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Spectroscopic and Antimicrobial Activities of Fractions Derived from Phyllanthus Amarus Schum and Thonn Aqueous Leaf Extract

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Abstract The purpose of study was to carryout spectroscopic study of four fractions obtained from Phyllanthus amarus Schum. and Thonn. aqueous leaf. extract and determine their antimicrobial activities. The plant leaves were dried, powdered and extracted with sterile distilled water. Aqueous extract obtained was fractionated with solvents of varying polarity through column chromatography. Fractions were characterized using UV-VIS, FTIR and GC/MS techniques. The UV-VIS profile indicated different peaks ranging from 200-800nm, Fraction A showed peaks at 661,491,396 and 272, fraction B, 660 and 600.50. Fraction C had only one peak, 651, and fraction D, two peaks 759,294. The IR spectrum showed hydroxyl (OH) absorption, methylene, methyl and carboxylic group absorptions. Compounds present in the various fractions included 1,2-benzenedicarboxylic acid dioctyl ester (phthalic acid), ricinoleic acid, pentadecanoic acid, tetradecanoic acid, Glycerin, 2-Coumaranone, n-hexadecanoic acid and others as detected by gas chromatography mass spectra. Compound obtained from the fractions were potent against *Escherichia coli, Staphylococcus aureus* and *Candida albicans*.The antimicrobial properties observed in these fractions are reflection of the compounds present in aqueous extract as detected by the spectroscopic analyses.

Keywords: *aqueous leaf extract, Chromatographic techniques, Spectroscopic methods. Phyllanthus amarus*

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1. Introduction

Phyllanthus amarus Schum. and Thonn. is an annual plant. It grows as weed in villages, cultivated fields and gardens. It can also be seen in waste ground, grassy shrub vegetation and dry deciduous forest. It thrives on sandy and humid soils at 1000 meters above the sea level [1]. *Phyllanthus amarus* has explosive seed capsules that propel the seeds some distance [2]. In Nigeria, *P. amarus* aqueous extract is used to eliminate waste from the body, build the blood and immune system. It is used to induce labour ,manage oedema and restore the liver [3,4] Though the plant has much medicinal properties which is exploited in ethnomedicine, little information is available on adverse effect, phytochemical,mineral,and proximate composition of aqueous extract of *P. amarus* [4]. The aim of study was to carryout spectroscopic and antimicrobial activities of fraction of *Phyllanthus amarus* aqueous leaf extract.

2. Materials and Methods

Dried powdered *Phyllanthus amarus* leaves (1.5kg) formerly collected from the wild and identified in Department of Botany, Faculty of Science, Delta State University, Abraka, were extracted with 5.8litres of distilled water by cold maceration method for three days.The crude extract was filtered with Whatman No. 1 Filter paper daily within the three days and evaporated in water bath regulated within 60-70°C. The weight of extract was noted and labelled and then stored at 4°C until required.

Fractionation of *Phyllanthus amarus* aqueous leaf extract.

The aqueous extract was packed to a column chromatography to separate the extract into various fractions. Silica gel and solvents with increasing polarity was used as described previously by other researchers

[5,6]. The lower part of the column was blocked with cotton wool using a class rod. Five grammes (5g) of aqueous extract of *P. amarus* obtained as described above was mixed with 10g of silica gel (60 – 120 mesh). The admixed was loaded in a column (5cm diameter × 50cm, height) packed with silica gel (150g) using hexane as the solvent. The column was eluted with a mixture of solvent with increasing polarity starting with chloroform, then ethylacetate and methanol The ratio of solvent combination used included Chloroform : ethylacetate, 1:0, 1:1, 1:9, Ethyl acetate: methanol 1:0 , 9:1.4:1,1:1,1:4, methanol 1:0. Each solvent/solvent combination volume was 100ml and 31 fractions were collected.

