ETHYL GALLATE AND PYROGALLOL FROM *ACALYPHA WILKESIANA VAR. LACE-ACALYPHA* (MUELL &ARG.)

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Acalypha wilkesiana var. lace-acalypha (Muell & Arg.) is a cultivated ornamental plant used in folkloric medicine for the treatment of fever, bacterial, skin fungal infections, wounds, tumors, inflammations and gastro-intestinal troubles. Silica gel column chromatography of the butanol fraction gave two polyphenolic compounds, designated as compounds **1** [m.p. 148-150 ^{0}C; R_f 0.67; $[n]^{20}_D$ 1.4118] and **2** [m.p. 130-132 ^{0}C; R_f 0.46; $[n]^{20}_D$ 1.4079]. The structures of **1** and **2** have been established to be ethyl 3, 4, 5-trihydroxybenzoate (ethyl gallate) and 1, 2, 3-benzenetriol (pyrogallol or fouramine brown) respectively using the ^{1}H NMR, ^{13}C NMR, MS and IR spectral techniques. Both polyphenols were strongly bacteriostatic against against *B. subtilis*, *S. aureus*, *E. coli*, *Ps. aeriginosa* and *S. typhi.* Furthermore, **2** was more suppressive of the bacterial strains than **1**. However, neither gave any anticandidal activity. The crude extract and butanol fraction demonstrated comparatively weaker antimicrobial activities than the two isolated compounds. The results of the antimicrobial screening have lent scientific credence to the traditional uses of the plant**.**

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INTRODUCTION

The genus, *Acalypha*, comprises of about 570 species[1] which are used in African, South American and Asian traditional medicine. *Acalypha wilkesiana var. lace-acalypha* (Muell & Arg.) is used in the treatment of fever, bacterial, skin fungal infections,[2-5] wounds, tumors, inflammations and gastro-intestinal disorders.[6] Extracts of this plant have demonstrated both hypotensive and hypoglycaemic activities.[7] The antiproliferative (antitumor) potential of this plant had been carried out on human lung carcinoma and fibroblast.[8] A pilot study carried out on six *Acalypha* species (including *lace-acalypha*) demonstrated that the antimicrobial activity was most pronounced in the butanol fractions of the species.[9] Hence, two tannins; corilagin and gerannin were obtained from butanol fractions of *A. wilkesiana var. red-acalypha* and *A. hispida.*[10] This present study was carried out with the aim of isolating chemical constituents in *A. wilkesiana var. lace-acalypha* and subsequently classifying compounds obtained therefrom as chemotaxonomic markers for this species and variety in particular and the genus, *Acalypha* in general.

MATERIAL AND METHODS

Collection of plant

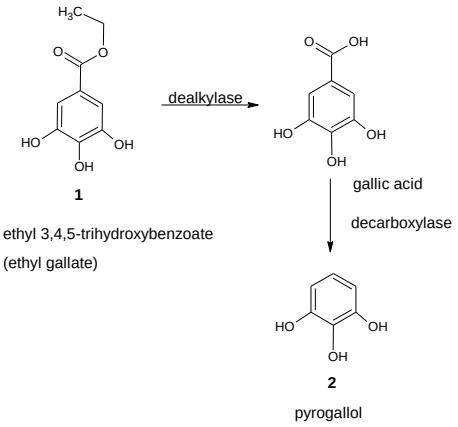
The fresh leaves of *A. wilkesiana var. lace-acalypha* were collected around the month of February, 2013 from a privately cultivated orchard at Nwaniba, Akwa Ibom State, Nigeria. The plant was identified by O. Etefia of the Department of Pharmacognosy and Natural Medicine, University of Uyo, Nigeria. The authentication by comparison was done with herbarium samples of the Forestry Research Institute of Nigeria (FRIN) and the National Institute of Horticulture (NIHORT), Nigeria. A voucher specimen of the plant (No H113) was deposited in the Herbarium Unit of the Faculty of Pharmacy. Immediately after collection, 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).

Bulk extraction and isolation

The dried powder (1.4 kg) was exhaustively extracted with 50 % EtOH (5x5 L) 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 prior to further use. 125 g of the extract was partitioned using H_2O:BuOH (7 x 500 mL). The obtained butanol fraction was evaporated to dryness to give a solid residue from which the two polyphenols were isolated. The butanol fraction (10 g) 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 20 % acetone:toluene (100 mL), 30 % acetone:toluene (100 mL), 40 % acetone:toluene (100 mL), 50 % acetone:toluene (100 mL) and 60 % acetone:toluene (100 mL). Fractions of 10 mL each were collected, monitored on silica plates in acetone: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 and four semi-pure residues coded 5A, 5B, 5C and 5D were obtained.

