Steroidal Alkaloids of *Veratrum lobelianum* Bernh. and *Veratrum nigrum* L.

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Twelve steroidal alkaloids were isolated from four populations of *Veratrum lobelianum* Bernh. and *Veratrum nigrum* L. Full NMR data for veralosinine (**1**), and extensive ^{1}H NMR data for veralosine (**3**) and teinemine (**5**) are presented here for the first time. (±)-15-*O*-(2-Methylbutyroyl)germine (**10**) is undescribed up to now. The antiproliferative activities of veranigrine, veralosinine, and neogermitrine have shown that they are a perspective for further studies.

Key words: *Veratrum lobelianum* Bernh., *Veratrum nigrum* L., Steroidal Alkaloids, Antiproliferative Activity

Introduction

The genus *Veratrum* (Liliaceae) comprises about 40 species (Huang *et al*., 2008) which can be found in many areas of the temperate northern hemisphere. *V. lobelianum* Bernh. and *V. nigrum* L. are very important because of their wide-spread and usage in folk medicine (Chinese medicine "Li-lu") (Zhao *et al*., 1991). Numerous phytochemical studies on the species have shown that the main active components are steroidal alkaloids (Zhao *et al*., 1991; Tezuka *et al*., 1998a; Cong *et al*., 2007). In an earlier paper concerning the alkaloid pattern of *V. nigrum* we described the structures of veramitaline and the new steroidal alkaloid veranigrine together with the assumption that they may possess cytotoxicity for the M-109 cell line (Christov *et al*., 2009).

Here we report the alkaloid pattern of *V. lobelianum* and *V. nigrum* from different populations as well as the antiproliferative activity of veranigrine, veralosinine, veratroylzygadenine, neogermitrine, and verabenzoamine.

Material and Methods

General

Melting points were determined on a Kofler microscope (uncorrected). Optical rotation was defined with a Perkin-Elmer 241 polarimeter. The NMR experiments were recorded in CD_3OD with TMS as internal standard on a Bruker Avance II+ 600 NMR spectrometer using standard Bruker software. HRCIMS was done with a Waters QToF Premier instrument (Hannover, Germany) with an ESI-ion source equipped with an Acquity UPLC console. Column chromatography (CC) was carried out on neutral Al_2O_3 Brockmann II (1:100) and the mobile phase was a petroleum ether/Me_2CO/MeOH gradient. TLC was performed on silica gel F_{254} (Merck) plates with the mobile phase dichloroethane/Me_2CO/EtOH (2:0.50:0.25) in vapours of NH_3, dichloroethane/MeOH/petroleum ether (2:0.5:0.2) in vapours of NH_3, and dichloroethane/Me_2CO/MeOH/25% NH_4OH (2:0.50:0.50:0.02). Preparative TLC was run on 20 × 20 cm plates with silica gel GF_{254} (1 mm thickness) Merck and above-mentioned mobile phases.

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Plant materials

Roots and rhizomes of *V. lobelianum* Bernh. were picked up from Tsagaan-Uur sumon, Khubsugul province, Northwest Mongolia (N 195) at the end of August 2008 and from Southwestern Bulgaria (SOM-11407) at the beginning of December 2008. The samples from roots and rhizomes of *V. nigrum* L. were collected from Bayan-Dun province, Dornod, East Mongolia (N 39) at the end of August 2007 and from Northwestern Bulgaria (SOM-11156) in the middle of September 2007. Prof. E. Ganbold, Dr. D. Zumberelmaa, and Dr. D. Dimitrov identified the plant materials. A voucher specimen from each sample is deposited at the Herbarium of the Institute of Botany, Mongolian Academy of Sciences and Institute of Botany, Bulgarian Academy of Sciences.

