SUPPLEMENTARY MATERIAL

A new antibacterial lupane ester from the seeds of *Acokanthera oppositifolia* Lam.

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Abstract

As a part of ongoing investigation of *Acokanthera oppositifolia* (Lam.) Codd., four compounds were isolated from its seeds, a new compound; lup-20(29)-en-3 β -O-(3'- β -hydroxy) palmitate (**1**), three known compounds; lupeol (**2**), a cardiac glycoside; acovenoside A (**3**), and a sterol; β -sitosterol (**4**). Their structures were investigated using 1D&2D- ^1H and ^{13}C NMR spectroscopy. Antimicrobial potential of the compounds was evaluated against ten microorganisms responsible for endocarditis. The minimum inhibitory concentration (MIC) of the compounds was determined using broth microdilution method. The new compound (**1**) evidenced significant antibacterial activity especially against *Pseudomonas aeruginosa* with (MIC 7.81 $\mu\text{g/ml}$). Lupeol (**2**) exhibited remarkable antimicrobial activity against Methicillin-resistant *Staphylococcus aureus*, *Aspergillus fumigates* and *Candida albicans* (MIC 3.9, 0.24 and 3.9 $\mu\text{g/ml}$, respectively). On the other hand, acovenoside A (**3**) inhibited the growth of *Escherichia coli* (MIC 0.98 $\mu\text{g/ml}$). We herein present the potential of *A. oppositifolia* as a cardioprotective agent against the microorganisms responsible for endocarditis.

Keywords: Cardenolide, lupeol ester, *Acokanthera*, antimicrobial, endocarditis.

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1. Experimental

General Experimental procedures

Material for chromatographic studies included pre-coated silica plates 60 GF₂₅₄, (20×20 cm) from Fluka (Sigma-Aldrich chemicals-Germany) for thin layer chromatography (TLC), silica gel 60 for normal phase column chromatography (CC), silica gel H for vacuum liquid chromatography (VLC) (Merck Darmstadt, Germany). The following solvent systems were used for developing the chromatograms; S₁: *n*-hexane- ethyl acetate (9 : 1 v/v), S₂: *n*-hexane-ethyl acetate (8 : 2 v/v), S₃: chloroform: methanol (9.5:0.5 v/v) and S₄: chloroform: methanol (9 : 1 v/v). Spots were visualized by spraying with *p*-anisaldehyde-sulphuric acid.

HR-ESIMS was measured in the JEOL JMX-AX 505, HAD mass spectrophotometer at an ionization voltage of 70 eV. IR spectra were observed as KBr discs using Jasco FT/IR-460 plus, Japan Infrared Spectrophotometer. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker high performance digital FT-NMR spectrophotometer operating at 400 (¹H) and 100 (¹³C) MHz in CDCl₃-d₆ as a solvent and chemical shifts were given in δ (ppm) relative to solvent as internal standard. Ultraviolet lamp (λ max = 254 and 330 nm, Shimadzu), a product of Hanovia lamps for localization of spots on chromatograms. Discs of ampicillin, gentamycin, vancomycin and amphotericin B 5 µg/ disc, Oxoid Chemical Co., UK

1.1 Plant Material

The seeds of *Akocanthera oppositifolia* (Lam.) Codd. were obtained from the trees growing in Bn Ghazy- Libya collected in November 2012. The plant was authenticated by Dr. Reem Samir Hamdy, Lecturer of Plant Taxonomy, Botany Department, Faculty of Science, Cairo University, Giza, Egypt. Voucher sample of the plant (No. 23102015) is deposited at the Museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

1.2 Extraction

The air-dried powdered seeds of *A. oppositifolia* (200 g) were extracted using cold method of extraction (percolation) in room temperature with 95% ethanol (4 x 500 mL) till exhaustion (for

one week). The 95% ethanol extract was filtered, distilled and evaporated under reduced pressure to give 20 g of greenish brown semi-solid residue. The dried residue was chromatographed on a VLC column 210 g silica gel (100 cm x 4.5 cm). Gradient elution with *n*-hexane, *n*-hexane-methylene chloride mixtures and chloroform-ethyl acetate mixtures and methanol was applied. Fractions 200 ml each were collected and the progress of separation was noted by thin layer chromatography (TLC) using $S_1 - S_4$ solvent system and *p*-anisaldehyde as detecting agent. Similar fractions were pooled together to obtain five major fractions.

