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One glucolipid from the ethyl acetate fraction of *Acalypha wilkesiana var. lace-acalypha* (Muell & Arg.)

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Abstract: Plant recipes are classified as medicinal when the biological activities of compounds obtained from them have been scientifically established. Before now, three compounds namely, ethyl gallate, pyrogallol and D-arabino-hex-1-enitol-1, 5-anhydro-2-deoxy have been isolated from the butanol fraction of *Acalypha wilkesiana var. lace-acalypha* (Muell & Arg.) and their antimicrobial activities evaluated, established and documented. In this study, the ethyl acetate fraction which resulted from solvent-partitioning of the aqueous crude extract with organic solvents of increasing polarities was subjected to silica-gel column chromatography. A glucolipid designated as H-2 [light brown oil; b.p. (391-393 ^{0}C); R_f (0.19); $[n]^{20}{}_D$ (1.3989)] was isolated. The structure of H-2 has been established to be ethyl α-D-glucopyranoside by a combination of ^{1}H NMR, ^{13}C NMR, MS and IR spectral techniques. H-2 was remarkably bacteriostatic against *B.subtilis*, *E.coli*, *K.pneumonia*, *Ps.aeriginosa* and *S. typhi*. However, it recorded no activitities against *S.aureus* and *C.albicans*. H-2 and other compounds previously obtained can be used to chemotaxonomically mark this plant and hence classify it as medicinal

Keywords: Ethyl acetate fraction; chromatography; glucolipid; ethyl α-D- glucopyranoside; *Acalypha wilkesiana var. lace-acalypha;* medicinal.

Introduction:

Plants are a great source of medicines, especially in traditional medicine[1-2] and are classified as medicinal only when their biological activities have been scientifically established.[3] The genus, *Acalypha* belongs to the Euphorbiaceae family.[4-7] Hundreds of compounds which include tannins, steroids, alkaloids and terpenes amongst many others[8-13] had beenisolated from plants in this family. Before now, three compounds namely, ethyl gallate, pyrogallol and D-arabino-hex-1-enitol had been obtained from the butanol fraction of *Acalypha wilkesiana var. lace-acalypha* (Muell &Arg.) and their antimicrobial activities documented[14-15] This present study examined the ethyl acetate fraction which resulted from the solvent-partitioning of the aqueous crude extract of the plant with organic solvents ofincreasing polarities for presence of more compound(s). Also, compound(s) to be isolated were expected to be screened for antimicrobial activities.

Materials and Methods:

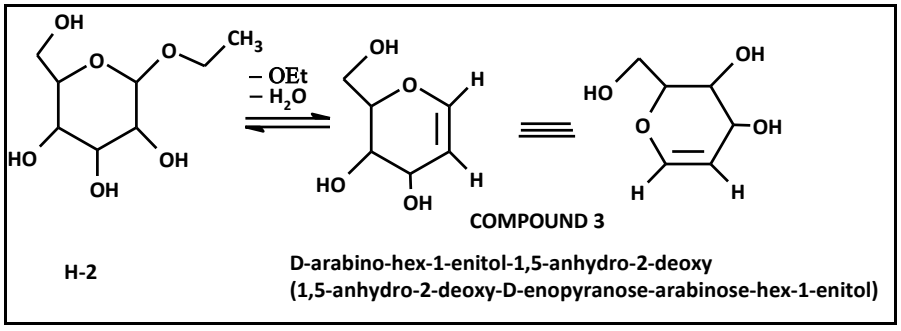
Collection of plant

A fresh collection of the leaves of *A. wilkesiana var. lace-acalypha* was carried out in the month of August, 2013 from an abandoned roadside farmland in Uyo, Akwa Ibom State, Nigeria. The identification of

the plant had previously been done as reported in an earlier Study[14]. The plant was dried in an oven (Gallenkamp, England) at 40 ^{0}C for 48 h and the resultant dried material powdered on an electric mill (Uniscope, England).

