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# Biological and Chemical Evaluation of the Extracts of the Leaf of *Phyllanthus amarus Schum*

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**Abstract:** The leaf of *Phyllanthus amarus* was investigated for its chemical and biological properties. The powdered leaf was extracted with 95% ethanol and the crude extract was partitioned successively with hexane, dichloromethane, ethyl acetate and 70 % aqueous methanol. Phytochemical analysis of the crude extract showed that it contained alkaloids, balsams, flavonoids, saponins, steroids, tannins, terpenoids, and anthraquinones. The proximate analysis revealed that *Phyllanthus amarus* leaf is a good source of carbohydrates, proteins and fibre. The crude extract and fractions were tested against *Pseudomonas aeruginosa ,Escherichia coli, Staphylococcous aureus,* and *Candida albicans* for their antimicrobial and antifungal activities. Flash column chromatographic separation of the antimicrobial dichloromethane fraction gave a partially pure isolate which on GC-MS analysis revealed the presence of 2- naphthyl-α- D-galactopyranoside and 9-octadecenoic acid in the leaf extract.
**Keywords :** *Phyllanthus amarus* leaf, antimicrobial activity, proximate analysis, chemical constituents.

## Introduction and Experimental

The genus *Phyllanthus* belongs to Euphorbiaceae which is a large family of upright or prostrate herbs or shrubs, often with milky acrid juice [1]. The *Phyllanthus* plants are monoecious or homogenous. The leaves are simple, alternate or opposite. The flowers are very small and diclinous, cluster in cup-shaped structures, greenish and often with glands. The fruit is a three-lobed capsule extending from the cup and the commonly long stalk [2]. In many countries around the world, plants of the genus *Phyllanthus* are used in folk remedies for the management of liver, kidney and bladder problems, diabetes and intestinal parasites. Some related species in this region with medicinal significance are *P. acuminantus, P. niruri, and P. urinaria* [2-8]. A number of species belonging to the genus *Phyllanthus* have been chemically and pharmacologically investigated and many compounds have been isolated and identified. Different classes of organic compounds of medicinal interest have been reported, including alkaloids, flavonoids, lactones, steroids, and terpenoids [7][9][10]. However, lignans, triterpenes, alkaloids, and tannins, are the most abundant compounds so far found in this genus. This work reports the antimicrobial activity using bioassay- directed fractionation and assesses the chemical constituents of the leaf of *Phyllanthus amarus*.

*Phyllanthus amarus (Schum & Thonn)* leaf was collected at Sheda, in Kwali Local Government Area Council, Federal Capital Territory, Abuja, Nigeria. The plant sample was authenticated at the National Institute for Pharmaceutical Research and development (NIPRD), Idu, Abuja, where a voucher specimen was deposited in the Herbarium.

All solvents used in this work were of standard grade and were redistilled before use. Thin-layer chromatography (TLC) was run on pre- coated Merck Kieselgel 60 F 254 with mesh of 0.25mm. The spots were visualised by exposure to UV light at 254/366nm and to iodine vapour. The media for antimicrobial

screening were the nutrient agar and the test organisms, *Pseudomonas aeruginosa (Pa), Escherichia coli (Ec), Staphyloccocus aureus (Sa) and Candida albicans (Ca)*, were clinical isolates obtained from Microbiology Laboratory, University of Abuja Teaching Hospital (UATH), Abuja, Nigeria. IR spectra were obtained on FTIR 8400S Shimadzu Fourier Transform Spectrophotometer using KBr disc and values were recorded in wave-numbers ($cm^{-1}$). The GC-MS analysis was carried out using Thermo-Scientific Trace GC ULTRA system equipped with an AS3000 auto sampler. The operating conditions were as follows: column DB-(optima-5), 30m x 0.25m i.d., o.25µm d. f. The oven temperature was maintained at 50 $^{o}$C for 1 min; then the temperature was increased to 250 $^{o}$C at 30 $^{o}$C per min., held for 5 mins. It was further increased to 180 $^{o}$C at 28 $^{o}$C/ min. and held for 3 mins. The injectioin temperature was 250 $^{o}$C and sample volume was1.0 µL. Helium was used as the carrier gas with a flow rate of 1mL/min.