Fraction **I** (0.37g), eluted with 10% methylene chloride in *n*-hexane was purified on a silica gel column using *n*-hexane: ethyl acetate (90 : 10 v/v) as eluent to obtain compound **1** (60 mg).

Fraction **II** (5.4 g), eluted with 30% methylene chloride in *n*-hexane, was purified on silica gel column using *n*-hexane: ethyl acetate (80 : 20 v/v) as eluent to obtain compound **2** (200 mg),

Fraction **III** (2.54 g), eluted with 5 - 10% ethyl acetate in methylene chloride was purified on silica gel column using 0.5 % methanol in methylene chloride mixture as eluent to yield white microcrystalline powder of compound **3** (50 mg)

Fraction **IV** (2.62 g), eluted with 30% ethyl acetate in methylene chloride, was purified on silica gel column using *n*-hexane-ethyl acetate (60 : 40 v/v) mixtures as eluent and revealed two spots. Further rechromatography on successive silica gel columns using *n*-hexane-chloroform mixtures, this fraction yielded compound **4** (20 mg).

^1H and ^{13}C NMR data of the isolated compounds are presented in Tables (1 -3)

1.3 Testing the antimicrobial activity

The antimicrobial activity testing was performed against eight selected bacterial and four fungal strains of standard properties, this work was performed in the Regional Center for Mycology and Biotechnology Al Azhar University. The tested Gram positive bacteria were *Staphylococcus aureus* (RCMB 010028), *Enterococcus faecalis* (RCMB 010084), *Streptococcus mitis* (RCMB 010039), *Lactobacillus acidophilus* (RCMB 010094) and Methicillin-resistant *Staphylococcus aureus* [MRSA](RCMB 010028) (obtained as clinical isolate). The Gram negative bacteria included *Pseudomonas aeruginosa* (RCMB 010043), *Escherichia coli* (RCMB 010052), *Mycobacterium tuberculosis* (RCMB 010120) and fungi [*Aspergillus fumigates* (RCMB 02568),

Syncephalastrum racemosum (RCMB 05922), *Geotricum candidum* (RCMB 05097) and *Candida albicans* (RCMB 05036)]. Bacteria were sub cultured on nutrient agar medium (Oxoid laboratories, UK) and fungi on Sabouraud's dextrose agar (Oxoid laboratories, UK). The isolates were separately tested against the selected strains at concentration of 1 mg/ml in DMSO (as the tested compounds are freely soluble in DMSO at this concentration) adopting agar well diffusion assay method as described by Holder and Boyce (1994). Ampicillin, gentamycin, and vancomycin were used as positive control for bacterial strain; amphotericin B was used as a positive control for fungi. The plates were done in triplicate. Bacterial cultures were incubated at 37°C for 24 h while the other fungal cultures were incubated at (25-30°C) for 3-7 days. Results are recorded as Mean zone of inhibition in mm $\pm$ standard deviation beyond well diameter (6 mm) produced on a range of environmental and clinically pathogenic microorganisms using (1 mg/ml) concentration of tested samples (Table S4) (Agwa *et al.*, 2000).

1.4 Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the samples was estimated for each of the tested organism in triplicates (Table S5). Varying concentrations of the samples (1000-0.007 μ g/ml), nutrient broth were added and then a loopful of the test organism previously diluted to 0.5 McFarland turbidity standard was introduced to the tubes. A tube containing broth media only was seeded with the test organisms to serve as control. Tubes containing tested organisms cultures were then incubated at 37°C for 24 h; while the other fungal cultures were incubated at (25-30°C) for 3-7 days. The tubes were then examined for growth by observing for turbidity (Doughari, 2006).

Statistical analysis

All data were expressed as mean $\pm$ SE and the statistical significance was evaluated using the ANOVA test followed by Duncan's multiple range tests. A probability value of less than 0.05 was considered statistically significant (P<0.05 was considered statistically significant).