Extraction and isolation

The dried powder (0.8 kg) was exhaustively extracted with 50 % EtOH (3 x 5L) at room temperature (27± 2 ^{0}C) for 72 h. The resultant crude extract was filtered, concentrated *in vacuo* on a rotary evaporator (R205D, Shensung BS & T, China), weighed and stored in a desiccator (Monsori, Scotland) prior to further use. 100 g of the crude extract was partitioned using H_2O:EtOAc (4 x 500 mL). The obtained ethyl acetate fraction obtained was evaporated to dryness to give a resin-like substance. The ethyl acetate fraction (8.7 g, viscous brown substance) was chromatographed on a silica-gel 254 column (Pyrex, USA; 10 g pre-swollen in 100 % toluene; 3 g concentration zone + 7 g separation zone; 17.5 x 4 cm) and eluted with a gradient of 10 % $(CH_3)_2$-CO:toluene (100 mL), 20 % $(CH_3)_2CO$:toluene (100 mL) and 30 % $(CH_3)_2CO$:toluene (100 mL). Fractions of 10 mL each were collected, monitored on silica plates (Merck, Germany) in $(CH_3)_2CO$:toluene:H_2O (10:20:1) using $FeCl_3/CH_3OH$ and vanillin-H_2SO_4 as spray reagents. Hence, fractions with similar TLC characteristics (R_f values, reaction with $FeCl_3$ reagent or vanillin-H_2SO_4 spray) were bulked to give two semi-pure residues coded E-1 and E-2. Further TLC examinations of these residues in $(CH_3)_2CO$:toluene:H_2O (10:20:1) indicated no materials especially in E-1. Subsequently, E-2 (0.13g) was purified on a much shorter silica gel 254 column (10 x 2 cm) by eluting successively with 100 % toluene (80 mL) and 10 % $(CH_3)_2CO$:toluene (50 mL) hence, resulting in the isolation of ethyl α-D-glucopyranoside **[H-2]** (light brown oil; R_f (0.19); 34 mg). The boiling point of **H-2** was determined by using the boiling point apparatus (Scientific Instruments, India) while the refractive index was measured at the wavelength (λ) of Na-D line (589.3 nm) at 20.5 ^{0}C [16-18] using the WAY-15 Abbe Refractometer (England).



Antimicrobial screening

The micro-organisms used in this study, namely; *Bacillus subtilis* (NCTC 8853), *Staphylococcus aureus* (NCTC 6872), *Escherichia coli* (NCTC 10764) *Pseudomonas aeriginosa* (ATCC 2654), *Samonella typhi* (NCTC 5438) and *Candida albicans* (NCYC 436) were clinically isolated from specimens of diarrheal stool, abscesses, necrotizing fascitis, osteomyelitis, urine, wounds and vaginal swabs obtained from the Medical Laboratory, University of Uyo Health Centre, Uyo. The clinical isolates were collected in sterile bottles, identified and typed by convectional biochemical tests[19-20] and then refrigerated at -5 ^{0}C at the Microbiology and Parasitology Unit, Faculty of Pharmacy prior to use. The hole-in-plate agar diffusion method was used observing standard procedure with Nutrient Agar-CM003, Mueller-Hinton-CM037 (Biotech Limited, Ipswich, England) and Sabouraud Dextrose Agar (Biomark, India) for the bacteria and fungus respectively. The inoculum of each micro-organism was introduced into each petri-dish (Pyrex, England). Cylindrical plugs were removed from the agar plates by means of a sterile cork borer (Simax, England) to produce wells with diameter of approximately 7 millimetres. The wells were equidistant from each other and the edge of the plate.[21-22] Concentrations of 20 mg mL^{-1} of crude extract, 10 mg mL^{-1} of ethyl acetate fraction, 2 mg mL^{-1} of **H-2** were introduced into the wells. Also, different concentrations of streptomycin (Fidson Chemicals, Nigeria), 1mg mL^{-1} of nystatin (Gemini Drugs, Nigeria) and 100 % methanol were introduced into separate wells as positive and negative controls respectively.[10, 23-24] The experiments were carried out in triplicates. The plates were left at room temperature for 2 h to allow for diffusion. The plates were then incubated at 37± 2 ^{0}C for 24 h. Zones of inhibition were measured in millimetre (mm).

