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Steroidal Saponins from *Vernonia amygdalina* Del. and Their Biological Activity

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Abstract: In the present study, four new steroidal saponins, namely vernoniamyoside A–D (1–4), together with the two known steroidal saponins vernoamyoside D (5) and vernonioside B₂ (6) were isolated from the ethanol extract of leaves of the African medicinal plant *Vernonia amygdalina* Del. (Asteraceae). Their structures were demonstrated by spectral analyses along with 1D and 2D nuclear magnetic resonance (NMR) techniques and mass spectrometry (MS). The cytotoxicity of the compounds was also tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method on the cell lines Hela, MCF-7, BT-549 and MDA-MB-231. Vernoniamyoside A, vernoniamyoside B, and vernonioside B₂ showed cytotoxicity towards BT-549 cell lines. Vernoniamyoside C, vernoniamyoside D and vernoamyoside D showed different levels of cytotoxic activities.

Keywords: *Vernonia amygdalina* Del.; vernoniamyoside; chemical constituents; cytotoxic activity

1. Introduction

Vernonia amygdalina Del. from the family Asteraceae is distributed throughout tropical Africa, especially in West Africa. It has received considerable scientific interest due to the observation that adult chimpanzees with malaria returned to normal activity after chewing the extract of the bitter juice of this species [1]. Over the years, several studies on the chemical components of this species, including flavonoids [2], sesquiterpene lactones [3], steroidal saponins [4–6], and fatty acids [7], have been performed. Previous studies have indicated different bioactivities of this species, including anti-inflammation [8], anti-malaria [9], anti-obesity [10,11], antioxidant [12,13], anti-tumor [14], and other activities [15]. Water and chloroform extracts of *V. amygdalina* (VA) interfere with the DNA synthesis of MCF-7 cells in breast cancer, affecting the activity of ERKs in vitro and inhibiting human breast cancer cells [16,17]. Via the MTT method, it has been verified that MCF-7 cells are inhibited in vitro and the DNA synthesis of BT-549 cells in breast cancer is disturbed by the addition of VA extract [18,19]. Moreover, three active fractions extracted from ethanol extracts of *V. amygdalina* have an inhibiting effect on tumor cells, the essential component being steroidal saponins [14]. So far, a certain amount of steroidal saponins has been obtained from *V. amygdalina*; however, studies on the anti-tumor activity are rare. It is therefore especially important to investigate the separation and biological activity of steroidal saponins in *V. amygdalina*.

In the present study, four new steroidal saponins, vernoniamyoside A–D (1–4) along with two known steroidal saponins vernoamyoside D (5) and vernonioside B₂ (6) were obtained. It is expected that these steroidal saponins also demonstrate anti-tumor activity, especially anti-breast cancer activity. Therefore, all compounds were evaluated for their cytotoxicity toward human Hela,

MCF-7, BT-549, and MDA-MB-231 cell lines by means of the MTT method. The isolation, structure identification, and biological activities of the aforementioned compounds are described.

2. Results and Discussion

2.1. Isolation, Characterization, and Structure Elucidation of Compounds

Compound **1** was obtained as a white powder, and its molecular formula $C_{35}H_{50}O_{11}$, determined by high resolution electrospray ionization-mass spectrum (HR-ESI-MS) at m/z 669.3240 $[M + Na]^+$ (calcd for $C_{35}H_{50}NaO_{11}$, 669.3206), has 11 degrees of unsaturation. In the 1H NMR spectrum of compound **1**, two olefinic proton signals at δ_H 5.40 (1H, br s, H-7) and 5.62 (1H, d, H-11), two angular methyl singlets at δ_H 0.52 (3H, s, CH_3 -18) and 0.87 (3H, s, CH_3 -19), two methyl doublets at δ_H 0.83 (3H, d, CH_3 -26), 0.91 (3H, d, CH_3 -27), and one methyl singlet 2.15 (3H, s, CH_3 -29) were observed. The ^{13}C -NMR and distortionless enhancement by polarization transfer (DEPT) spectra indicated that compound **1** contained 35 carbon signals, including five methyls (at δ_C 13.8 (C-18), 19.4 (C-19), 17.1 (C-26), 17.0 (C-27) and 29.2 (C-29)), eight methines (including cyclic olefinic carbon signals at δ_C 121.9 (C-7), 133.1 (C-8), 143.9 (C-9), and 117.6 (C-11)), eight quaternary carbons (including two carbonyl carbon signals at δ_C 213.0 (C-28) and 214.2 (C-16) and a lactone carbon signal at δ_C 177.3 (C-21)), and 14 methylenes. Particularly, an obvious carbonyl signal at δ_C 214.2, corresponding to δ_H 2.63 (1H, H-17), 2.98 (H-20), 2.02 and 2.34 (H-15), in 1H -detected heteronuclear multiple-bond correlation (HMBC) was observed, suggesting a carbonyl group is connected to C-16. The 1H and ^{13}C NMR analysis (see Table 1) supported the hypothesis that compound **1** might be $\Delta^{7,9(11)}$ stigmastane-type steroid derivative, with the same skeleton as vernonioside A₃ [5]. Moreover, two carbon signals at δ_C 83.0 (C-24), 33.5 (C-25) and obvious HMBC correlations of δ_H 0.83 (3H, d, CH_3 -26) with δ_C 83.0, 33.5, 17.0 and δ_C 0.91 (3H, d, CH_3 -27) with δ_C 83.0, 33.5, 17.1 were observed. According to the NMR data and analysis, we suggest that compound **1** contains a-HOCCH(CH_3)₂ group. A carbonyl signal at δ_C 213.0 (C-28) also showed HMBC correlations with a methyl signal at δ_H 2.15 (3H, s, CH_3 -29). In the HMBC spectrum, δ_H 2.63 (H-17) has correlations of δ_C 26.0 (C-22), 36.8 (C-20), δ_H 4.63 (H-23) has correlations of δ_C 26.0 (C-22), 36.8 (C-20), 177.3 (C-21).