Sample 5B (1.8 g, dirty green) was purified on a much shorter silica gel 254 column (10 x 2 cm) successively with 100 % toluene (130 mL) and 20 % acetone:toluene (50 mL) resulting in ethyl 3, 4, 5-trihydroxybenzoate (ethyl gallate) **1** (amorphous white solid; R_f (0.67); 165 mg). Likewise, 5C (2.4 g, greenish brown substance) was also cleaned on a short silica gel 254 column using 30 % acetone:toluene (210 mL) which furnished 1,2,3-benzenetriol (pyrogallol or fouramine brown) **2** (light brown crystals; R_f (0.46); 243 mg).

Efforts to equally purify 5A and 5D were abortive. Hence, they were not procesed any further in this study. The melting points of the two compounds were determined using the melting point apparatus (Electrothermal, England) while the refractive indices were obtained using WAY-15 Abbe refractometer (England).



Antimicrobial tests

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[11-12] 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 and Sabouraud Dextrose Agar (Oxoid, England) 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 (Pyrex, England) to produce wells with diameter of approximately 5 mm.

The wells were equidistant from each other and the edge of the plate.[13-14] Concentrations of 20 mg mL^{-1}of crude extract, 10 mg mL^{-1} of butanol fraction, 2 mg mL^{-1} of **1** and **2** were introduced into the wells. Also, different concentrations of 10 µg mL^{-1} streptomycin (Fidson Chemicals, Nigeria), 1 mg mL^{-1} of nystatin (Gemini Drugs, Nigeria) and 100 % methanol were introduced into separate wells as positive and negative controls respectively.[9-10,15-16]

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 Shimadzu FTIR 8400S, ^{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.

Compound **1:** $C_9H_{10}O_5$; amorphous white solid; m.p. 148-150 ^{0}C; R_f 0.67; $[n]^{20}_D$ 1.4118; MS [ES$^+$-MS] m/z (relative intensity): 198 $[M]^+$ (48.45 %), 183 $[M- CH_3]^+$ (5.24 %), 170 $[M-(CH_2)_2]^+$ (32.67 %), 154 $[M- OC=O]^+$ (20.49 %), 153 $[M- OC_2H_5]^+$ (100.00 %) (base peak), 125 $[M-(CH_2)_2$ -3OH +2H$]^+$ (26.27 %), 79 [M- 3OH-74+ 6H$]^+$ (15.27 %) and 51[M-198+3OH] 12.87 %; IR [FTIR] cm^{-1}: 773 (finger print), 1648 (Ar CH=CH), 1720 (-C=O) and 3647 (Ar-OH); ^{1}H NMR δ (ppm): 1.12, 1.41, 6.15, 7.45, 7.54 and 7.66 ; ^{13}C NMR δ (ppm): 19.45 (methyl- C), 34.42 (methylene-C), 100.45, 105.23 (hydroxylated-C), 123.32 (Ar- C) and 161.84 (ester -C).

Compound **2:** $C_6H_6O_3$; light brown crystals; m.p. (130-132 ^{0}C); R_f 0.46; $[n]^{20}_D$ 1.4079; MS [ES$^+$-MS] m/z (relative intensity): 126 $[M]^+$ (100.0 %), 108 $[M-OH_2]^+$ (62.82 %), 97 [M-2OH +5$]^+$ (31.67 %), 80 [M- 3OH +5$]^+$ (52.77 %), 68 [M-3OH-7$]^+$ (14.84 %) and 52 [M-75+3OH+1$]^+$ (21.53 %); IR [FTIR] cm^{-1}: 1641 (Ar CH=CH) and 3528 (Ar-OH); ^{1}H NMR δ (ppm): 6.45, 7.65 and 7.84; ^{13}C NMR δ (ppm): 106.15, 107.28 (hydroxylated-C), 122.56 and 123.32 (Ar-C).

Collection and processing of plant

The rules governing plant collection and extraction were observed thereby guaranting the intergrity of the extract and fractions.[17-18] 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. Furthermore, the antibacterial and antifungal activities resided in the butanol fraction.[9-10] In addition, the H_2O/BuOH partition extracted the largest amount of plant constituents, hence the choice of the BuOH fraction for column chromatography from where the isolates **1** and **2** were obtained.

Elucidation of structures of compounds 1 **and** 2

Some physical constants of compounds **1** and **2** such as the refractive index measured at the λ 589.3nm (Na-D light) and 20 ^{0}C and melting point were determined. The results highlighted (Experimental Section) show that compound **1** with a higher refractive index (1.4118) is denser than compound **2** (1.4079). This is not surprising because **1** (ethyl gallate) has a molecular weight of 198 while **2** (pyrogallol or fouramine brown) has a molecular weight of 126. The higher the molecular weight or the more the atomic species in a compound, the higher the refractive index.[19-21] The melting points of **1** and **2** are 149-151 ^{0}C and 131-133 ^{0}C respectively in literature. These values are consistent with those obtained in this study. The structures of **1** and **2** were established by a combination of spectroscopic techniques as highlighted above.