**Extraction and fractionation**

The powdered leaf (1500g) was extracted with 95% ethanol (500mL) using a soxhlet extractor for 48 h. The extract was filtered and evaporated *in vacuo* with a rotary evaporator to yield the dry crude extract (71.30g; 4.75%). The crude extract (60.0g) was re-dissolved in 50% aqueous methanol and successively fractionated into hexane (11.47g), dichloromethane (27.50g), and ethyl acetate (10.53g) in a separating funnel. The aqueous methanol residue on evaporation to dryness weighed 8.90g.

**Phytochemical screening**

The crude ethanol and aqueous methanol extracts were screened for the presence of some classes of natural products using standard procedures [11] [12].

**Antimicrobial Screening**

The antimicrobial analysis was carried out using the disc-diffusion method as described by Bauer *et al.* [13]. Standard 6 mm diameter discs were produced from Whatman filter paper using office perforator. Stock solutions of samples were prepared by taking 20 mg of extracts and dissolving it in a mixture of dimethylsulphoxide (DMSO) and water (1:1). Each stock solution (2.0 ml) solution was then used to prepare the following concentrations by appropriate dilutions: 10,000, 1000, and 100 µg/ml. Then 0.2 ml of each concentration was added to 20 discs each separately. For the positive control discs, amoxicillin and tetracycline capsules were mixed with water in ratio (1:4), respectively, and for the negative control a mixture of DMSO and water (1:1) was used in place of the experimental solutions.

Nutrient agar (28g) was dissolved in distilled water and made up to the mark in a standard flask. The mixture was then sterilized in an autoclave for 15 minutes at 121°C and allowed to cool to 47°C. The prepared agar was then poured into a petri dish and allowed to cool to room temperature. The petri dishes were then inverted and allowed to dry. The bottom of each petri dish was marked using a marker.

The microbial suspensions were swabbed over the surface of the agar media using a sterile cotton swab to ensure uniform inoculation. The plates were divided into segments of 10,000, 1000 and 100 µg/ml. The negative disc was placed in the same segment as 100µg/ml. The centre in each plate was used for the positive control disc. The set up was then placed in an incubator for 24hrs.The experiment was carried out in triplicate. After about 24hrs of incubation, they were removed from the incubator and examined. The zone of inhibition was measured using a transparent ruler in mm. Activity or otherwise was scored by comparing with the negative control where the tracks should be flourishing. Activity was scored by writing the lowest concentration which prevented growth, called the minimum inhibitory concentration (MIC).

**Proximate analysis of the leaf**

The ash and moisture contents were determined as described by AOAC[14] (Association of official analytical chemists; official methods of analysis, 15$^{th}$ Edition, Washington DC, 808:831-835 (1990). The crude fat was extracted by the soxhlet method with petroleum ether 40-60°C for 8hrs. The total Nitrogen was determined using the micro Kjeldahl method and converted to crude protein content by multiplying with 6.25. The carbohydrates content was determined by the percentage difference of the various other proximate compositions summed together. Determinations were done in triplicate and results expressed as average of percentage values on dry weight basis.

**Isolation and identification of components**

The initial spotting of the dichloromethane fraction obtained on TLC plates showed more than one component hence the need for proper separation with column chromatography. A glass column was washed, dried and clamped vertically on to a retort stand. The glass column was then packed with silical gel of 60 (0.063-0.200 mm) meshes was slowly poured into the column with the tap opened such that it gently settled on a uniform fashion. The column was constantly tapped to get rid of any trapped air bubbles and also ensure a levelled layer of the absorbent. The solvent system used in eluting the column started with 100% hexane, hexane/ethyl acetate(9:1),(8:2),(6:4),(5:5),(2:8), 100% ethyl acetate and ethyl acetate/methanol (9:1), (8:2), (6:4), (5:5). A partially pure pale white compound was obtained (SCO3, 40 mg, 100 % ethyl acetate).The isolate SCO3 was subjected to IR and GC-MS analyses.

## Results and Discussion

The dry leaf powder was extracted with ethanol and fractionated into various solvents. The crude ethanol extract and the aqueous methanol fraction were subjected to phytochemical screening. The results are shown in Table 1. The results of the phytochemical studies of the extract of *Phyllanthus amarus* leaves showed the presence of alkaloids in the aqueous and ethanol extracts in Table 2. This is in agreement with literature [4]. It was also observed that flavonoids, tannins and terpenoids were present. This supports the result reported by Morton, [14] and Foo, [2]. The presence of anthraquinones, balsams, saponins and steroids were observed in the aqueous methanol fraction and ethanol extract as shown in Table 2. These compounds have been found to interact with key enzymes such as aldose reductase, angiotensin-converting enzyme, mitrochondria and ATPase [15].

