The ethyl acetate produce higher activity on average than the rest. The diameter of zones of inhibition for the ethyl acetate fraction was between 18- 25 mm, that of the n-butanol fraction ranged from 15-22 mm while that of the aqueous fraction was between 16.5-21 mm. The Minimum Inhibitory Concentration of this fraction for *B. subtilis, E. coli, P. aeruginosa, S.typhi, S. aureus* and *C. albicans* were *3.125, 6.25, 25, 25 3.12* and 1.565 mg/ml respectively while the minimum bactericidal concentrations for the test organisms were found to be 6.25, 100, 50, 100, 6.25 and 6.25 mg/ml respectively. These zones of inhibition compared favourably with the sensitivity of the test organisms against standard antibiotic (Ofloxacin disc) which was 23 to 27mm. The inhibitory activity of the extract against the test organisms provides scientific support for the traditional uses of the plant for the treatment of infections especially those caused by the test organisms. The study also showed that the plant is practically non toxic and that it has good immuno-stimulatory properties on cellular immunity which explains its wide spread use in herbal preparations as blood tonic. Further investigation will be required on chronic toxicity profile of the plant.

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