References

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Table 1S. ¹HNMR (400MHz, CDCl₃) of compound 1, 2 and 4

Position	δH ppm		
	Compound 1	Compound 2	Compound 4
1	2.49 (m), 2.54 (d, J=12.8 Hz)	2.25 (m), 3.08 (d, J=13.4 Hz)	2.22 (m), 2.98 (d, J=12.8 Hz)
2	1.90 (m), 2.00 (m)	1.81 (m), 2.15 (m)	1.78 (m), 2.14 (m)
3	4.53 (1H,m)	3.23 (1H,m)	3.54 (1H,m)
5	1.42 (m)	1.40 (m)	-
6	-	-	5.37 (1H, br.s)
18	-	-	0.70 (3H,s)
19	2.36 (1H, t)	2.36 (1H,t)	1.03 (3H,s)
21	-	-	0.84 (3H, d, J=6.3 Hz)
23	0.80 (3H, s)	0.78 (3H, s)	-
24	0.86 (3H, s)	0.81 (3H, s)	-
25	0.87 (3H, s)	0.85 (3H, s)	-
26	0.91 (3H, s)	0.96 (3H, s)	0.86 (3H, d, J=6.7 Hz)
27	0.96 (3H, s)	0.99 (3H, s)	0.93 (3H, d, J=6.7 Hz)
28	1.05 (3H, s)	1.05 (3H, s)	-
29	4.58 (1H, br.s, H-29a) 4.70 (1H, br.s, H-29b)	4.59 (1H, br.s, H-29a) 4.70 (1H, br.s, H-29b)	0.81 (3H, t, J=6.7 Hz)
30	1.70 (3H,s)	1.70 (3H,s)	-
1 ^ˆ	--		
2 ^ˆ	2.4-2.5 (2H, m)	-	-
3 ^ˆ	4.00 (1H, m)	-	-
4 ^ˆ -15 ^ˆ	1.27 (12H, br s)	-	-

16	1.38 (3H, t)	-	-
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Table 2S. ¹³CNMR (100MHz, CDCl₃) of compound 1, 2 and 4

Position	δC ppm		
	Compound 1	Compound 2	Compound 4
1	38.2	38.7	37.2
2	23.6	27.4	31.6
3	81.4	79.0	71.8
4	37.7	38.8	42.3
5	55.3	55.3	140.7
6	18.2	18.3	121.6
7	34.1	34.6	31.9
8	40.8	40.8	31.9
9	50.3	50.4	50.1
10	37.8	37.1	36.5
11	20.9	21.1	21.1
12	25.4	25.1	39.8
13	38.0	38.0	42.3
14	42.8	42.8	56.7
15	27.4	27.6	24.3
16	35.5	35.5	28.2
17	42.9	43.0	56.0
18	48.2	47.9	11.9
19	48.0	48.3	19.3
20	150.9	150.9	36.1
21	29.8	29.9	18.9
22	39.6	40.0	33.9
23	28.0	28.0	26.2
24	16.6	15.3	45.2
25	16.1	16.1	29.2
26	15.9	15.9	19.0
27	14.5	14.5	19.8
28	18.0	18.0	23.1
29	109.3	109.3	11.9
30	19.3	19.3	

1 ⁺	172.8		
2 ⁺	41.6		
3 ⁺	68.2		
4 ⁺ -12 ⁺	29.3 – 29.7		
13 ⁺	31.9		
14 ⁺	22.7		
15 ⁺	14.13		
16 ⁺	25.0		

Table 3S. ¹HNMR and ¹³CNMR (400MHz, 100MHz, CDCl₃) of compound 3

Position	δH ppm	δC ppm
1	3.7 d (<i>J</i> =8.8 Hz)	72.4
2	1.89, 1.95	31.7
3	4.26	72.3
4	1.83, 1.62	28.2
5	1.9	30.4
6	1.81, 1.36	25.9
7	1.72, 1.25	20.9
8	1.62	41.9
9	1.49	37.6
10	--	40.2
11	1.35, 1.36	21.1
12	1.30, 1.53	39.9
13	--	49.4
14	--	85.3
15	2.1, 1.73	33.1
16	2.14, 1.89	26.9
17	2.79	50.8
18	0.91	15.8
19	1.12	18.8
20	--	174.3
21	4.80, 4.98 (d, <i>J</i> =17.8 Hz)	73.4
22	5.9	117.8
23	--	174.1
1⁻	5.0	97.6
2 ⁻	3.88	68.4
3 ⁻	3.37	75
4 ⁻	3.88	69.7
5 ⁻	3.98	66.7
6 ⁻	1.36 (d, <i>J</i> =6.4 Hz)	16.5
3 ⁻ -OCH ₃	3.51	55.6

The tested microorganisms	The tested samples			
Fungi	Compound 1	Compound 2	Compound 3	<i>Amphotericin B</i>