**Table 1**: **Phytochemical screening of *Phyllanthus amarus* leaf extracts**

| Phytochemicals | Aq. methanol extract | Ethanol extract |
|---|---|---|
| Alkaloids | + | + |
| Anthraquinone | + | + |
| Balsams | + | + |
| Flavonoids | + | + |
| Glycosides | - | - |
| Phlobatanins | - | - |
| Saponins | + | + |
| Tannins | + | + |
| Terpenoids | + | + |

**Key: (+) = present (-) = absent**

The proximate analysis of the leaf was also determined. The results are presented in Table **2.** Literature gave no information on the use of *Phyllanthus amarus* leaf as a vegetable. However, its use as tonic has being reported [3][16][17]. From the results in Table 2, the leaf contains a high percentage of carbohydrates 43.30% and crude fibre 21.50%. This may be the reason for its use as tonic. The present investigation also indicated that *P. amarus* contains 10.1 0% moisture, 8.45% protein 7.15% fat and 6.50 % ash content. The high composition of the proximate parameters indicates a possibility of the plant being used as a vegetable plant.

**Table 2: Proximate analysis of *Phyllanthus amarus* leaf**

| Components | % Composition |
|---|---|
| Moisture | 10.10 |
| Ash | 6.50 |
| Fat | 7.15 |
| Protein | 8.45 |
| Fibre | 21.50 |
| Carbohydrate | 43.30 |

The extractives from *Phyllanthus amarus* were subjected to antimicrobial screening against some bacteria and a fungus, *Candida albicans*. The results (Tables 3 **and 4**) showed that the dichloromethane fraction

had activity against all the test organisms with MIC at 100µg/ml while the hexane, ethyl acetate and aqueous methanol fractions showed no activity against the organisms. The results of antifungal study (Table **5**) showed that the fractions have activity against the organism *Candida albicans* as the plates did not show growth of any organism after incubation at $37^0$C for 21days. This suggests that the fraction is active against the fungus, *Candida albicans*. This is in agreement with a previous report by Foo and Wong [15]. The present result showed that the dichloromethane fraction has a "broad" spectrum activity when compared to the standards. Ericson H.C.*et al*. had reported activity of methanol extract of the plant against some organisms [18].

**Table 3**: **Antimicrobial activity of extractives from *Phyllanthus amarus* leaf**

| Extractives | Pa | Ec | Sa |
|---|---|---|---|
| Hexane | + | + | + |
| Dichloromethane | - | - | - |
| Ethyl acetate | + | + | + |
| Methanol | + | + | + |
| Amoxicillin (AMO) | + | + | - |
| Tetracycline (TET) | + | + | - |

**Key: (+) = inactive: (-) = active , Sa = *Staphylococcus aureus* , Pa = *Pseudomonas aeruginosa* , Ec = *Escherischia Coli***

**Table 4**: **Results of Minimum Inhibition Concentration (MIC) of dichloromethane fraction (mm)**

| Conc. (µg/ml) | Zones of Inhibition (mm)/organisms | | | |
|---|---|---|---|---|
| | Pa | Ec | Sa | Ca |
| 10,000 | 18.00 | 14.00 | 20.10 | 20.10 |
| 1,000 | 14.60 | 12.30 | 11-20 | 11 .40 |
| 100 | 11.30 | 10.10 | 11.10 | 9.30 |
| AMO (25) | 34.54 | 20.60 | 28.31 | 25.55 |
| TET (25) | 36.70 | 23.70 | 32.00 | 28.30 |

**Key: *Pseudomonas aerugenosa* (Pa), *Escherischia coli* (Ec), *Staphylococcus aureus* (Sa), *Candida albicans* (Ca), Amoxicillin (AMO), Tetracycline (TET)**

**Table 5: Results of Antifungal screening of extractives from the leaf of *Phyllantus amarus***

| Extractives | Sabourauds dextrose agar | Sabourauds glucose agar |
|---|---|---|
| Crude Extract | - | - |
| Dichloromethane fraction | - | - |
| Ethyl acetate fraction | - | - |
| Aq. methanol fraction | - | - |

