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PHYTOCHEMICAL CONSTITUENT AND ANIMICROBIAL PROPERTIES OF PHYLAMENTHUS AMARUS (SHUM & THONN) CHAKPA HEIKRU IN MANIPUR

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ABSTRACT
The chemical composition of the leave extracts of Phyllanthus amarus (Schum and Thonn) of the family Euphorbiaceae from Nigeria was analyzed by GC-MS. The extracts were also examined for their potential to inhibit the growth of clinical isolates following standard procedure. The major compounds identified in the hexane extract are a flavonoid, flavone 4,5,7-triethoxy-3,3,6- trimethoxy (20.23%) and a triterpenoid 17-(1,5-Dimethylhexyl)-6-hydroxy-5-methylestr-9-en-3-yl acetate (19.02%) while bufalin (18.71%) and tetratetracontane (12.91%) were the major compounds detected in the methanol extract. Steroidal triterpenoids are the major compounds present in the extracts as it accounted for 47% of the total detectable content in the hexane extract and 52% in the methanol extract. The steroidal triterpenoids which exist primarily as acetate in the hexane extract include cycloeucalenyl acetate, ergosta-5,7,22-trien-3-ol acetate, macdougallin, 17-(1,5-Dimethylhexyl)-6-hydroxy-5-methylestr-9-en-3-yl acetate, stigmasterol and B-sitosterol while the methanol extract contains 6,7-epoxypregn-4-ene-9,11,18-triol-3,20-dione, 11,18-diacetate, bufalin, olean-13(18)-ene, methyl ursolate, barringenol R1 and 7,8- epoxylanostan-11-ol,3-acetoxy. Hexane extract of the plant exhibited antifungal activity on Candida albicans while methanol extract revealed significant antibacterial activity on Pseudomonas aeruginosa and Staphylococcus aureus at all concentrations of the extract between 12.5 and 100 mg/mL, the activity being comparable to the standard antibacterial drug, Oxacillin. The leaves of Phyllanthus amarus is a potential source of steroidal triterpenoids which could serve as biomarker for the plant species. The extracts of the plant may also serve as a natural source of antimicrobial agents for the treatment of some microbial infections.

KEYWORDS: Phytochemicals, antimicrobial, leaf extracts, phyllathus amarus.

INTRODUCTION
The Genus Phyllanthus (family: Phyllantheae) consists of approximately 1000 species, spread over the American, African, Australian and Asian Continents (Tasheen and Mishra, 2013). All the major habits are trees, shrubs and herbs are seen amongst the Phyllanthus species. Most of the herbs, belonging to the Phyllanthus, afford various secondary metabolites with important medicinal properties. Bioactives such as alkaloids, flavonoids, lignins, phenols, tannins and terpenes have been isolated from these plants (Calixto, 1998; Bahar et al, 2011).

Phyllanthus amarus (Schum and Thonn) is one of the most pharmacologically important species of the Phyllanthus family. It is a medicinally important plant belonging to Euphorbiaceae otherwise known as “stone breaker”, “carry me seed” etc. In Nigeria, the plant is called “geron tsuntsaye” (Hausa), “eyin- olobe” (Yoruba) and “ngwu” (Igbo). Phyllanthus amarus is an erect annual herb of not more than one and half feet tall. It has small leaves and yellow flowers. It is commonly found in forest areas, arid land, savannah areas, leached and exhausted soil in many countries including China, India, Nigeria, Cuba and Philippines amongst others (Burkill, 1994; Bharatiya, 1992).