Table 4S. Antimicrobial activity of the isolated compounds

<i>Aspergillus fumigatus</i> (RCMB 02568)	20.2 ± 0.58 85.2%	22.8 ± 0.44 96.2%	16.3 ± 1.2 68.7%	23.7 ± 0.10 100%
<i>Candida albicans</i> (RCMB 05031)	NA 0.0%	18.8 ± 0.44 94.9%	NA 0.0	19.8 ± 0.20 100%
Gram + ve bacteria				Ampicillin
<i>Staphylococcus aureus</i> (RCMB 010028)	21.7 ± 0.22 79.1%	23.4 ± 0.22 85.4%	17.4 ± 0.72 63.5%	27.4 ± 0.18 100%
<i>Enterococcus faecalis</i> [RCMB 010084(6)]	24.2 ± 0.58 91.6%	21.2 ± 0.58 80.3%	20.3 ± 1.2 76.8%	26.4 ± 0.34 100%
<i>Streptococcus mitis</i> (RCMB 010039)	NA 0.0%	17.3 ± 0.58 71.1%	NA 0.0	24.3 ± 0.44 100%
<i>Lactobacillus acidophilus</i> (RCMB 010094)	NA 0.0%	24.2 ± 0.58 96%	NA 0.0	25.2 ± 0.58 100%
Gram - ve bacteria				Gentamicin
<i>Pseudomonas aeruginosa</i> (RCMB 010043)	NA 0.0%	NA 0.0%	18.3 ± 1.5 106%	17.3 ± 0.15 100%
<i>Escherichia coli</i> (RCMB 010052)	20.6 ± 0.58 92.3%	18.3 ± 0.58 82.0%	19.8 ± 0.72 88.7%	22.3 ± 0.18 100%
<i>Mycobacterium tuberculosis</i> (RCMB 010120)	15.4 ± 0.44 83.6%	NA 0.0%	NA 0.0	18.4 ± 0.58 100%
Gram+ ve bacteria				Vancomycin
Methicillin-resistant <i>Staphylococcus aureus</i> [MRSA](RCMB 010028(3) Clinical isolate	16.3 ± 0.63 83.1%	18.9 ± 0.63 96.4%	14.6 ± 1.5 74.4%	19.6 ± 0.58 100%

The test was done using the agar diffusion technique, Well diameter: 6.0 mm (100 µl Was tested), RCMB: Regional Center for Mycology and Biotechnology Antimicrobial unit test organisms *NA : No activity, data are expressed in the form of mean ± SD.

Table 5S. Antimicrobial Activity as MICS (µg / ml) of the isolated compounds against tested microorganisms:

Tested microorganisms	Minimum inhibitory concentration (µg/ml)			
Fungi	Compound 1	Compound 2	Compound 3	Amphotericin B

<i>Aspergillus fumigatus</i> (RCMB 2568)	32.25	0.24	1.95	0.12
<i>Candida albicans</i> (RCMB 05031)	NA	3.9	NA	1.95
Gram + ve bacteria				<i>Ampicillin</i>
<i>Staphylococcus aureus</i> (RCMB 010028)	15.63	0.49	0.49	0.06
<i>Enterococcus faecalis</i> (RCMB 010084(6))	3.9	0.12	0.12	0.03
<i>Streptococcus mitis</i> (RCMB 010039)	NA	15.63	NA	0.12
<i>Lactobacillus acidophilus</i> (RCMB 010094)	NA	0.12	NA	0.03
Gram - ve bacteria				<i>Gentamicin</i>
<i>Pseudomonas aeruginosa</i> (RCMB 010043)	7.81	NA	NA	15.63
<i>Escherichia coli</i> (RCMB 010052)	3.9	7.81	0.98	0.49
<i>Mycobacterium tuberculosis</i> (RCMB 010120)	NA	NA	62.5	7.81
Gram + ve bacteria				<i>Vancomycin</i>
Methicillin-resistant <i>Staphylococcus aureus</i> [MRSA](RCMB 010028(3) (clinical isolate)	62.5	3.9	31.25	1.95

Fig. 1S. ^1H -NMR spectrum of compound 1

Fig. 2S. ^{13}C -NMR spectrum of compound 1

Compound 1

Fig. 3S. Selected HMBC correlations of compound 1

Fig. 4S. HMQC spectrum of compound 1

Fig. 5S. NOESY spectrum of compound 1

Fig. 6S. HSQC spectrum of compound 1