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Vernodalidimer L, a sesquiterpene lactone dimer from *Vernonia extensa* and anti-tumor effects of vernodalin, vernolepin, and vernolide on HepG2 liver cancer cells

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ABSTRACT

Vernonia extensa, known as "Phim Phai Lin" in Thai, is distributed in most regions of Thailand. The plant has been used in Ayurveda and traditionally used to treat malaria and cancer, and possesses several sesquiterpene lactones. This study aimed to investigate and identify the active constituents by bioactivity-based analysis, as well as to evaluate the cytotoxic activity of *V. extensa* by MTT or XTT assays in ten cancer cell lines (Liver HepG2 and S102; Bile duct HuCCA-1; Leukemia HL-60 and MOLT-3; Lung A549 and H69AR; Breast MDA-MB-231 and T47D; Cervical HeLa). Bioactivity-guided fractionation and semi-preparative HPLC purification were used to separate the bioactive constituents. Apoptosis-inducing activity and cell cycle inhibitory effect of selected active compounds were determined on HepG2 cells by flow cytometric analysis. Bioactivity-guided fractionation of the CH_2Cl_2 extract and chemical investigation of the cytotoxic fractions led to the isolation of a new sesquiterpenoid pseudo-dimer named vernodalidimer L, together with eight known sesquiterpenoids from the aerial part of *V. extensa*. The structures of the isolates were elucidated based on spectroscopic analysis, including 1D and 2D NMR and HRMS. Vernolide has potent broad-spectrum cytotoxicity with IC_{50} values in the range of 0.91–13.84 μM, against all ten cancer cell lines. The annexin-V flow cytometric analysis showed that vernodalin, vernolepin, and vernolide induced apoptosis on HepG2 cells in a dose dependent manner and these effects correlated with G2/M phase cell cycle arrest. Our results indicated that vernodalin, vernolepin, and vernolide have potential to be used as lead compounds in the development of a therapeutic natural product for treatment of liver cancer.

1. Introduction

The *Vernonia* genus is characteristically known to contain large amounts of sesquiterpene lactones, with several reported biological activities [1–3]. *Vernonia extensa* (var. *amygdalina* syn. *Gymnanthemum extensum*) with the common name of "Phim Phai Lin[1]" in Thai, belongs to the family of Asteraceae. This plant is a shrub with a characteristic odor and a bitter taste, growing up to 5 m in height, and has been cultivated for medicinal uses, including treatment of diarrhea, skin wounds, fever, malaria, hepatitis, worm infection, inflammation, vitiligo, and cancer [4–7]. In particular, cytotoxicity activity has been reported in leaf extract against HepG2, SNU-182, and SNU-449 liver cancer cell lines [8]. The current studies and previous phytochemical research indicated that this plant produces several biologically active sesquiterpene lactones [9–12], and other diverse groups of compounds including flavonoids [13,14], steroids [7,15], and triterpenes [16–18]. Preliminary bioactivity-guided fractionation experiments carried out in our group revealed that only the CH_2Cl_2 aerial part extract (30 μg/mL) possessed strong cytotoxic effect against A549, HuCCA-1, HepG2, and MOLT-3 cell lines with inhibition values of 89, 91, 96, and 100%, respectively. Additionally, the crude extract also expressed a high inhibitory effect on aromatase activity (85% inhibition), which requires

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[1] According to a taxonomic revision for Flora of Thailand, the plant "Phim Phai Lin" is now in the genus of *Gymnanthemum*.

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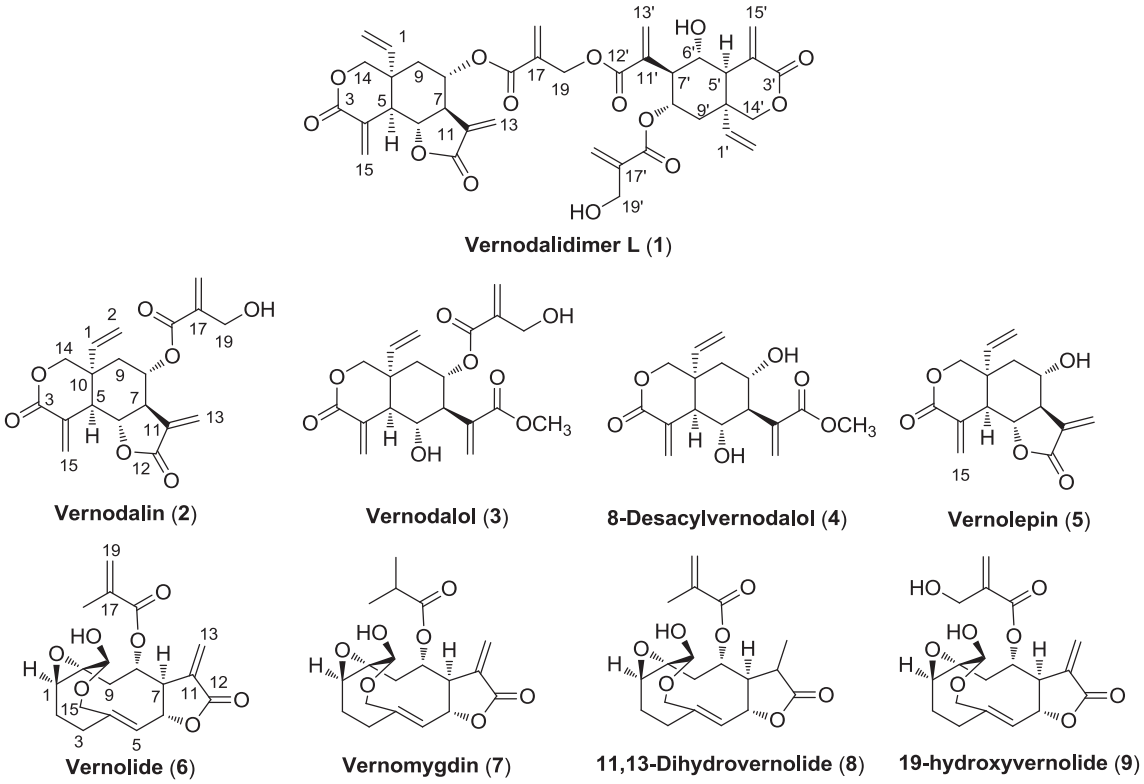

Fig. 1. Structural compounds (**1–9**) isolated from *V. extensa.*

further investigations.

