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Research Article

Isolation, Characterization and Antimicrobial Analysis of Ethyl succinate and Ethyl β-riboside from *Acalypha wilkesiana var. golden-yellow* (Muell & Arg.)

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Background: The genus, *Acalypha* is one of the many genera in the Euphorbiaceae family. *A. wilkesiana var.* ***golden-yellow*** **(**Muell & Arg.) syn. *A. wilkesiana var. tropical tempest* is a species and variety employed in traditional medicine for the treatment of gastrointestinal disorders, inflammations, bacterial and skin fungal infections amongst many others. Before now, some biological investigations had been done but very limited chemical studies have been carried out on the plant.

Objectives: This study was principally done to isolate chemical compound(s) inherent in the plant and also evaluate their antimicrobial potential.

Methodology: The leaves were to extracted cold with 50% ethanol and the resultant aqueous crude extract partitioned with butanol. Isolates obtained from the silica-gel column chromatography of the butanol fraction were analysed for antimicrobial activities using the hole-in agar diffusion technique. The compounds were screened for both antibacterial and antifungal activities using the microbes namely, *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhi, V. cholerae* and *C. albicans.*

Results: Two compounds, designated as compounds **W-1** [R_f **(**0.76); [n]^{20}D (1.4333)] and **W-2** [R_f (0.58)] were isolated. The structures of **W-1** and **W-2** have been established to be 4-ethoxy-4-oxobutanoic acid (ethyl succinate) and 2-ethoxy-5(hydroxymethyl)-oxalane-3,4-diol (ethyl β-riboside) respectively 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*. Interestingly, **W-1** and **W-2** demonstrated very minimal activity against *P. aeruginosa*, *S. typhi and V. cholerae.* However, neither gave any anticandidal activity.

Conclusion: The two isolates (though generally weakly active) would serve as chemotaxonomic markers for this species and variety in particular and the genus, *Acalypha* in general.

Keywords: Ethyl succinate**;** ethyl β-riboside; *A. wilkesiana var.* ***golden-yellow*** **(**Muell & Arg.)

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1. Introduction

Acalypha wilkesiana is named after the American scientist and explorer Admiral Chas Wilkes (1801-1877) (Bailey, 1951). *A. wilkesiana var.* ***golden-yellow*** **(**Muell & Arg.) syn. *A. wilkesiana var. tropical tempest* is characterized by bright lime, yellow and green speckled leaves which add a bright punch to the environment. It is cultivated from cuttings as an ornamental foliage wild shrub in gardens, greenhouses, orchards and parks

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(Hutchinson and Dalziel, 1958; Uphof, 1959; Burkill, 1985; Watt and Breyer-Brandwijk, 1962).). 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 for the treatment of malaria, wounds, tumours, inflammations, gastrointestinal disorders, bacterial and skin fungal infections (Oliver, 1958; Oliver, 1959; Akinde and Odeyemi, 1987; Alade and Irobi, 1993; Bussing et al, 1999; Ikewuchi et al, 2008). Before now, 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* (Adesina et al, 2000) while ethyl gallate, pyrogallol, D-arabino-hex-1-enitol and ethyl α-D-glucopyranoside had been obtained from *A. wilkesiana var. lace-acalypha* (Oladimeji and Igboasoiyi, 2014; Oladimeji and Udom, 2014; Oladimeji and Johnson, 2015).

This present study was carried out by subjecting the butanol fraction and sub-fractions obtained from this variety to column chromatography with a view to isolating chemical constituents therein and evaluating their antimicrobial potential. It is hoped that the obtained compounds will serve as chemotaxonomic markers for this species and variety in particular and genus, *Acalypha* in general.

2. Materials and Methods

2.1 Collection of plant material

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. The plant was identified at the Department of Botany and Ecology and Department of Pharmacognosy and Natural Medicine respectively, both of University of Uyo, 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 (Gallenkamp, England) at 40 ^{0}C for 48 h and the resultant dried material powdered on an electric mill (Uniscope, England).