Fractions were spotted on commercial precoated silica gel 254 adsorbent (0.25mm thick) plates. Precoated thin layers plates were dried in air and developed in the chromatographic tanks using different solvent systems. Fractions of 15ml were collected and each sample was subjected to thin layer and paper chromatography. Samples that had similar peaks after visualization under ultra violet light at 242 and 254nm and development under suitable solvents were bulked together. Four fractions were obtained from thirty – one fractions eluted. The eluates were concentrated to dryness, weighed and subjected to antimicrobial and spectroscopic studies.

Characterization of aqueous leaf extract of *P. amarus* fractions using Spectroscopic Techniques

The Ultra violet –visible spectroscopy (UV-VIS) was measured on UV-2500PC Series and Infra red spectra on FTIR -8400S Fourier Transform Infrared Spectrophotometer. The GC-MS analysis was done using Shimadzu QP 2010 EI.

The fractions were analyzed in UV-Visible range between 200-800 nm using UV-Visible Spectrophotometer.

Fourier transform infrared spectrophotometer was used to analyze the compounds obtained from *Phyllanthus amarus.* The spectrum was focused on IR range between 500-4500 cm-^{1} by the KBr pellet technique. A small quantity (0.1g) of sample and 0.025g of dry potassium bromide (KBr) were homogenized using mortal and pestle. A portion of the homogenized mixture was placed on the disc and pressed using a mini heat press to form a KBr thin film and the disc was placed in the FT-IR spectrophotometer in which spectra was measured, accumulating 64 scans at 4 cm-^{1} resolution in the spectra range of 4500-500cm-1. Percentage transmittance was plotted against wavelength. The FT-IR spectra were used to identify the functional groups of active metabolites based on the peak values in the infra red region

One microlitre aliquot of the sample solution was injected into the GC-MS equipment, for Gas chromatography/mass spectrometry detection. An electron ionization system with ionization energy of 70ev was used. The carrier gas was Helium (99.99%), used at a constant flow rate of 1ml/min, injector and mass transfer line temperature were set at 250°C and 200°C respectively, and an injection volume of 1µl was employed (split ratio 10:1), the oven temperature was programmed from 80°C (isothermal for 2min), with an increase of 9°C/min to 200°C for 4min, 10°C/min to 280°C, ending with a 5min isothermal at 280°C. The MS operating parameters were as follow : ionization energy, 70eV; ion source temperature, 200°C, solvent cut time, 3.0min, relative detector gain mode, scan speed 1428µ/sec; scan range 50-700Da, the interface temperature was 250°C. The total running time of GC-MS was 30min. The relative percentage of the extract was expressed as percentage with peak area normalization.

Antimicrobial Activities of the Fractions using Kirby-Bauer Method.

Source of Bacteria and fungi isolates used

The bacteria used for the study were clinical isolates from urine and urethral swab formerly identified [7,8] They included *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The isolates were revived by culturing in Muller Hinton and Saboraud dextrose broths (oxoid, UK). The organisms were diluted based on MacFarland standard. The plate were inoculated with the test organisms using sterile swab stick. Disk impregnated with different fractions (different concentrations 25mg/ml, 12.5mg/ml, 6,25mg/ml, 3.125mg/ml) were introduced into plates in equidistant positions and then allowed to diffuse on laboratory bench for an hour, plates were then incubated for 24-48 hours at 37°C. Inhibition zone were measured using a meter rule.

Determination of Inhibitory/Bactericidal/ Fungicidal Concentrations

Minimum inhibitory, bactericidal and fungicidal concentrations of fractions were determined using broth dilution method [9]. The reconstituted compound was diluted (double dilution) in Mueller-Hinton and Saboraud broth. Duplicate tubes of each dilution 25mg/ml - 3.125mg/ml were used. The broth was inoculated with 5 X10^{8} cells (cfu/ml) of the test bacteria/ fungus cultures, incubated at 37°C for 24hours for bacterial and 48hours for fungus. Minimum inhibitory concentration was taken as the highest dilution (least concentration) of compound showing no detectable growth.

The minimum bactericidal / fungicidal concentration was determined by streaking out from the tubes of MIC on the Mueller Hinton agar and Saboraud dextrose agar. The lowest concentration that prevented bacteria / fungi growth after incubation, indicated the minimum bactericidal / fungicidal concentration.