Results and Discussion:

Spectroscopic data:

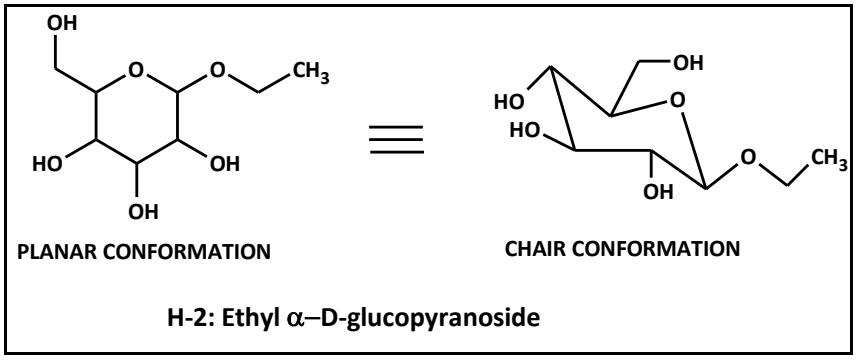
The data were obtained thus: ES+ - MS on Kratos MS 80, IR on Perkin-Elmer FT-IR, ^{1}H and ^{13}C NMR on Bruker AC 250 operating 300 MHz for proton and 75 MHz for carbon-13 using CD_3OD as solvent and TMS as internal standard.

H-2: $C_8H_{16}O_6$; light brown oil; b.p. (391-393 ^{0}C); R_f (0.19); $[n]^{20}{}_{D}$ (1.3989); MS [ES+-MS] m/z (relative intensity): 208 $[M]^+$ (2.10 %), 177 $[M\text{-}CH_2OH]^+$ (2.94 %), 159 $[M\text{-}3OH+2H]^+$ (2.51 %), 144 $[M\text{-}OCH_2CH_3\text{-}OH\text{-}2H]^+$ (1.94 %), 131 $[M\text{-}OCH_2CH_3\text{-}CH_2OH\text{-}1H]^+$ (3.87 %), 116 $[M\text{-}OCH_2CH_3\text{-}3OH+4H]^+$ (7.97 %), 98 $[M\text{-}OCH_2CH_3\text{-}CH_2OH\text{-}2OH]^+$ (12.87 %), 88 $[M\text{-}OCH_2CH_3\text{-}CH_2OH\text{-}3OH+7H]^+$ (21.43 %) and 60 $[M\text{-}C_8H_{16}O_6 + CH_2OH + CH_2CH_3]^+$ (100.00 %) (base peak); IR [FTIR] cm-1: 789 (finger print), 1073 (-C-O-C) and 3450 (-OH); ^{1}H NMR δ (ppm): 0.97{CH_3 (t)}, 1.36{CH_2 (q)} and 1.48{CH_2 (s)}; ^{13}C NMR δ (ppm): 19.72 (methyl-C), 35.82 (methylene-C) and 104.93 (hydroxylated-C).

Collection and processing of plant materials

The rules governing plant collection and extraction were observed thereby preserving the chemical composition of the crude extract and fraction.[25-26] Two previous studies had reported that the crude extract of *A.wilkesiana var. lace-acalypha* contained saponins, tannins, flavonoids, terpenes and cardiac glycosides while alkaloids, anthraquinones and cyanogenic glycosides were absent. Apart from the butanol fraction which recorded the highest antimicrobial activities against selected microbes, the ethyl acetate fraction equally afforded remarkable antibacterial and antifungal activities[10,12] which neccesitated its choice for column chromatography from where **H-2** was obtained.

Elucidation of structure of H-2



H-2: Ethyl α–D-glucopyranoside

Physical constants such as optical rotation, optical density, refractive index and boiling point are used in the qualitative and quantitative analyses of substances. Also, these parameters are employed to confirm the purity, identity, integrity of active substances and as well as monitor the progress of reactions.[16-18] The physical examination of **H-2** showed that it was an oily substance. In this study, the refractive index was measured at the wavelength (λ) of Na-D light (589.3 nm) and a temperature of 20.5 ^{0}C. The boiling point was equally determined. The refractive index of a substance is an indication of the number, type of atoms and chemical groups (species) in the substance. Each atom or group in the substance contributes to its refractivity which adds eventually to the refractive index of the substance. Furthermore, refractive index can be used to monitor the progress of chromatographic separation by measuring the refractive indices of the effluent solvents employed.[16-18] **H-2** recorded a refractive index of 1.3989 while it gave a boiling point of 391-393 ^{0}C which is particularly consistent with that in literature. The structure was established by a combination of spectroscopic techniques as highlighted above. The obtained MS data were matched with library data of organic compounds [27] hence, **H-2** was identified to be ethyl α-D-glucopyranoside. Due to the nature of the matrix, many fragmented ions appeared in the MS of this compound but those that could readily be identified include; $[M]^+$ at m/z 208 (2.51 %) while the base peak at 60 (100.00 %) represents the disintegration of $[M]^+$save for ethyl and methylene alcohol units. However, the ion at 177 (2.89 %) represents the loss of only methylene alcohol units. Furthermore, the peak at 144 (1.96 %) indicates the loss of ethoxy and hydroxyl groups. Other noticeable fragments at 133 (3.76 %), 116 (7.93 %), 98 (12.76 %), 73 (38.76 %) and 42 (48.65 %) represent *quazi-peaks* [28-31] in the spectrum. The IR spectrum of **H-2** shows diagnostic stretchings at 1073 and 3450 cm^{-1} for ether linkage

