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INHIBITORY EFFECTS OF *PHYLLANTHUS AMARUS* EXTRACTS ON THE GROWTH OF SOME PATHOGENIC MICROORGANISMS

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ABSTRACT

This study evaluated the inhibitory effects of *Phyllanthus amarus* extracts on *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Candida albicans.* These effects were compared with those of ampicillin, gentamicin and pefloxacin. Phytochemical analysis showed that the plant contained flavonoids, steroids, terpenes, alkaloids, benzenoids, saponins and lipids. This plant was found to have remarkable inhibitory effects on the growth of all the organisms tested; *S. aureus* was the most susceptible (MIC 20ug/ml) while *Pseudomonas aeruginosa* and *C. albicans* were the least susceptible (MIC 30ug/ml). The organisms were inhibited in a dose-dependent manner, the inhibition was almost directly proportional to the extract concentration. The aqueous extract had no significant increase inhibitory effects compared to the ethanol extract ($p > 0.05$). The standard antibiotics had no greater inhibitory effects on the test organisms in relation to the plant extracts ($p>0.05$). The *in vitro* analysis revealed that *Phyllanthus amarus* possesses an antimicrobial activity comparable with those of standard antibiotic discs. Further works is recommended to determine its suitability in chemotherapy.

Keywords: Inhibitory effects, *Phyllanthus amarus* extract, Pathogenic microorganisms.

DES EFFETS INHIBITEURS SUR DES EXTRAITS AMARUS SUR LA CROISSANCE DE CERTAINS MICRO – ORGANISMS PATHOGENES DE PHYLLANTHUS

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RESUME

Cette étude a évaluée les effets inhibiteurs des extraits de Phyllanthus amarus sur Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogènes, Pseudomonas aeruginosa et Candida albicans. Ces effets ont été comparés à ceux de l'ampicilline, gentamicine et l'ofloxacine.

Analyse phytochimique a montré que la plante a contenu des flavonoïdes, des stéroïdes, des terpènes, des alcaloïdes, des benzénoïdes, des saponines, et des lipides.

Cette plante a été trouvée d'avoir des effets inhibiteurs remarquables sur la croissance de tous les organismes testés ; S. aureus était le plus sensible (MIC 20ug/ml) tandis que Pseudomonas aeruginosa et C. albicans étaient les moins sensibles (MIC 30ug/ml). Les organismes ont été inhibés d'une manière dépendante de la dose, l'inhibition était presque directement proportionnelle à la concentration de l'extrait. L'extrait aqueux n'a eu aucun effet inhibiteur d'augmentation significative par rapport aux extraits d'éthanol (p>0,05). Les antibiotiques standard n'a eu aucun effet d'inhibition plus importante sue les organismes d'essai par rapport aux extraits de plantes (p>0,05).

L'analyse in vitro a révélé que Phyllanthus amarus possède une une activité anti · microbienne comparable à celle des disques antibiotiques standards. La poursuite des travaux est recommandée de déterminer son aptitude à la chimiothérapie.

Mots clés: Effets inhibiteurs, extrait de Phyllanthus amarus, micro –organismes pathogènes.

INTRODUCTION

Clinical microbiologists are faced today with growing resistance of microorganisms to conventional antimicrobial agents. This has led to an intensive search for new antibiotics from herbs. As many bacteria, especially *P. aeruginosa* are resistant to some of the orthodox antibacterial agents resulting to prolonged illness and waste of money, the need for alternative approach to such cases has been advocated. Certainly, the discovery of naturally occurring agents and herbs that are well tolerated in the body and at the same time treating the related disease would be a welcome development in the practice of medicine. The environment where a plant is growing may affect the phytochemicals present in a plant and may affect its functions. There is paucity of information on antimicrobial activity of *Phyllanthus amarus* against *Pseudomonas aeruginosa, Streptococcus pyogrnes, Streptococcus pneumoniae* and *Candida albicans.* This study aims at investigating the antimicrobial effects of extracts of *Phyllanthus amarus* on these microorganisms and how these extracts compare with some standard antibiotics. Effects of the extracts on *Staphylococcus aureus, were also studied.*

Phyllanthus amarus belongs to the family Euphobiaceae. It is a small, erect, annual herb that grows up to 30-40cm in height. It is called Carry me go seed in English, Eyin Olobe in Yoruba, Geeron-Tsuntsaayee (Bird's millet) in Hausa and Enyikwonwa in Ibo. It grows well in moist and shady places. Each little leaf of the branch carries in the angle of the flower, the fruit. *Phyllanthus amarus* is popularly used by traditional healers for diverse purposes especially in the treatment of malaria, scalp and various skin diseases, gastrointestinal disturbances and sexually transmitted diseases.