3. Result and Discussion

UV-VIS analysis

The UV-VIS analysis was performed for identification of phytoconstituents present in the various fractions of aqueous leaf extract of *Phyllanthus amarus*. The UV-VIS profile of fractions were taken at the wavelength of 200-800nm. The fraction A showed peaks at 661,491,396 and 272. With absorption 0.109.0.132,0.222 and 2.501. Two peaks (660 and 600.50, with absorption of 0.384, 0.311) were showed in fraction B (Figure 2). Fraction C had only one peak 651, and absorption 0.0084 (Figure 3), while fraction D showed two peaks 759,294, absorption 0.081 and 2.994 respectively. The use of UV-VIS spectroscopy for elucidation of chemical structure is limited because of difficulty in assigning the absorption peaks to any particular constituents in the system hence the need for

FTIR and GCMS for definite characterization of molecular skeleton and functional groups in isolation and identification of chemical compounds in plants [6].

IR analysis

The IR spectrum showed hydroxyl (OH) absorption at 3434; methylene, methyl at 2926 and carboxylic group absorption at 2351 and absorption characteristic of its aromatic nature were also discernable in the spectrum (1722, 1461,1274 cm^{-1}). For fraction B, IR spectrum showed hydroxyl absorption of 1701 cm^{-1}, an absorption characteristic of presence of esters. The IR spectrum showed hydroxyl OH absorptions of 3398 methylene, methyl at 2946 and ester absorption at 2063 for C and the IR spectrum for D showed hydroxyl (OH) absorption at 3384 and 2940, carbonyl absorption at 1703cm-1. Spectroscopic and chromatographic methods are used to determine and estimate the presence of phytoconstituents of plants of medicinal importance. Fourier transform infrared spectroscopy y is used to determine the functional properties and structural information of a compound. From the results in Figure 5 - 8, the various fractions showed absorption band and the prominent peaks are described in Table 1. The peaks at 3434, 3386, 3398, 3384cm-1 of various fractions are assigned to O-H stretching vibration. Methylene; 2926, 2936, 2946, 2940cm-1 were also present. The presence of carboxylic group 2351,2352, 2063, 1703cm-1, thus depicting that carboxylic compounds were present in the fractions molecular skeleton, Absence or presence of functional groups within plant and plant products have been characterized using characteristic peaks in FTIR spectrum [10]. The medicinal properties of plants are due to functional groups present which are confirmed with FTIR [11]. Fourier transform infrared spectroscopy are also used to identify the concrete structure of certain plant secondary metabolites [12,13,14].

Table 1. IR Spectra Band Assignment for Chlorofom,Ethylacetate, Ethylacetate+Methanol and Methanol of Aqueous leaf Extract of *Phyllanthus amarus*

CHLOROFORM fraction A	ETHYLACETATE fraction B	ETHYLACETATE+METHANOLfraction C	METHANOL fraction D	Band assignment
3434.39	3386.15	3398.69	3384.22	O-H
2926.11	2936.75	2946.36	2940.58	CH2,CH3
2351.30	23.52.27	2063.90	1703.20	0=C-O
1722.49	1701.27	1715.74	1627.01	C=O
1461.13	1620.26	1398.44	1409.05	CH2,CH3 or O-H bending
1274.03	145.09	12.29.66	1236.41	O-H bending
1091.75	1030.99	1051.24	1057.99	C-O stretching
436.89/836.17 base	440/836.17 base	437/914.2	462.93/931.65	Aromatic ring, Fingerprint region

