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Di-alkyl substituted phenol from *Acalypha wilkesiana var. golden-yellow* (Muell & Arg.)

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Abstract
Acalypha wilkesiana var. golden-yellow **(**Muell & Arg.) is a species and variety used in native medicine for the management or treatment of headache, gastrointestinal disorders, wounds, tumors and skin infections amongst many others. Before now, two compounds namely, 4-ethoxy-4-oxobutanoic acid (ethyl succinate) and 2-ethoxy-5(hydroxymethyl)-oxalane-3, 4-diol (ethyl β-riboside) have been obtained from the butanol fraction. This present study was carried out by subjecting one of the residues obtained from the processing of plant to column chromatography. A phenol designated as W-3(I) [R_f (0.42); m.pt. (88-90 ^{0}C] was isolated whose identity has been established to be 3, 5-Di-t-butyl phenol (3, 5-Di-t-butyl hydroxy benzene) using the ^{1}H NMR, ^{13}C NMR, MS and IR spectral techniques. W-3(I) was non-suppressive of *B. subtilis, S. typhi and V. cholerae* but recorded minimal activity against *S. aureus, E. coli* and *Ps. aeruginosa*. However, the compound gave no anticandidal activity. The compound would serve as chemotaxonomic marker for this species and variety in particular and the genus, *Acalypha* in general.

Keywords: Phenol; Chemotaxonomic; *Acalypha wilkesiana var. golden-yellow* (Muell & Arg.)

INTRODUCTION

The use of plants is of huge importance in traditional medicine especially in the remedy of various diseases (Bako *et al.*, 2005). Furthermore, the medicinal value of plants is contained in the constituent phytochemicals such as alkaloids, tannins, flavonoids and terpenes amongst many others which elicit definite physiological actions in the human body (Hill, 1952). Also, plants are classified as medicinal only when their biological properties have been scientifically established (Elujoba, 1997). One of such plants is *A. wilkesiana var. golden-yellow* (Muell & Arg.). Before now, two compounds namely, 4-ethoxy-4-oxobutanoic acid (ethyl succinate) and 2-ethoxy-5(hydroxymethyl)-oxalane-3,4-diol (ethyl β-riboside) (Oladimeji *et al.*, 2016) have been obtained from the butanol fraction. This present study was carried out by subjecting the remaining semi-pure residue obtained from the processing of plant to column chromatography with a view to isolating chemical constituent(s) therein and evaluating its/their antimicrobial potential.

EXPERIMENTAL

Isolation. Sample C-3 (3.6 g, dirty white), a multi-component semi-pure residue had been obtained previously from the

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chromatographic separation of the butanol fraction (Oladimeji *et al.*, 2016). It was purified on a much shorter silica-gel 254 column (Merck, Germany; 8 g pre-swollen in 100 % toluene; 2 g concentration zone + 8 g separation zone; 10 x 3 cm) by gradient elution with 30 % $(CH_3)_2CO$:toluene (60 mL), 40 % $(CH_3)_2CO$: toluene (60 mL) and 50 % $(CH_3)_2CO$: toluene (60 mL). Fractions of 7 mL each were collected, monitored on silica plates in $(CH_3)_2CO$:toluene:H_2O (10:20:1) and $(CH_3)_2CO$:EtOAc (35:65) using $FeCl_3$ /CH_3OH and vanillin-H_2SO_4 as spray reagents. Hence, two sub-fractions coded W-3(I) and W-3-(II) with different TLC characteristics (R_f values, reaction with $FeCl_3$ reagent or vanillin-H_2SO_4 spray) were bulked. Further TLC examinations of these sub-fractions in $(CH_3)_2CO$:toluene:H_2O (10:20:1) and $(CH_3)_2CO$: Et OAc (35:65) indicated a single spot in W-3(I) (amorphous off-white; R_f (0.42); 0.22 mg) which spectral analyses had identified to be 3, 5-Di-t-butyl phenol (3, 5-Di-t-butyl hydroxy benzene). W-3 (II) on the other hand had three spots with complex overlapping TLC profiles. Further work was suspended on W-3 (II) in the course of this study.

Structure elucidation. The mass spectrum of W-3(I) was obtained on Kratos MS 80 (Germany) while the infra-red data were obtained on Shimadzu FTIR 8400S (Japan). The ^1H and ^{13}C NMR spectral analyses were done 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. The melting point of the compound was determined using the melting point apparatus (Electrothermal, England).

Antimicrobial tests. The micro-organisms used in this study *viz.*, *Bacillus subtilis* (NCTC 8853), *Staphylococcus aureus* (ATCC 25723), *Escherichia coli* (ATCC 25173), *Pseudomonas aeruginosa* (ATCC 2654), *Samonella typhi* (NCTC 5438), *Vibro cholerae* and *Candida albicans* 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 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 7 millimeters. 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-3(I) 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 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 millimeters (mm) with the aid of a ruler.