**Key: (-) = active**

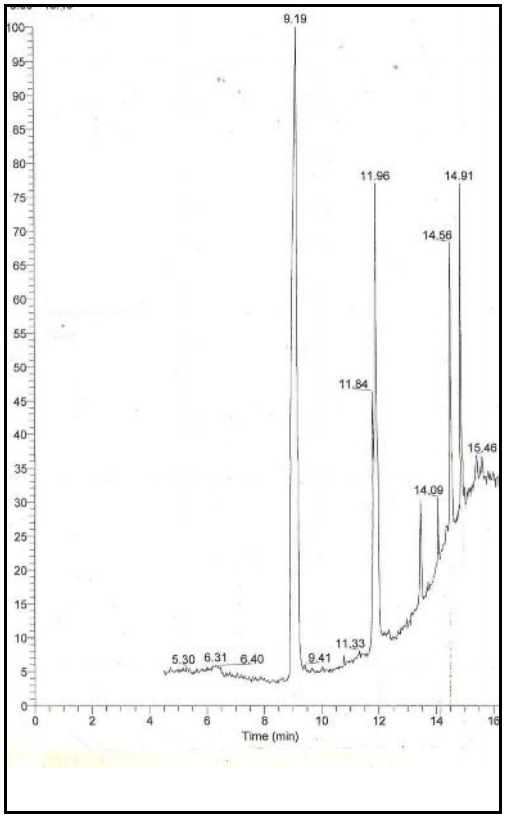
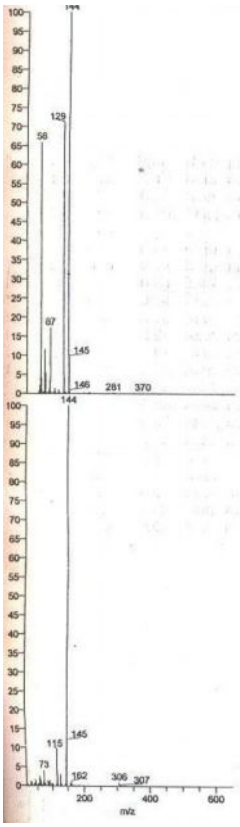



**Figure 1: Gas chromatogram of column fraction SCO-3**

**($A_1$)**




**($A_2$)**

**Figure 2: Mass Spectrum of SCO-3-1($A_1$) compared to computer data for 2- naphthyl-α-D-galactopyranoside($A_2$)**

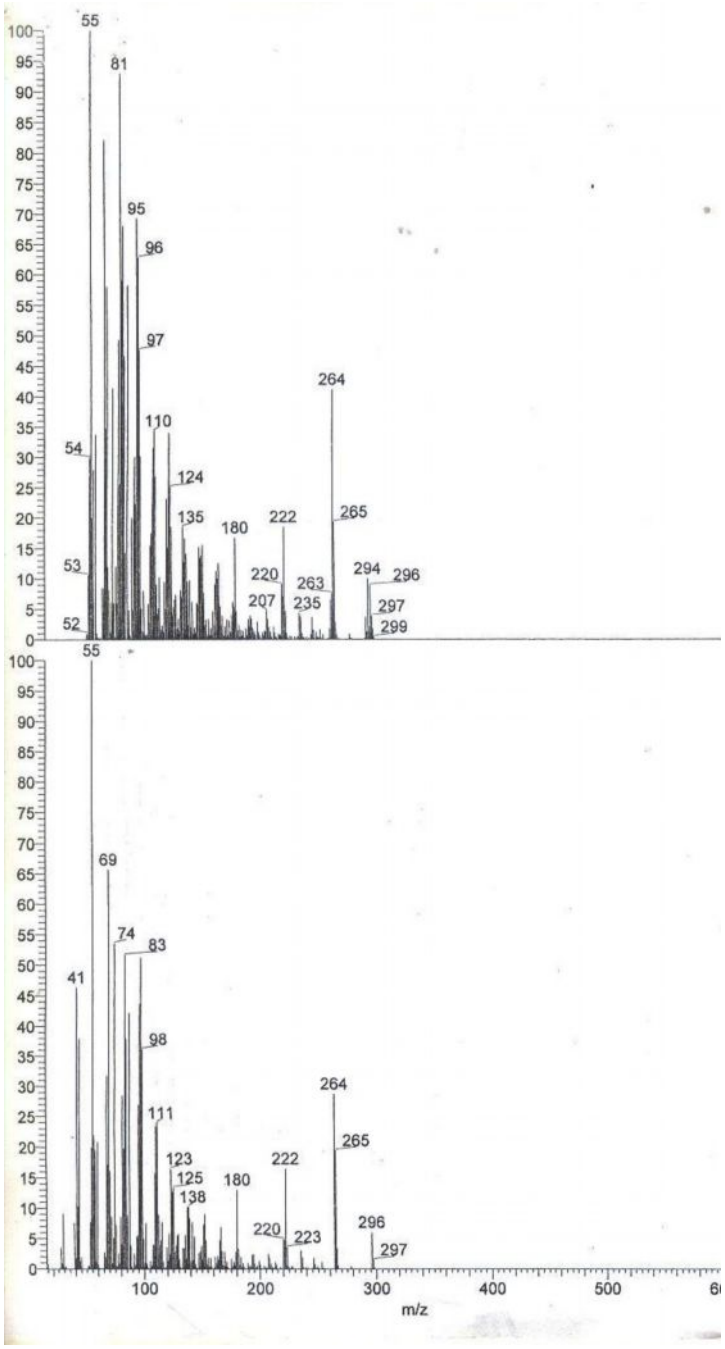
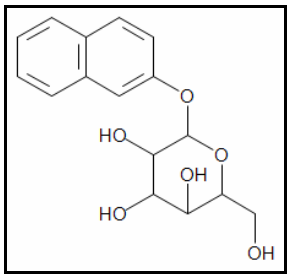