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Table 1. Antimicrobial screening of crude extract, butanol fraction and isolates **1** and **2** at different concentrations on test microbes in 100 % MeOH.

Test microbe	LA 20 mg mL^{-1}	BU 10 mg mL^{-1}	1 2 mg mL^{-1}	2 2 mg mL^{-1}	Streptomycin 10 µg mL^{-1}	Nystatin 1 mg mL^{-1}	100 % MeOH
B. subtilis (NCTC 8853)	14	15	13	21	22	5	5
S. aureus (NCTC 6872)	13	21	20	31	37	5	5
E. coli (NCTC 10764)	5	5	14	18	18	5	5
Ps.aeriginosa (ATCC 2654)	7	18	12	18	5	5	5
S. typhi (NCTC 5438)	12	8	14	19	19	5	5
C. albicans (NCYC 436)	11	5	5	5	5	28	5

Key: The zone diameter recorded is zone of inhibition + size of cup (zone of inhibition +5) **in** mm; **LA** = crude ethanolic extract; **BU** = butanol fraction; **1** = Ethyl 3,4,5-trihydroxybenzoate (ethyl gallate); **2** = 1,2,3-benzenetriol (pyrogallol); **NCTC** - National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9, UK; **NCYC** - National Collection of Yeast Cultures, UK; **NCYC** - National Collection of Yeast Cultures, UK; **ATCC** - American Type Culture Collection**,** Washington, DC.

The obtained MS data were matched with library data of organic compounds,[22] hence, **1** and **2** were identified to be ethyl 3,4,5-trihydroxybenzoate (ethyl gallate) and 1,2,3-benzenetriol (pyrogallol or fouramine brown) respectively. The ES$^+$-MS of **1** showed diagnostic peaks such as $[M]^+$ at m/z 198 (48.45 %) while 183 (5.24 %), 154 (20.49 %) and the base peak at 153 (100.00 %) represent the losses of -CH_3, -COO and -OC_2H_5 units respectively from the $[M]^+$. Due to the nature of the matrix, many fragmented ions also appeared in the MS of **2** but those that could readily be identified include: $[M]^+$ at m/z 126 (also base peak) (100.00 %) while 108 (62.82 %) indicates the loss of -OH_2. However, the peak at 52 (21.53 %) represents the disintegration of $[M]^+$ save for the 3 OH groups. The IR spectra of the two compounds show diagnostic Ar-CH=CH at 1641 and 1648 cm^{-1} and Ar-OH at 3528 and 3647 cm^{-1} respectively. In addition, the -C=O absorption at 1720 cm^{-1} observed in **1** was equally very diagnostic. Also, the obtained ^{1}H and ^{13}C NMR spectra of both compounds are consitent with those in literature.[10,22] **1** and **2** are polyhydroxyl benzenoid compounds. It is very probable that **2** could have arisen *in-situ* from biogenetic chemical transformations of **1** in the presence of enzymes in the plant. These possible transformations are provided in the Scheme.

Antimicrobial screening

The results of the antimicrobial tests presented in **Table 1** show that the two compounds were strongly bacteriostatic against against *B. subtilis*, *S. aureus*, *E. coli*, *Ps. aeriginosa* and *S. typhi*. Interestingly, the two compounds were remarkably active against gram negative strains such as *E. coli* and more especially *Ps. aeriginosa*. These bacteria are well known for their unique resistance to antimicrobial 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.[23] Furthermore, compound **2** was observed to be more suppressive of the bacterial strains than **1**. This observation was unexpected because both compounds are polyphenols with 3 OH groups each which confer hydrophilic character on them and as well as some level of antimicrobial activity. Polyphenols (tannins) obtained in previous studies have demonstrated antimicrobial activities.[10,24] However, the C_2H_5 group attached to the -COO link in **1** is expected to make it more lyphophilic and consequently more antimicrobial.[20]

However, the contrary was obtained in this study. It would be interesting to know the mechanisms of the antimicrobial activities demonstrated by these compounds. Also, both compounds demonstrated no antifungal activity against *C. albicans.* This particular observation was not surprising because fungal strains such as *Candida spp*. limit the permeation of substances because of their integral structures which are pleomorphic and facultative in nature hence, resembling those of higher plants.[23]

CONCLUSIONS

This study reports for the first time the isolation of ethyl gallate and pyrogallol from the butanol fraction of *A. wikesiana var. lace-acalypha.* These two compounds are expected to serve as chemotaxonomic markers for this species and variety in particular and the genus, *Acalypha* in general. Also, the observed antimicrobial activities of the isolated compounds have lent credence to the folkloric uses of the plant especially in the treatment of diseases of microbial origin.

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