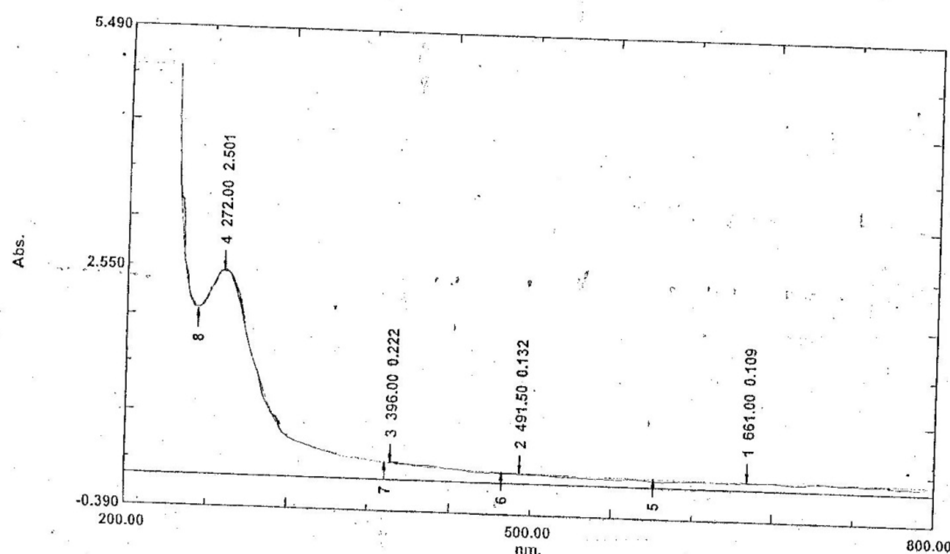

Figure 1. The UV spectrum of Fraction A of *Phyllanthus amarus* aqueous leaf extract

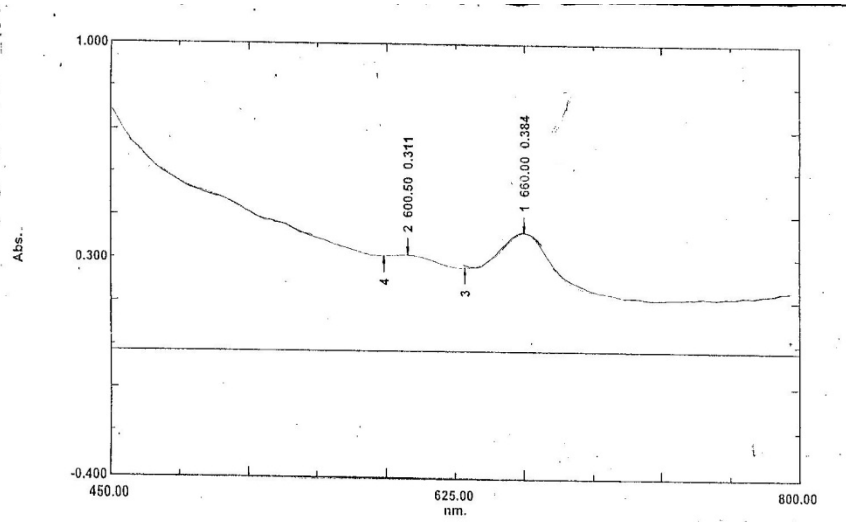

Figure 2. The UV spectrum of Fraction B of *Phyllanthus amarus* aqueous leaf extract

