Marwa Elsbaey*, Kadria F.M. Ahmed, Mahmoud F. Elsebai, Ahmed Zaghloul, Mohamed M.A. Amer and Mohamed-Farid I. Lahloub

Cytotoxic constituents of *Alocasia macrorrhiza*

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Abstract: An indole alkaloid, 2-(5-hydroxy-1*H*-indol-3-yl)-2-oxo-acetic acid (**1**) isolated for the first time from nature, in addition to the nine known compounds 5-hydroxy-1*H*-indole-3-carboxylic acid methyl ester (**2**), alocasin B (**3**), hyrtiosin B (**4**), α-monopalmitin (**5**), 1-*O*-*β*-D-glucopyranosyl-(2*S*, 3*R*, 4*E*, 8*Z*)-2-[(2(*R*)-hydroctadecanoyl) amido]-4,8-octadecadiene-1,3-diol (**6**), 3-*epi*-betulinic acid (**7**), 3-*epi*-ursolic acid (**8**), *β*-sitosterol (**9**) and *β*-sitosterol 3-*O*-*β*-D-glucoside (**10**) were isolated from the rhizomes of *Alocasia macrorrhiza* (Araceae). Their structures were elucidated by 1D and 2D NMR spectroscopic data. Of these compounds, **6** exhibited the strongest cytotoxicity against the four tested human cancer cell lines (IC_{50} of about 10 μM against Hep-2 larynx cancer cells).

Keywords: *Alocasia macrorrhiza*; *epi*-triterpene; indole alkaloids.

1 Introduction

The genus *Alocasia* of the Araceae family comprises about 60–70 species of tropical herbs. *Alocasia macrorrhiza* L. Schott (Syn. *A. indica*) is native to India and Malaya [1]. It is a widely known ornamental, characterized by its arrow-shaped leaves [2]. The underground stem and root stocks are edible if cooked for a time long enough to destroy the calcium oxalate raphides contained in them [3]. Reviewing the literature, it is considered a medicinal plant in the folklore of South Asia. The different plant parts are traditionally used for iron deficiency, against poor eyesight, colic, constipation, rheumatoid arthritis, jaundice and inflammation. The rhizome of *A. macrorrhiza* is reported to have potent antihyperglycemic, moderate antioxidant and mild cytotoxic activity. The leaf is reported to have antidepressant, laxative and diuretic as well as hepatoprotective activities [4–7]. Despite this multitude of bioactivities, only few phytochemical investigations of *A. macrorrhiza* are found in the literature. The isolation of two ceramides [8] and of seven indole alkaloids, *viz*. alocasin A-E, hyrtiosin B and hyrtiosulawesine [9] has been reported. Therefore this study was undertaken to identify more of the phytoconstituents of *A. macrorrhiza*.

***Corresponding author: Marwa Elsbaey,** Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt, Phone: +00201005437480, Fax: +02(050)2213101, E-mail: marwaelsbaey@yahoo.com
Kadria F.M. Ahmed, Mahmoud F. Elsebai, Mohamed M.A. Amer and Mohamed-Farid I. Lahloub: Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt
Ahmed Zaghloul: Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt; and Pharmacognosy Department, Faculty of Pharmacy, Salman Bin Abdulaziz University, Riyadh 11451, Saudi Arabia

2 Results and discussion

2.1 Isolation and identification of compounds from *A. macrorrhiza* rhizome

Compound **1** was isolated as brown orange powder. It gave a positive Dragendroff's reaction, indicating its alkaloidal nature [10]. The molecular formula was deduced to be $C_{10}H_7NO_4$ based on HR-ESI-MS which exhibited a positive molecular ion peak $[M+Na]^+$ at *m/z* 228.0809 (calcd. 228.1566) and a MS fragment ion at *m/z* 160.0548 corresponding to $[M-COOH]^+$.

