ETHYL SUCCINATE AND ETHYL-β-RIBOSIDE FROM *ACALYPHA WILKESIANA VAR. GOLDEN-YELLOW* (MUELL & ARG.)

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Studies on chemical constituents of *A. wilkesiana var. golden-yellow* **(**Muell & Arg.) syn. *A. wilkesiana var. tropical tempest* used in traditional medicine, was carried out with solvent-partitioning of the aqueous extract of the plant with various organic solvents and subjecting the organic phases to silica gel column chromatography. Two compounds could be isolated from the butanol fraction which were assigned as 4-ethoxy-4-oxobutanoic acid (ethyl succinate) and 2-ethoxy-5(hydroxymethyl)-oxalane-3,4-diol (ethyl β-riboside) using the ^{1}H NMR, ^{13}C NMR, MS and IR spectral techniques. Both compounds were inactive against *B. subtilis*, *S. aureus* and *E. coli*. and showed minimal activity against *Ps. aeruginosa*, *S. typhi* and *V. cholerae*, and they had no anticandidal activity. The two isolates would serve as chemotaxonomic markers for this species and variety in particular and the genus, *Acalypha* in general.

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INTRODUCTION

Acalypha wilkesiana is named after the American scientist and explorer Admiral Chas Wilkes (1801-1877).[1] *A. wilkesiana var. golden-yellow* **(**Muell & Arg.) syn. *A. wilkesiana var. tropical tempest* is characterized by bright lime, yellow and green speckled leaves.[2-5] This variety possesses same morphological features as red acalypha variety which is predominately red and mottled with purple colourations. Different preparations of this plant are employed in folklore medicine in the treatment of malaria, wounds, tumours, inflammations, gastrointestinal disorders, bacterial and skin fungal infections.[6-11] Earlier, corilagin, geraniin, gallic acid, quercetin 3-O-rutinoside and kaempferol 3-O-rutinoside had been isolated from *A.wilkesina var. red acalypha* and *A. hispida* [12] while ethyl gallate, pyrogallol, D-arabino-hex-1-enitol and ethyl α-D-glucopyranoside had been obtained from *A. wilkesiana var. lace-acalypha.*[13-15]

In the present work studies have been carried out to isolate chemical constituents of the butanol fraction obtained from aqueous extract of the plant by column chromatography and also evaluate their antimicrobial potential. It is hoped that the obtained compounds may serve as chemotaxonomic markers for this species and variety in particular and genus, *Acalypha* in general.

Experimental

Collection of plant

The fresh leaves of *A. wilkesiana var.* golden-yellow **(**Muell & Arg.) were collected in the month of December, 2014 from a greenhouse facility located within the University of Uyo Town Campus, Uyo, Akwa Ibom State, Nigeria. A voucher specimen of the plant (No. H122) was deposited in the Herbarium Unit of the Faculty of Pharmacy. The plant was dried in an oven 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 (0.85 kg) was exhaustively extracted with 50 % EtOH (4 x 5 L) at room temperature (27 ± 2 ^{0}C) for 72 h. The obtained crude extract was filtered, concentrated *in vacuo* on a rotary evaporator weighed and stored in a desiccator prior to further use. 107 g of the extract was partitioned using H_2O:1-butanol (6 x 500 mL). The resultant butanol fraction was evaporated to dryness to give a solid green residue. The butanol fraction (11.6 g) was chromatographed on a silica gel 254 (Merck, Germany) glass column (Techmel, USA; 10 g pre-swollen in 100 % toluene, 3 g concentration zone + 7 g separation zone, 16.5 x 3 cm) and eluted with a gradient of 10 % $(CH_3)_2CO$:toluene (60 mL), 20 % $(CH_3)_2CO$:toluene (60 mL), 30% $(CH_3)_2CO$:toluene (60 mL), 40 % $(CH_3)_2CO$:toluene (60 mL) and 50% $(CH_3)_2CO$: toluene (60 mL). Fractions of 8 mL each were collected, monitored on silica plates (Model No 64271, 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.

Subsequently, fractions with similar TLC characteristics (R_f values, reaction with $FeCl_3$ reagent or vanillin-H_2SO_4 spray) were combined and three semi-pure residues coded C-1, C-2 and C-3 were obtained. C-1 (1.4 g, deep green) was purified on a much shorter glass column (9.6 x 2 cm) isocratically with 100 % toluene (60 mL) resulting in 4-ethoxy-4-oxobutanoic acid (ethyl succinate) (olive green) coded **W-1** (R_f (0.76); 65 mg). Similarly, C-2 (1.1g, faintly greenish substance) was also cleaned on a short glass column using 20 % $(CH_3)_2CO$:toluene (80 mL) which yeilded 2-ethoxy-5-hydroxymethyloxalane-3,4-diol (ethyl β-riboside) (golden brown) **W-2** (R_f (0.58); 28 mg). C-3 (3.6 g, dirty white), a multi-component semi-pure residue was not processed any further in the course of the present study.