Presently, we report the isolation of a new elemanolide sesquiterpene lactone pseudo-dimer named vernodalidimer L (**1**), together with eight known compounds from the aerial part CH_2Cl_2 extract of *V. extensa* (Fig. 1). Exceptional cytotoxicity of some isolated compounds, vernodalin, vernodalol, 8-desacylvernodalol, and vernolepin, on A549, HeLa, HL-60, and MDA-MB-231 cell lines has already been reported [19–21]. However, there has been no report of their cytotoxicity against HuCCA-1, HepG2, H69AR, T47-D, S102, HL-60, MOLT-3, and MRC-5 cell lines (data retrieved from SciFinder Scholar, January 2019). Therefore, we performed the primary screening of the cytotoxic activities of these compounds in a panel of ten cancer cell lines (A549, H69AR, HuCCA-1, HepG2, S102, HeLa, HL-60, MOLT-3, T47-D, and MDA-MB-231) and a normal human fibroblast cell line, MRC-5, by using *in vitro* assays. Described herein are the isolation, structural elucidation, and the cytotoxicity of the isolated compounds. Among them, vernodalin (**2**), vernolepin (**5**), and vernolide (**6**) were chosen for investigating the mechanism underlying the cytotoxic effects on the HepG2 liver cancer cell line. Furthermore, the effects of the active compounds on cell morphology changes, apoptosis, and cell cycle progression of HepG2 cells were analyzed.

2. Experimental section

2.1. General experimental procedures

Optical rotations were determined on a JASCO P-1020 polarimeter. CD spectra were recorded on a JASCO J-180 CD spectrometer. FTIR spectra were recorded on a PerkinElmer Spectrum One spectrometer using UATR technique. The ^{1}H and ^{13}C NMR spectra were measured on a Bruker AVANCE 400 and 600 MHz spectrometers in $CDCl_3$ or CD_3OD with TMS as the internal standard. HPLC purification was performed using Waters Delta 600 pumps, equipped with a Waters 2996 photodiode array detector. HRESIMS analyses were recorded on a Bruker Compact qTOF and Bruker Daltonics MicroTOF$_{LC}$ mass spectrometers.

2.2. Plant material

The aerial part of *Vernonia extensa* (*Gymnanthemum extensum*) was collected in Lak-Si District, Bangkok (coordinates of the site 13°52′36.08″N 100°34′28.66″E), in March 2018. The plant was taxonomically identified by Prof. Dr. Wongsatit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. A voucher specimen (GE2018ST) was deposited at the Laboratory of Natural Products, Chulabhorn Research Institute, Bangkok, Thailand. The samples were dried under the open-sun and shade to a constant weight and then ground.

2.3. Extraction and isolation

The dried aerial part of *V. extensa* (1.3 kg) was extracted successively at room temperature with CH_2Cl_2 (3 × 15 L), CH_2Cl_2/MeOH (1:1) mixture (3 × 15 L), and MeOH (3 × 15 L), respectively. The solvents were filtrated and evaporated under reduced pressure, yielding CH_2Cl_2 (29.2 g), CH_2Cl_2/MeOH (5.4 g), and MeOH (50.8 g) extracts.

The CH_2Cl_2 extract (29.2 g) was further separated on silica gel column chromatography (CC), eluted with a stepwise gradient of hexane-CH_2Cl_2 (100:0–0:100, v/v) and CH_2Cl_2-MeOH (100:0 to 0:100), to provide four fractions (A–D). The portion of 4.1 g of fraction B was applied to Sephadex LH-20 column chromatography (CC) using CH_2Cl_2-MeOH (20:80) to give four subfractions (B1–B4). Subfraction B3 (2.5 g) was fractionated on silica gel column, eluting with hexane-EtOAc (95:5–0:100, v/v) to obtain four subfractions (B31–B34). Subfraction B32 (1.4 g) was reapplied to a Sephadex LH-20 CC eluted with 100% MeOH to give five subfractions (B321 − B325). Subfraction B324 (21 mg) was purified by TLC pre-coated plate RP-18, using MeCN-H_2O (55:45) to give **2** (6 mg, vernodalin). Subfraction B323 (1.1 g) was purified by using semi-preparative HPLC (YMC) with a gradient elution (30–100% MeCN in H_2O over 60 min, at a flow rate of 10 mL/min, and UV monitoring at wavelength of 210 nm) to give three subfractions (B3231 − 3233) and **2** (662.5 mg, 2.3% crude extract, $[\alpha]_D^{26}$ + 89.7 (*c*

0.78, $CHCl_3$), vernodalin, t_R 17.9 min). Subfractions B3231 (48 mg) was purified by HPLC using a gradient elution (30–100% MeCN in H_2O) to give **4** (2.5 mg, 0.01% crude extract, $[\alpha]_D^{27}$ + 28.3 (*c* 0.24, $CHCl_3$), 8-desacylvernodalol, t_R 7.5 min), **5** (23 mg, 0.08% crude extract, $[\alpha]_D^{25}$ + 96.4 (*c* 0.41, $CHCl_3$), vernolepin, t_R 10.9 min.), subfraction B3232 (44 mg) yielded **3** (15.4 mg, 0.05% crude extract, $[\alpha]_D^{25}$ + 126.1 (*c* 0.17, $CHCl_3$), vernodalol, t_R 11.9 min), and subfraction B3233 (44 mg) yielded **6** (27 mg, 0.09% crude extract, $[\alpha]_D^{25}$ = + 222.5 (*c* 1.34, $CHCl_3$), vernolide, t_R 19.9 min), respectively. Subfraction B3234 (20 mg) was further purified by HPLC (YMC) using an isocratic elution (30% MeCN in H_2O over 60 min, flow rate of 10 mL/min, and UV monitoring at 210 nm) to give **7** (4 mg, 0.01% crude extract, $[\alpha]_D^{27}$ = +80.3 (*c* 0.35, $CHCl_3$), vernomygdin, t_R 44.7 min) and **8** (4 mg, 0.01% crude extract, $[\alpha]_D^{27}$ = +111.1 (*c* 0.27, $CHCl_3$), 11, 13-dihydrovernolide, t_R 48.7 min). Subfraction B33 (283 mg) was fractionated again over silica gel RP-18 eluting with MeCN-H_2O (30:70–100:0) to give **3** (42 mg, 0.14% crude extract), and an impure compound **1**. The impure compound **1** (5.5 mg) was further purified by HPLC (YMC) using a gradient elution (30–100% MeCN in H_2O) to yield **1** (2 mg, 0.01% crude extract, vernodalidimer L, t_R 23.5 min).

Fraction C (3.5 g) was applied to Sephadex LH-20 column (100% MeOH) to give eight subfractions (C1–C8). Subfraction C4 (223 mg) was purified by semi-preparative HPLC (YMC) using a gradient elution (20–100% MeCN in H_2O over 60 min, flow rate of 10 mL/min, and UV monitoring at 210 nm) to give **9** (34 mg, 0.12% crude extract, $[\alpha]_D^{27}$ = +189.4 (*c* 0.30, $CHCl_3$), 19-hydroxyvernolide, t_R 15 min).