The ^{1}H-NMR signal at δ 8.46 (1H, *d*, *J* = 3.2 Hz) was correlated through HSQC to the methine carbon signal at δ 138.4 (Table 1). It showed COSY correlation (Figure 1) with the nitrogenous proton signal at δ 11.87 (1H, *d*, *J* = 3.2 Hz). It also showed HMBC correlation with the quaternary carbon signals at δ 112.1, 127.8 and 130.7. The values of the carbon signals at δ 138.4, 112.1, 127.8 and 130.7 are well matched with those of C-2, C-3, C-3a and C-7a of a 3-monosubtituted 5-hydroxy-indole nucleus [11]. The ^{1}H-NMR signals at δ 7.54 (1H, *d*, *J* = 2.28 Hz), 6.65 (1H, *dd*, *J* = 8.68, 2.34 Hz) and 7.22 (1H, *d*, *J* = 8.58 Hz) were assigned to H-4, H-6 and H-7, respectively, based on their splitting pattern as well as their COSY correlations (H-4/H-6 and H-6/H-7). The 3-monosubtituted 5-hydroxy-indole moiety was further confirmed by HMBC correlations of H-4 to the carbons C-3, C-5, C-6 and C-7a at δ 112.1, 154.1, 113.4 and 130.7.

Table 1: ^{1}H (600 MHz), ^{13}C (150 MHz)-NMR, DEPT, COSY, HSQC and HMBC spectral data of compound **1** in DMSO-d_6.

H/C no.	^{1}H-NMR	^{13}C-NMR/DEPT	HSQC	COSY	HMBC
1	11.87 (1H, *d*, *J* = 3.2)	–		H-1/H-2	
2	8.46 (1H, *d*, *J* = 3.2)	138.4 (CH)	C-2		C-3, C-3a, C-7a
3	–	112.1 (Q)			
3a	–	127.8 (Q)			
4	7.54 (1H, *d*, *J* = 2.28)	106.3 (CH)	C-4	H-4/H-6	C-3, C-5, C-6, C-7a, C-8
5	–	154.1 (Q)			
6	6.65 (1H, *dd*, *J* = 8.68, 2.34)	113.4 (CH)	C-6	H-6/H-7	C-4, C-5, C-7a
7	7.22 (1H, *d*, *J* = 8.58)	113.4 (CH)	C-7		C-3a, C-4, C-5
7a	–	130.7 (Q)			
8	–	183.0 (Q)			
9	–	166.6 (Q)			
O<u>H</u>	9.03 (1H, *s*)	–			C-4, C-5, C-6
COO<u>H</u>	7.92 (1H, *s*)	–			C-9

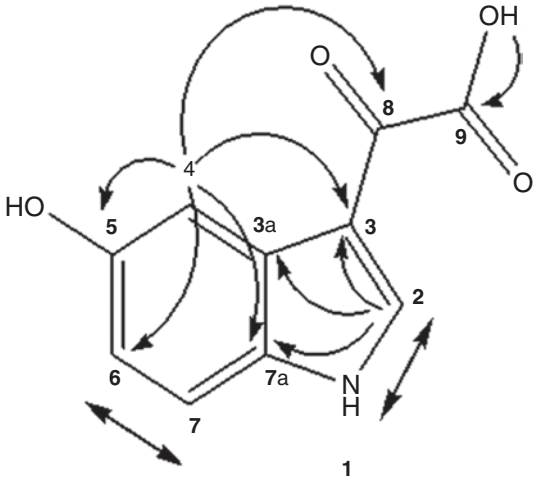

Figure 1: Key HMBC and COSY correlations of compound **1**.

Beside the 3-monosubtituted 5-hydroxy-indole partial structure, the ^{13}C-NMR spectrum revealed the presence of two quaternary carbon signals at δ 183.0 (C-8) and 166.6 (C-9) which were assigned to a carbonyl and a carboxylic group, respectively [12]. The up-field shift of the proton signal at δ 7.92 (H, *s*) suggested the presence of an α-keto acid moiety (CO-COO<u>H</u>) [13]. The latter proton signal showed HMBC correlation to C-9 (COO<u>H</u>/C-9) confirming the previous suggestion. The HMBC correlation between the methine proton at δ 7.54 (H-4) and the carbons at δ 112.1 (C-3) and 183.0 (C-8) suggested that this moiety is located at C-3. Compound **1** was thus determined to be 2-(5-hydroxy-1*H*-indol-3-yl)-2-oxo-acetic acid. To the best of our knowledge, this compound is reported here for the first time from a natural source, but it has been previously synthetically prepared from indole and its derivatives [14].