Extraction and isolation

3.60 kg (Mongolian) and 0.75 kg (Bulgarian) dried and powdered plant materials of *V. lobelianum* and 6 kg (Mongolian) and 0.50 kg (Bulgarian) of *V. nigrum* were extracted exhaustively with EtOH. After evaporation to dryness the combined EtOH extracts were acidified with 5% CH_3COOH, filtered, and extracted with $CHCl_3$. The latters were evaporated to brown resins which contained no alkaloids. The acid aqueous solutions were made alkaline to pH 9 with 25% NH_4OH. The alkaline solutions were extracted exhaustively with $CHCl_3$ to give crude alkaloid mixtures (CAMs). For additional purification CAMs were subjected to further acid-alkaline procedures as above mentioned to give 850 mg (Mongolian) and 151 mg (Bulgarian) CAMs from *V. lobelianum* samples and 1090 mg (Mongolian) and 240 mg (Bulgarian) CAMs from *V. nigrum* samples. The latters were chromatographed over neutral Al_2O_3 Brockmann II. The fractions were monitored by TLC. Pure alkaloids were obtained after recrystallization in Me_2CO or by preparative TLC of mixed fractions.

(–)-Veralosinine (**1**) (Khashimov *et al.*, 1971; Moiseeva *et al.*, 1976): Yield 13.5 mg from 3.6 kg *V. lobelianum*. – White solid. – $[\alpha]_D^{20}$ –145.07° (*c* 0.0128, MeOH). – HRCIMS: *m/z* (positive mode) = 456.3474 $[M+H]^+$ (calcd. for $C_{29}H_{46}NO_3^+$, $[M+H]^+$, 456.3478). – ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz): see Table I.

Isoveralosinine (**2**): Yield 1.2 mg solid phase from 3.6 kg *V. lobelianum* (as a mixture with **1** in the ratio 1:1.5 according to the ^{1}H NMR spectrum). – ^{1}H NMR: see Table I.

(–)-Veralosine (havanine, **3**) (Khashimov *et al.*, 1970; Basterechea *et al.*, 1984): Yield 3.6 mg from 3.6 kg *V. lobelianum* (as a mixture with **4** in the ratio 2:1 according to the ^{1}H NMR spectrum). – Pale yellow amorphous solid. – $[\alpha]_D^{20}$ –36.91° (*c* 0.0018, MeOH). – ^{1}H and ^{13}C NMR: see Table I.

Isoveralosine (**4**): Yield 3.6 mg from 3.6 kg *V. lobelianum* (as a mixture with **3** in the ratio 1:2). – ^{1}H and ^{13}C NMR: see Table I.

(–)-Teinemine (**5**) (Gaffield *et al.*, 1982): Yield 3.3 mg from 3.6 kg *V. lobelianum*. – White amorphous solid. – $[\alpha]_D^{20}$ –19.018° (*c* 0.0016, MeOH). – HRCIMS: *m/z* (positive mode) = 416.3523 $[M+H]^+$ (calcd. for $C_{27}H_{46}NO_2^+$, $[M+H]^+$, 416.3529). – ^{1}H NMR (600 MHz, CD_3OD): δ_H = 5.34 (1H, br d, $J_{5,6}$ = 5.3 Hz, H-6), 4.06 (1H, td, $J_{15,16}$ = 7.8 Hz, $J_{15,16}$ = 1.5 Hz, H-16), 3.39 (1H, tt, $J_{3,4a}$ = $J_{2a,3}$ = 11.0 Hz, $J_{3,4e}$ = $J_{2e,3}$ = 5.0 Hz, H-3), 3.04 (3H, overlapped, H-22, 2H-26), 2.23 (1H, ddd, $J_{4a,4e}$ = 13.0 Hz, $J_{3,4e}$ = 5.0 Hz, $J_{4e,6}$ = 2.1 Hz, H-4*e*), 2.21 (1H, m, H-4*a*), 2.07 (1H, overlapped, H-25), 2.01 (1H, dt, *J* = 12.5, 3.2 Hz, H-12), 2.0 (1H, overlapped, H-8), 1.95 (1H, overlapped, H-20), 1.9–1.5 (10H, 2H-2, 2H-11, H-14, 2H-15, H-23, 2H-24), 1.35 (1H, overlapped, H-17), 1.32 (1H, overlapped, H-12), 1.10 (2H, overlapped, H-9, H-1), 1.11 (3H, d, *J* = 7.0 Hz, CH_3-27), 1.08 (3H, d, *J* = 7.4 Hz, CH_3-21), 1.02 (3H, s, CH_3-19), 0.77 (3H, s, CH_3-18). – ^{13}C NMR (150 MHz, CD_3OD): δ_C = 142.6 (C-5), 122.4 (C-6), 77.3 (C-16), 72.8 (C-3), 64.0 (C-22), 62.3 (C-17), 55.6 (C-14), 52.0 (C-26), 51.8 (C-9), 45.7 (C-13), 43.3 (C-4), 41.3 (C-12), 39.3 (C-20), 38.7 (C-1), 37.9 (C-10), 36.8 (C-15), 33.1 (C-8, C-7), 32.5 (C-2), 30.2 (C-24), 27.6 (C-25), 24.5 (C-23), 22.1 (C-11), 20.1 (C-19), 17.1 (C-27), 16.6 (C-21), 13.8 (C-18).