Vernodalidimer L (**1**): White powder; $[\alpha]_D^{25}$ + 228.7 (*c* 0.03, $CHCl_3$); CD (1.94×10^{-4} μM, MeOH) λ_{max} nm ($\Delta\varepsilon$): 208 (+9.5162), 212 (+10.1589), 222 (+16.2477), 252 (+3.4080); ^{1}H NMR ($CDCl_3$, 600 MHz), and ^{13}C NMR ($CDCl_3$, 150 MHz) data, listed in Table 1; (+) HR-ESI-MS *m/z* 743.2292 $[M+Na]^+$ (calcd for $C_{38}H_{40}O_{14}Na$, 743.2310, Δ = 2.5 ppm).

Table 1
^{1}H (600 MHz) and ^{13}C (125 MHz) NMR data of compound **1** in $CDCl_3$.

Position	δ_H, mult, (*J* in Hz)	δ_C	Position	δ_H, mult, (*J* in Hz)	δ_C
1	5.73, dd (17.4, 10.8)	139.8	1′	5.69, dd (17.4, 10.8)	140.4
2α	5.32, d (11.2)	117.0	2′α	5.25, d (17.5)	116.1
2β	5.29, d (17.9)		2′β	5.24, d (11.0)	
3		163.1	3′		163.4
4		130.0	4′		132.5
5	3.05, d (11.5)	46.8	5′	2.48, d (10.2)	51.6
6	4.09, dd (11.2, 11.2)	78.0	6′	4.17, ddd (10.3, 10.3, 4.0)	68.5
7	2.98, ddd (11.2, 11.0, 4.0)	50.5	7′	2.68, t (10.9)	54.9
8	5.23, ddd (11.0, 10.1, 4.7)	69.2	8′	5.41, ddd (11.4, 11.0, 5.0)	68.8
9α	2.22, dd (14.2, 4.6)	38.9	9′α	2.04,dd (13.8, 4.9)	37.8
9β	1.66, dd (14.2, 10.3)		9′β	1.64, dd (13.7, 11.8)	
10		41.0	10′		39.6
11		135.7	11′		136.4
12		168.3	12′		165.1
13	6.21, d (3.1) 5.62, d (2.9)	121.2	13′	6.35, br s 5.77, br s	130.5
14α	4.55, d (12.4)	70.6	14′α	4.61, d (11.9)	70.9
14β	4.28, dd (12.0, 1.9)		14′β	4.31, dd (12.0, 1.9)	
15α	6.77, br s	135.7	15′	6.65, br s	133.4
15β	5.96, br s			5.75, br s	
16		164.1	16′		165.3
17		134.4	17′		139.1
18α	6.45, s	130.5	18′α	6.17, s	126.1
18β	6.04, d (1.0)		18′β	5.82, d (1.0)	
19	4.95, d (13.3) 4.85, d (13.3)	63.0	19′	4.27, dd (14.0, 6.0) 4.22, dd (14.0, 6.0)	62.3
			OH-6′	2.11, d (4.2)	
			OH-19′	2.16, t (6.0)	

2.4. Cytotoxicity assay

Cytotoxic activity of tested compounds against human cancer cell lines, including HL-60 (acute promyelocytic leukemia), MOLT-3 (*T*-cell acute lymphoblastic leukemia), A-549 (lung adenocarcinoma), HeLa (cervical carcinoma), HuCCA-1 (cholangiocarcinoma derived from a Thai patient), S102 (hepatocellular carcinoma derived from a Thai patient), HepG2 (hepatocellular carcinoma), MDA-MB-231 (triple-negative breast cancer), T-47D (hormone-dependent breast carcinoma), H69AR (multidrug-resistant small-cell lung carcinoma), and MRC-5 (normal embryonic lung fibroblast). The cells were incubated with tested compounds for 48 h. The number of surviving cells at the end of the treatment was measured by MTT or XTT assays, as previously described [22,23]. IC_{50} values were obtained by serial dilution of the compounds (50, 10, 2.0, 0.40, 0.08, and 0.016 μg/mL). Doxorubicin was used as positive control in cytotoxic assays. All experiments were carried out three times with triplicates. IC_{50} values were calculated by linear regression.

2.5. Apoptosis and cell cycle analysis

Flow cytometric techniques using Muse Cell Analyzer (Merck Millipore, Hayward, CA, USA) were used to determine apoptotic cell death and cell cycle distribution as previously described [24,25]. Briefly, HepG2 cells were incubated with 10 μM of tested compounds for 48 h. After treatment, the cells were harvested by trypsinization and subjected to apoptosis detection and cell cycle analysis using Muse Annexin-V & Dead Cell kit and Muse Cell Cycle kit (Merck Millipore), respectively, according to the manufacturing protocols. Percentage of early and late apoptotic cell populations were combined and reported as total apoptosis. Data are expressed as mean ± SD from three independent experiments.

3. Results and discussion

3.1. Chemical identification of the bioactive compounds from the CH_2Cl_2 fractions

A bioassay-guided separation of the CH_2Cl_2 fractions, using a combination of chromatographic methods and HPLC purification, afforded the new elemanolide sesquiterpenoid lactone dimer, named vernodalidimer L (VDi, **1**), together with eight known compounds identified by comparison of their spectroscopic data with those reported in the literatures and HRMS analysis, as vernodalin (VDa, **2**) [26], vernodalol (VDol, **3**) [27], 8-desacylvernodalol (8Des, **4**) [28], vernolepin (VLe, **5**) [29], vernolide (VLi, **6**) [30], vernomygdin (Vmy, **7**) [26,31], 11,13-dihydrovernolide (11,13DLi, **8**) [32,33], and 19-hydroxyvernolide (19HLi, **9**) [32] (Fig. 1).

We have provided the complete and unambiguous ^{1}H- and ^{13}C NMR assignment of all isolated compounds (see Supplementary data), especially for the vernomygdin (**7**) which was first reported by Kupchan [26]. The original drawing structure of this compound was misleading, but the ^{1}H & ^{13}C NMR data in the text were correct. The later papers [34–36] had reported this compound by referring to Kupchan's paper causing confusion in the correct structure of vernomygdin (**7**). Herein, we reported both the correct structure of vernomygdin and complete spectroscopic data for clarification.