RESULTS

Spectroscopic data:

W-3(I): $C_{14}H_{20}O$; off-white solid; R_f (0.42); 0.22 mg; m.pt. (88-90 ^{0}C);

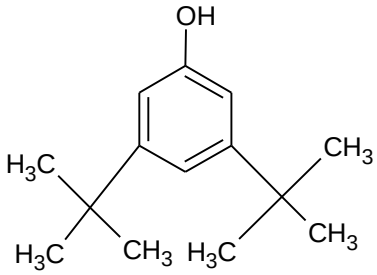
MS [ES+-MS] m/z (relative intensity): 206 $[M]^+$ (31.01%), 191 $[M\text{-}CH_3]^+$ (100.00 %) (base peak), 161 $[M\text{-}3CH_3]^+$ (0.52 %), 147 $[M\text{-(t-butyl)-}1]^+$ (0.27 %), 131 $[M\text{-(t-butyl)-OH})]^+$ (14.60 %), 115 $[M\text{-(t-butyl)-}2CH_3\text{-}3]^+$ (0.62 %), 107 $[M\text{-Ph-}CH_3\text{-}6]^+$ (11.92 %), 91 $[M\text{-Ph-}2CH_3\text{-}7]^+$ (12.21 %), 77 $[M\text{-2(t-butyl)-}13]^+$(11.93 %), 57 $[M\text{-2(t-butyl)-OH-}16]^+$ (51.63%), 55 $[M\text{-Ph-(t-butyl)-}CH_3]^+$ (0.45 %), 41 $[M\text{-Ph-(t-butyl)-}2CH_3\text{+H}]^+$ (19.06 %), and 27 $[M\text{-Ph-(t-butyl)-}2CH_3\text{-}13]^+$ (0.43 %);

IR [8400S-FTIR] cm^{-1}: 3319 (-OH); 1662 (Ar-C=C-) and 683, 798 (substitution on benzene);

^{1}H NMR δ (ppm): 2.1 (dd) and 6.42 (s);

^{13}C NMR δ (ppm): 21.35 (methyl-C), 122.87 (Ar-C=C) and 143.89 (hydroxylated C).

Chemical structure of **W-3(I)**



3,5-di-t-butylhydroxybenzene

W-3

Table: Antimicrobial screening of crude extract, butanol fraction, W-3(I) at different concentrations on test microbes in 50 % MeOH

Test microbe	CE/ 20 mg/mL	BT/ 10 mg/mL	W-3(I/ II) mg/Ml	50 % MeOH	CP/10 μg mL^{-1}	NY/ 1mg/mL
B. subtilis (NCTC 8853)	7	7	7	7	36	7
S. aureus (ATCC 25723)	7	7	8	7	33	7
E. coli (ATCC 25173)	7	7	8.6	7	37	7
Ps. aeruginosa (ATCC 26154)	8.6	8.8	8.8	7	30	7
S. typhi (NCTC 5438)	7	7	7	7	30	7
V. cholerae	7	7	7	7	39	7
C. albicans	7	7	7	7	7	40

Key: The zone diameter recorded is zone of inhibition + size of cup (zone of inhibition +7) mm;
CE = Crude ethanolic extract; BT = Butanol fraction; CP = Chloramphenicol; NY =Nystatin; W-3 = W-3(I) = 3, 5-Di-t-butyl phenol (3, 5-Di-t-butyl hydroxy benzene); 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.

DISCUSSION

Structural elucidation. 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, W-3(I) was isolated in the solid form, hence its melting point was determined to be 88-90 ^{0}C which is particularly consistent with 87-89 ^{0}C in literature. The chemical structure of the compound was 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-3(I) has been identified to be 3, 5-Di-t-butyl phenol (3, 5-Di-t-butyl hydroxy benzene). Due to the nature of the matrix, many fragmented ions appeared in the MS of the compound but those that could readily be identified include; $[M]^+$ at m/z 206 (31.01%) while fragments at 191 (100.00 %) (base peak), 161 (0.52 %), 147 (0.27 %), 131 (14.60 %), (115 (0.62 %), 91 (12.21 %) and 77 (11.93 %) which corresponded to the excisions of a methyl group, 3 methyl groups, t-butyl radical, t-butyl radical and hydroxy units, t-butyl radical, phenyl and methyl units and 2 t-butyl radical units respectively from the molecular ion. Other noticeable peaks at 107 (11.92 %), 57 (51.63%), 55 (0.45 %), 41 (19.06 %) and 27 (0.43 %) were also considered in identifying W-3(1). The IR spectrum of W-3(I) revealed diagnostic stretchings at 3319 and 1662 cm^{-1} which indicated Ar-OH and Ar-C=C entities respectively. Also, the obtained ^{1}H and ^{13}C NMR spectra of the compound are as expected though the assignments of the protons and carbon atoms would have unambiguous had the 2D-NMR HECTOR and NOESY experiments been carried out. It is remarkable to note that tannins (polyphenols) have been isolated from other *Acalypha* species. Gallic acid, corilagin and geraniin have both been obtained from *Acalypha wilkesiana var. red acalypha* and *Acalypha hispida* (Adesina *et al.*, 2000) while ethyl gallate and pyrogallol have been isolated from *Acalypha wilkesiana var. lace-acalypha* (Oladimeji and Igboasoiyi, 2014). The isolation of 3, 5-Di-*t*-butyl phenol from *Acalypha wilkesiana var. golden-yellow* is not surprising since phenols have proved to be of chemotaxonomic importance to the genus, *Acalypha.*

Antimicrobial screening. The sensitivity tests were carried out with micro-organisms reflecting the entire spectrum for antimicrobial activities. The results displayed in the Table show that both the crude extract and butanol fraction were non-suppressive of the micro-organisms except *Ps. aeruginosa*. W-3(I) was inactive against *B. subtilis*, *S. typhi* and *V. cholerae* while it demonstrated some antibacterial activity (though minimal) against *S. aureus*, *E. coli* and *Ps. aeruginosa.* The activities given by especially the gram negative bacteria such as *Ps. 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). Furthermore, W-3(I) recorded no antifungal activity against *C. albicans.* This 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).

Conclusion. In this study, 3, 5-Di-t-butyl phenol (3, 5-Di-t-butyl hydroxy benzene) was isolated from *A. wilkesiana var. golden-yellow* (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 weakly active against some of bacterial strains while it recorded no antifungal activity against the *C. albicans*.

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