(±)-15-O-(2-Methylbutyroyl)germine (**10**): Yield 10.3 mg from 6 kg *V. nigrum*. – Pale yellow amorphous solid. – $[\alpha]_D^{20}$ ±0° (*c* 0.0032, MeOH). – HRCIMS: *m/z* (positive mode) = 594.3641 $[M+H]^+$ (calcd. for $C_{32}H_{52}NO_9^+$, $[M+H]^+$, 594.3642). – ^{1}H and ^{13}C NMR spectra and comparisons with the data in literature: Zhao *et al.* (1991).

The known alkaloids veratroylzygadenine (**6**), 3-*O*-(*β*-D-glucopyranosyl)etioline (**7**), (–)-jervine

(**8**), rubijervine (**9**), (±)-neogermitrine (**11**), (–)-germidine (**12**), (+)-verabenzoamine (**13**), and zygadenine (**14**) were identified using ^{1}H, ^{13}C, DEPT, 2D-NMR spectra, HRCIMS, physical constants like melting point and optical rotation, and comparison with the literature data (Kadota *et al.*, 1995; Ripperger, 1996a; Tezuka *et al.*, 1998a, b; Sayed *et al.*, 1995; Han *et al.*, 1991). The alkaloids **6**, **7** and **11**, **12**, **13** were from Mongolian populations of *V. lobelianum* and *V. nigrum*, while the alkaloids **8**, **9** and **6**, **14** were isolated from *V. lobelianum* and *V. nigrum* from Bulgarian populations.

Cell cultures

L5178 mouse T-cell lymphoma cells (U.S. FDA, USA) were transfected with pHa MDR1/A retrovirus (Cornwell *et al.*, 1987). The *mdr1*-expressing cell line was selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. L5178 (parental) mouse T-cell lymphoma cells and the human *mdr1*-transfected subline were cultured at 37 ºC in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine, and antibiotics. The mouse lymphoma cell line was maintained in a 5% CO_2 atmosphere.

Assay of antiproliferative effect

The effects of increasing concentrations of the drugs alone and their combinations with resistance modifiers on cell growth were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in two steps in a volume of 50 μl to a final concentration of 25 μg/ml. A total of 10^4 cells in 0.1 ml of medium were then added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C for 72 h, at the end of which 20 μl of MTT solution (thiazolyl blue solved in PBS to a final concentration of 5 mg/ml) were added to each well. After further incubation at 37 °C for 4 h, 100 μl of sodium dodecyl sulfate (SDS) solution (10%) were added to each well, and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of cell growth was determined as the percentage according to the formula:

$$\text{inhibition (\%)} = 100 - \left[\frac{\text{OD(sample)} - \text{OD(medium control)}}{\text{OD(cell control)} - \text{OD(medium control)}}\right] \cdot 100,$$

where OD(sample) is the optical density of the sample, OD(medium control) is the optical density of the medium control, and OD(cell control) is the optical density of the cell control. The ID_{50} value was defined as the concentration of compound, which inhibited 50% of cell proliferation.

Results and Discussion

Phytochemical studies

Twelve steroidal alkaloids have been isolated from four populations of *V. lobelianum* and *V. nigrum*. They are from verazine, cevanine, jervine, and solanidine types.