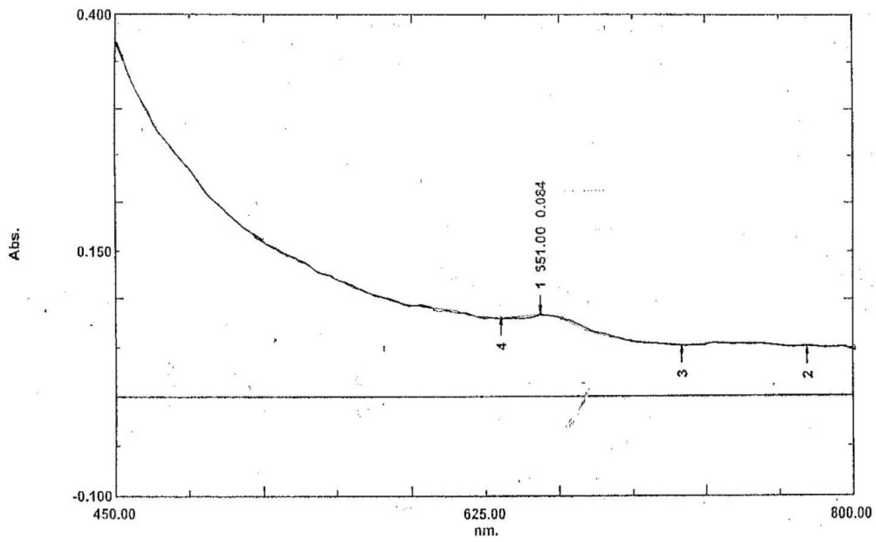

Figure 3. The UV spectrum of Fraction C of *Phyllanthus amarus* aqueous leaf extract

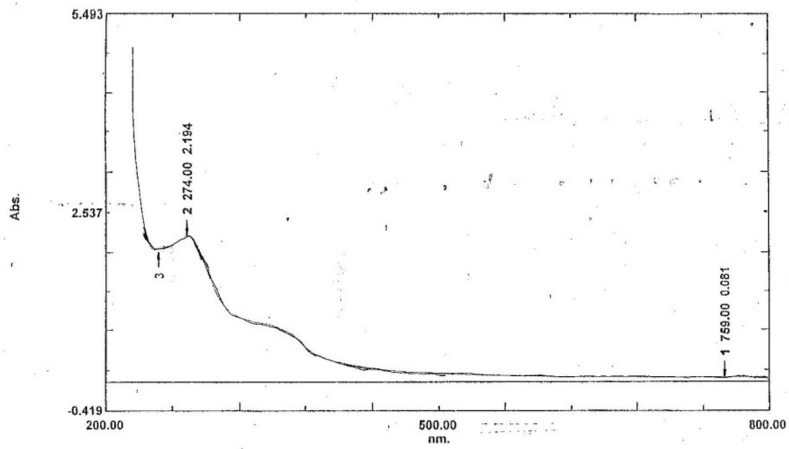

Figure 4. The UV spectrum of Fraction D of *Phyllanthus amarus* aqueous leaf extract

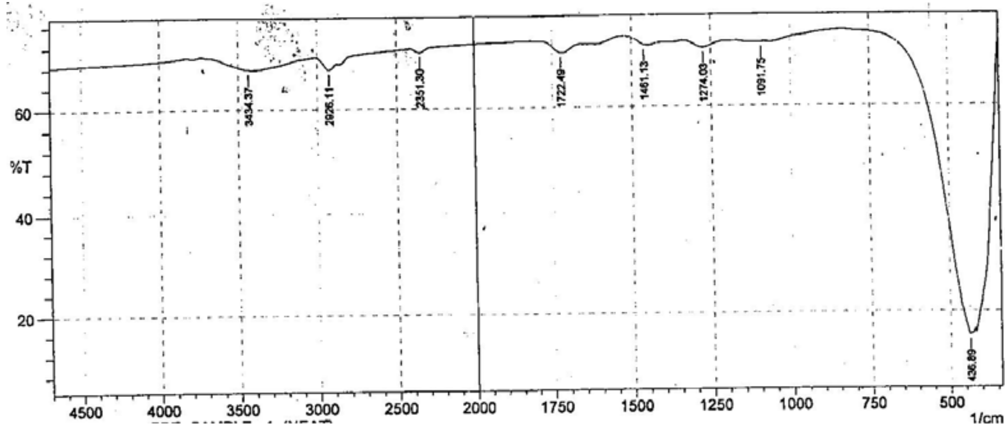

Figure 5. FTIR spectrum of Fraction A of *Phyllanthus amarus* aqueous leaf extract