The structures of compounds **2–10** (Figure 2) were elucidated by comparing their spectral data with those reported in the literature as 5-hydroxy-1*H*-indole-3-carboxylic acid methyl ester (**2**) [11, 15], alocasin B (**3**) [9], hyrtiosin B (**4**) [16], α-monopalmitin (**5**) [17], 1-*O*-*β*-D-glucopyranosyl-(2*S*, 3*R*, 4*E*, 8*Z*)-2-[(2(*R*)-hydroctadecanoyl) amido]-4,8-octadecadiene-1,3-diol (**6**) [18], 3-*epi*-betulinic acid (**7**), 3-*epi*-ursolic acid (**8**) [19], *β*-sitosterol (**9**) [20], *β*-sitosterol 3-*O*-*β*-D-glucoside (**10**) [12] (Tables S1–S6 in Supplementary Material). It is worth noting that apart from compounds **3** and **4**, all other compounds have been isolated here for the first time from *A. macrorrhiza*, while compounds **2**, **7**, **8** have been isolated for the first time from the family Araceae. In our study, we were interested in those phytoconstituents that had not been previously isolated [8, 9].

2.2 Cytotoxicity assay

The total extract was rather cytotoxic aganist the Hep-2 and HCT-116 cell lines with IC_{50} values of about 7 and 8 µg/mL, respectively, as compared to 5-FU (IC_{50} of 5 and 6 µg/mL, respectively), but less so against the other two cell lines (HepG2: 16, MCF-7: 18 µg/mL). An extract from *A. macrorrhiza* was previously reported to have mildly toxic activity in the brine shrimp assay [8].

Almost all tested compounds had IC_{50} values lower than that of 5-FU against the HepG2, Hep-2 and HCT-116 cell lines, while against the MCF-7 cell line, only compounds **4** and **6** had activities somewhat higher than that of 5-FU (Table 2). Compound **6** was the most active of the tested compounds aganist all four tested cell lines. This