Table 1. 1H (600 MHz) and ^{13}C (150 MHz) NMR data of **1–4** in CD_3COCD_3 .

No.	1 ^a		2 ^a		3 ^a		4 ^a	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	34.1	1.27, 2.0, m	34.3	1.23, 1.96, m	34.3	1.24, 1.93, m	34.3	1.24, 1.94, m
2	29.2	1.48, 1.88, m	29.3	1.45, 1.87, m	29.2	1.44, 1.84, m	29.2	1.46, 1.87, m
3	76.3	3.57, m	76.3	3.56, m	76.2	3.56, m	76.2	3.56, m
4	33.7	1.22, d(11.6) 1.83, m	33.7	1.19, d(11.6) 1.80, m	33.7	1.18, d(11.7) 1.79, m	33.7	1.18, d(11.7) 1.76, m
5	38.7	1.35 ^a	38.7	1.29 ^a	38.6	1.31 ^a	38.6	1.31 ^a
6	29.4	1.26, 1.88 ^a	29.5	1.23, 1.87 ^a	29.5	1.24, 1.84 ^a	29.4	1.23, 1.87 ^a
7	121.9	5.40, br s	120.1	5.37, br s	120.7	5.36, br s	120.7	5.39, br s
8	133.1		135.8		135.1		135.7	
9	143.9		143.3		143.6		143.7	
10	35.8		35.5		35.6		35.9	
11	117.6	5.62, d(5.8)	119.0	5.53, d(6.2)	117.9	5.47 ^a	118.3	5.51 d(6.2)
12	39.0	2.27, m	40.8	2.01, d(7.4) 2.84, m	39.6	1.83, d(6.6) 2.17, m	39.4	1.94, d(6.6) 2.16, m
13	40.3		41.8		43.6		43.4	
14	45.0	2.65 ^a	45.1	1.94 ^a	48.7	2.54 ^a	46.0	2.52 ^a
15	37.0	2.02, 2.34 ^a	28.0	1.47, 2.09 ^a	34.5	1.66, 1.92 ^a	26.5	1.91 ^a
16	214.2		22.8	1.39, 1.76 ^a	74.3	4.40 ^a	23.1	1.49, 1.85 ^a
17	61.2	2.63, d(2.9)	50.9	2.19 ^a	56.6	2.41, d(7.5)	50.8	2.27 ^a
18	13.8	0.52, s, 3H	11.8	0.54, s, 3H	13.2	0.30, s, 3H	12.1	0.32, s, 3H

Table 1. Cont.

No.	1 ^a		2 ^a		3 ^a		4 ^a	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
19	19.4	0.87, s, 3H	19.4	0.85, s, 3H	19.3	0.81, s, 3H	19.3	0.82, s, 3H
20	36.8	2.98, ddd, (12.2,8.7,3.2)	47.6	2.76, dd, (10.8, 6.6)	133.9		134.9	
21	177.3		175.4		173.3		172.9	
22	26.0	1.88, 2.16 ^a	79.1	4.58, t(6.0*2)	146.8	7.49, br s	146.9	7.44, br s
23	80.6	4.63, d(5.5)	78.7	4.62, d(5.1)	82.8	5.24, m	82.7	5.22, m
24	83.0		82.6		83.3		83.3	
25	33.5	2.07, m	30.8	1.91, m	32.9	2.16, m	32.8	2.16, m
26	17.1	0.83, d(7.0)	16.5	0.87, d(6.8)	16.9	0.82, d(6.9)	16.9	0.81, d(6.8)
27	17.0	0.91, d(7.0)	17.6	1.03, d(6.8)	16.7	0.97, d(6.9)	16.6	0.96, d(6.8)
28	213.0		106.9		211.2		211.1	
29	29.2	2.15, s, 3H	15.0	1.26, s, 3H	28.1	2.09, s, 3H	28.1	2.09, s, 3H
Glu								
1'	100.9	4.24, d(8.1)	100.9	4.23, d(8.1)	100.9	4.22, d(8.0)	100.9	4.22, d(8.1)
2'	73.5	2.90 ^a	73.5	2.90 ^a	73.5	2.89 ^a	73.5	2.89 ^a
3'	76.7	3.10 ^a	76.7	3.10 ^a	76.8	3.10 ^a	76.8	3.11 ^a
4'	70.1	3.03 ^a	70.1	3.02 ^a	70.1	3.02 ^a	70.1	3.02 ^a
5'	76.8	3.10 ^a	76.8	3.10 ^a	76.8	3.10 ^a	76.8	3.11 ^a
6'	61.1	3.42, 3.65 ^a	61.1	3.41, 3.65 ^a	61.2	3.41, 3.65 ^a	61.1	3.41, 3.65 ^a
OCH ₃			49.5	3.15, s, 3H				

^a Resonance pattern unclear due to overlapping.

The typical carbon signal δ_C 100.9 has a ¹H-detected heteronuclear single quantum correlation (HSQC) correlation of δ_H 4.24 (1H, d, H-1') and in the HMBC spectrum, obvious correlations from δ_H 4.24 to δ_C 76.3 and δ_H 3.57 to δ_C 100.9 suggest a glucose moiety in compound **1**. The signal δ_C 100.9 is caused by anomeric carbon and the glucose moiety connected to C-3. The other carbon signals δ_C 61.1 and a series of signals between δ_C 70.0 and 80.0, along with proton signals between δ_H 2.90 and 3.90, are a better validation of the previous assumption. Through acid hydrolysis and a comparison of the retention time to standard D-glucose, the glucose moiety was confirmed to be D-glucose. Compared to the known compounds, compound **1** has the same side chain as vernoamyoside D [6].