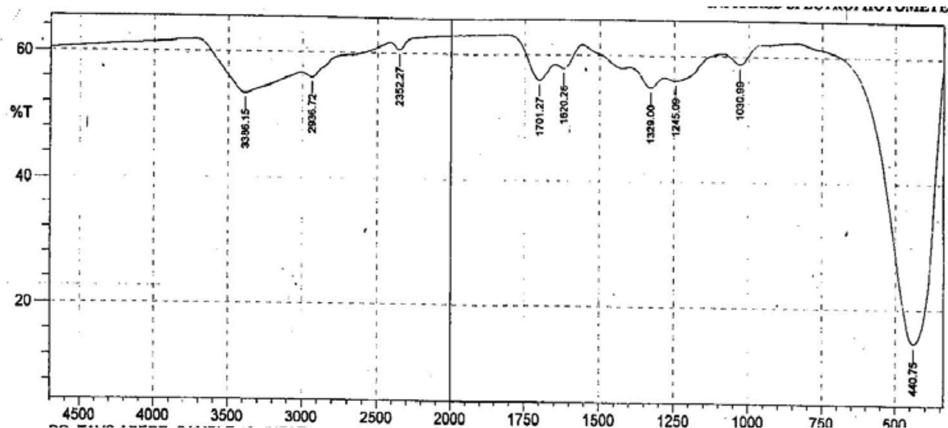

Figure 6. FTIR spectrum of Fraction B of *Phyllanthus amarus* aqueous leaf extract

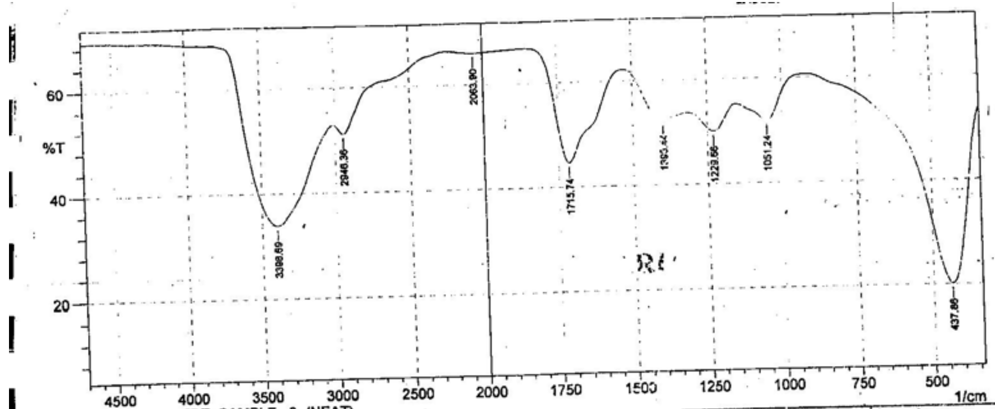

Figure 7. FTIR spectrum of Fraction C of *Phyllanthus amarus* aqueous leaf extract

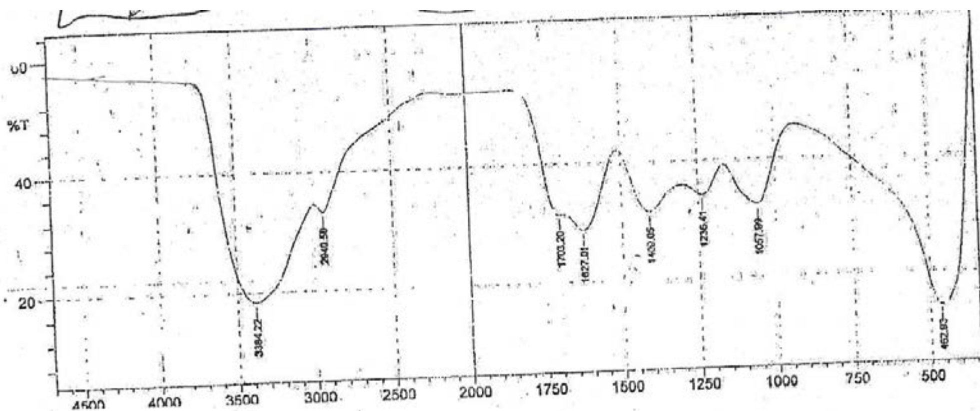

Figure 8. FTIR spectrum of Fraction D of *Phyllanthus amarus* aqueous leaf extract