The decoctions of various parts of the herbs are used traditionally for the treatment of hepatic, urinary and sexually transmitted diseases, diabetes, hypertension and cancer. In folk medicine, P. Amarus herb has found applications in the management of several health problems such as diarrhea, dysentery, dropsy. Jaundice, intermittent fevers, urinogenital disorders, scabies and wounds. Literature indicated the application of the plant in the treatment and management of kidney problems, appendix inflammation, prostate problems, pain, gonorrhea, diabetes and chronic dysentery. Topically, it is used for several skin-related problems ranging from skin ulcers, sores, swelling, itchiness, wounds, bruises, scabies, ulcers, sores, edematous swellings, tubercular ulcers, ringworm, scabby and crusty lesions (Khartoon et al., 2004; Sen and Batra, 2013; Ushie et al, 2013). Other reported pharmacological activities of the plant indude

anticancer, antioxidant, antileptospiral, antimicrobial, anti-diabetic, and inflammatory and anti-convulsant activities (Rajeshkumar et al, 2002; Lim and Murtijaya, 2007; Chandan et al, 2012; Oluwafemi and Debiri, 2008; Bahar, 2011; Evi and Degbeku, 2011; Saranraj and Sivasakthivelan, 2012; Adeolu and Sunday, 2013; Manikkoh et al., 2011; Uander et al., 1995). Numerous phytochemicals such as alkaloids, flavonoids, tannins, lignans and polyphenolics as well as tetracyclic triterpenoids have identified and isolated from the plant (Foo and Wong, 1992; Foo, 1993 and 1995; Leite et al., 2006; Londhe et al., 2009; Moronkola et al., 2009). Based on the numerous folkloric applications of Phyllanthus amarus that have not been scientifically proven, this study aimed at identifying the chemical composition of the non-polar and polar extracts of P. Amarus leaves and evaluate the antimicrobial activities of the extracts as well.

MATERIALS AND METHODS

Materials and Reagents

Solvents used were of analytical grade, and when necessary, solvents were redistilled before use. Freshly prepared phytochemical screening reagents were used. Thin layer chromatography was performed on aluminum sheet 20 x 20 cm already pre-coated with silica get 60-120 mesh with a thickness of 200 um Merck, Germany and spots were viewed under UV lamp model SAFE Germany. The organisms used for the antimicrobial testing were clinical bacterial and fungal isolates from the Medical Laboratory of the Microbiology and Parasitology Unit of the University of Ilorin Teaching Hospital, Ilorin, Nigeria.

Sample Collection and Preparation

Fresh leaves of P. amarus were collected in the month of September, 2014 from Tanke-Bubu in Ilorin South Local Government Area of Kwara State, Nigeria. The plant was identified and authenticated at Herbarium of the Department of Plant Biology University of Ilorin, Nigeria, with the voucher number UIH002/884. The fresh leaves collected were air-dried for two weeks and thereafter pulverized to powder using a mortar and pestle and kept in a cellophane bag in a cool place until further work.

Extraction of Plant Material

Air-dried pulverized leaves sample of P. Amarus (150 g) was cold extracted in distilled n-hexane, ethylacetate and methanol separately for 3 days each by agitating and decanting for three successive extractions. The extracts were filtered using Whatman No. 2 filter paper. The filtrate was evaporated to dryness using rotatory evaporator. The obtained crude extracts were stored in a screw cap glass vials in refrigerator below 10°C for further analysis (Dada et al., 2014).

Phytochemical Screening

Phytochemical tests were carried out on the crude n-hexane and methanol extracts of the leaves using standard procedures to identify the constituents (Trease and Evans, 1988; Harbone, 1998; Sofowora, 1993a and 1993b).

Gas Chromatography Mass Spectrometry (GC- MS)

The crude extract of the leaves of P. Amarus (n-hexane and methanol) were subjected to GC-MS analysis using Agilent 19091S-433 equipped with column of 30 m long (0.25 µm x 250 µm). The carrier gas was helium with a flow rate of 4°C/min using a split less injector mode (at 250°C). About 1 µL of the sample was injected by an auto sampler, over the oven temperature programme: From 40 to 300°C and a total run-time of 95 minutes. The relative percentage constituents were evaluated based on an estimate of the depicted area of the peak in the total ion chromatogram. Identification of the compounds was done by comparing the mass spectra fragmentation pattern with NIST database (NIST, 2009; Adams, 1995).