3.2. Structural elucidation of the new compound, vernodalidimer L (1)

Vernodalidimer L (**1**) was obtained as a white powder. Its molecular formula was determined to be $C_{38}H_{40}O_{14}$ deduced from HRESIMS at *m/z* 743.2292 $[M+Na]^+$ (calcd for $C_{38}H_{40}O_{14}Na$ 743.2310), implying nineteen degrees of unsaturation. The assignments of ^{1}H and ^{13}C NMR spectroscopic data of compound **1** are summarized in Table 1 based on HSQC, HMBC, and COSY spectra (Fig. 2). The ^{13}C NMR and DEPT

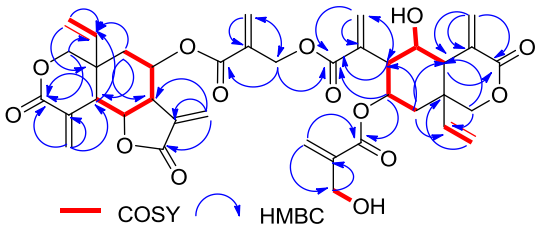

Fig. 2. Selected ^{1}H-^{1}H COSY and HMBC correlations of **1**.

spectra showed thirty eight carbon signals including six ester carbonyls at δ_C 168.3 (C-12), 165.3 (C-16′), 165.1 (C-12′), 164.1 (C-16), 163.4 (C-3′), and 163.1 (C-3) as well as other carbon signals including six sp^3 and eight sp^2 methylenes, eight sp^3 and two sp^2 methines, and two sp^3 and six sp^2 quaternary carbons. The ^{1}H NMR and ^{13}C NMR data of **1** indicated that there were two parts as moiety A and B, whose spectra were very similar to those of vernodalin [26] and vernodalol [27] or vernodalinol [37]. The ^{1}H-^{1}H COSY correlations of H-1/H-2, H-5/H-6, H-6/H-7, H-7/H-8, and H-8/H-9, and the HMBC correlations between H-5 and C-1/C-3/C-4/C-10/C-14/C-15, H$_2$-14 and C-1/C-3/C-5, H$_2$-13 and C-7/C-11/C-12, H-1/H-2 and C-10, suggested that the moiety A had structure close to that of vernodalin. Likewise, the moiety B could be assigned to either vernodalol or vernodalinol based on the ^{1}H-^{1}H COSY correlations of H-1′/H-2′, H-5′/H-6′, H-6′/H-7′, H-7′/H-8′, H-8′/H-9′, H-7′/H-13′, OH-6′/H-6′, and OH-19′/H$_2$-19′. Furthermore, The ^{1}H–^{13}C HMBC NMR correlations between H-2′ and C-1′/C-10′, H-5′ and C-1′/C-3′/C-6′/C-14′, H-8′ and C-16′, H$_2$-9′ and C-5′/C-7′/C-8′/C-10′, H$_2$-13′ and C-7′/C-11′/C-12′, and H$_2$-15′ and C-3′/C-5′ indicated that C-7′ in the cyclohexane ring was connected to a prop-2-enoic acid (2-methyl ester) moiety through its 2 position whereby the propenoic acid (2-methyl ester) was composed of C-11′, C-12′, C-13′, and H-13′a/b, and an ester side chain located at C-8′ same as that in moiety A. The deshielded oxygenated methylene proton resonances (δ_H 4.95 and 4.85, both d, J = 13.3 Hz, H$_2$-19) and the molecular formula obtained from MS data indicated that compound **1** is not a mixture of the two sesquiterpenes, but a conjugate of these two sesquiterpenoid units forming a dimer. This was fully supported by the HMBC correlation between H$_2$-19 and C-12′.

Relative stereochemistry of **1** was based on NOESY spectrum and coupling constant between protons. Both H-6 and H-6′ were β–orientation because of large coupling constants between H-5/5′ and H-6/6′ and between H-6/6′and H-7/7′ ($J_{5,6}$ = $J_{6,7}$ = 10–11 Hz) as well as the H-7/7′ coupled with H-6/6′ and H-8/8′ ($J_{6,7}$ = $J_{7,8}$ = 10–11 Hz). This implied trans-diaxial orientation, i.e. H-5α, H-6β, H-7α, and H-8β-oriented, which was a characteristic feature for naturally occurring sesquiterpenoids [38]. The NOESY correlations of H-6/H-8 and H-7/H-5 indicated that the relative configuration of moiety A was the same as those of vernodalin. For the moiety B, the NOESY correlations of H-6′/H-8′, H-7′/H-5′, and H-5′/H-1′ indicated β–orientation. Upon these data, the structure of **1** is established (Fig. 1).

The absolute configuration (AC) of compound **1** was further supported by the results obtained from the electronic circular dichroism (ECD) spectra (Fig. 3). The ECD calculations were performed using time-dependent density functional theory (TDDFT) method at the B3LYP/6–31 + G(d,p) level by using the solvent model IEFPCM in CH_3OH (see supplementary data for details). The calculated CD spectrum of the isomer **1a** with 5*R*, 6*R*, 7*S*, 8*S*, 10*R*, 5′*R*, 6′*R*, 7′*S*, 8′*S*, 10′*R* and the CD spectrum of **1** were in good agreement. Therefore, the AC of **1** was assigned to be 5*R*, 6*R*, 7*S*, 8*S*, 10*R*, 5′*R*, 6′*R*, 7′*S*, 8′*S*, 10′*R* as shown in Fig. 1. It is noteworthy that compound **1** had a similar structure to that of venodalidimer G [20], but containing vernodalin and vernodalol or vernodalinol moieties instead. Aisa and coworkers [20] determined the AC of vernodalidimer G to be of the 5*S*, 6*S*, 7*R*, 8*R*, 10*S*, 5′*S*, 6′*S*, 7′*R*, 8′*R*, 10′*S* configuration. Therefore, we performed the calculated ECD spectrum of compound **1b** with 5*S*, 6*S*, 7*R*, 8*R*, 10*S*, 5′*S*, 6′*S*, 7′*R*, 8′*R*, 10′*S* configuration to see the possibility of this configuration. The calculated ECD spectrum of **1b** using TDDFT ECD at the B3LYP/6–31 + G(d,p) and B3LYP/TZVP levels do not match the experimental ECD spectrum of **1** (see supplementary data). Additionally, compound **1** displayed positive optical rotation ($[\alpha]_D^{26}$ + 228.7), while vernodalidimer G had negative optical rotation ($[\alpha]_D^{25}$ –0.68), indicating that the AC of **1** is likely different from that in vernodalidimer G. However, attempts to further determine the accuracy of the AC of compound **1** by X-ray crystallography or other approaches were hampered due to an insufficient amount of material. The biosynthetic pathway of vernodalidimer L (**1**) was possibly *via* a trans esterification process of the vernodalin with either vernodalinol or vernodalol (Scheme S1, Supplementary data).

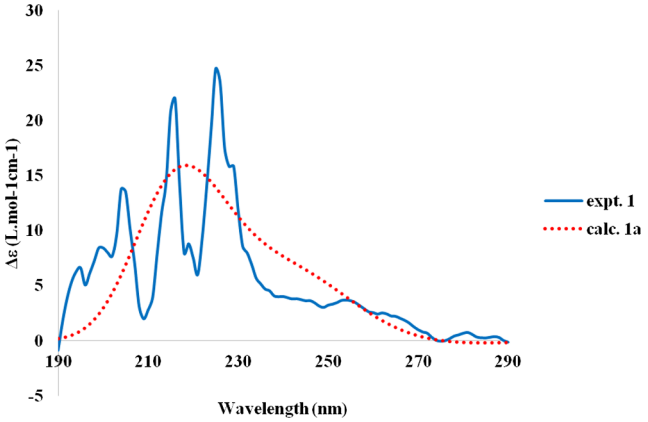

Fig. 3. Experimental CD and calculated ECD spectra of **1**.

