

Alkaloids and Flavone Acyl Glycosides from *Acanthus arboreus*

Masouda E. Amer^a, Mohamed I. Abou-Shoer^a, Maged S. Abdel-Kader^{*,a}, Amina M.S. El-Shaibany^b
and Nabil A. Abdel-Salam^a

^aDepartment of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt

^bDepartment of Pharmacognosy, Faculty of Medicine, Sana'a University, Sana'a, Yemen

O estudo fitoquímico de *Acanthus arboreus* resultou no isolamento de 3 novos alcalóides: 6-hidróxi-2-benzoxazolinona, 4-hidróxi-acantamina (3,4-di-hidróxi-1,4-benzoxazino-2-ona) e acantaminosídeo (3-O-glicopiranosídeo-1,4-benzoxazino-2-ona). Além destes alcalóides foi também isolado o novo flavonóide 7-O-β-D(6''-trans-p-cumaroil)3''-O-acetilglicopiranosídeo apigenina e os seguintes compostos de estruturas já conhecidas: apigenina, 7-O-β-D(6''-trans-p-cumaroil)3''-O-glicopiranosídeo apigenina, ácido vanílico, lupeol, estigmasterol e 3-β-glicopiranosídeo sitosterol. As estruturas dos compostos foram determinadas por métodos espectroscópicos e transformações químicas.

Phytochemical study of *Acanthus arboreus* resulted in the isolation of three novel alkaloids: 6-hydroxy-benzoxazolinone, 4-hydroxyacanthamine and acanthaminoside. In addition, a new acyl flavonoid apigenin-7-O-β-D-(6''-trans-p-coumaroyl)-3''-O-acetyl glucopyranoside was also isolated. The known compounds were identified as apigenin, apigenin-7-O-β-D-(6''-trans-p-coumaroyl)-glucoside, vanillic acid, lupeol, stigmasterol and sitosterol glucoside. The structures were determined by physical, chemical and spectral techniques.

Keywords: *Acanthus arboreus*, Acanthaceae, alkaloids, flavone acyl glycosides, antimicrobial activity

Introduction

The Acanthaceae is a large family with more than 250 genera and 2700 species.¹ Chemical investigation of genus *Acanthus* resulted in the isolation of flavonoids, alkaloids, triterpenoids and sterols.²⁻⁵ *A. ilicifolius* is used as anticonvulsant, hypnotic and skeletal muscle relaxant due to the presence of benzoxazolinone; an alkaloid with CNS depressant activity.^{6,7}

Benzoxazolinone also exhibited antiprotozoal activity against *Leishmania donovani in vitro*; while its ribose derivatives were active as anticancer and antiviral agents.^{5,8}

Results and Discussion

The HRCI-MS of **1** showed an M⁺+1 at m/z 579.151 for the molecular formula C₃₀H₂₆O₁₂. Physical and spectral data of **1** were identical with those reported for apigenin O-β-D-(6''-trans-p-coumaroyl)-glucoside isolated from *Pogostemon cablin*.⁹ However, Singh *et al* in 1986 reported the same compound as a novel product from *Echinops*

echinatus under the name echinacin.¹⁰ Based on COSY and HMQC experiments, complete assignments for the sugar protons and all carbons were achieved. Our assignments were in complete agreement with first publication.⁹ However, some of the ¹³C-NMR assignments in the latter publication¹⁰ must be revised.

The HRCI-MS (M⁺+1 at m/z 621.161 for the molecular formula C₃₂H₂₈O₁₃) and other spectral data of **4** (see experimental) indicated an additional acetyl group compared with **1**. Complete assignments of the sugar protons could be achieved by a combination of COSY and HMQC experiments. A major difference was observed in the chemical shift of H-3'' (δ 4.97, t) compared to that of **1** (δ 3.30, m), indicating that the acetyl group is located at C-3''. Further evidence for the position of the acetyl group was obtained from the ¹³C-NMR, where a substantial downfield shift Δ_{δC-3''} = 1.20 ppm and the upfield shift of C-2'' and C-4'' (Δ_{δC-2''} = 1.66 ppm and Δ_{δC-4''} = 1.67 ppm) relative to those of **1** were observed.¹¹ A literature search revealed that **4** (apigenin 7-O-β-D(6''-trans-p-coumaroyl)3''-O-acetylglucopyranoside) is a previously unreported natural compound.