Antimicrobial Activity

Cultures of two human pathogenic bacteria were used for the antibacterial assay. These were: Staphylococcus aureus and Pseudomonas aeruginosa while two fungi which were also utilized for antifungal assay were Candida albicans and Aspergillus flavus. All the microorganisms used were clinical strains from the Medical Microbiology Department, University Teaching Hospital, Ilorin, screened in the Laboratory of Microbiology Department, University of Ilorin. Nutrient agar, Sabouraud dextrose agar, nutrient broth and tryptone soya agar were used in this study. N-hexane and methanol were also used in solubilizing the extracts and as negative controls in the assays. Oxacillin (antibacterial) and Griseofluvin (antifungal) were employed as standard reference drugs in the study (Ghuman et al., 2016; Prasad et al., 2013).

Determination of Antimicrobial activity

The anti-bacteria activity was done with Agar Diffusion Ditch method. An overnight culture of each organism was prepared by taken two wire-loop of the organism from the stock, each inoculated into 5ml of sterile nutrient broth and incubated for 24 hr at 37°C. From the cultured organisms, 0.1 ml of each organism was taken from the overnight culture and put into the 9.9 mL of sterile distilled water to obtained 102 inoculum concentration of the test organism. Subsequently, 0.2 ml was taken from the diluted test organism (10) into the prepared sterile nutrient agar cooled to about 45°C, then poured into sterile Petri dishes and allowed to solidify for 60 min. A sterile cork-borer of 8mm diameter was used to make 8 wells on the media according to the number of the diluted extracts for the experiment. The graded concentrations (6.25 – 200 mg/ml) of the extracts were put into each well and separated from the controls. The studies were done in duplicates to ascertain the results obtained. The plates were left on the bench for about 2 hr to allow the extract diffuse properly into the nutrient agar

l.e. pre-diffusion. The plates were incubated for 24 hr at 37°C (Bauer et al., 1996).

Agar diffusion-Surface method was used for the antifungal assay, a sterile sabouraud dextrose agar was prepared accordingly and aseptically poured into the sterile plates in triplicates and solidified properly, then 0.2 ml of the 102 inoculum concentration of the test organism was spread on the surface of the agar using a sterile Petri-dish to cover all the surface of the agar. Eight wells were bored by using a sterile cork- borer of 8mm diameter. The graded concentrations of the extracts were put into each well separately with the controls.

All the plates were left on the bench for 2hr to allow the extract diffuse properly into the agar i.e. pre- diffusion. The plates were incubated at 25°C for 72 hr (Oshima et al., 1995; El et al., 2016)

Minimum Inhibition Concentration (MIC)

Minimum inhibitory concentration (MIC) of n-hexane and methanol extracts with standard antimicrobial drugs such as oxacillin (antibacterial) and griseofulvin (antifungal) were determined by preparing solution of the samples at various concentrations of 12.5, 25, 50,100 and 125 mg/ml. Exactly 9 ml of sterile peptone water was dispensed into each test tube, then 1ml of each of the extract at different concentrations were introduced and mixed in a test tube. 0.1 ml of inocula was added to each tube. The tubes were incubated aerobically at 37°C for 24 hrs for bacteria and 48hrs for fungi. The growth of the inoculum in the broth is indicated by turbidity or cloudiness of the broth and the lowest concentration of the extract, which inhibited the growth of the test organism was taken as the Minimum Inhibitory Concentration (MIC) (El *et al.,* 2016).

Two control tubes were maintained for each test batch. These included antibiotic control (tube containing extract and the growth medium without the inocula) and organism control (the tube containing the growth medium and the inocula only). The lowest concentration (higher dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tube was regarded as minimum inhibitory concentration (MIC) (Fawole and Oso, 2007).

RESULTS AND DISCUSSION

The result of the phytochemical screening (Table 1) indicate that alkaloids, tannins, terpenoids, saponins, phenolics, flavonoids and steroids were present in the n-hexane extract of the plant of *P. amarus*. Glycosides and carbohydrate are not within the detectable range. However, all the class of tested phytochemicals with the exception of saponins was detected in the methanol extract. The result corroborates previous report which indicates that the phytochemicals are responsible for the activities of *P. amarus* (Foo and Wong, 1992).