In earlier papers **1** was identified mainly by chemical transformations and CD (Khashimov *et al.*, 1971; Moiseeva *et al.*, 1976). Here we report for the first time full ^{1}H and ^{13}C NMR data of **1** (Table I). The assignment was made with the aid of 1D- and 2D-NMR spectra and comparison with the NMR data of etioline (Ripperger, 1996a). The NMR spectral data confirmed the *axial* and α orientation of H-3 at δ 3.39 tt (J = 11.4, 4.50 Hz) as well as the β orientation of H-16 because of its NOESY correlation with CH_3-18. The *S* configuration at C-20 was determined by comparison with the NMR data for solafloridine [(20*S*,25*R*)-22,26-epiminocholest-22(*N*)-ene-3β,16α-diol] and 20-isosolafloridine [(20*R*,25*R*)-22,26-epiminocholest-22(*N*)-ene-3β,16α-diol]. The values of the coupling constants of H-20 at δ 2.50 dq (J = 11.4, 6.4 Hz), the NOE effect between CH_3-21 and CH_3-18, and the absence of NOE between CH_3-21 and H-16 are in agreement with the first structure (Ripperger, 1996b).

The 25*S* configuration was unambiguously determined with the help of CD spectra (Moiseeva *et al.*, 1976) of veralosidine and its derivatives, among them veralosinine. The coupling constant of H-26α in *axial* orientation at δ 2.70 with H-25 at δ 1.57 (J = 10.9 Hz) indicated the *axial* position of H-25 and that CH_3-27 is in *equatorial* position. This was also confirmed by the NOE effect of H-26α and CH_3-27.

In the course of isolation of **1** from *V. lobelianum* we obtained a fraction (1.2 mg) containing two compounds. As it can be seen in Table I,

Table I. ^{1}H and ^{13}C NMR data of **1**, **2**, **3**, and **4** in CD_3OD; δ in ppm (J in Hz).

	^{13}C NMR			^{1}H NMR			
No.	**1**	**3**	**4**	**1**	**2**	**3**	**4**
1 CH_2	38.7	38.7		*e* 1.87 dt (13.3, 3.5) *a* 1.07 ddd (13.3, 9.3, 4.1)		1.88[b], 1.1[b]	
2 CH_2	32.6	31.1		1.80[b]		1.80[a]	
3 CH	72.6	79.8		3.39 tt (11.4, 4.5)		3.38 tt (11.0, 6.0)	
4 CH_2	43.3	40.3		2.22 m		2.00[b]	
5 C	142.6	142.3					
6 CH	122.4	122.7		5.33 br d (5.3)	5.33 br d (5.3)	5.36 br d (5.2)	
7 CH_2	33.1	33.0		1.90[b], 1.40[b]		1.90[b], 1.50[b]	
8 CH	32.8	32.8		1.60[b]		1.90[b]	
9 CH	51.7	51.7		1.00[b]		0.99[b]	
10 C	38.0	38.2					
11 CH_2	22.2	22.1		1.60[b]		1.90[b]	
12 CH_2	41.3	41.2		*e* 2.04 dt (12.7, 3.1) *a* 1.42[b]		2.04[b] 1.42[b]	
13 C	44.9	45.1	44.9				
14 CH	55.6	55.5	55.1	1.40[b]		1.40[b]	
15 CH_2	35.8	35.7		1.40[b]		1.70[b], 1.40[b]	
16 CH	80.8	80.7	80.3	4.91[c]	4.96[c]	4.89[c]	4.97 br t (7.8)
17 CH	60.2	61.1	59.4	1.70[b]		1.80[b]	1.90[a]
18 CH_3	14.0	13.8	14.6	0.81 s	0.76 s	0.83 s	0.77 s
19 CH_3	20.2	20.2		1.02 s	1.00 s	1.03 s	
20 CH	47.0	46.7		2.50 dq (11.4, 6.4)	2.55 dq (11.6, 7.0)	2.58 dq (11.5, 6.9)	2.61 dq (11.5, 6.9)
21 CH_3	17.7	17.5		1.12 d (6.9)	1.14 d (6.9)	1.16 d (6.8)	1.16 d (6.8)
22 C	178.2	179.0					
23 CH_2	28.9[a]	28.4[a]		1.80[b]			
24 CH_2	29.0[a]	28.0[a]		1.10[b]			
25 CH	28.9	28.5	28.4	1.57 dd (10.9, 7.0)		1.66[b]	1.71[b]
26 CH_2	57.6	56.5	56.6	*e* 3.60 ddd (17.0, 4.1, 2.1) *a* 2.70 dd (17.0, 10.9)	3.63 ddd (17.0, 4.1, 2.1) 2.99 dd (17.0, 9.8)	*e* 3.62[b] *a* 2.78 dd (16.3, 10.8)	*e* 3.64[b] *a* 3.01 dd (16.5, 9.5)
27 CH_3	20.0	19.8	19.5	0.91 d (7.0)	0.94 d (6.6)	0.94 d (6.7)	0.96 d (6.7)
CH_3CO	172.4	172.4	172.8	1.98 s	2.00 s	1.99 s	2.01 s
	22.0	22.0	21.6				
Glc							
1' CH		102.8				4.40 d (8.0)	
2' CH		75.4				3.16 dd (8.0)	
3' CH		78.4				3.37 dd (8.5)	
4' CH		72.0				3.29[b]	
5' CH		78.2				3.28[b]	
6' CH_2		63.1				3.87 br d (12.6), 3.67[a]	