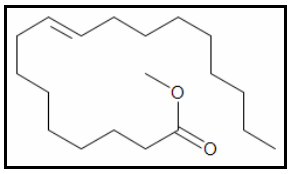

**Figure 3: Mass spectrum of SCO-3-2($B_1$) compared to computer MS data for 9-octadeccenoic acid, methyl ester (oleic acid, methyl ester)($B_2$)**

Flash Column Chromatography of the dichloromethane fraction gave an active fraction SCO3 which was subjected to IR spectral analysis and GC/MS analysis. The IR spectrum of SCO-3 showed a characteristic band at 3336 which is due to the presence of OH function. The band at 1614$cm^{-1}$ is attributable to aromatic C=C bond. The gas chromatogram of the isolate SCO3 (Figure **1)** showed several peaks indicating that it was impure, but with the peak at retention time 9.19 min (SCO-3-1) being the most prominent. The MS of this component(Figure **2)** gave a base peak at m/z 144, corresponding to naphthol and a very weak molecular ion ($M^+$) peak at m/z 306, corresponding to a galactoside of naphthol. Other significant peaks were observed at m/z 162($M^+$-144) and 145(m/z144+H). These fragmentations are consistent with 2-naphthyl-α-D-galactopyranoside ($C_{16}H_{18}O_6$).Thus, based on the above spectral characteristics and by direct comparism with computer MS data, compound SCO-3-1 is probably 2-naphthyl-α-D-galactopyranoside, **1**. Another significant peak in the GC of SCO-3 was component SCO-3-2 with retention time14.91 mins (Figure **1**). The MS (Figure **3)** of this component showed a molecular ion peak at m/z 296 and other peaks including m/z 297[M +H]$^+$ and m/z 264 corresponding to loss of methanol from the molecular ion(296-32). These MS spectral characteristics are

suggestive of oleic acid methyl ester, **2** and are supported by comparison with the standard computer MS data (Fig.4).




**1**




**2**

Oleic acid and its derivatives have been found to possess significant antimicrobial activity against several species of bacteria [19] and are known to play a role in the activation of different intercellular pathways in carcinoma cell development. This role has been suggested as the basis for their reported anti-tumor activity in clinical studies [20]. Some natural naphthalene glycosides such as stelladerol from edible Daylily (*Hemerocallis*) flowers have been found to possess antioxidant property [21][22]. This may further confer anticancer property on the leaf of *Phyllanthus amarus*

## Conclusion

The results obtained from the present work showed that the leaf of *Phyllanthus amarus* contained alkaloids, anthraquinone, balsam, flavonoids, saponins, steroids, tannins, terpenoids, and in particular 2-naphthyl-α-D-galactopyranoside and 9-octadecenoic acid methyl ester( oleic acid methyl ester). The presence of these chemical components may account for the traditional medicinal uses of the leaves of *Phyllanthus amaru*s to manage a number of bacterial and fungal infections.

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