3.3. Cytotoxicity evaluation

The cytotoxicity of the CH_2Cl_2, CH_2Cl_2/MeOH, and MeOH fractions of the aerial part of *V. extensa* were primarily screened at a concentration of 30 μg/mL with HepG2, HuCCA-1, A549, and MOLT-3 cell lines. The fractions which showed less than 50% cytotoxicity were considered to be inactive. Only the CH_2Cl_2 fraction exhibited strong cytotoxicity in all tested cell lines, while MeOH fraction was inactive in all cell lines. The CH_2Cl_2/MeOH mixture (1:1) showed potent cytotoxicity against MOLT-3 leukemia cells with percent inhibition of 98%, but was inactive in the other three cancer cell lines (Table 2). As a result, the CH_2Cl_2 fraction was chosen for investigation and was therefore subjected to further purification.

Nine compounds, including a new dimeric sesquiterpene lactone (**1**) were isolated from the CH_2Cl_2 fraction, with the percent isolated yields of the nine compounds shown in Table 3. Of these, the highest isolated yield was obtained for vernodalin (**2**) (2.3%), followed by vernodalol (**3**) and 19-hydroxyvernolide (**9**) (0.2% and 0.12%), vernolide (**6**) and vernolepin (**5**) (0.09% and 0.08%), while isolated yields for compounds **1**, **4**, **7**, and **8** were all only 0.01% percent (Table 3), These results

Table 2
Cytotoxic effects of the fractions of the aerial part of *Vernonia extensa* against four human cancer cell lines.[a]

Fraction	% Cytotoxicity at 30 μg/mL			
	HepG2	HuCCA-1	A549	MOLT-3
CH_2Cl_2	95.6	91.0	89.0	100
CH_2Cl_2/MeOH (1:1) mixture	22.9	41.0	17.0	98
MeOH	0.0	5.0	7.0	17

[a] HepG2: human hepatocellular carcinoma cells; HuCCA-1: Thai human cholangiocarcinoma cells; A549: human lung carcinoma cells; MOLT-3: human acute lymphoblastic leukemia cancer cells.

Table 3
Results of cytotoxicity of isolated compounds towards different human cancer cell lines.

Cpds	% yield	Cell lines, IC_{50} (μM), [SI]										
		A549	HeLa	HuCCA-1	HepG2	H69AR	T47-D	MDA-MB-231	S102	HL-60	MOLT-3	MRC-5
VDi (**1**)	0.01%	ND	ND	ND	41.77 ± 3.14 [1.7]	ND	ND	74.42 ± 3.28	I	ND	ND	I
VDa (**2**)	2.3%	13.61 ± 0.25 [5.1]	**3.81** ± 0.36 [18.3]	***8.17*** ± 0.17 [8.5]	15.47 ± 1.69 [4.5]	21.47 ± 0.22 [3.3]	***6.86*** ± 0.28 [10.1]	16.14 ± 1.89 [4.3]	21.47 ± 1.03 [3.3]	**2.28** ± 0.17 [30.6]	**3.92** ± 1.19 [17.8]	69.83 ± 6.78
VDol (**3**)	0.2%	81.45 ± 3.85	10.82 ± 0.52 [6.9]	41.81 ± 1.41 [1.8]	62.58 ± 5.18 [1.2]	66.91 ± 2.33[1.1]	42.65 ± 1.03 [1.8]	70.3 ± 3.83 [1.1]	97.60 ± 4.36	26.05 ± 0.46 [2.9]	16.66 ± 2.99 [4.5]	75.13 ± 4.26
8Des (**4**)	0.01%	I	24.77 ± 3.54 [6.6]	ND	83.21 ± 16.40 [2.0]	ND	57.24 ± 0.26 [2.8]	74.71 ± 3.73 [2.2]	I	ND	ND	I
VLe (**5**)	0.08%	13.73 ± 2.86 [6.7]	11.27 ± 0.22 [8.1]	***9.31*** ± 0.40 [9.8]	20.15 ± 1.88 [4.5]	***8.70*** ± 0.69 [10.5]	***9.31*** ± 0.40 [9.8]	19.35 ± 1.74 [4.7]	30.58 ± 4.24 [3.0]	**3.84** ± 0.18 [23.8]	***5.29*** ± 1.45 [17.3]	91.49 ± 8.55
VLi (**6**)	0.09%	***6.91*** ± 0.69 [2.8]	**0.91** ± 0.14 [20.9]	***6.22*** ± 0.97 [3.1]	**2.57** ± 0.47 [7.4]	***5.94*** ± 0.08 [3.2]	**1.27** ± 0.11 [15.0]	**3.29** ± 0.47 [5.8]	13.84 ± 0.39 [1.4]	**1.52** ± 0.14 [12.5]	**1.77** ± 0.28 [10.8]	19.06 ± 5.25
Vmy (**7**)	0.01%	ND	ND	ND	17.61 ± 0.82 [1.1]	11.43 ± 0.06 [1.7]	ND	ND	69.04 ± 1.76	**2.86** ± 0.19 [6.9]	ND	19.84 ± 8.76
11,13DLi (**8**),	0.01%	ND	ND	ND	I	I	ND	ND	I	26.07 ± 2.42 [3.8]	ND	97.80
19HLi (**9**)	0.12%	72.22 ± 6.75 [1.8]	11.75 ± 1.24[11.3]	36.51 ± 1.11 [3.6]	61.24 ± 1.96 [2.2]	25.40 ± 0.40 [5.2]	22.54 ± 1.51 [5.9]	64.44 ± 4.31 [2.1]	77.01 ± 2.70 [1.7]	12.72 ± 1.46 [10.4]	12.04 ± 1.46 [11.0]	I
DOX		**0.40** ± 0.007 [6.3]	**0.67** ± 0.071 [3.8]	**0.76** ± 0.017 [3.3]	**0.53** ± 0.034 [4.8]	31.03 ± 2.43 [0.08]	**0.79** ± 0.051 [3.2]	**2.02** ± 0.034 [1.3]	**1.67** ± 0.017 [1.5]	**0.17** ± 0.00 [14.9]	**0.012** ± 0.007 [210]	**2.53** ± 0.24

DOX = Doxorubicin; [SI] = Selectivity index, IC_{50} normal cell/IC_{50} cancer cell, SI value ≥ 3.0 indicating highly cancer-selective; I = Inactive at 50 μg/ml; ND = Not determine; VDi = Vernodalidimer L; VDa = Vernodalin; VDol = Vernodalol; 8Des = 8-Desacylvernodalol; VLe = Vernolepin; VLi = Vernolide; Vmy = Vernomygdin; 11,13DLi = 11,13-Dihydrovernolide; 19HLi = 19-Hydroxyvernolide; **IC_{50}** < 2 μM; **IC_{50}** < 5 μM; ***IC_{50}*** < 10 μM.

suggest that vernodalin (**2**) was the major constituent of the CH_2Cl_2 fraction.