Contreras and Gamara (1) reported *Phyllanthus niruri* (synonym of *P. amarus*) to have antibacterial effect over *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis*. The herb was equally reported to have remarkable effects on chronic viral hepatitis B, recovery of liver functions and inhibition of the replication of hepatitis B virus (2). A chemical compound has been isolated in the plant with reverse transcriptase inhibition activity. This compound was named niruriside (3). In 1992, the HIV-1 reverse transcriptase inhibitory properties of *Phyllanthus niruri* was reported (4). The plant was reported to have complex biochemical compounds which include major compounds like lignanes terpenes, flavonoids, lipids, benzenoids, alkaloids, steroids, tannins and saponins (5, 6). The antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, neuroprotective and diuretic properties of *Phyllanthus amarus* has been discovered (5).

MATERIALS AND METHODS

Plant collection, preservation and storage

Fresh, clean and dry plants of about 45cm tall were collected from Nnewi, Anambra State, Nigeria and identified at the Department of Botany, Nnamdi Azikiwe University, Awka, Nigeria. The leave of plant was washed thoroughly with tap water and dried through progressive elimination of moisture for seven days by spreading in a thin layer over white paper and turning the plant over twice daily. Dried plant was ground into powder (7) using grinding machine, blended and stored in brown bottle in a cupboard until ready for extraction.

Extraction of active ingredients

Extraction of active ingredients from the leaves of the plant was done in biochemistry Department of Nnamdi Azikiwe University, Awka, Anambra State. The pulverized plant leave was subjected to ethanol and distilled water extraction as follows: 300g of

powdered leave was extracted with 1litre of ethanol for 7 hours in soxlet extraction apparatus by applying heat (8). After extraction, the ethanol was allowed to evaporate until a semi solid, brownish, syrupy substance was obtained (9). This resulting deposit was freeze-dried and weighed. The same quantity of powdered plant leave was extracted with distilled water overnight, filtered and heated at 60^{o}C to evaporate the solvent. The extract was then freeze dried and weighed.

Preparation of antimicrobial disc
Preparation and impregnation of discs with the diluted extracts was done at Mega Diagnostic Laboratories, Nnewi. Disks of 6 mm diameter (10) were cut from Whatman number 1 filter paper using a perforator. These were arranged in glass Petri dishes and sterilized in a hot air oven at 160^{o}C for 1 hour (11). After cooling, 0.02ml (11) of various dilutions of 1000 mg/ml of aqueous extract in sterile distilled water were carefully dropped on each set of discs such that sets of discs containing 1.25 mg, 2.5 mg, 5.0 mg and 10 mg extracts were prepared. This process was repeated for ethanol extract. The extract-impregnated discs (aqueous and ethanol) were dried at 40^{o}C, packed and stored at 8^{o}C prior to use (11).

Test and control organisms
Pure cultures of *S. aeruginosa, S. aureus, S. pyogenes, S. pneumoniae* and *C. albicans* were obtained from the Microbiology Laboratory of Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Anambra State. Standard control strains of *S. aureus* (NCTC 6571) and *P. aeruginosa* (NCTC 10662) were obtained from Microbiology Department of Obafemi Awolowo University, Ile-Ife.

Antimicrobial sensitivity testing
Antimicrobial sensitivity testing was done at Mega Diagnostic Laboratories, Nnewi. Stokes Disc Diffusion Method (12) was adopted for the sensitivity testing using Mueller-Hinton Medium (oxoid). 5% red cells were incorporated in the medium for testing the Streptococci while Sabouraud agar was used for *C. albicans*. Each test organism and control were tested against 10 mg discs of both ethanol and aqueous extracts from quarter strength Ringers solution previously inoculated with colonies of the test organisms to give a density similar to an overnight broth culture (12). The plates were incubated overnight at 37^{o}C and observed after 24 and 48 hours incubation. Selected organisms were also tested against graded concentrations of both extract.

Determination of minimum inhibitory concentration (MIC)
The tube method described by Baker and Breach (12) with little modification was used in the determination of MIC of the extracts. From extract concentration of 2000µg/ml in sterile distilled water, test concentrations of 400, 200, 100, 50 and 25µg/ml was used for preliminary MIC determination. Further concentrations of 60, 55, 50, 45, 40, 35, 30, 25, 20 and 15µg/ml in Brain-heart infusion (BHI, Oxoid) was used for more accurate determination of MIC. A drop of 1 in 250 dilution of an 18 hour broth culture (in BHI) was used to inoculate the tubes (11). Control tubes used were seeded BHI without extract to show the suitability of the broth for growth of organism, unseeded BHI and unseeded BHI with extract both served as control for the clarity and sterility of the broth (12). MIC of C. *albicans* was done using Sabouraud liquid medium and inoculated from an overnight Sabouraud broth culture. The lowest concentration of the extracts that did not permit visible growth (turbidity) in broth was taken as the MIC.