[a] Interchangeable. [b] Overlapped signals. [c] Overlapped with the signal of the solvent.

the two compounds showed doubling of some signals, and most probably they are diastereomers. The ^{1}H NMR spectrum of the first compound corresponded to **1**. There were no differences in the signals for the rings A, B, and C. The coupling constants of H-20 were also the same for the two compounds. The main difference was in the signal of H-26α, δ 2.70 dd (J = 17.0, 10.9 Hz) and δ 2.99 dd (J = 17.0, 9.8 Hz) for **1** and **2**, respectively. By analogy with etioline and isoetioline (Ripperger, 1990), a 25*R* configuration could be assigned to the second compound. That means most probably it is (20*S*,25*R*)-16α-acetoxy-22,26-epiminocholest-5,22(*N*)-dien-3β-ol (isoveralosinine, **2**) (Fig. 1.) The ratio veralosinine/isoveralosinine was 1.5:1.

The ^{13}C NMR spectrum of **3** showed differences in the chemical shifts for some of the carbon atoms, and doubling of the signals for the protons H-16, H-17, H-18, H-20, H-25, H-26$_a$, H-26$_e$, CH_3-27 and $C\underline{H}_3CO$ in the ^{1}H NMR spectrum. The main differences were in the chemical shifts of H-26α at δ 2.78 dd (J = 16.3, 10.8 Hz) and δ 3.01 dd (J = 16.5, 9.5 Hz) for **3** and **4**, respectively, or in other words there were again two isomers: (20*S*,25*S*)-*O*(3)-β-D-glucopyranosyl-16α-acetoxy-22,26-epiminocholest-5,22(*N*)-dien-3β-ol (veralosine, **3**) and (20*S*,25*R*)-*O*(3)-β-D-glucopyranosyl-16α-acetoxy-22,26-epiminocholest-5,22(*N*)-dien-3β-ol (isoveralosine, **4**) (Fig. 1) in approximately the ratio 1:1. To our knowledge this is the first report for the presence of **2** and **4** in plants. The exhaustive ^{1}H NMR data of **3** are presented here for the first time.

From the biogenetic point of view there is no inconsistency with the presence of **2** and **4** because the former is the aglycone of the latter one as well as **1** is the aglycone of **3**. Teinemine (**5**) (Fig. 1) was identified by 1D- and 2D-NMR spectra and comparison with the data in the literature (Gaffield *et al.*, 1982). Here we present exhaustive ^{1}H NMR data of **5** for the first time. (±)-15-*O*-(2-Methylbutyroyl)germine (**10**) (Fig. 1) has not been isolated up to now.