All nine compounds were subjected to cytotoxicity evaluation in a panel of ten cancer cell lines (breast, liver, lung, cervical, and leukemia) as well as normal human lung cells (MRC-5) (Table 3). Doxorubicin was used as positive control. Selective cytotoxicity of tested compounds in cancer cells *versus* normal cells was analyzed and expressed as selectivity index (SI). Compounds with SI value ≥ 3.0 have been considered to possess cancer-selective cytotoxicity [39]. The results showed that most isolated compounds showed low toxicity to the MRC-5 cells (Table 3). Moreover, comparison of the IC_{50} values of these sesquiterpene lactones in tested cell lines revealed important structural requirements for the observed cytotoxic activity.

The isolated compounds examined in this study could be classified into two groups, elemanolide and germacrolide sesquiterpenes. The elemanolide sesquiterpenes include vernodalidimer L (**1**), vernodalin (**2**), vernodalol (**3**), 8-desacylvernodalol (**4**), and vernolepin (**5**). The germacrolide sesquiterpenes include vernolide (**6**), vernomygdin (**7**), 11,13,-dihydrovernolide (**8**), and 19-hydroxyvernolide (**9**). In the group of elemanolide sesquiterpenes, vernodalin (**2**) and vernolepin (**5**) showed more potent cytotoxicity than other compounds. Comparison of cytotoxic activities indicated that compound **2** has higher toxic to cells than the related structure **3** in all tested cell lines, and similarly, compound **5** had higher cytotoxic activity than the related structure **4**. The major difference in these two sets of structures was the bis-α-methylene lactone in **2** and **5** and the methylene methyl ester in **3** and **4**, suggesting that the bis-α-methylene lactone moiety plays an important role in increasing cytotoxic activity. Additionally, when comparing compounds **2** and **5**, compound **2** displayed a slightly greater cytotoxicity than compound **5** towards almost all tested cell lines except H69AR, and the same trend was also observed in comparison of compounds **3** and **4**. This greater activity could be due to the 2-(hydroxymethyl)acryl moiety present at C-8 in **2** and **3**, and its absence in **5** and **4** (Fig. 2). Vernodalidimer L (**1**), only available in minute amounts, was tested with HepG2, MDA-MB-231, S102, and MRC-5, with the results showing little or no activity with these cell lines.

In the group of germacrolide sesquiterpenes, vernolide (**6**) showed highly potent cytotoxic activity with IC_{50} in the range of single digit to sub micromolar level in almost all cancer cell lines except for S102. Replacement of the double bond in **6** with a single bond (compound **8**) led to loss of cytotoxic activity in the tested cell lines (HepG2, H69AR, S102, HL-60, and MRC-5), suggesting that the bis-α-methylene lactone moiety was important for the activity. Compounds **6**, **7**, and **9**, with the same core structure, but different side chains, showed cytotoxicity in HepG2, H69AR, S102, HL-60, and MRC-5 in the order **6** > **7** > **9**. In other cell lines, the cytotoxicity of **6** was also more potent than **9**. This suggested that at the C-8 position, the methacryl moiety in **6** yielded higher cytotoxicity than the isobutyryl group in **7** and the 2-(hydroxymethyl) acryl group in **9**.

As shown in Table 3, among the tested compounds, compounds **2**, **5**, and **6** displayed much more potent inhibitory properties. We speculated that the bis-α-methylene lactone present in these structures made a strong contribution to cytotoxic activity, because decrease in cytotoxic activity was observed in the absence of the bis-α-methylene lactone in the related structures (**2** vs **3**, **5** vs **4**, and **6** vs **8**). An important determinant for activity of the bis-α-methylene lactone was likely to be due to its role as a Michael acceptor [40–42].

Vernodalin (**2**) and vernolepin (**5**) have been reported to show antiproliferative activities [43] against HeLa, A549, and MDA-MB-23 cell lines, with IC_{50} values of [0.20, 0.40, and 0.30 μM] and [2.20, 16.9, and 1.90 μM], respectively. Our results confirmed the cytotoxicity of both compounds in these three cell lines, although with a much lower cytotoxicity (higher IC_{50}) than the previous report. Moreover, we also found that compounds **2** and **5** showed selective toxicity to leukemia cancer cells, HL-60 (SI = 30.6 and 23.8, respectively) and MOLT-3 (SI = 17.8 and 17.3, respectively) with the IC_{50} values of **2** and **5** being 2.28 and 3.83 μM in HL-60 cells and 3.92 and 5.29 μM in MOLT-3 cells, respectively. Among all the tested compounds, vernolide (**6**), showed the strongest cytotoxic activity against all tested cancer cell lines. Interestingly, **5** and **6** displayed significantly more potent cytotoxic activity against H69AR than that of doxorubicin (IC_{50} value of 8.70 μM and 5.94 μM vs 31.03 μM) and more importantly both compounds had promising selectivity index of 131 and 40 times greater than doxorubicin (SI = 10.5 and 3.2 vs 0.08).

Vernodalin (**2**), vernolepin (**5**), and vernolide (**6**) were very attractive compounds since they exhibited strong cytotoxicity and high cancer-selectivity towards the panel of ten cancer cell lines. Vernodalin (**2**) was the most cytotoxic among the compounds in the elemanolide group with IC_{50} less than 5 μM in three cancer cell lines and less than 10 μM in another two cell lines. Moreover, vernolide (**6**) was the most cytotoxic compound in the germacrolide group with IC_{50} less than 5 μM in three cancer cell lines and less than 10 μM in another six cell lines. Vernolepin (**5**) also showed interesting cytotoxic activity with IC_{50} less than 10 μM in five cancer cell lines, so it should not be disregarded.

As shown in Table 3, the isolated yields of compounds **2**, **5**, and **6** present in the cytotoxic CH_2Cl_2 fraction were significantly high, suggesting that these three compounds are active cytotoxic constituents of *V. extensa*. It is therefore possible that these compounds may be responsible for the anti-tumor effect in the ethnomedical uses of this plant for cancer treatment.

Of further interest is that the cancer-selective cytotoxicity or SI values of these three compounds were higher than 3.0 in almost all cancer cell lines. Vernodalin (**2**) and vernolepin (**5**) displayed SI ≥ 3.0 in all ten cancer cell lines, and vernolide (**6**) showed SI ≥ 3.0 in eight cancer cell lines (Table 3), suggesting their highly selective cytotoxic activity toward cancer cells. This remarkable property of compounds **2**, **5**, and **6** make them worthy of further study for the mechanism of their cytotoxicity.