2.2 Extraction and Isolation

The dried powder (0.85 kg) was exhaustively extracted with 50 % EtOH (4 x 5L) at room temperature (27± 2 ^{0}C) for 72 h. The obtained crude extract was filtered, concentrated *in vacuo* on a rotary evaporator (R205D, Shensung BS & T, China), weighed and stored in a desiccator (Monsorief, Scotland) prior to further use. 107 g of the extract was partitioned Using (1:4) H-$_2$O:CH_3CH_2CH $_2CH_2OH$ (6 x 200 mL). The resultant butanol fraction was evaporated to dryness to give a solid green residue which later yielded the two compounds. 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 % $(CH_3)_2$-CO: toluene (60 mL), 20 % $(CH_3)_2$CO: toluene (60 mL), 30 % $(CH_3)_2$CO: toluene (60 mL), 40 % $(CH_3)_2$CO:toluene (60 mL) and 50 % $(CH_3)_2$CO: toluene (60 mL). Fractions of 8 mL each were collected, monitored on silica plates (Model No 64271, Merck, Germany) in $(CH_3)_2$CO:toluene:H_2O (10:20:1) using $FeCl_3$/CH_3OH and vanillin-H_2 SO_4 as spray reagents. Subsequently, fractions with similar TLC characteristics (R_f values, reaction with $FeCl_3$ reagent or vanillin-H_2 SO_4 spray) were bulked 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 % $(CH_3)_2$ CO:toluene (80 mL) which yeilded 2-ethoxy-5(hydroxymethyl)-oxalane-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.

2.3 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 (Olaniyi, 1989; Olaniyi and Ogungbamila, 1991; Olaniyi, 2000) using the WAY-15 Abbe Refractometer (England).

2.4 Antimicrobial Sensitivity Screening

The micro-organisms used in this study namely, *Bacillus subtilis* (NCTC 8853), *Staphylococcus aureus* (ATCC 25723), *Escherichia coli* (ATCC 25173), *Pseudomonas aeruginosa* (ATCC 2654), *Samonella typhi* (NCTC 5438), *Vibro cholerae* (ATCC 25032) and *Candida albicans* (NCYC 436) were 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 isolates were collected in sterile bottles, identified and typed by convectional biochemical tests (Gibson and Khoury, 1986; Murray et al, 1995). These 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 each 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 (Washington, 1995; NCCLS, 2003). 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), 1mg mL^{-1} of nystatin (Gemini Drugs, Nigeria) and 50 % methanol were introduced into separate wells as positive and negative controls respectively (Oladimeji, 1997; Oladimeji, 2012; Oladimeji and Igboasoiyi, 2014; Oladimeji and Udom, 2014; Oladimeji and Johnson, 2015).

The experiments were carried out in triplicates. The plates were labelled 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.

3. Results and Discussion

3.1 Processing of Extract, Fraction and Sub-fractions

The collection and identification of the plant was done by observing basic rules of phytomorphology. Also, the principles governing extraction and processing of extracts were observed, thus preventing any changes to the chemical composition of the crude extract (Odebiyi and Sofowora, 1978; Odebiyi and Sofowora, 1979). Previous studies on the crude extract of this plant revealed the presence of alkaloids, saponins, tannins, terpenes and cardiac glycosides while flavonoids, anthraquinones and cyanogenic glycosides were absent (Oladimeji, 1997; Oladimeji and Usifoh, 2015).

Also, Oladimeji (1997), observed that the antibacterial and antifungal activities resided in the butanol fraction of the plant. In addition, the water/butanol partition extracted the largest amount of plant constituents, hence the choice of the butanol fraction for column chromatography. This exercise gave three semi-pure residues (C-1, C-2 and C-3) but C-1and C-2 were further processed, thereby yielding the isolates **W-1**and **W-2**. However, C-3 which showed a multi-component TLC profile was not processed any further.

3.2 Elucidation of Structures

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.

W-1: C_6 $H_{10}O_4$; amorphous olive green solid; R_f (0.76); 65 mg; $[n]^{20}$ $_D$ (1.4333); 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 [8400S-FTIR] cm^{-1}: 719, 864 (finger print), 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).

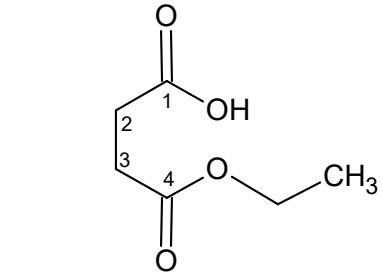
W-2: C_7H_{14} O_5; golden brown substance*;* R_f (0.58); 28 mg; MS [ES+-MS] m/z (relative intensity):178 $[M]^+$ (0.12 %), 147 $[M-CH_2OH]^+$ (0.67 %), 133 $[M-OC_2H_5]^+$ (0.54 %), 114 $[M-OC_2H_5-OH-2]^+$ (0.25%), 101 $[M-OC_2H_5-CH_2OH-1]^+$ (0.46%), 88 $[M-CH_2OH-2OH-25]^+$ (10.78%), 71 $[M-CH_2OH-2OH-C_2H_5-13]$ $^+$ (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 [8400S-FTIR] cm^{-1}: 634, 756, 843 (finger print) 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).