First, the planar structure of compound **1** was determined. The nuclear overhauser effect spectroscopy (NOESY) correlation between H-3 (δ_H 3.57) and H-5 (δ_H 1.35), H-5 (δ_H 1.35) and H-14 (δ_H 2.65), Me-18 (δ_H 0.52) and Me-19 (δ_H 0.87), as well as Me-18 (δ_H 0.52) and H-20 (δ_H 2.98) indicated that compound **1** contains trans rings of nuclear parent and H-3, H-5 and H-14 were α -configured, while Me-18, Me-19, and H-20 were β -configured. A NOESY correlation from H-17 (δ_H 2.63) to H-14 (δ_H 2.65) was observed, which determined that H-17 has an α -orientation. Peaks from H-23 (δ_H 4.63) to H-20 (δ_H 2.98) indicated that the proton H-23 was β -configured. The configuration of C-24 could not be determined by NOESY; thus, we compared the carbon signals between compound **1** and vernoamyoside D [6] with the same fragment. Thus, compound **1** was determined and named vernoniamyoside A (see Figures 1–3).

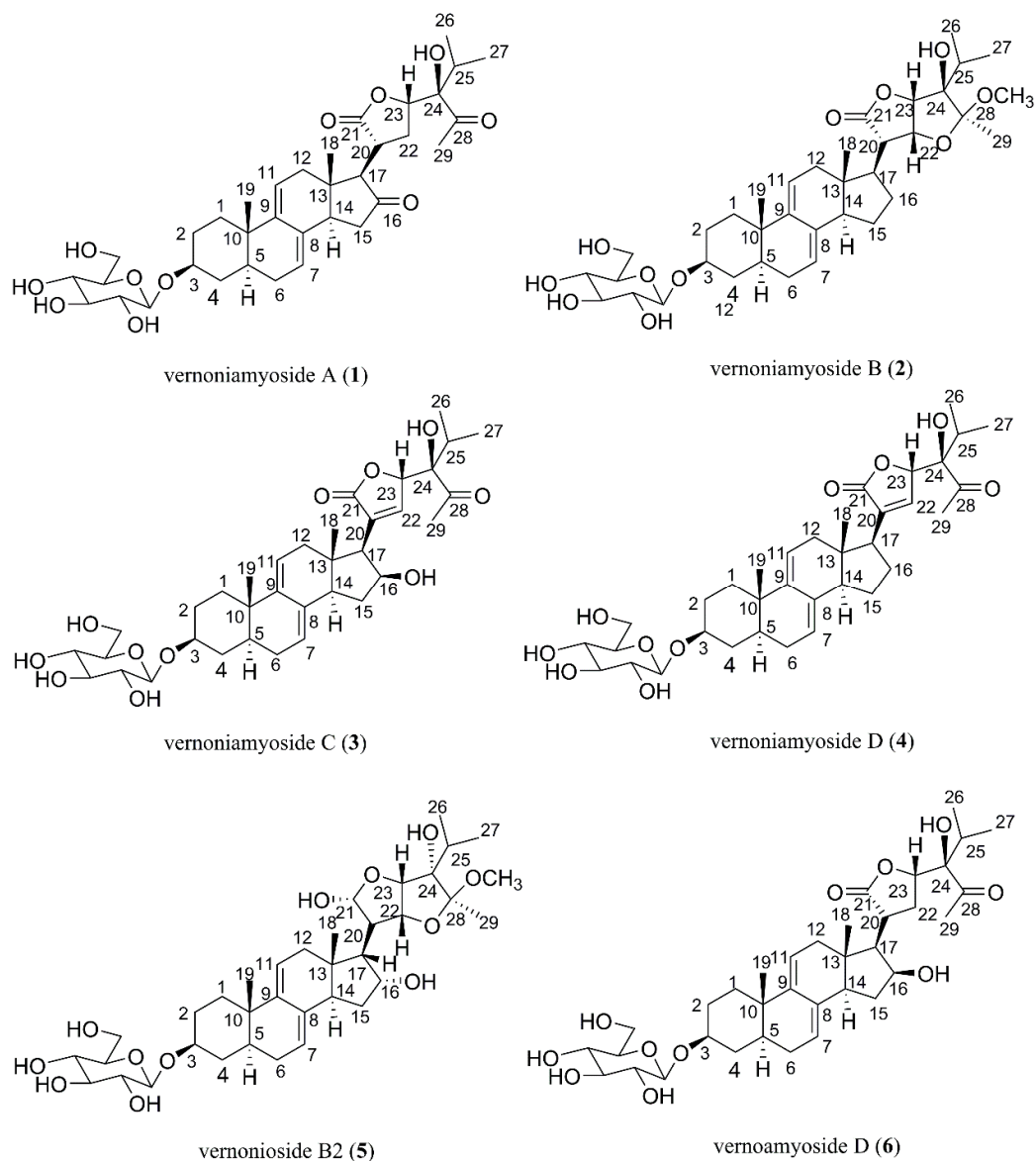


Figure 1. Structures of compounds 1–6.

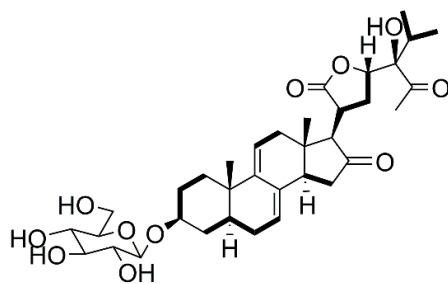


Figure 2. Major ^1H - ^1H correlation spectroscopy (^1H - ^1H COSY) correlations of compound 1 (bold lines).