*3.4. Mechanistic studies of cell death induced by vernodalin (VDa, **2**), vernolepin (VLe, **5**), and vernolide (VLi, **6**) on HepG2 liver cancer cells*

Liver cancer (hepatocellular carcinoma) ranks in the top-three most cancers found in Thailand. Two liver cancer cell lines were used in our cytotoxic screening, HepG2 and S102. However, we decided to investigate the mechanism underlying cytotoxicity of vernodalin (**2**), vernolepin (**5**), and vernolide (**6**) on the HepG2 cell line because this cell line was more sensitive to the selected compounds than the S102 cell line. At a concentration of 10 μM, percent cytotoxicity of **2**, **5**, and **6** on HepG2 cells, as determined by MTT assay, were 38%, 30%, and 86%, respectively (Scheme S2, Supplementary data). This concentration was used for mechanistic studies of cell death.

To analyze whether the selected compounds induce cell death through the apoptosis mechanism, HepG2 cells were incubated with **2**, **5**, and **6** at 10 μM for 48 h; subsequently, the cells were subjected to apoptosis detection using flow cytometry. As shown in Fig. 4A, all three compounds induced morphological changes and cell detachment from the tissue culture plates, similarly. Analysis of apoptosis revealed an increase in Annexin-V positive cell populations in cells treated with all three compounds, compared with untreated control (Fig. 4B). Proportions of apoptotic cells (both early and late apoptosis) obtained after treatments with **2**, **5**, and **6**, were 10.9%, 20.0%, and 31.2%, respectively, while the basal apoptosis level in untreated cells was 6.7% (Fig. 4C). These results suggested that all the three compounds exerted their cytotoxic effect through inducing apoptosis, and **6** was the most potent among the three compounds, consistent with their cytotoxicity.

We also determined the effect of compounds **2**, **5**, and **6** on cell cycle progression on HepG2 cells under the same conditions as for study of apoptosis. Interestingly, after 48 h treatment, all three compounds caused a decrease in proportions of cells in G0/G1 and S phases while the proportions of cells in G2/M phase were considerably elevated (Fig. 5). The percentages of cells in G2/M phase after treatment with **2**,

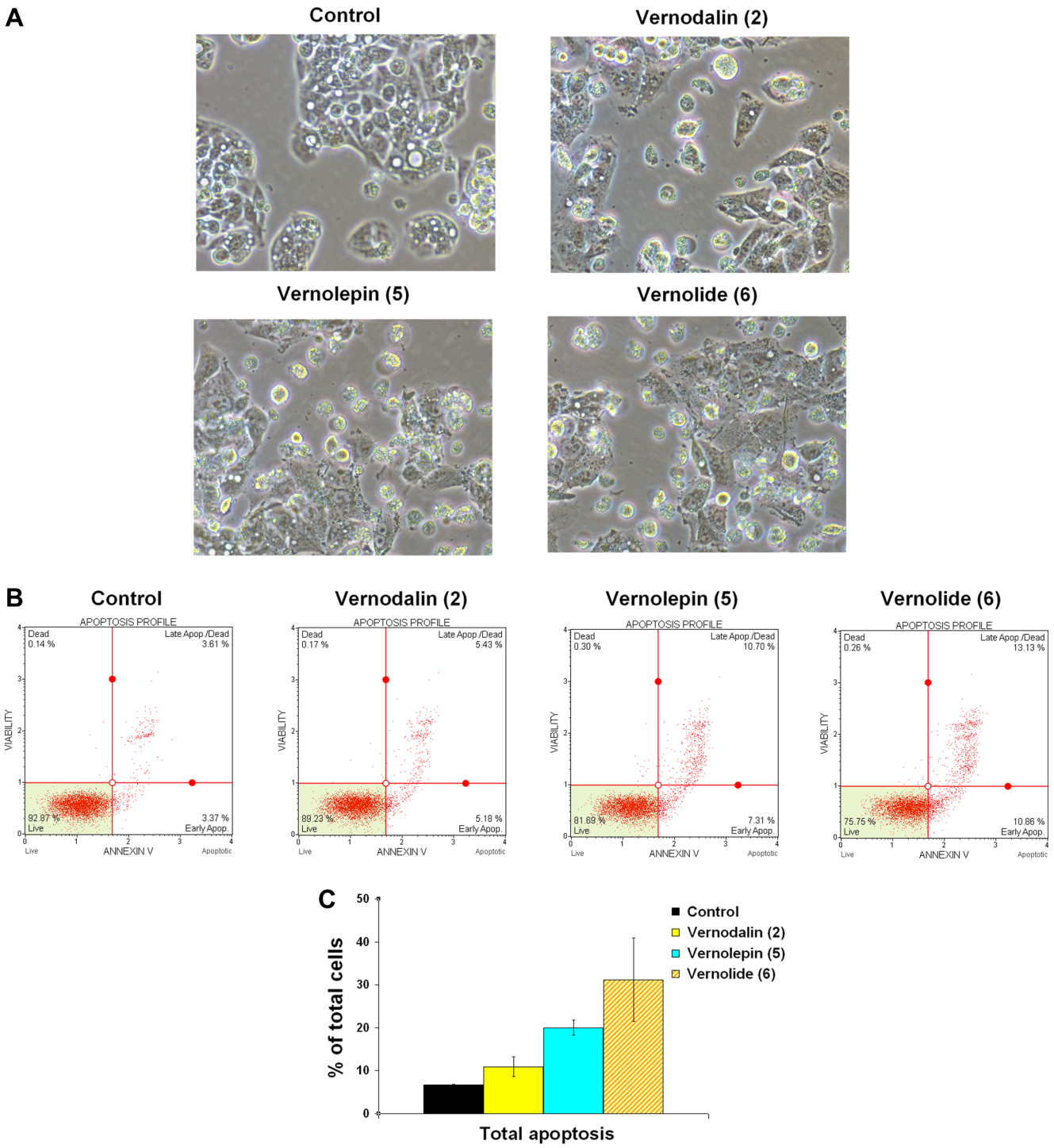

Fig. 4. Effect of vernodalin (**2**), vernolepin (**5**), and vernolide (**6**) on cell morphology and apoptosis induction on HepG2 cells. Cells were incubated in the absence or presence of 10 μM of tested compounds for 48 h and photographs were taken, after which cells were analyzed for apoptosis by using Muse cell Analyzer with Muse Annexin-V and Dead Cell kit. (A) Cell morphology changes, original magnification of 200×. (B) Dot plots recording Annexin-V and 7-AAD positive cells in a representative experiment. Quadrants: lower left, live cells; lower right, early apoptotic cells; upper left, the necrotic cells; and upper right, late apoptotic cells. (C) Quantitative analysis of percent cell populations. Data are reported as average values from three independent experiments with standard deviations (SD).