Determination of minimum cidal concentration (MCC)
Following incubation and reading of the MIC test, the first tube that showed growth (turbidity) and all tubes that showed no evidence of growth was sub cultured on solid media (12). The agar plates were incubated at 37^{o}C and read after 24 hour and 48 hour incubation (12). The minimum cidal concentration was read as the lowest extract concentration which yielded no growth upon subculture.

Testing orthodox antibiotics and plant extracts against test and control organisms
30µg ampicillin, 10µg gentamicin and 10µg pefloxacin all commercially prepared standard disks (oxoid) and 50µg disc of *Phyllanthus amarus* extract (MCC of *P. aeruginosa*) were tested against test and control organisms.

Phytochemical studies
Qualitative tests for flavonoids using cyaniding test (13,14), alkaloids using Dragerdorff's reagent (13,14), tannins (13), saponin using hemolytic test (13), terpenes and steroids using Leibermann-Burchard and Selzowski's tests respectively, and lipids using Sudan III solution were carried out.

RESULTS
The yield of ethanol extraction was 15.4 grams while that of aqueous extract was 12.6 grams. The result of the photochemical analysis is shown in Table 1.

Preliminary sensitivity tests showed that the test and control organisms were inhibited by 10 mg discs of ethanol and water extracts except *C. albicans* that was resistant to the aqueous extract but sensitive to ethanol extract. All the organisms tested against graded aqueous and ethanol extracts were clearly inhibited. *C. albicans* was not tested against water extract (Tables 2 and 3). The organisms were inhibited in a dose-dependent manner (Tables 2 and 3). *S. aureus* was the most sensitive organism to the extract (MIC 20 µg/ml and MCC 35µg/ml) while *P. aerugenosa* was the least sensitive (MIC 30µg/ml and MCC 50µg/ml. The MIC and MCC results are shown in Table 4.

A concentration of 50µg discs of both aqueous and ethanol extracts (MCC of *P. aeruginosa*) showed a comparable activity against the test organisms in relation to the commercially prepared disks of ampicillin gentamicin, and pefloxacine (Table 5) A percentage sensitivity of 100 was observed in both extracts against all the organisms while pefloxacine had 20% and 30% against *S. pyogenes* and *S. pneumoniae* respectively. *S. aureus* and *P. aeruginosa* were significantly inhibited by pefloxacine (100% and 80% respectively). Gentamicin showed a poor activity against *S. pyogenes* and *S. pneumoniae* (15% and 25% respectively) but *S. aureus* and *P. aeruginosa* were significantly inhibited (100% and 75% respectively). Ampicillin showed no activity against *S. aureus* and *P. aeruginosa* but significantly inhibited *S. pyogenes* and *S. pneumoniae* (100% and 95% respectively.

TABLE 1: PHYTOCHEMICAL ANALYSIS OF *PHYLLANTHUS AMARUS*

Phytochemicals	Aqueous extract	Ethanol extract
Flavonoids	+	+
Tanins	+	+
Saponin	+	+
Alkaloids	+	+
Terpenes	+	+
Steroids	+	+
Lipids	-	+

Key: + = detected; - = Not detected

TABLE 2: ANTIMICROBIAL ACTION OF GRADED AQUEOUS EXTRACT OF *PHYLLANTHUS AMARUS* AGAINST SELECTED TEST AND CONTROL ORGANISMS

Pathogens	Diameter (mm) of growth inhibition by graded aqueous extract			
	1.25 mg	2.50 mg	5.0 mg	10.0 mg
P. aeruginosa	6.0	8.0	11.0	14.35
P. aeruginosa NCTC 10662	4.0	6.0	7.5	10.85
S. aureus	10.0	13.0	15.0	18.20
S. aureus NCTC 6571	6.5	9.0	12.0	13.39
S. pyogenes	7.0	10.0	13.0	16.80
S. pneumonia	9.0	12.0	14.0	17.60
C. albicans	NT	NT	NT	NT

Key: NT – Not Tested

DISCUSSION

This study showed that both aqueous and ethanol extracts of *Phyllanthus amarus* possess antimicrobial activities against the tested *P. aeruginosa, S. aureus, S. pyogenes* and *S. pneumoniae.* Only the ethanol extract was able to inhibit *C. albicans*. This may be due to the presence of the antifungal agents in the plant which is only appreciably soluble in ethanol. Analysis of the results showed that the test organisms were all significantly inhibited when compared with the control organisms in both aqueous and ethanol extracts ($P < 0.05$). There were no significant differences between the antimicrobial activities of aqueous extract and ethanol extracts ($P > 0.05$).