The new alkaloid **2** gave positive reactions with FeCl₃, Lassaigne and ninhydrin reagents indicating its phenolic

* e-mail: mpharm101@hotmail.com

nitrogenous nature. HREI-MS (experimental) showed an M^+ at m/z 151.027 for the molecular formula $C_7H_5O_3N$. It also showed characteristic mass fragments at m/z 105, 95, 91 and 77, suggesting the presence of 2-benzoxazolinone.¹² In the ^{13}C -NMR spectrum (Table 1) the seven carbons were resolved as three aromatic methines (δ 99.1, 111.0 and 111.6) assigned to C-7, C-5 and C-4 respectively, three quaternary aromatic carbons (δ 129.9, 146.1 and 154.8) attributed to C-9, C-8 and C-6 respectively, and a fourth quaternary carbon at δ 157.7 assigned to the lactone carbonyl. The 1H -NMR spectrum (Table 1) showed an ABX system at δ 6.68 (1H, d, J 2.3 Hz), 6.85 (1H, d, J 8.5 Hz) and 6.60 (1H, dd, J 2.3, 8.5 Hz) indicating a trisubstituted aromatic system. Compound **2** was identified as the demethyl derivative of 6-methoxy-benzoxazolinone i.e. 6-hydroxy-benzoxazolinone. Even the known 6-methoxy-benzoxazolinone is a rare natural product isolated only once from Corn plants.¹³

Chemical reactions and solubility in NaOH, followed by recovery after acidification, suggested that **3** is a nitrogenous compound with a lactone function. This was confirmed by the IR absorption bands for carbonyl (1670 cm^{-1}), hydroxy and/or NH groups (3325 cm^{-1}). The HRCI-MS showed an $M^+ + 1$ at m/z 182, and an M^+ at m/z 181.144 for the molecular formula $C_8H_7O_4N$. The ^{13}C -NMR spectrum (Table 1) showed five methine signals and three quaternary carbons. In the 1H -NMR spectrum the coupling pattern of the 4 aromatic protons suggested the presence of an *O*-disubstituted benzene ring. In the 1H -NMR spectrum the singlet at δ 5.68, diagnostic for a proton flanked by two electronegative atoms, was assigned for H-3. This was supported by a ^{13}C -NMR signal which appeared at δ 93.7 and was attributed to the oxymethine

carbon. The remaining three quaternary carbon absorptions at δ 129.7, 142.5 and 160.1 were assigned to C-10, C-9 and the carbonyl group respectively. The violet colour with $FeCl_3$ ¹⁴ as well as the M^+ of the diacetate derivative **3a** at m/z 265 indicated the presence of a hydroxylamine group. The exact positions of the carbonyl and hydroxyl groups were established from alkaline hydrolysis and by the inability of **3** to give *O*-aminophenol after fusion with KOH. This clearly distinct the new alkaloid **3** (3,4-dihydroxy-1,4-benzoxazine-2-one) from blepharigenin (2-hydroxy-1,4-benzoxazine-3-one) a compound with very close 1H -NMR data.¹⁵

The HRCI-MS of the third new alkaloid **5** showed M^+ at m/z 327.095 for the molecular formula $C_{14}H_{17}O_8N$. The