Structural elucidation

The mass spectra of the two compounds were obtained on Kratos MS 80 (Germany) while the infra-red analyses were done on Shimadzu FTIR 8400S (Japan). The ^{1}H and ^{13}C NMR spectra were obtained on Bruker AC 250 (Germany) operating 300 MHz for proton and 75 MHz for carbon-13 using CD_3OD as solvent and TMS as internal standard. Efforts were made to obtain the refractive indices of the compounds at the wavelength (λ) of Na-D line (589.3 nm) and at 20.5 ^{0}C [16-18] using the WAY-15 Abbe Refractometer (England).

Antimicrobial sensitivity screening

The microorganisms used in this study viz., *Bacillus subtilis* (NCTC 8853), *Staphylococcus aureus* (ATCC 25723), *Escherichia coli* (ATCC 25173), *Pseudomonas aeriginosa* (ATCC 2654), *Samonella typhi* (NCTC 5438), *Vibro cholerae* (ATCC 25032) 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 conventional biochemical tests.[19-20] These clinical microbes were then refrigerated at -5 ^{0}C at the Microbiology and Parasitology Unit, Faculty of Pharmacy prior to use. The agar plates used were prepared by adhering to the manufacturer's instructions. The media and plates were sterilized in an autoclave at 121 °C for 15 min. 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 separate petri-dish (Pyrex, England). Cylindrical plugs were removed from the agar plates by means of a sterile cork borer (Simax, India) to produce wells with diameter of approximately 6 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 butanol fraction, 2 mg mL^{-1} of **W-1** and **W-2** were introduced into the wells. Also, different concentrations of 10 µg mL^{-1} chlorampenicol (Gemini Drugs, Nigeria), 1 mg mL^{-1} of nystatin (Gemini Drugs, Nigeria) and 50 % methanol were introduced into separate wells as positive and negative controls respectively.[13-15,23-24] The experiments were carried out in triplicates. The plates were labeled on the underside and left at room temperature for 2 h to allow for diffusion. The plates were then incubated at 37 ± 2 ^{0}C for 24 to 48 h. Zones of inhibition were measured in millimetres (mm) with the aid of a ruler.

Spectral data

W-1: $C_6H_{10}O_4$; amorphous olive green solid; R_f (0.76); 65 mg; $[n]^{20}_D$ (1.4333, 20.5 ^{0}C); MS [ES+-MS] m/z (relative intensity): 147 $[M+H]^+$ (0.34%) , 146 $[M]^+$ (0.76 %), 128 $[M-H_2O]^+$ (14.74 %), 119 $[M-C_2H_5)]^+$ (0.84 %), 101 $[M-COOH]^+$ (100.00 %) (base peak), 84 $[M-OC_2H_5-OH]^+$ (0.78 %), 73 $[M-OC_2H_5-CO]^+$ (27.12 %), 56 $[M-OC_2H_5-COOH]^+$ (12.77 %), 55$[M-OC_2H_5-COOH-1]^+$ (28.47 %), 29 $[M-COOC_2H_5-OC=O]^+$ (49.65 %) and 27 $[M-COOC_2H_5-COOH-1]^+$ (21.54 %); IR cm^{-1}: 719, 864 (fingerprint), 1721(-C=O) and 3219 (-OH); ^{1}H NMR δ (ppm): 0.98 (t) and 1.42 (q); ^{13}C NMR δ (ppm): 19.45 (methyl-C), 34.42 (methylene-C), 160.43 (carbonyl-C) and 162.59 (ester-C).

W-2: $C_7H_{14}O_5$; golden brown substance; R_f (0.58); 28 mg; MS [ES+-MS] m/z (relative intensity):178 $[M]^+$ (0.12 %), 147 $[M-CH_2OH]^+$ (0.67 %), 133 $[M-OC_2H_5]^+$ (0.54 %), 114 $[M-OC_2H_5-OH-2]^+$ (0.25 %), 101 $[M-OC_2H_5-CH_2OH-1]^+$ (0.46 %), 88 $[M-CH_2OH-2OH-25]^+$ (10.78 %), 71 $[M-CH_2OH-2OH-C_2H_5-13]^+$ (48.67 %), 60 $[M-CH_2OH-3OH-C_2H_5-7]^+$ (100.00 %) base peak), 47 $[M-CH_2OH-3OH-OC_2H_5-4]^+$ (40.87 %); IR cm^{-1}: 634, 756, 843 (fingerprint), 1052 (-C-O-C), 2937 and 3376 (-OH); ^{1}H NMR δ (ppm): 1.26 (t), 1.47 (q) and 5.15(s); ^{13}C NMR δ (ppm): 29.52 (methyl-C), 36.48 (methylene-C), 145.63 and 146.45 (hydroxylated-C).

The $[n]^{20}_D$ value of **W-1** was found to be 1.4333 which is particularly consistent with the literature value of 1.4328. The insufficient amount of **W-2** sample prevented establishing the refractive index unambiguously.