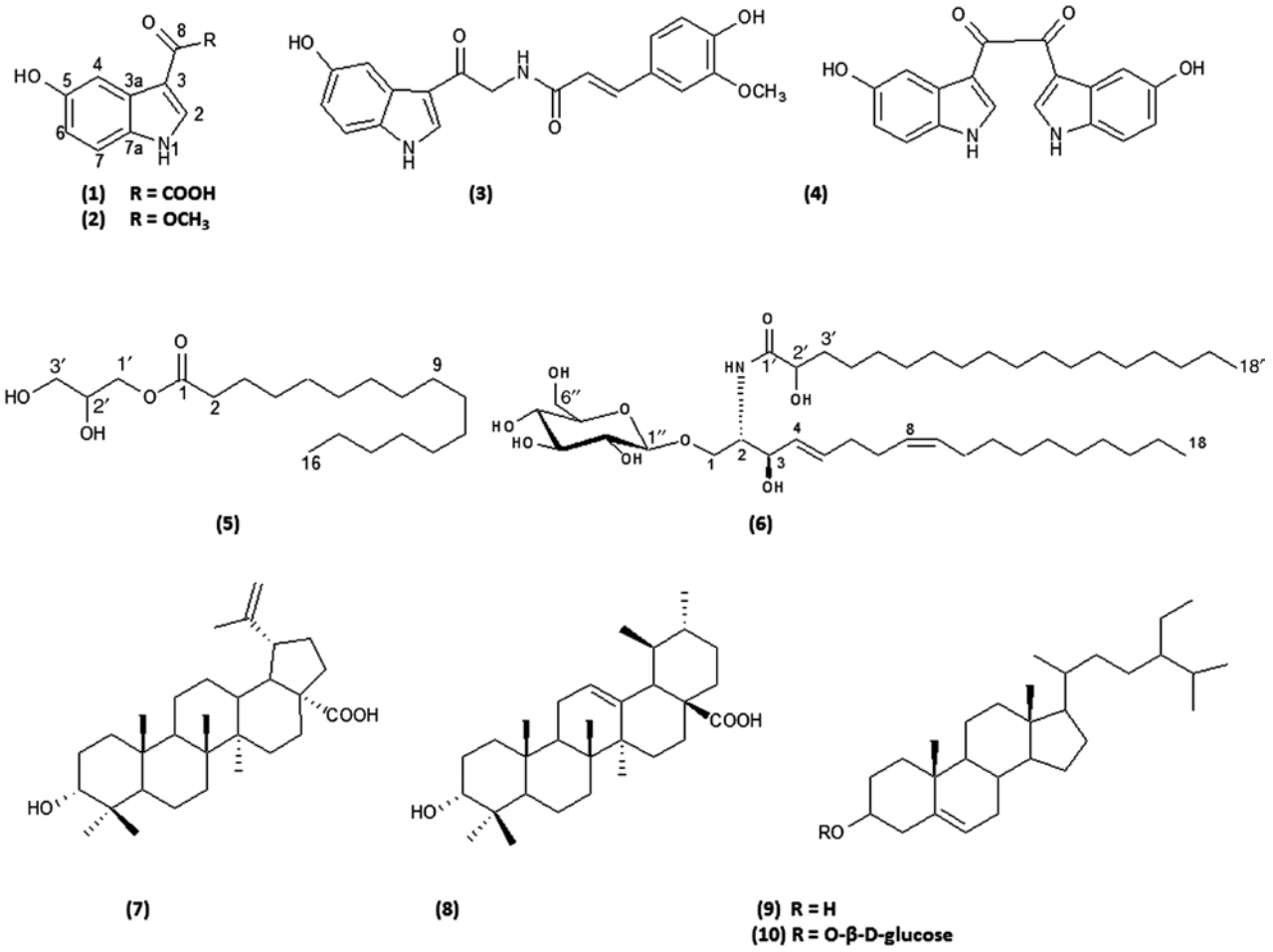

Figure 2: Structures of compounds **1–10** isolated from *A. macrorrhiza*.

Table 2: Cytotoxicity of the total extract and some of the compounds isolated from *Alocasia macrorrhiza* according to the MTT assay.

Tested compounds	*In-vitro* cytotoxicity IC_{50} (μg/mL)			
	HePG2	Hep-2	HCT-116	MCF-7
Total extract	15.81 ± 0.89	6.94 ± 0.53	7.99 ± 1.34	17.73 ± 0.43
5-Flurouracil	8.14 ± 0.12	5.22 ± 0.16	6.25 ± 0.76	4.95 ± 1.48
				In-vitro cytotoxicity IC_{50} (μM)
5-Flurouracil	62.57 ± 0.12	40.12 ± 0.16	48.04 ± 0.76	38.05 ± 1.48
Compound 4	28.22 ± 0.64	14.51 ± 0.16	19.60 ± 0.4	33.87 ± 0.9
Compound 5	92.28 ± 0.26	36.76 ± 0.57	36.24 ± 0.09	85.05 ± 0.34
Compound 6	22.51 ± 0.59	9.94 ± 0.49	11.66 ± 1.16	22.65 ± 0.36
Compound 7	61.83 ± 0.98	24.69 ± 1.24	25.20 ± 0.08	57.30 ± 0.63
Compound 8	40.07 ± 1.1	26.64 ± 0.26	19.33 ± 0.2	44.90 ± 0.65
Compound 9	55.55 ± 2.13	23.24 ± 0.06	25.68 ± 0.56	53.91 ± 0.24
Compound 10	54.34 ± 0.36	30.89 ± 0.33	15.82 ± 0.85	52.17 ± 0.76

All data are expressed as mean ± SD of three independent experiments; each assay was performed in triplicate.

high activity could be related to the ability if sphingolipids to affect cellular processes such as causing cell cycle arrest and apoptosis by modulation of protein kinases and other signaling pathways. They have been reported to act as tumor-suppressor lipids [21] and to have antihepatotoxic activity [18]. Compound **6** was previously tested against a number of human cancer cell lines but found to have no significant cytotoxic activity [18].

Compound **4** was next in activity against the HepG2, Hep-2 and MCF-7 cell lines; this was expected as bis-indole alkaloids are well known for their cytotoxic activity which is associated with their ability to inhibit tubulin polymerization [22].

Compound **10** was third in its activity against the HCT-116 cell line. The cytotoxicity of β-sitosterol 3-*O*-β-D-glucoside was previously reported as moderate against HCT-116 cells and less so against MCF-7 cells as compared to doxorubicin [23, 24].

3 Conclusion

The rhizome of *A. macrorrhiza* afforded the indole alkaloid **1**, isolated for the first time from a natural source, together with nine known compounds, seven of which have been isolated for the first time from this plant species. Compounds **4**, **6** and **10** have remarkable cytotoxic activity.