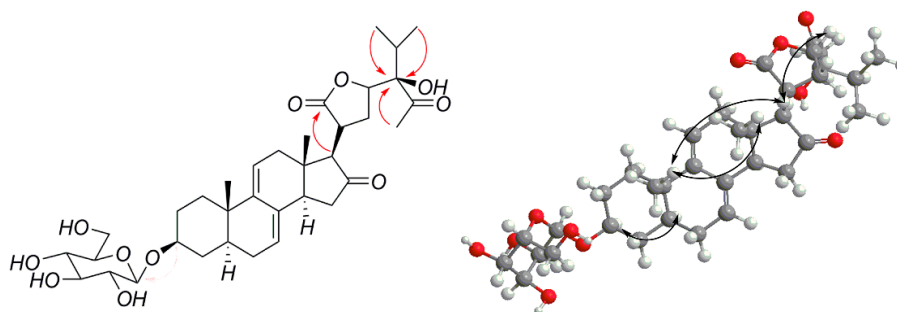


Figure 3. Major HMBC and NOESY correlations of compound 1.

Compound 2 was obtained as a white powder, and its molecular formula $C_{36}H_{54}O_{11}$, determined by HR-ESI-MS at m/z 685.3554 $[M + Na]^+$, (calcd for $C_{36}H_{54}NaO_{11}$, 685.3553), has 10 degrees of unsaturation. The 1H NMR spectrum of compound 2 showed the following specific signals: two methyl doublets at δ_H 0.87 (3H, d, CH_3 -26), 1.03 (3H, d, CH_3 -27), and two methyl singlets at 1.26 (3H, s, CH_3 -29) and 3.15 (3H, s, OCH_3). The ^{13}C NMR spectra indicated 36 carbon signals and showed the following typical five methyl signals: (at δ_C 11.8 (C-18), 19.4 (C-19), 16.5 (C-26), 17.6 (C-27), and 15.0 (C-29)). In the HMBC spectrum, we observed correlations between δ_H 1.39 and 1.76 with δ_C 22.8 (C-16), δ_H 1.39 with δ_C 28.0 (C-15), 50.9 (C-17), 135.8 (C-8), and δ_H 1.76 with δ_C 41.8 (C-13), 50.9 (C-17), indicating that the signal δ_C 22.8 is connected to C-16. We therefore infer that compound 2 is $\Delta^{7,9(11)}$, a stigmastane-type steroid derivative with the same skeleton as vernonioside B1 [1] by comparison with 1H -NMR and ^{13}C -NMR (see Table 1).

A large number of ^{13}C -NMR signals were between δ_C 70.0 and 90.0, indicating that there is a highly oxidized side chain. In the HMBC spectrum, the correlations of H-25 (δ_H 1.91), H-26 (δ_H 0.87), and H-27 (δ_H 1.03) to the carbon signals at δ_C 82.6 (C-24) and H-22 (δ_H 4.58), H-23 (δ_H 4.62), H-29 (δ_H 1.26) to δ_C 106.9 (C-28), and H-22 (δ_H 4.58), H-23 (δ_H 4.62), H-20 (δ_H 2.76) to δ_C 175.4 (C-21) indicate that the side chain is similar compared to that of vernoamyoside C [6]. The difference between compound 2 and vernoamyoside C is C-21, the absence of signals at δ_H 5.45 and δ_C 100.0, and the presence of signals at δ_C 175.4, indicating that the side chain is highly oxidized. This helped us to determine the planar structure of compound 2. The correlation between H-3 (δ_H 3.56) and H-5 (δ_H 1.29), H-5 (δ_H 1.29) and H-14 (δ_H 1.94), Me-18 (δ_H 0.54) and Me-19 (δ_H 0.85), and Me-18 (δ_H 0.54) and H-20 (δ_H 2.76) indicated that compound 2 has the trans rings of the nuclear parent and H-3, H-5 and H-14 adopted an α -configuration, while Me-18, Me-19 and H-20 were β -configured. This is similar to compound 1. The NOESY spectrum from H-17 (δ_H 2.19) to H-14 (δ_H 1.94) was observed, which determined that the H-17 has α -orientation. Peaks from H-23 (δ_H 4.62) to H-20 (δ_H 2.76) indicated that proton H-23 was β -configured. The significant signals from H-20 (δ_H 2.76) to H-18 (δ_H 0.54), H-22 (δ_H 4.58) and H-23 (δ_H 4.62) indicated that H-22 (δ_H 4.58) and H-23 (δ_H 4.62) were β -configured. The correlation between H-29 (δ_H 1.26) and H-14 (δ_H 1.94) suggested that CH_3 -29 has an α -configuration [6]. Thus, compound 2 was determined and named vernoniamyoside B (see Figures 1 and 4).

Compound 3 was obtained as a white powder, and its molecular formula $C_{35}H_{50}O_{11}$, determined by HR-ESI-MS at m/z 669.3250 $[M + Na]^+$ (calcd for $C_{35}H_{50}NaO_{11}$, 669.3206), has 11 degrees of unsaturation. The proton signal is at δ_H 4.40 (H-16), which has correlation with δ_C 48.7 (C-14) in the HMBC spectrum. The correlations between H-17 (δ_H 2.41) and the carbon signal at δ_C 74.3 in the HMBC spectrum confirm that the δ_C 74.3 is connected to C-16. The 1H NMR and ^{13}C NMR signals (see Table 1) of compound 3 indicated that it is a $\Delta^{7,9(11)}$ stigmastane-type steroid derivative with the same skeleton as vernoamyoside D [6]. According to the HMBC, the signal at δ_H 7.49 has a correlation with δ_C 56.6 (C-17), 173.3 (C-21), 82.8 (C-23), while δ_H 2.41 (H-17) and 5.24 (H-23) have correlations with δ_C 133.9. Therefore, there is a double bond between C-20 (δ_C 133.9) and C-22 (δ_C 146.8). Thus, the planar structure of compound 3 could be determined. Compared to the 1H NMR and ^{13}C NMR signals, the side chain is the same as in vernonioside A4 [20]. The significant nuclear overhauser effect (NOE)

correlations between H-16 (δ_{H} 4.40) and Me-18 (δ_{H} 0.30) suggested that OH-16 has an β -configuration. Therefore, compound **3** was determined and named vernoniomyoside C (see Figures 1 and 5).