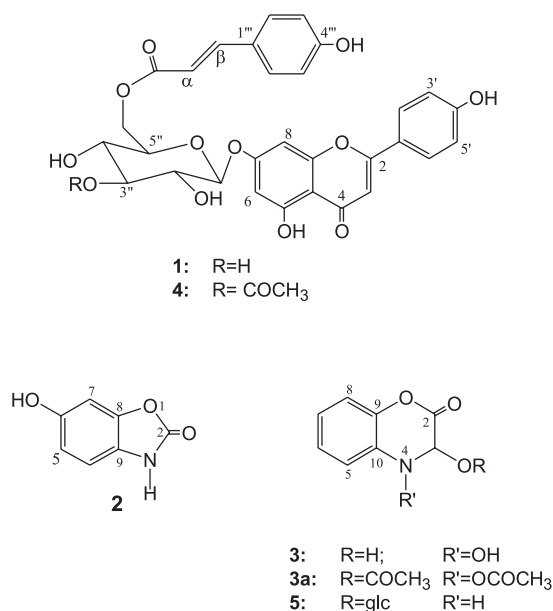


Table 1. 1H - and ^{13}C -NMR spectral data of compounds **2**, **3** and **5** (coupling constants in Hz)^a

#	2 ^b		3 ^b		5 ^c	
	1H	^{13}C	1H	^{13}C	1H	^{13}C
2	-	157.7	-	160.1	-	162.6
3	-	-	5.68 s	93.7	5.78 s	96.4
4	6.85 d J 8.5	111.6	-	-	-	-
5	6.60 dd, J 2.3, 8.5	111.0	7.36 dd J 1.6, 7.8	114.4	7.12 m	119.1
6	-	154.8	7.08 m	125.6	7.05 m	124.9
7	6.68 d J 2.3	99.1	7.08 m	123.8	7.05 m	124.2
8	-	146.1	7.01 dd J 1.6, 7.8	118.5	6.99 m	116.9
9	-	129.9	-	142.5	-	142.0
10	-	-	-	129.7	-	127.4
1'	-	-	-	-	4.70 d J 7.8	104.0
2'	-	-	-	-	3.28 m	74.9
3'	-	-	-	-	3.30-3.34 m	78.0
4'	-	-	-	-	3.30-3.34 m	71.1
5'	-	-	-	-	3.30-3.34 m	78.6
6'	-	-	-	-	3.83 d J 11.9	62.5
					3.68 dd J 3.6, 11.9	

^a Assignments are based on HMQC experiments; ^b Spectra were measured in CD_3OD ; ^c Spectra were measured in $CD_3OD/DMSO$ mixture

14 carbon signals were clear in the ^{13}C -NMR spectrum (Table 1), of which 8 signals were accounted for the aglycone part, while the remaining 6 carbon signals were assigned for the sugar moiety. The 1,4-benzoxazine-2-one skeleton was assigned to **5** rather than the 1,4-benzoxazine-3-one as indicated from its reaction with KOH solution and fusion test.¹⁵ The spectral data of **5** aglycone (Table 1 and Experimental) showed a close similarity to **3**. However, the CI-MS and the negative reaction with FeCl_3 suggested the absence of a hydroxyl group attached to nitrogen atom. Consequently, the only possible site for glycosylation is the C-3 hydroxyl. The identity of the sugar moiety was established as glucose by spectral evidences and by TLC comparison after acid hydrolysis. On the basis of the chemical shift and coupling constant of the anomeric proton ($J_{1,2}$, 7.8 Hz), the glucosidic linkage should have the β -orientation. The identity of **5** was therefore established as 1,4-benzoxazine-2-one-3-*O*-glucoside.

The known compounds were identified by direct comparison with reference materials (Aldrich).

Compounds **1** and **3-5** were subjected to antimicrobial testing using 10 microorganisms. Only compounds **1** and **4** were active against *Bacillus subtilis* with an MIC 64 and 128 $\mu\text{g}/\text{mL}$ respectively.

Experimental

General procedure

The CI-MS of **3a** was measured on a Finnigan SSQ7000 mass spectrometer. NMR spectra were recorded on a JEOL 500 NMR instrument at 500 MHz for ^1H and 125 MHz for ^{13}C . Other experimental conditions were as previously described.¹⁶

Plant material

The whole plants of *Acanthus arboreus* Forssk. growing wild in Wadi Dhar, Sana'a, Yemen was collected during the flowering stage in August 1998 and was identified by Prof. Nabil El-Hadidy, Department of Plant Taxonomy, Faculty of Science, Cairo University. A voucher sample (YA1) is preserved in the Department of Pharmacognosy, Faculty of Pharmacy, University of Alexandria, Egypt.