Table 1: Phytochemical constituents of hexane and methanol leaf extracts of *Phyllanthus amarus*

Class of Compound	Hexane Extract	Methanol Extract
Carbohydrate	_	+
Alkaloids	+	+
Tannins	+	+
Saponins	+	-
Steroid	+	+
Terpenoids	+	+
Phenolics	+	+
Flavonoids	+	+
Glycosides	_	+

+ = present; - = absent

The chemical compositions of hexane and methanol leave extracts of *P. amarus* were determined by subjecting the extracts to GC-MS analyses. The compounds were identified by marching their respective mass spectrum of each compound with the library. The total ion chromatogram indicated ten peaks which correspond to ten compounds of which eight (Table 2) were identified and two unidentified in the hexane extract. Nineteen peaks corresponding to nineteen compounds were observed in the methanol extract. Seventeen compounds (Table 3) were identified and two compounds could not be identified. The major class of compound in both extracts is the steroidal triterpenoid (Figs. 2 and 3) which adds credence to the result obtained for the phytochemical screening obtained *ab initio*. The steroidal triterpenoids (Figs. 1 and 2) accounted for 47% of the total detectable content in the hexane extract and 52% in the methanol extract. The major compounds identified in the hexane extract are a flavonoid, flavone, 4′,5,7-triethoxy-3,3′,6-trimethoxy (20.23%) and a triternoid 17-(1,5-Dimethylhexyl)-6-hydroxy-5-methylestr-9-en-3-yl acetate (19.02%) while bufalin (18.71%) and tetratetracontane (12.91%) were the major compounds detected in the methanol extract.

Alpha-Tocopherol was present in significant amount in the methanol extract (9.41%). Alpha-Tocopherol is known to play lipophilic antioxidant role in plants. It has been isolated from series of plants including corn, olive, soyabean, sunflower, grape seeds and seedlings of pine (Isidorov *et al.,* 2008; Swiglo *et al.,* 2007). Bufalin (18.71%) which was the most prominent compound in the methanol extract is also a steroidal triterpenoid. It is a cardiotonic steroid originally isolated from the Chinese toad venom. It is a component found in many Chinese traditional medicine. Bufalin has an antitumor effect against various malignancies such as hepatocellular and lung carcinoma (Zhang, 2014; Wu, 2014; Qiu *et al.,* 2013*).* The flavonoids identified in the n-hexane extract have been detected in other plants (Foo and Wong, 1992; Foo, 1993 and 1995). Macdougallin (2.96%) observed in the hexane extract have been isolated from *Myrtillocactus geometrizans* and tested for anti-inflammatory activity (Salazar *et al.,* 2011).

Although, there have being no report of the isolation of steroidal compounds from the plant, most of the identified compounds are steroidal triterpenoids, which supports the phytochemical results as both hexane and methanol extracts tested positive for both steroids and terpenes.

Table 2: Compounds detected in the hexane crude leaf extracts of *Phyllanthus amarus*

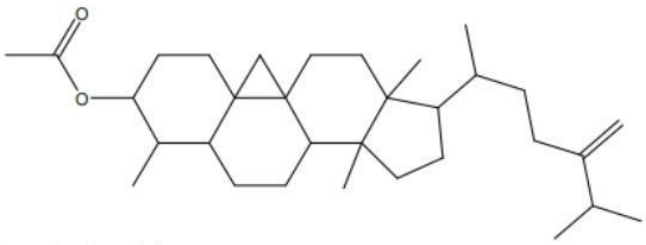
S/N	RT	Compounds	% RA
1	65.40	Cycloeucalenyl acetate	5.51
2	66.45	Unidentifiable	0.86
3	67.22	2′,3′-O-p-Anisylideneguaanosine	9.96
4	67.9	Unidentifiable	21.86
5	68.08	Macdougallin	2.96
6	68.19	Ergosta-5,7,22-trien-3-ol, acetate	16.21
7	69.13	Flavone, 4′,5,7-triethoxy-3,3′,6-trimethoxy	20.23
8	70.31	17-(1,5-Dimethylhexyl)-6-hydroxy-5-methylestr-9-en-3-yl acetate	19.02
9	72.91	Stigmasterol	0.81
10	73.78	β-sitosterol	2.58
		Total Unidentified compounds	**22.72**
		Triterpenoids	**47.09**
		Non-Triterpenoids	**52.91**

RT indicates retention time on the column in minutes. %RA indicates percentage relative abundance (peak area relative to the total peak area).