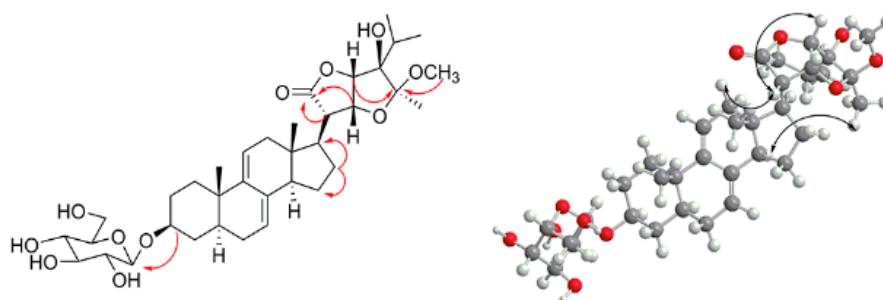


Figure 4. Major HMBC and NOESY correlations of compound 2.

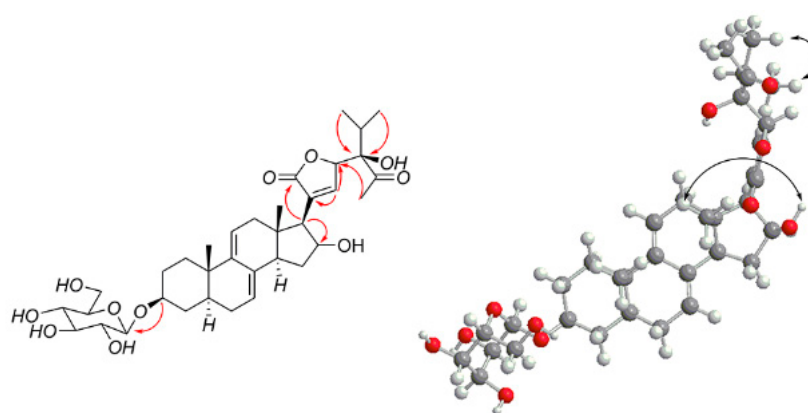


Figure 5. Major HMBC and NOESY correlations of compound 3.

Compound **4** was obtained as a white powder, and its molecular formula $\text{C}_{35}\text{H}_{50}\text{O}_{10}$, determined by HR-ESI-MS at m/z 653.3303 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{35}\text{H}_{50}\text{NaO}_{10}$, 653.3257), has 11 degrees of unsaturation. The ^{13}C NMR spectra indicated 35 carbon signals. Compared with the ^1H NMR and ^{13}C NMR signals (see Table 1) of compound **2**, compound **4** is also a $\Delta^{7,9(11)}$ stigmasterane-type steroid derivative with a glycoside. As for the side chain, δ_{H} 5.22 (H-23) has correlations with δ_{C} 135.7 in the HMBC spectrum. Compound **4** had similar ^1H , ^{13}C NMR, and NOESY data when compared with compound **3**. Thus, compound **4** was determined and named vernoniomyoside D (see Figures 1 and 6).

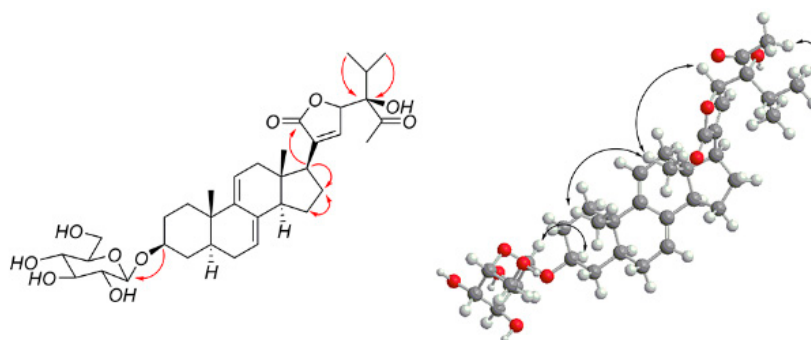


Figure 6. Major HMBC and NOESY correlations of compound 4.

According to the literature, the two known compounds are identified as vernoamyoside D (5) [6] and vernonioside B₂(6) [20] (see Figure 1).

2.2. Results of the Cytotoxicity Test

According to a previous study, MCF-7 cells are inhibited in vitro, and the DNA synthesis of BT-549 cells in breast cancer is disturbed by the application of *V. amygdalina* extract [18,19]. A series of steroidal saponins exhibited anti-tumor activity, especially anti-breast tumor activity [14]. In this study, the cytotoxicity of compounds 1–6 was tested on BT-549, MDA-MB-231, MCF-7, and HeLa cell lines. The anti-tumor activity of $\Delta^{7,9(11)}$ stigmastane-type steroidal saponins was introduced for the first time. As seen in Table 2, the inhibition against the BT-549 cell line of compound 1 could reach up to 63.61%, while compound 2 and 6 also showed cytotoxicity towards the BT-549 cell line (inhibition = 62.17 and 51.14%). This leads us to infer that compounds 1, 2, and 6 are highly toxic towards BT-549 cell lines, while they showed a general cytotoxicity to cell lines MDA-MB-231, MCF-7, and HeLa. Based on this, these compounds might play a certain role in the treatment of breast cancer. The cytotoxicity activities of compound 3, 4, and 5 showed different levels against the tested cell lines. Moreover, further studies are necessary to confirm whether these compounds are also toxic towards other tumor cell lines. Both compounds have the same sugar chains; therefore, the different activities might be due to the side chain and the nuclear parent. Furthermore, it should be highlighted that compounds 1–6 had a different selectivity for tumor cell lines.