Extraction and isolation

The air-dried powdered whole plant of *Acanthus arboreus* Forssk (3.5 kg) were extracted by 95% ethanol at room temperature. The concentrated ethanolic extract was

partitioned between CHCl_3 (1L) and water (1L). The CHCl_3 fraction (80 g) was again partitioned between 90% MeOH (1L) and petroleum ether (1L). The aqueous fraction was extracted with EtOAc (3x500 mL), then with *n*-butanol (3x500 mL).

A sample (10 g) of the 90% methanolic extract (26 g) was chromatographed on silica gel column (200 g, 4 cm) eluted with petroleum ether- CH_2Cl_2 (1:1) with increasing content of CH_2Cl_2 , then methanol. Fractions of 250 ml each were collected, screened by TLC and similar fractions were combined. Fractions 6-15 (1.9 g, petroleum ether/ CH_2Cl_2 40:60) afforded lupeol (800 mg) upon crystallization from petroleum ether. Crystallization of fractions 16-20 (0.8 g, petroleum ether/ CH_2Cl_2 25:75) from methanol gave stigmaterol (100 mg). Fractions 36-42 (0.9 g, CH_2Cl_2 /MeOH 92.5:7.5) were rechromatographed on silica gel column (30 g, 1 cm) eluted with a mixture of EtOAc/MeOH with increasing proportion of MeOH. Fractions of 50 ml each were collected. Fractions 5-10 were further purified by PTLC on silica gel plates developed with CHCl_3 /MeOH (9:1)(double development) and the zone with an R_f value of 0.54 was scraped off, eluted with a mixture of chloroform and methanol (1:1) to afford **1** (160 mg). Fractions 43-47 (1.1 g, CH_2Cl_2 /MeOH 90:10) gave sitosterol glucoside (230 mg) on crystallization from methanol.

The EtOAc extract (12 g) was fractionated on silica gel column (400 g, 3 cm) eluted with CH_2Cl_2 and CH_2Cl_2 /MeOH mixtures with gradual increase of methanol content. Ninety fractions (150 mL each) were collected. Repeated crystallization of fraction 12 (0.85 g, CH_2Cl_2 /MeOH 98:2) from methanol afforded vanillic acid (200 mg). Crystallization of fractions 25-29 (0.78 g, CH_2Cl_2 /MeOH 97:3) from methanol afforded apigenin (10 mg); while PTLC of the supernatant on silica gel plates developed with CHCl_3 /MeOH (8:2) gave 5 mg of **2** (R_f = 0.58). Fractions 36-45 (2.1 g, CH_2Cl_2 /MeOH 96:4) were crystallized from methanol to give **3** (850 mg) (R_f = 0.34 EtOAc/MeOH/ H_2O 30:5:2). Crystallization of fractions 49-55 (1.6 g, CH_2Cl_2 /MeOH 94:6) from methanol gave **4** (620 mg) (R_f = 0.30 EtOAc/MeOH/ H_2O 30:5:2). Additional quantity of **1** (210 mg) was obtained by crystallization of fractions 78- 81(1.4 g, CH_2Cl_2 /MeOH 90:10) from MeOH.

A sample (8 g) of the *n*-butanol extract (30 g) was chromatographed of a silica gel column (160 g, 2 cm) eluted with CH_2Cl_2 and CH_2Cl_2 /MeOH mixtures with a gradual increase of methanol content. Twenty five fractions (100 mL each) were collected. Fractions 13-16 (0.7 g, CH_2Cl_2 /MeOH 80:20) afforded **5** (120 mg) (R_f = 0.47 EtOAc/MeOH/ H_2O 30:5:4) on crystallization from MeOH.

Apigenin 7-O- β -D-(6''-trans-p-coumaroyl) glucoside (1). White crystals, mp 267 °C (lit.⁹ mp 260-264 °C).