Table 1. Results of antimicrobial screening of crude extract, butanol fraction, **W-1** and **W-2** at different concentrations on test microbes in 50 % MeOH

Test microbe	CE, 20 mg mL^{-1}	BT, 10 mg mL^{-1}	W-1, 2 mg mL^{-1}	W-2, 2 mg mL^{-1}	50 % MeOH	CP, 10 µg mL^{-1}	NY,1 mg mL^{-1}
B. subtilis (NCTC 8853)	6	6	6	6	6	34	6
S. aureus (ATCC 25723)	6	6	6	6	6	35	6
E. coli (ATCC 25173)	6	6	6	6	6	36	6
Ps. aeriginosa (ATCC 26154)	7.6	7.8	7.8	7.8	6	31	6
S. typhi (NCTC 5438)	6	6	7.3	7.5	6	28	6
V. cholerae (ATCC 25032)	6	6	7.8	7.7	6	38	6
C. albicans (NCYC 46)	6	6	6	6	6	6	39

Key: The zone diameter recorded is zone of inhibition + size of cup (zone of inhibition +6) mm; **CE** = Crude ethanolic extract; **BT** = Butanol fraction; **CP** = Chloramphenicol; **NY** =Nystatin; **W-1 =** 4-ethoxy-4-oxobutanoic acid (ethyl succinate); W-**2 =** 2-ethoxy-5-(hydroxymethyl)oxalane-3,4-diol (ethyl β-riboside); **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.

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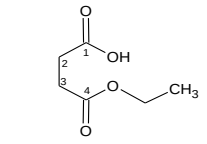
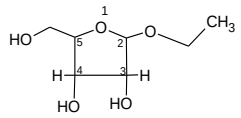
RESULTS AND DISCUSSION

The chemical structures of the compounds were established by a combination of spectroscopic techniques as mentioned above. The ^{1}H and ^{13}C NMR spectra of **W-1** and **W-2** were found to be consistent with literature values for the two known organic compounds namely 4-ethoxy-4-oxobutanoic acid (ethyl succinate) and 2-ethoxy-5-hydroxymethyloxalane-3, 4-diol (ethyl β-riboside),[28], respectively.



Ethyl succinate
(4-ethoxy-4-oxobutanoic acid)

W-1



Ethyl β–riboside
(2-ethoxy-5(hydroxymethyl) oxalane-3,4-diol)

W-2

Due to the chemical nature of the matrices, a lot of fragmented ions appeared in the MS spectra of both compounds. In the MS of **W-1,** those that could readily be identified include; $[M]^{+}$ at m/z 146 (2.51 %) while fragments at 119 (0.84 %), 101 (100.00 %) (base peak), 73 (27.12 %) and 56 (12.77 %) corresponded to the losses of ethyl group, carboxylate group, ethoxy and carbonyl units and ethoxy and carboxylate groups, respectively. Other noticeable ions at 84 (0.78 %), 55 (28.47 %), 29 (49.65 %) and 27 (21.54 %) were *quasi*-peaks. Similarly, **W-2** showed numerous peaks in its MS matrix but there were easily identifiable ions including $[M]^{+}$ at m/z 178 (0.12 %), while fragments at 147 (0.67 %) and 133 (0.54 %) represented the excisions of hydroxy methyl and ethoxy units from the **W-2** molecule, respectively. Other ions found in its spectrum at 114 (0.25 %), 101(0.46 %), 88 (10.78 %), 71 (48.67 %), 60 (100.00 %) (base peak) and 47 (40.87 %) were *quasi*-peaks.

The IR spectrum of **W-1** showed diagnostic stretchings at 1721 and 3219 cm^{-1} which indicated -C=O and -OH groups respectively. The IR spectrum of **W-2** indicated diagnostic signals at 1052, 2937 and 3376 cm^{-1} which represented -C-O-C (ether linkage) and -OH functional groups respectively.

Antimicrobial tests

The microbes employed in the sensitivity tests reflected the entire antimicrobial spectrum encompassing gram positive, gram negative and fungal strains. The results displayed in the Table 1 show that both the crude extract and butanol fraction were largely inactive against the microorganisms. Furthermore, **W-1** and **W-2** recorded no activity against *B. subtilis*, *S. aureus* and *E. coli* while the two compounds demonstrated very minimal activity against *Ps. aeriginosa*, *S. typhi* and *V. cholerae*. The non-reactivity shown towards especially the gram negative bacteria such as *Ps. aeriginosa* was not surprising because 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 possess a sophisticated three-layered envelope which does not allow permeation of external agents. 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.[29]

CONCLUSION

This study reports isolation of 4-ethoxy-4-oxobutanoic acid (ethyl succinate) and 2-ethoxy-5-hydroxymethyloxalane-3,4-diol (ethyl β-riboside) from the *A. wilkesiana var. golden-yellow*. These compounds are expected to serve as chemotaxonomic markers for the species and variety in particular and the genus, *Acalypha* in general. However, both compounds were generally inactive against microbes employed.

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