4 Experimental

4.1 Instrumentation

1D and 2D NMR spectroscopy was performed on a Bruker Avance DPX spectrometer (Bruker Daltonics, Bremen, Germany). HRESI-MS experiments were performed on a Synapt G2 HDMS mass spectrometer (Waters, Manchester, UK) capillary voltage was 3000 V and cone voltage 20 V. Leu-enkaphaline was used as the lock mass. The UPLC-MS (ultra-performance liquid chromatography MS) system was operated with Mass Lynx 4.1 software. Silica gel G60-230 (Merck, Darmstadt, Germany) and phase-bonded octadecylsilyl-silica gel (RP-C18, Merck) were used for column chromatography. Thin-layer chromatography was performed on silica gel F254 plates (Merck) using vanillin–sulfuric acid and Dragendroff's spray reagents. The solvents used were of reagent grade (El-Nasr Co., Abu Zaabal – Kalyoubia, Cairo, Egypt).

4.2 Plant material

The rhizomes of *A. macrorrhiza* were collected in June 2012 from Mansoura University gardens, Mansoura, Egypt. The identity of the plant was confirmed by Dr. Mahmoud Makram Kassem, Department of Vegetables and Ornamentals, Faculty of Agriculture, Mansoura University, Egypt.

4.3 Extraction and isolation of compounds

The air-dried powdered rhizomes (3.5 kg) were extracted by maceration in a glass jar with cold distilled methanol (9×5 L). The combined methanolic extract was concentrated to a syrupy consistency under reduced pressure and then allowed to dry in a desiccator over anhydrous $CaCl_2$ to a constant weight (200 g). The extract was fractionated over a silica gel column (20×8.5 cm, 550 g). It was eluted first with CH_2Cl_2 in petroleum ether [25% (3 L), 50% (3 L) and 100% (4 L) v/v], then with EtOAc in CH_2Cl_2 [25% (4 L), 50% (4 L), 75% (4 L) and 100% (4 L) v/v] and finally with MeOH in EtOAc [25% (3 L), 50% (3 L), 75% (3 L) and 100% (2 L) v/v]. Eluted fractions of 1000 mL each were separately concentrated and monitored by TLC; the developed chromatograms were heated with vanillin/sulfuric acid and Dragendroff's spray reagents. Similar fractions were pooled, affording six major groups (Supplementary flowchart S1). Group (1) afforded compound **9**, group (2) compounds **7** and **8**, group (3) **2** and **5**. Group (4) {fractions 20–21}, eluted with 50% EtOAc in CH_2Cl_2, was subjected to column chromatography (70×1.5 cm i.d., 40 g) using 2% MeOH in CH_2Cl_2; 20 mL fractions were collected. Fractions 34–50 afforded a pure brown orange powder which was washed with CH_2Cl_2 several times to obtain pure compound **1**; group (5) afforded compounds **3** and **4**; finally group (6) afforded compounds **6** and **10** (Supplementary flowcharts S2–S3). All yields are found in the flowcharts in Supplementary Materials.

Compound **1**: R_f = 0.26; CH_2Cl_2:MeOH (95:5); ^{13}C and ^1H-NMR: (see Table 1). HR- ESI-MS: *m/z* 228.0809 $[M+Na]^+$ and 160.0548 $[M\text{-}COOH]^+$ (calcd. 228.1566 and 160.1494, respectively).

4.4 MTT assay

Human cancer cell lines from liver (HePG-2), larynx (Hep-2), colon (HCT-116), or breast (MCF-7) originated from ATCC (Manassas, VA, USA) and were obtained from VACSERA (Cairo, Egypt). Cells were observed under an inverted microscope (Olympus 1×70, Tokyo, Japan). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) , dimethylsulfoxide (DMSO), 5-fluorouracil (5-FU), and RPMI-1640 medium were obtained from Sigma-Aldrich (St. Louis, MO, USA), 10% fetal bovine serum from GIBCO, Paisley, UK). Samples were dissolved in DMSO and diluted with PBS to concentrations of 400, 200, 100, 50 and 25 µg/mL. In all experiments, control cells were exposed to DMSO alone. The assay was carried out according to Mauceri et al. [25]. A BioTeck® microplate

reader (Winooski, VT, USA) was used to determine optical densities. Statistical analysis of the data was performed using Microsoft Excel software version 2010.

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