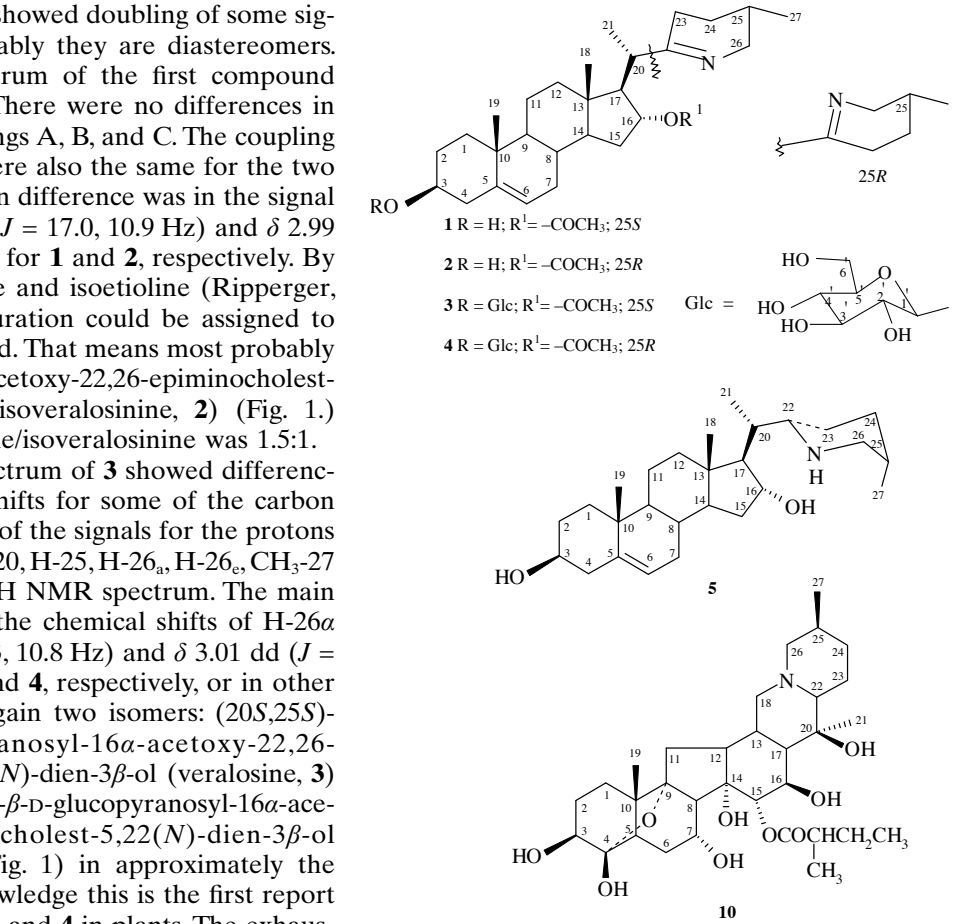

Fig. 1. Chemical structures of the steroidal alkaloids veralosinine (**1**), isoveralosinine (**2**), veralosine (**3**), isoveralosine (**4**), teinemine (**5**), and 15-*O*-(2-methylbutyroyl)germine (**10**).

Biological assays

Among the mechanisms by which cancer cells evade chemotherapy multidrug resistance (MDR) is certainly the best known. MDR is characterized by cross-resistance between numerous natural products used in cancer treatment especially antibiotics and plant alkaloids (Robert, 1996; Lavie

Table II. Antiproliferative effects of alkaloids on MDR cell line.

Compound	ID_{50} [μg/ml][a]
Veranigrine	20.76
Veralosinine (**1**)	22.69
Veratroylzygadenine (**6**)	24.86
Neogermitrine (**11**)	21.76
Verabenzoamine (**13**)	26.07
DMSO	25.95

[a] ID_{50}, concentration of the compound inhibiting 50% of cell proliferation; each value is the mean from parallel experiments (n = 2).

et al., 2001). In this connection and with respect to our previous assumptions (Christov *et al*., 2009), we decided to examine some of the steroidal alkaloids for their antiproliferative activity. From the isolated alkaloids we have chosen veranigrine, veralosinine (**1**), veratroylzygadenine (**6**), neogermitrine (**11**), and verabenzoamine (**13**) as they are in pure form and in sufficient quantity. They were tested by the MTT test against the human MDR1 gene-transfected mouse lymphoma cells. All of them displayed close cell growth inhibitory potency. The preliminary investigations demonstrated that veranigrine had the lowest ID_{50} value (20.76 μg/ml) while verabenzoamine showed the highest ID_{50} value (26.07 μg/ml) (Table II).

These data are valuable for development of this kind of plant alkaloids as possible antiproliferative agents of cancer cells and for design and modification for new anticancer agents.

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