Some phytochemicals found in *Phyllanthus amarus* (5) were also discovered in this study. Tannins which have been reported to be well known for their antimicrobial properties (15, 16, 17) were also detected. The antibacterial properties of flavonoids have equally been reported (18, 19). These phytochemicals are among the active ingredients detected in *P. amarus* and are active against microorganisms. The pattern of inhibition of the organisms and controls in various concentrations of the extracts showed inhibition of the organisms in a dose-dependent manner.

TABLE 3: ANTIMICROBIAL ACTION OF GRADED ETHANOL EXTRACTS OF *PHYLLANTHUS AMARUS* AGAINST SELECTED TEST AND CONTROL ORGANISMS

Pathogen	Diameter (mm) of growth inhibition by graded ethanol extract			
	1.25 mg	2.50 mg	5.0 mg	10.0 mg
P. aeruginosa	5.0	8.0	12.0	13.86
P. aeruginosa NCTC 10662	5.0	6.0	8.0	10.94
S. aureus	8.0	12.0	14.0	17.82
S. aureus NCTC 6571	7.0	10.0	12.5	13.84
S. pyogenes	6.5	9.0	14.0	16.58
S. pneumonia	10.0	13.0	16.0	16.79
C. albicans	6.0	9.0	14.0	14.36

TABLE 4: MIC AND MCC OF THE EXTRACTS AGAINST TEST AND CONTROL ORGANISMS

Organisms	MIC		MCC	
	Aqueous extract (µg/ml)	Ethanol extract (µg/ml)	Aqueous extract (µg/ml)	Ethanol extract (µg/ml)
S. aeruginosa	30	30	50	50
P. aeruginosa NCTC 10662	30	35	45	50
S. aureus	20	20	35	35
S. aureus NCTC 6571	20	20	35	35
S. pyogenes	25	20	40	40
S. pneumonia	20	20	40	40
C. albicans	NT	30	NT	45

Key – NT means Not Tested

TABLE 5: MEAN PERCENT SENSITIVITY OF DIFFERENT ISOLATES OF EACH ORGANISM AGAINST AMPICILLIN, GENTAMICIN, PEFLOXACIN AND *PHYLLANTHUS AMARUS*

Test organisms	Ampicillin 30 µg	Genticin 10 µg	Peflacine 10 µg	Aqueous extract 50 µg	Ethanol extract 50 µg
p. aeruginosa	0	75	80	100	100
S. aureus	0	100	100	100	100
S. pyogenes	100	15	20	100	100
S. pneumonia	95	25	30	100	100

This study is consistent with the findings of other workers who claimed that *P. amarus* has antibacterial properties. Contreras and Gamara (1) reported that the plant's extract at concentrations of 30, 50, 100, 200, and 300µg/ml exhibited antibacterial actions on *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis*. Farouk (20) also found out that the plant is active against Staphylococcus, Micrococcus and Pasteurella. The results of this research agree with an earlier report that *Phyllanthus amarus* inhibit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (21). There is paucity of reports on the activity of the plant against *Streptococcus pyogenes*, *Streptococcus pneumoniae and Candida albicans*. It is may be interesting to know that this study indicated that *P. amarus* is active against the multiresistant *P. aeruginosa*.

The traditional uses of the plant in fever, stomach pains, eye infections, urogenital pains and scratches, and various skin diseases appear to be supported by the outcome of this study (antibacterial and antifungal properties). However aqueous extraction may not favour the extraction of active ingredients in *Phyllanthus amarus* against *Candida albicans*. We conclude that the growth environment may only have minimal effects on the preponderance of the phytochemicals present in *Phyllanthus amarus* growing in Nnewi, Anambra State, Nigeria. *P. amarus* possesses antimicrobial actions against *P. aeruginosa, S. aureus, S. pyogenes, S. pneumoniae* and *C. albicans*. This antimicrobial activity is comparable with that of conventional antibiotics such as ampicillin, genticin and peflacine. A further study is recommended to determine the suitability of *Phyllanthus amarus* extracts in clinical trials.

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