5, and **6** were 41.6%, 51.5%, and 58.8%, respectively, compared with 38.4% for control (Table 4). These results indicated that the three compounds were able to induce cell cycle arrest at G2/M phase in HepG2 cells. Furthermore, the potential of the three compounds to induce cell cycle arrest was consistent with their apoptosis-inducing potential.

Apoptosis is a mechanism of cell death that controls proper number of cells in the body, however all cancer cells are able to avoid apoptosis [44]. Many therapeutic sesquiterpene lactones, such as dehydroleucodine obtained from *Gynoxys verrucosa* and parthenolide derived from feverfew (*Tanacetum parthenium*) could induce apoptosis in cancer cells [45,46]. Additionally, parthenolide has also been reported to induce G2/M cell cycle arrest in cancer cells [47]. In the present study, we showed that vernodalin (**2**), vernolepin (**5**), and vernolide (**6**) were potent cytotoxic sesquiterpene lactone compounds. Our mechanistic studies revealed that these three compounds possessed apoptosis-

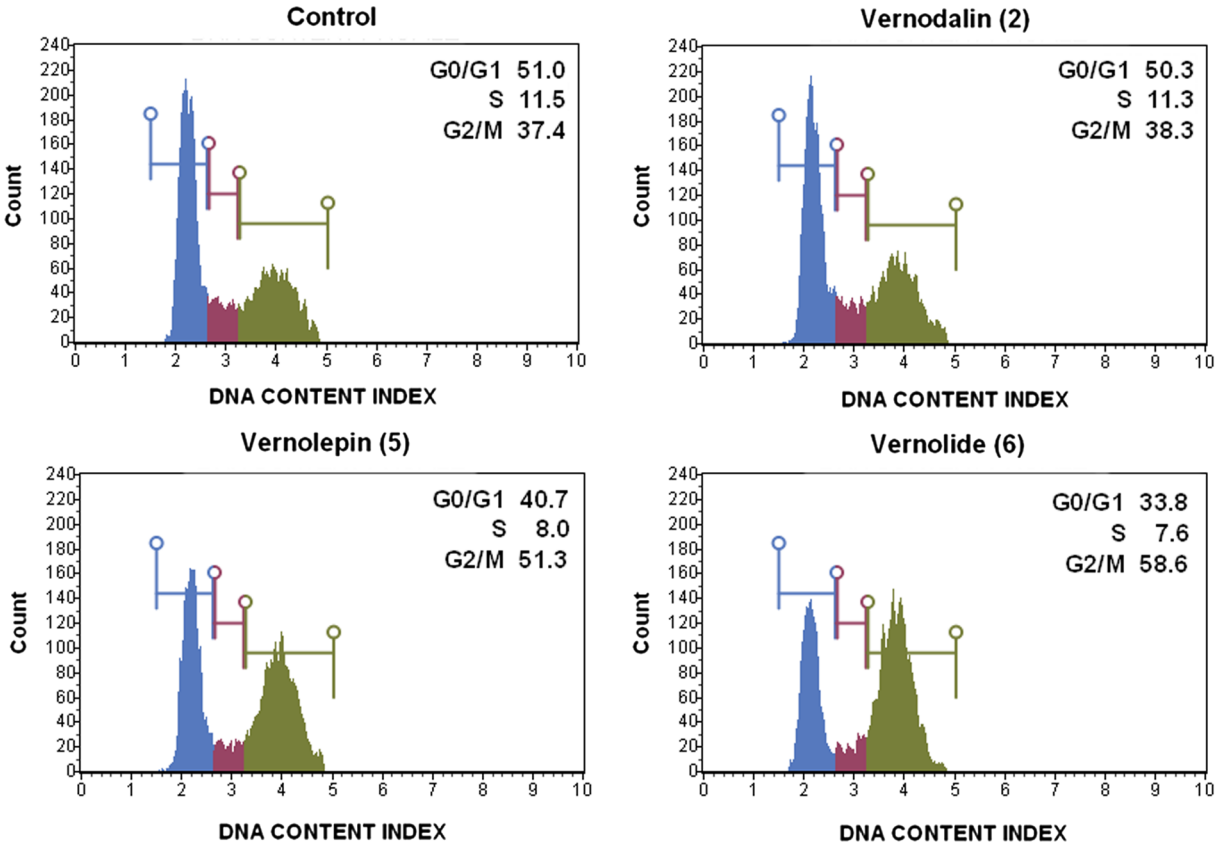

Fig. 5. Analysis of cell cycle distribution of HepG2 cells. Cells were incubated in the absence or presence of 10 μM of tested compounds for 48 h. The percentages of cells in the G0/G1, S, and G2/M phases were determined by using Muse Cell Analyzer with Muse Cell Cycle kit. Data from a representative experiment are shown. The experiment was performed in triplicate.

Table 4
Cell cycle distribution of HepG2 cells after 48 h treatment with 10 μM of each compound.

Treatment	Cell cycle distribution (%)[a]		
	G0/G1	S	G2/M
Control	49.8 ± 0.9	11.6 ± 0.2	38.4 ± 0.8
Vernodalin (**2**)	47.9 ± 3.3	10.3 ± 0.8	41.6 ± 3.8
Vernolepin (**5**)	40.4 ± 2.0	8.0 ± 0.5	51.5 ± 2.5
Vernolide (**6**)	34.1 ± 2.3	6.9 ± 0.5	58.8 ± 2.5

[a] Data are expressed as average ± SD from three independent experiments.

inducing activity and also caused cell cycle arrest at the G2/M phase in HepG2 liver cancer cell line, and these activities might contribute to their cytotoxicity, similar to the mechanisms found with the sesquiterpene lactone parthenolide.

4. Conclusions

Sesquiterpene lactones are a class of compounds found in many plants and possess a wide range of biological activities, including antibacterial, anti-inflammatory, antiparasitic, and cytotoxicity [48–52]. With the aim of identifying their anti-tumor activity, activity-guided fractionation was performed using the CH_2Cl_2 extracts from the aerial part of *V. extensa* followed by determination of cytotoxicity. A new pseudo-disesquiterpenoid named vernodalidimer L (**1**) possibly formed by esterification of two sesquiterpenoids, and eight known sesquiterpenoids (**2**–**9**) were isolated from *V. extensa*. The cytotoxicity of the isolated compounds was confirmed using a panel of ten cancer cell lines. The mechanism underlying the cytotoxic activity of *V. extensa* extract was also determined. Vernolide (**6**) was so far the most promising agent from this plant, and has high potential for development as an anti-cancer agent.

Our findings provided experimental evidence supporting the claimed ethnomedical uses of *V. extensa* in the treatment of various cancers and also confirm the anticancer potential of this plant. In order to fully exploit the plant's medicinal importance, further study is recommended.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103197.

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