CI-MS m/z (rel. Int.): 579 (6, $[M+1]^+$), 489 (2), 433 (4), 417 (16), 416 (8), 350 (2), 311 (6), 309 (18), 299 (30), 271 (100), 270 (25) 192 (8), 165 (78), 147 (40), 121 (18), 99 (5).). HRCI-MS m/z 579.151 (M^++1), calcd for $C_{30}H_{26}O_{12}$, 579.150. 1H -NMR of sugar protons (ppm, DMSO- d_6) δ 3.30 (1H, m, H-3''), 3.32 (1H, m, H-4''), 3.36 (1H, m, H-2''), 3.83 (1H, dd, J 7.8, 9.4 Hz, H-5''), 4.17 (1H, dd, J 4.8, 10.3 Hz, H-6''b), 4.45 (1H, d, J 10.3 Hz, H-6''a), 5.16 (1H, d, J 8.0 Hz, H-1''). ^{13}C -NMR of sugar carbons (ppm, DMSO- d_6) δ 63.4 (C-6''), 70.0 (C-4''), 72.9 (C-2''), 73.8 (C-5''), 76.2 (C-3''), 99.5 (C-1'').

6-Hydroxy-2-benzoxazolinone (2). Yellowish white crystals, mp 260 °C, UV λ_{max}/nm (MeOH): 340, 305, 302, 268. EI-MS m/z (rel. Int.) 152 (6, $[M+1]^+$), 151 (25, M^+), 149 (38), 142 (28), 130 (5), 122 (3, $[M-(HCO)]$), 117 (9), 107(5) 105 (21), 95 (21, $[M^+ -2CO]$), 91 (14), 84 (22), 77 (14), 66 (30), 55 (38). HREI-MS m/z 151.027 (M^+); Calc. for $C_7H_5O_3N$, 151.026. 1H - and ^{13}C -NMR (Table 1).

4-Hydroxyacanthamine (3,4-dihydroxy-1,4-benzoxazine-2-one) (3). Yellow needles, mp 135°C. IR (KBr): ν_{max}/cm^{-1} : 3325 (OH), 3050, 2823, 1670 (CO), 1550, 1345. UV λ_{max}/nm (MeOH): 290, 279, 253. EI-MS m/z (rel. Int.): 182 (46, $[M+1]^+$), 181 (14, M^+), 165 (12), 164 (100, $[M^+-OH]$), 136 (25), 108 (10). HRCI-MS m/z 181.144 (M^+); Cald. for $C_8H_7O_4N$, 181.145. 1H - and ^{13}C -NMR (Table 1).

Alkaline hydrolysis of 3. A sample (5 mg) of **3** was dissolved in MeOH/3N NaOH (1:1) and heated for fifteen minutes on water bath. The resulting solution was first extracted with EtOAc, acidified with dil. HCl and extracted with EtOAc. The EtOAc fraction, after acidification, showed a TLC spot with same R_f value of material **3**.

Potassium hydroxide fusion test of 3. A mixture of **3** (3 mg) and KOH (15 mg) was fused in an oil bath for 30 min. The reaction mixture was then allowed to cool, diluted with water and filtered. The filtrate was neutralized with dil. HCl and extracted with EtOAc. TLC revealed that the EtOAc extract resulting from the KOH fusion test was devoid of an *ortho*-aminophenol spot.

Acetylation of 3. A sample (4.0 mg) of **3** in pyridine (2.0 mL) was treated with Ac_2O (0.2 mL) for 24 h at room temperature. Evaporation of the mixture under a stream of nitrogen yielded chromatographically homogeneous **3a** (4.0 mg). CI-MS m/z (rel. Int.): 266 (4, $[M+1]^+$), 265 (10, M^+), 223 (16), 206 (100) 164 (169), 136 (20), 79 (5).