Table 2. GC-MS Analysis of Various fractions from aqueous leaf extract of *Phyllanthus amarus*

S/No	Retention Time	Name Of Compound	Molecular Formulae	Molecular Weight	Area%
1	16.69	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.96
2	19.06	Hexadecanoic acid	$C_{17}H_{34}O_2$	270	2.70
3	19.31	1,2-Benzenedicarbxylic acid	$C_{16}H_{22}O4$	278	1.60
4	20.41	Pentadecanoic acid	$C_{15}H_{30}O_2$		21.44
5	22.01	13-Hexyloxacyclotridec-10-en-2-one	$C_{18}H_{32}0_2$	280	2.69
6	22.30	9-octadecenoic acid	$C_{19}H_{36}O_2$	298	4.39
7	22.64	Octadecanoic acid methyl ester	$C_{19}H_{38}O_2$	296	3.12
8	23.31	18-Nonadecenioc acid	$C_{19}H_{36}O_2$	312	43.40
9	24.94	Ricinoleic acid methyl ester	$C_{19}H_{36}O_2$	184	3.50
10	25.70	Oxacyclododecan-2-one	$C_{11}H_{20}O_2$	338	4.94
11	26.76	(E)-13-Docosenoic acid	$C_{22}H_{42}O_2$	210	3.68
12	27.46	13-tetradecenal	C14H26O	390	5.80
13	28.72	Di-n-octylphthalate	$C_{24}H_{38}O_4$	224	1.77
		Fraction B			
1	12.86	1,2,3-Benzenetriol	$C_6H_6O_3$	126	22.00
2	16.68	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.72
3	19.07	Pentadecanoic acid14-methyl-methyl ester	$C_{17}H_{34}O_2$	270	1.11
4	20.45	Tridecanoic acid	$C_{13}H_{26}O_3$	214	17.31
5	22.02	13-Heyloxacyclotridec-10-en-2-one	$C_{18}H_{32}O_2$	280	1.58
6	22.31	8-octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	2.91
7	23.35	6-octadecenoic acid	$C_{18}H_{36}O_3$	282	43.02
8	24.95	Methy ricinoleate	$C_{19}H_{34}O_3$	312	1.48
9	25.74	Ricinoleic acid	$C_{18}H_{34}O_3$	298	7.59
10	26.76	9-octadecenoic acid	$C_{57}H_{104}O_6$	884	2.28
		Fraction C			
1	7.01	Hydrazine,1-methy-1-thioacetyl	$C_3H_8N_2S$	104	5.09
2	7.98	Butanedioic acid, monomethyl ester	$C_5H_8O_4$	132	11.64
3	11.69	1,2-propanediol,3-(methylthio)-	$C_4H_{10}O_6S$	122	8.64
4	12.99	1,2,3-Benzenetriol	$C_6H_6O_3$	126	7.08
5	14.77	Butanedioic acid, dimethyl ester	$C_6H_{10}O_4$	146	1.05
6	19.07	Hexadecanioc acid methyl ester	$C_{17}H_{34}O_2$	270	0.95
7	20.46	Tridecanoic acid	$C_{13}H_{26}O_2$	214	14.24
8	22.31	9-octadecenoic acid	$C_{19}H_{36}O_2$	296	2.54
9	23.36	Decanoic acid,10-(2-hexylcyclopropyl	$C_{19}H_{36}O_2$	296	34.25
10	23.76	Ricinoleic acid	$C_{18}H_{34}O_3$	298	14.32
		Fraction D			
1	6.19	Glycerin	$C_3H_{18}O_3$	92	1.86
2	9.46	2-Coumaranone	$C_8H_{16}O_2$	134	1.13
3	19.26	Pytrolidine-2-carboxylic acid, methyl-phenyl-amide	$C_{12}H_{16}N_{20}$	204	5.77
4	20.45	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	23.74
5	22.30	9-octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	3.53
6	23.01	6-octadecanoic acid	$C_{18}H_{34}O_2$	282	49.00
7	25.75	Ricinoleic acid	$C_{18}H_{34}O_3$	298	14.98