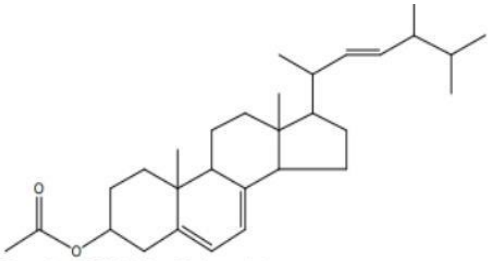
Table 3: Compounds detected in the methanol crude leaf extracts of *Phyllanthus amarus*

S/N	RT	Compounds	% RA
1	42.98	Hexadecanoic acid	1.00
2	47.09	Methyl linolenate	0.99
3	47.46	Phytol	1.05
4	48.60	Linolenic acid	2.56
5	62.21	Tetratetracontane	12.91
6	63.38	α-Tocopherol	9.41
7	65.58	6,7-Epoxypregn-4-ene-9,11,18-triol-3,20-dione, 11,18-diacetate	8.20
8	69.06	Bufalin	18.71
9	69.42	2,3,3′,4′-tetramethoxy-5-(3-methoxyprop-1-enly)stilbene	6.50
10	70.97	Olean-13(18)-ene	2.70
11	72.75	7,8-Epoxylanostan-11-ol,3-acetoxy	3.76
12	73.88	Docosanoic acid,1,2,3-propanetriyl ester	2.69
13	75.47	Methyl ursolate	7.11
14	75.80	Barringenol R1	7.86
15	77.24	7,8-Epoxylanostan-11-ol,3-acetoxy	3.92
16	77.25	Rhodopin	1.91
17	78.55	Unidentifiable	3.98
18	79.51	Tetrahydrospirilloxanthin	3.77
19	82.99	Unidentifiable	0.97
		Total Unidentified Compounds	**4.95**
		Steroidal Triterpenoids	**52.26**
		Non- Steroidal Triterpenoids	**47.74**

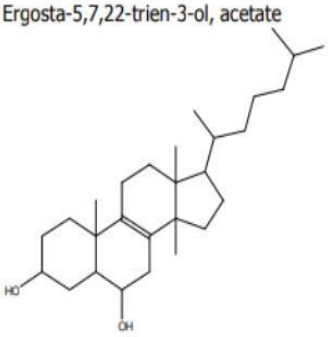
RT indicates retention time on the column in minutes. %RA indicates percentage relative abundance (peak area relative to the total peak area).