Apigenin 7-O- β -D-(6''-trans-p-coumaroyl)3''-O-acetylglucopyranoside (4). Pale yellow crystals, mp 223°C. IR (KBr): ν_{max}/cm^{-1} : 3395 (OH), 3050, 2820, 1670 (CO), 1560, 1320. UV λ_{max}/nm (MeOH): 316, 268, (NaOMe) 368, 300, 272, ($AlCl_3$) 375, 325, 319, 298, 276, ($AlCl_3/HCl$) 375, 325, 318, 298, 276, (NaOAc) 382, 318, 268. CI-MS m/z (rel. Int.): 621 (4, $[M^++1]$), 517 (1), 475 (2), 417 (3),

414 (3), 351 (51), 313 (7), 299 (15), 271 (52), 267 (6), 187 (13), 165 (35), 121 (100), 99 (18), 61 (84), HREIMS m/z 621.161 (M^++1); Calc. for $C_{32}H_{28}O_{13}$, 621.160. 1H -NMR (ppm, DMSO- d_6): δ 2.07 (3H, s, $COCH_3$), 3.48 (1H, m, H-2''), 3.98 (2H, m, H-4'', 5''), 4.20 (1H, dd, J 5.3, 11.7 Hz, H-6''b), 4.43 (1H, H-6''a), 4.97 (1H, t, J 9.6 Hz, H-3''), 5.30 (1H, d, J 7.8 Hz, H-1''), 6.35 (1H, d, J 16 Hz, H- α), 6.50 (1H, d, J 1.9 Hz, H-6), 6.66 (2H, d, J 8.5 Hz, H-3''', 5'''), 6.84 (1H, d, J 1.9 Hz, H-8), 6.85 (1H, s, H-3), 6.92 (2H, d, J 8.9 Hz, H-3', 5'), 7.39 (2H, d, J 8.5 Hz, H-2''', 6'''), 7.49 (1H, d, J 16 Hz, H- β), 7.94 (2H, d, J 8.9 Hz, H-2', 6'). ^{13}C -NMR (ppm, DMSO- d_6): δ 21.1 ($COCH_3$), 63.3 (C-6''), 68.3 (C-4''), 71.3 (C-2''), 73.9 (C-5''), 77.4 (C-3''), 95.1 (C-8), 99.8 (C-1'', C-6), 103.4 (C-3), 105.9 (C-10), 114.0 (C- α), 115.9 (C-3''', 5'''), 116.2 (C-3', 5'), 121.4 (C-1'), 125.4 (C-1'''), 128.8 (C-6'), 130.3 (C-2''', 6'''), 145.4 (C- β), 157.3 (C-9), 160.1 (C-4'), 161.4 (C-4'''), 161.7 (C-5), 162.8 (C-7), 164.7 (C-2), 166.8 (C=O), 170.2 ($COCH_3$), 182.3 (C-4).

Acanthaminoside (1,4-benzoxazine-2-one-3-O-glucoside) (5). Colourless needles, mp 213-214 °C. IR (KBr) ν_{max}/cm^{-1} : 3475 (OH), 3125, 2960, 1700 (CO), 1600, 1375. UV λ_{max}/nm (MeOH): 283, 275, 250. CI-MS m/z (rel. Int.): 328 (14, $[M+1]^+$), 327 (14, M^+), 166 (100), 165 (15.5), 164 (16, aglycone), 163 (13, glucosyl), 148 (100); 145 (30), 136 (18). HRCI-MS m/z 327.095 (M^+), calcd for $C_{14}H_{17}O_8N$, 327.095. 1H - and ^{13}C -NMR (Table 1).

Acid hydrolysis of 5. A sample (6 mg) of **5** was dissolved in methanol (10 mL)/2N HCl (1 mL) and heated under reflux for 1 hour. After cooling, the aqueous solution was extracted with EtOAc (3x5 mL) and neutralized with 5% Na_2CO_3 . TLC identified the sugar in the aqueous layer as glucose using $CHCl_3/MeOH$ (6:4) as developing system and thymol/ H_2SO_4 as spray reagent.

Antimicrobial testing

The MIC were determined for compounds **1** and **3-5** using a procedure described elsewhere.¹⁷ Twelve microorganisms: *Bacillus subtilis*, *Micrococcus luteus*, *Sarcina lutea*, *Staphylococcus aureus*, *Bordetella bronchiseptica*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Serratia marcescens* and *Shigella sonnie* were used in the study. The antibiotics ampicillin, ciprofloxacin, erythromycin and gentamicin were used as controls.

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