Table 2. Results of the cytotoxicity assay.

Sample	BT-549		MDA-MB-231		MCF-7		Hela	
	Average Absorbance	Inhibition (%)	Average Absorbance	Inhibition (%)	Average Absorbance	Inhibition (%)	Average Absorbance	Inhibition (%)
Compound 1	0.2748	63.61	0.6102	28.97%	0.4217	46.54	0.5212	42.05
Compound 2	0.2836	62.17	0.6196	27.78%	0.4893	37.07	0.6063	31.64
Compound 3	0.4546	34.18	0.5803	32.74%	0.4728	39.38	0.6464	26.73
Compound 4	0.3946	44.00	0.5899	31.53%	0.5301	31.36	0.5957	32.93
Compound 5	0.4410	36.41	0.5734	33.61%	0.3990	49.72	0.6893	21.48
Compound 6	0.3510	51.14	0.5961	30.75%	0.4750	39.08	0.5737	35.63
Positive control	0.1515	83.79	0.1789	83.39%	0.0734	95.32	0.0996	92.70
Negative control	0.6634		0.8399		0.7540		0.8648	
Blank	0.0525		0.0470		0.0400		0.0477	

3. Materials and Methods

3.1. General Experimental Procedures

Optical rotations were measured with an Automatic polarimeter (Hackettstown, NJ, USA). UV spectra were recorded by a Shimadzu UV-2600 PC spectrophotometer (Suzhou, Jiangsu, China). IR (KBr-disks) spectra were measured using a Bruker Alpha (Karlsruhe, Germany). The HR-ESI-MS spectra were recorded by a Thermo Scientific Q Exactive Plus Orbitrap LC-MS/MS system (Waltham, MA, USA); NMR spectra were recorded by a Bruker AVANCE III 600 MHz spectrometer (Zurich, Switzerland) in CD_3COCD_3 , with TMS as internal standard. The preparative high-performance liquid chromatography (HPLC) system consisted of an LC-6AD intelligent prep. pump (Kyoto, Japan), an SPD-20A intelligent UV/VIS detector (Kyoto, Japan), and a YMC-Park ODS-A column (5 μ m, 250 \times 10 mm I.D., YMC Co. Ltd., Ishikawa, Japan). Silica gel GF₂₅₄ for thin-layer chromatography (TLC) and silica gel (200–300 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Factory (Qingdao, Shandong, China). Sephadex LH-20 (Merck, Darmstadt, Germany) was suited for size-exclusion chromatography. The cell lines BT-549 HeLa, MCF-7, and MDA-MB-231 were obtained from China Center for Type Culture Collection (Wuhan, Hubei, China); MTT were obtained from Sigma Company. All solvents were purchased from Sinopharm Chemical Reagents (Shanghai, China). Methyl alcohol used for HPLC analysis was of chromatographic grade (Sigma, St. Louis, MO, USA). All aqueous solutions were prepared with double-distilled water.

3.2. Plant Material

Dried leaves of *V. amygdalina* were collected in Xiamen, Fujian Province, China, and identified by Qing Chen, Assistant Professor from the School of Pharmaceutical Sciences, Xiamen University.

3.3. Extraction and Isolation

The dried leaves (7.5 kg) were extracted with 95% ethanol and concentrated under vacuum to obtain crude extract. Subsequently, the crude extract was suspended in H₂O and partitioned in petroleum ether, dichloromethane (CH₂Cl₂), ethyl acetate, and *n*-butanol in sequence to obtain the petroleum ether fraction (170 g), the dichloromethane fraction (224 g), the ethyl acetate fraction (80 g), and the *n*-butanol fraction (60 g), respectively. The dichloromethane fraction (224 g), a dark green syrup, was subjected to macroporous resin column chromatography (CC) (4 kg, D101) and eluted with gradient methyl alcohol (MeOH)-H₂O (8:2) and MeOH. After removing the solvents under vacuum, the MeOH-H₂O extract (110 g) was subjected to silica gel column chromatography (CC) (1 kg, 200–300 mesh) and eluted with gradient CH₂Cl₂-MeOH (50:1 to 1:1 *v/v*) and MeOH to obtain fractions A (9.8 g), B (14.2 g), C (21.7 g), D (22.2 g), E (15.7 g), F (14.7 g), and G (8.5 g) after deducting the solvents. Fraction C was re-chromatographed on a silica gel CC eluted with CH₂Cl₂-MeOH, using a gradient (15:1 to 5:1 *v/v*) to obtain five fractions (Ca-Ce). Fraction Cb was further purified by Sephadex LH-20 eluted with MeOH to obtain 32 fractions, which were pooled together using TLC to obtain the three sub-fractions Cba-Cbc. The fraction Cbb (922 mg) was separated by preparative HPLC, using a YMC-Park ODS-A column (5 μm, 250 × 10 mm I.D.) with a flow rate of 5 mL/min and a mobile phase of MeOH-H₂O (75:25) to obtain the five sub-fractions Cbba-Cbbe. Sub-fraction Cbba (100 mg) was purified by preparative HPLC, using a YMC-Park ODS-A column (5 μm, 250 × 10 mm I.D.) with a flow rate of 5 mL/min and a mobile phase of MeOH-H₂O (58:42) to yield compound 1 (49 mg). Sub-fraction Cbbd (18 mg) was purified by preparative HPLC, using a YMC-Park ODS-A column (5 μm, 250 × 10 mm I.D.) with a flow rate of 5 mL/min and a mobile phase of MeOH-H₂O (70:30) to yield compound 4 (5.1 mg). Sub-fraction Cbbe (40 mg) was purified by preparative HPLC, using a YMC-Park ODS-A column (5 μm, 250 × 10 mm I.D.) with a flow rate of 5 mL/min and a mobile phase of MeOH-H₂O (75:25) to yield compound 2 (21 mg). Fraction D was re-chromatographed on silica gel CC eluted with CH₂Cl₂-MeOH, using a gradient (12:1 to 1:1 *v/v*) which was pooled together using TLC to obtain six fractions (Da-Df). Fraction Dd (828 mg) was purified by preparative HPLC, using a YMC-Park ODS-A column (5 μm, 250 × 10 mm I.D.) with a flow rate of 5 mL/min and a mobile phase of MeOH-H₂O (57:43) to yield compounds 3 (22 mg) and 5 (16 mg). Fraction Db was further purified by preparative HPLC, using a YMC-Park ODS-A column (5 μm, 250 × 10 mm I.D.) with a flow rate of 5 mL/min and a mobile phase of MeOH-H₂O (71:29) to obtain two sub-fractions. Sub-fraction Dbb (40 mg) was further purified by preparative HPLC, using a YMC-Park ODS-A column (5 μm, 250 × 10 mm I.D.) with a flow rate of 5 mL/min and a mobile phase of MeOH-H₂O (73:27) to obtain compound 6 (27 mg).