Physical constants such as optical rotation, optical density, refractive index, melting point 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 (Olaniyi, 1989; Olaniyi and Ogungbamila, 1991; Olaniyi, 2000). In this study, efforts were made to measure the refractive index of **W-1** and **W-2** at the wavelength (λ) of Na-D light (589.3 nm) and a temperature of 20.5 ^{0}C. 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. **W-1** recorded a refractive index of 1.4333 which is particularly consistent with the literature value of 1.4328 while that of **W-2** could not be unambiguously established because of insufficient amount of sample.

The chemical structures of the compounds were established by a combination of spectroscopic techniques as highlighted above. The obtained data were matched with library data of organic compounds (Lopez-Avila, 1987), hence, **W-1** and **W-2** have been identified to be 4-ethoxy-4-oxobutanoic acid (ethyl succinate) and 2-ethoxy-5(hydroxymethyl)-oxalane-3,4-diol (ethyl β-riboside) respectively (**Figure 1**).

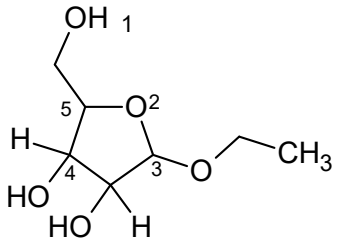
Due to the nature of the matrices, many fragmented ions appeared in the MS spectra of the compounds. In the MS of **W-1,** those that could readily be identified include; $[M]^+$ at m/z 146 (2.51 %) while fragments at 119 (0.84 %), 101 (100.00 %) (base peak), 73 (27.12 %) and 56 (12.77 %) correspond to the losses of ethyl group, carboxylate group, ethoxy and carbonyl units and ethoxy and carboxylate groups from **W-1** respectively. Other noticeable ions at 84 (0.78 %), 55 (28.47 %), 29 (49.65%) and 27 (21.54%) are *quazi*-peaks. The IR spectrum of **W-1** shows diagnostic stretchings at 1721 and 3219 cm^{-1} indicating -C=O and -OH groups respectively. Also, the obtained ^{1}H and ^{13}C NMR spectra of the compound are as expected.

Equally, **W-2** showed numerous peaks in its MS matrix but the easily identifiable ions include $[M]^+$at m/z 178 (0.12 %), while fragments at 147 (0.67 %) and 133 (0.54 %) represent 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 %) represent *quazi*-peaks. The IR spectrum of **W-2** shows diagnostic signals at 1052, 2937 and 3376 cm^{-1} representing -C-O-C (ether linkage) and -OH functional groups respectively. Also, the ^1H and ^{13}C NMR spectra of **W-2** are consistent with literature values.



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

W-1



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

W-2

Figure 1: Structures of **W-1** and **W-2**

Table: 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	Zones of inhibition (mm)						
	CE 20 mg/mL	**BT 10 mg/mL**	**W-1 2 mg/mL**	**W-2 2 mg/mL**	**50 % MeOH**	**CP 10 µg/mL**	**NY 1mg/mL**
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
P. 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, UK.
NCYC- National Collection of Yeast Cultures, UK.
ATCC- American Type Culture Collection**,** Washington, DC.

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3.3 Antimicrobial Tests

The microbes employed in the sensitivity tests reflected the entire antimicobial spectrum encompassing gram positive, gram negative and fungal strains. The results displayed in **Table 1** show that both the crude extract and butanol fraction (which was supposed to be relatively purer than the former) were largely inactive against the micro-organisms except *P. aeruginosa*. 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 *P. aeruginosa, S. typhi* and *V. cholerae.*

The activities given by especially the gram negative bacteria such as *P. aeruginosa* were 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 (Brown, 1975).

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 (Brown, 1975).

4. Conclusion

This study reports for the first time the isolation of 4-ethoxy-4-oxobutanoic acid (ethyl succinate) and 2-ethoxy-5(hydroxymethyl)-oxalane-3,4-diol (ethyl β-riboside) from the butanol fraction of 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.

Conflict of Interest Declaration

The authors declare no conflict of interest.

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