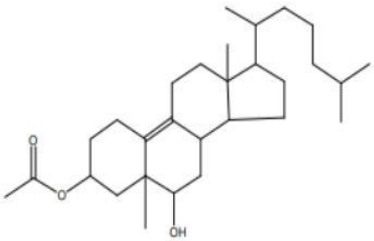
Cycloeucalenyl acetate



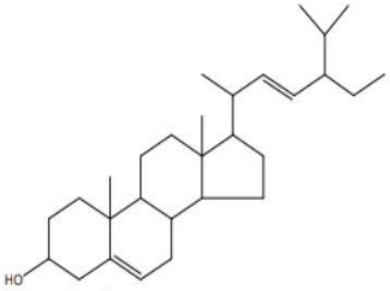
Ergosta-5,7,22-trien-3-ol, acetate



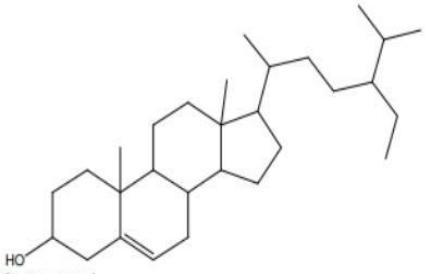
Macdougallin



17-(1,5-Dimethylhexyl)-6-hydroxy-5-methylestr-9-en-3-yl acetate

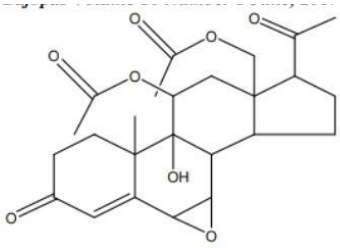


Stigmasterol

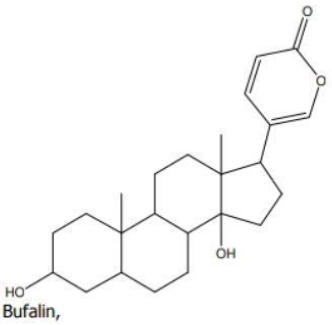


β-sitosterol

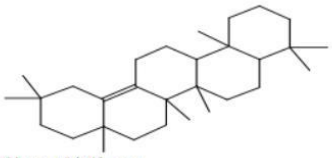
Figure 1: Steroidal triterpenoids detected in the n-hexane extract of *Phyllanthus amarus*



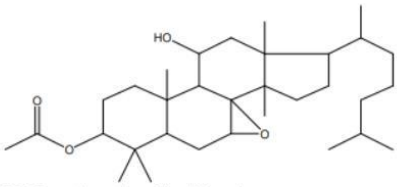
6,7-Epoxypregn-4-ene-9,11,18-triol-3,20-dione, 11,18-diacetate



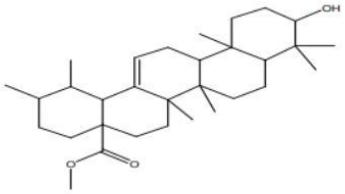
Bufalin,



Olean-13(18)-ene



7,8-Epoxylanostan-11-ol,3-acetoxy



Methyl ursolate

Table 4: Antimicrobial activity of hexane extract of the leaves of *Phyllanthus amarus*

Organisms	Concentrations (mg/ml)	Zone of inhibition (mm)		
		Methanol	*n*-Hexane	Oxacillin
Bacteria				
Pseudomonas aeruginosa	12.5	16	0	15
	25	19	0	16
	50	20	0	19
	75	22	12	26
	100	26	13	32
Staphylococcus aureus	12.5	14	0	15
	25	21	0	17
	50	24	0	18
	75	25	0	27
	100	25	11	31
Fungi				Griseofulvin
Candida albicans	12.5	0	9	17
	25	0	12	17
	50	0	16	18
	75	0	15	25
	100	19	10	30
Aspergillus flavus	12.5	0	0	16
	25	0	0	25
	50	0	0	18
	75	14	0	27
	100	15	19	31

CONCLUSION

Phytochemical composition of the leaves extracts of Phyllanthus amarus (Schum and Thonn) of the family Euphorbiaceae from Manipur has been established. The GC-MS result o the hexane extract showed ten compounds while nineteen compounds were indicated in the methanol extract. Steroidal triterpenoids represent the primary constituents in both hexane and methanol extracts. The major triterpenoids include cycloeucalenyl acetate, ergosta-5,7,22-trien-3- ol acetate, macdougallin, 17-(1,5-Dimethylhexyl)-6- hydroxy-5-methylestr-9-en-3-yl acetate, stigmasterol and B-sitosterol in the hexane extract and 6,7- epoxypregn-4-ene-9,11,18-triol-3,20-dione, 11,18- diacetate, bufalin, olean-13(18)-ene, methyl ursolate, barringenol R1 and 7,8-epoxylanostan-11-ol,3-acetoxy in the methanol extract. Hexane extract possesses antifungal property against Candida albicans while methanol extract of the plant exhibited significant antibacterial activity against Pseudomonas aeruginosa and Staphylococcus aureus at both low and high concentrations of the extract. The plant is a rich source of uncommon triterpenes and thus could be a viable source of natural anti-microbial agents.

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