3.3.1. Vernoniomyoside A (1)

White amorphous powder; HR-ESI-MS *m/z* 669.3240 [M + Na]⁺ (calcd for C₃₅H₅₀NaO₁₁, 669.3206); [α]_D²⁵: −0.32 (c 0.05, CH₃OH); UV (DMSO): λ_{max}: 252.0, 208.8 nm; IR (KBr) *v*_{max}: 3392, 2933, 2361, 1770, 1704, 1354, 1025 cm^{−1}; ¹H and ¹³C NMR data: see Table 1.

3.3.2. Vernoniomyoside B (2)

White amorphous powder; HR-ESI-MS *m/z* 685.3554 [M + Na]⁺ (calcd for C₃₆H₅₄NaO₁₁, 685.3553); [α]_D²⁵: +0.40 (c 0.10, CH₃OH); UV (DMSO): λ_{max}: 252.0, 207.2 nm; IR (KBr) *v*_{max}: 3395, 2937, 2360, 1775, 1382, 1025 cm^{−1}; ¹H and ¹³C NMR data: see Table 1.

3.3.3. Vernoniomyoside C (3)

White amorphous powder; HR-ESI-MS m/z 669.3250 $[M + Na]^+$ (calcd for $C_{35}H_{50}NaO_{11}$, 669.3206); $[\alpha]_D^{25}$: -0.20 (c 0.05, CH_3OH); UV (DMSO): λ_{max} : 252.0, 208.8 nm; IR (KBr) ν_{max} : 3735, 3421, 2933, 2361, 1750, 1716, 1507, 1024 cm^{-1} ; 1H and ^{13}C NMR data: see Table 1.

3.3.4. Vernoniomyoside D (4)

White amorphous powder; HR-ESI-MS m/z 653.3303 $[M + Na]^+$ (calcd for $C_{35}H_{50}NaO_{10}$, 653.3257); $[\alpha]_D^{25}$: -0.24 (c 0.05, CH_3OH); UV (DMSO): λ_{max} : 252.0, 208.8 nm; IR (KBr) ν_{max} : 3735, 3421, 2933, 2361, 1750, 1716, 1558, 1507, 1024 cm^{-1} ; 1H and ^{13}C NMR data: see Table 1.

3.3.5. Vernoamyoside D (5)

White amorphous powder; 1H and ^{13}C NMR data comparable to published data [6]. ^{13}H NMR (600Hz, CD_3COCD_3): δ_H 5.33 (1H, br s, H-7) and 5.51 (1H, d, H-11), 0.44 (3H, s, CH_3 -18), 0.83 (3H, s, CH_3 -19), 0.84 (3H, d, CH_3 -26), 0.91 (3H, d, CH_3 -27) and 2.16 (3H, s, CH_3 -29). ^{13}C NMR (150Hz, CD_3COCD_3): δ_C 33.7 (C-1), 29.3 (C-2), 76.3 (C-3), 34.2 (C-4), 38.7 (C-5), 29.8 (C-6), 121.9 (C-7), 135.1 (C-8), 143.8 (C-9), 35.6 (C-10), 118.8 (C-11), 40.7 (C-12), 42.4 (C-13), 48.1 (C-14), 34.0 (C-15), 73.5 (C-16), 59.1 (C-17), 12.8 (C-18), 19.4 (C-19), 39.4 (C-20), 177.8 (C-21), 27.4 (C-22), 80.4 (C-23), 83.8 (C-24), 33.4 (C-25), 17.2 (C-26), 17.0 (C-27), 213.5 (C-28), 29.4 (C-29), 100.9 (C-1'), 73.5 (C-2'), 76.7 (C-3'), 70.1 (C-4'), 76.7 (C-5'), 61.2 (C-6').