Table 3. Solvent System, Weight and Antibacterial Activities of Fractions Isolated from Aqueous leaf Extract of *Phyllanthus amarus*

Fractions/ compounds	Solvent system	Weight (g)	Relative flow	Antibacterial Activity		
				E.coli	*S. aureus*	*C albicans*
A	Chloroform	0.44	0.71	-	++	++
B	Ethylacetate+Methanol	0.55	0.40	++	++	++
C	Ethylacetate+ Methanol	0.63	0.32	-	-	-
D	Methanol	0.65	0.65	++	-	++

Key: - negative, + slightly active ++, very active

Table 4. Inhibitory/Bactericidal/Fungicidal Concentration of Various of Fractions (Mg/mL)

Fractions/ compounds	*E.coli*	*S. aureus*	*C. albicans*
	MIC/MBC	MIC/MBC	MIC/MFC
1,2-Benzene-dicarboxylic acid	12.50/25.00	12.50/25.00	12.50/25.00
Ricinoleic acid methyl ester	12.50/25.00	12.50/25.00	12.50/25.00
Ricinoleic acid	12.50/25.00	12.50/25.00	12.50/25.00
6-octadecenoic acid.	12.50/25.00	12.50/25.00	12.50/25.00

GCMS Analysis

Previous study on qualitative and quantitative analysis of aqueous plant extracts showed that extract contained various phytochemical compounds [15]. The GCMS results of the fractions are presented in Table 2. The compounds present in the various fractions included, 1,2-benzendicarboxylic acid diocytyl ester (phthalic acid), pentadecanoic acid, tetradecanoic acid, Glycerin 6-octadecenoic acid, 2-Coumaranone and n-hexadecanoic acid. Hexadecanoic acid and pentadeconoic acid were also detected in previous studies on GCMS analysis of *Phyllanthus amarus* leaves [5,16]. Similarly, 1,2 Benzene dicarboxylic diocytyl ester was isolated using chloroform. It is worthy to note that these compounds were different from previous compounds derived from *P. amarus* [17,18] Diocytyl ester was also detected in this study. This compound has been reported to have antimicrobial activity [19]. The Ricinoleic acid methyl ester was present in fraction A while Ricinoleic acid was present in fractions B, C, and D. Ricinoleic acid is a major component of castor oil, accounting for 90% of the total compound [20]. Ricinoleic acid has anti-inflammatory property like capsaicin while topical capsaicin has shown therapeutic property in the treatment of cutaneous disorder, postherpetic neuralgia, painful diabetic neuropathy, pruritus, psoriasis, post-mastectomy, pain syndrome and vulva-vestibulitis [21,22] but its utility is limited due to its irritant properties. Ricinoleic acid does not have the pungent effect of capsaicin but maintain its anti-inflammatory activities in mouse and guinea pigs [20]. Ricinoleic acid has been isolated from *Phyllanthus niruri* seed oil and *ocimum gratissimum* [23,24]. Table 3 and Table 4 show the antimicrobial activities of the fractions. The minimum inhibitory, bactericidal and fungicidal concentrations were 12.5/25mg/ml for *Escherichia coli, Staphylococcus aureus* and *Candida albicans.*

Conclusion

Aqueous leaf extract of *Phyllanthus amarus* was fractionated using chloroform, ethylacetate and methanol. Spectroscopic (UV-VIS, FTIR and GC/MS) analyses revealed compounds such as, 1, 2-benzenedicarboxylic acid diocytyl ester (phthalic acid), pentadecanoic acid, tetradecanoic acid, Glycerin, 2-Coumaranone, n-hexadecanoic acid, ricinoleic acid and others. The various fractions were potent against microbial isolates including gram positive, gram negative and fungi. *Phyllanthus amarus* Schum. and Thonn. aqueous leaf extract contained compounds with pharmacological properties.

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