and OH functional groups respectively. Also, the obtained ^{1}H and ^{13}C NMR spectra of the compound are as expected and consitent with those in literature. **H-2** is presented both in the planar and chair conformations.[32] Interestingly, one of the three compounds previously isolated from the butanol fraction of *A. wilkesiana var. lace-acalypha* was a yellow oil (compound **3)** which was identified as D-arabino-hex-1-enitol-1, 5-anhydro-2-deoxy (1, 5-anhydro-2-deoxy-D-enopyranose-arabino-hex-1-enitol).[15] In this present study, **H-2 (**ethyl α-D-glucopyranoside) was obtained as a brown oil and its structure bears some resemblance to that of **3** which is re-presented for emphasis in this report. **H-2** and **3** both have the pyran moiety in their structures. A closer examination of the chemical structures of the two compounds reveals that **3 (**obtained from the BuOH fraction) could probably had arisen *in-situ* from the losses of OEt group and H_2O molecule in biogenetic chemical transformations in **H-2 (**obtained from the EtOAc fraction).

Antimicrobial screening

The crude extract, ethyl acetate fraction and **H-2** were screened for antibacterial and antifungal activities using *B.subtilis*, *S.aureus*, *E.coli*, *K.pneumonia*, *Ps.aeriginosa*, *S. typhi* and *C.albicans* to represent a desirable spectrum of microbes. The results of the antimicrobial tests displayed in **Table 1** show that **H-2** was strongly bacteriostatic against *B. subtilis*, *E.coli*, *K.pneumonia*, *Ps.aeriginosa* and *S. typhi*. However, it recorded no activitities against *S.aureus* and *C.albicans*. Surprisingly, it was observed that this compound was remarkably suppressive of gram negative bacterial strains such as *E.coli*, *K.pneumonia*, *Ps. aeriginosa* and *S.typhi*. This particular observation was unique because these microbes are well known for their resistance to antibacterial agents. This resistance is believed to be due to the nature of the cell envelope of these organisms which unlike gram positive organisms, posses a sophisticated three-layered envelope which does not allow permeation of external agents.[33]

Table 1: Antimicrobial screening of crude extract, ethyl acetate fraction and H-2 at different concentrations on test microbes in 100 % MeOH

Test microbe	CE 20 mg L^{-1}	ET 10 mg mL^{-1}	H-2 2 mg L^{-1}	Streptomycin 10 μg mL^{-1}	Nystatin 1 mg mL^{-1}	100 % MeOH
B. subtilis (NCTC 8853)	16	16	20	25	7	7
S. aureus NCTC 6872)	16	22	7	26	7	7
E. coli (NCTC 10764)	7	7	19	21	7	7
Ps. aeriginosa (ATCC 2654)	20	20	12	7	7	7
S. typhi (NCTC 5438)	15	9	19	20	7	7
C. albicans (NCYC 436)	13	7	7	7	28	7

Key: The zone diameter recorded is zone of inhibition + size of cup (zone of inhibition +7) mm;
CE = Crude ethanolic extract;
ET = Butanol fraction;
H-2 = Ethyl α-D-glucopyranoside;
NCTC - National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9, UK.
NCYC- National Collection of Yeast Cultures, UK.
ATCC- American Type Culture Collection, Washington, DC.

Conclusions:

In this study, ethyl α-D-glucopyranoside has been isolated from the ethyl acetate fraction of *A. wilkesiana var. lace-acalypha* (Muell &Arg.). It is expected that this compound would serve as a chemotaxonomic marker for this species and variety in particular and the genus, *Acalypha* in general. The isolated compound was remarkably active against gram negative bacterial strains while it demonstrated no antifungal acvtivity against the candidal strain employed.

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