3.3.6. Vernonioside B₂ (6)

White amorphous powder; 1H and ^{13}C NMR data: comparable to published data [20]. ^{13}H NMR (600Hz, CD_3COCD_3): δ_H 5.36 (1H, br s, H-7) and 5.47 (1H, d, H-11), 0.48 (3H, s, CH_3 -18), 0.83 (3H, s, CH_3 -19), 0.85 (3H, d, CH_3 -26), 0.88 (3H, d, CH_3 -27), 1.33 (3H, s, CH_3 -29) and 3.12 (3H, s, OCH_3). ^{13}C NMR (150Hz, CD_3COCD_3): 35.2 (C-1), 29.7 (C-2), 77.1 (C-3), 34.9 (C-4), 38.9 (C-5), 29.9 (C-6), 121.8 (C-7), 135.7 (C-8), 143.7 (C-9), 36.0 (C-10), 118.6 (C-11), 41.1 (C-12), 43.1 (C-13), 48.5 (C-14), 34.9 (C-15), 76.7 (C-16), 54.9 (C-17), 14.3 (C-18), 19.7 (C-19), 47.9 (C-20), 98.4 (C-21), 80.2 (C-22), 90.2 (C-23), 81.3 (C-24), 31.7 (C-25), 17.5 (C-26), 18.4 (C-27), 112.4 (C-28), 17.5 (C-29), 101.4 (C-1'), 75.5 (C-2'), 80.2 (C-3'), 72.9 (C-4'), 77.2 (C-5'), 61.6 (C-6'), 48.2 (OCH_3).

3.4. Acid Hydrolysis of Compounds

Compounds 1–4 (1 mg each) were dissolved in 2 M HCl (5.0 mL) and stirred at 90 °C for 4 h. The reaction solution was neutralized with NH_4OH and partitioned between EtOAc and H_2O . The residue was obtained from the water layer under vacuum distillation and dissolved in pyridine (1 mL), which was added to 0.1 M L-cysteine methyl ester hydrochloride in pyridine (1 mL). After being heated to 60 °C for 1 h, the mixture was added to phenyl isothiocyanate in pyridine (1 mL) and stirred at 60 °C for an additional 1 h. After removal of the solvent, the residue was dissolved in MeOH and analyzed by HPLC, with the mobile phase CH_3CN-H_2O (30:80, v/v) containing 0.1% formic acid; the flow rate was 1 mL/min. The standard D-glucose derivative was prepared in the same way. We then compared the retention times of sugar derivatives obtained from compounds with standard D-glucose derivatives (D-glucose: 19.5 min).

3.5. Cytotoxicity Assay

The cytotoxicity of compounds 1–6 was tested on cell lines MCF-7, BT-549, MDA-MB-231, and Hela via the MTT method. Cells were seeded into 96-well microplates at 100 μL per well (MCF-7, MDA-MB-231, and A549 were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution, while BT-549 was cultured in Roswell Park Memorial Institute (RPMI) (according to previous experiments, the DMSO concentration we used showed no interference with the experimental results). After 48 h, we added MTT (0.5 mg/mL) dissolved

in 100 μ L of fetal calf serum per well. After 4 h, we removed the MTT reagent and added 150 μ L DMSO to each well. Doxorubicin was used as the positive control. The sample without test compounds was used as the negative control. Absorbance was measured at 490 nm in an automated microplate reader. All experiments were performed in triplicate. Inhibition was calculated via the following equation:

$$\text{Inhibition} = \left[\frac{A_{\text{test sample}} - A_{\text{blank}}}{A_{\text{negative control}} - A_{\text{blank}}} \right] \times 100\%$$

$A_{\text{test sample}}$ is the test sample absorbance, A_{blank} is the blank absorbance, $A_{\text{negative control}}$ is the negative control absorbance.

4. Conclusions

We obtained four new steroidal saponins, namely vernoniamyoside A–D (1–4), together with the two known steroidal saponins vernoamyoside D (5) and vernonioside B₂ (6) from *V. amygdalina*. Of these, 1, 2, and 6 showed an excellent cytotoxicity on BT-549 cell lines in the cytotoxicity activity assay (Table 2), as expected. It is worth noting that 1 and 2 were selective for different tumor cell lines. Our results indicate that substances 1 and 2 were toxic to BT-549 cell lines. Further studies should consider the screening of more types of tumor cells.

The novelty of the saponins 1–4 is represented by the highly oxidized groups in the side chain, which may be associated with their cytotoxic expression. Saponin 1 has a strong cytotoxicity to BT-549 cell lines, including two ketones and one ester group. It is assumed that the cytotoxicity of saponin 1 may be derived from the C=O group. This group, present in the highly oxidized side chains of saponins 2–4, may provide a basis for the cytotoxicity for BT-549. However, the different inhibitory effects may be due to the relationships between the different structures and spatial configurations. The specific mechanisms and verifications offer a new direction for further experiments.

The separated steroidal saponins from the dichloromethane extraction of *V. amygdalina*, are mainly hypoglycemic steroidal saponins. However, it is essential to separate polysaccharide steroidal saponins, which always exist in ethyl acetate and *n*-butanol extraction, from *V. amygdalina*. In addition to the steroidal saponins, *V. amygdalina* contains a series of active substances such as sesquiterpene lactones and flavonoids. Therefore, the separation and identification of these chemical components should be one of the most promising trends in the study of *V. amygdalina*.

Our results provide a scientific basis for the use of *V. amygdalina* in anti-tumor research, with a potential value in the treatment of cancer.

Supplementary Materials: The following ¹H NMR, ¹³C NMR, 2D-NMR, and HR-ESI-MS spectra are available as supporting data. Supplementary materials are available online.

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Conflicts of Interest: We wish to confirm that there are no known conflicts of interest associated with this publication and that there has been no significant financial support for this work that could have influenced its outcome.

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Sample Availability: Samples of the compounds vernoniamyoside A–D, vernoamyoside D and vernonioside B₂ are available from the authors.



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