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Chemical constituents of Euphorbia tangutica

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Chemical constituents of *Euphorbia tangutica*

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Sixteen known compounds isolated from the whole plants of *Euphorbia tangutica*, including phorbol-13-actate (**1**) previously synthesised and obtained from a natural source for the first time, were evaluated *in vitro* against a panel of human cancer cell lines using the MTT method. Among them, ergosterol (**6**) exhibited significant cytotoxic activity against HL-60 cell line with an IC_{50} value of 3.3 μM, and 3β,5α-dihydroxy-15β-cinnamoyloxy-14-oxolathyra-6*Z*,12*E*-diene (**7**) also displayed moderate activity.

Keywords: Euphorbiaceae; *Euphorbia tangutica*; phorbol-13-actate; ergosterol; cytotoxic activity

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1. Introduction

The genus *Euphorbia* is the largest in the plant family Euphorbiaceae, comprising about 2000 known species, more than 80 of which are distributed in China (Ma & Tseng, 1997) and range from annuals to trees. Detailed study of the profile of secondary metabolites could contribute to taxonomic subdivision of this complex genus. Many secondary metabolites with specific types of diterpene skeleton in the genus have been found to possess a number of interesting biological activities (Ahmad et al., 2005; Lal, Cambie, Rutledge, & Woodgate, 1990; Ravikanth et al., 2002; Vasas et al., 2011), especially antioxidant (Basma, Zakaria, Latha, & Sasidharan, 2011), anti-HBV (Tian, Sun, Li, Liu, & Dong, 2011a), anticancer (Kumar, Sun, Li, Liu, & Dong, 2011; Mayur, Ravirajsinh, Menaka, Ranjitsinh, & Sonal, 2011; Milica et al., 2011) and antiviral activities (Kumar et al., 2011). *Euphorbia tangutica* Proch., a perennial herbaceous plant, with a milky juice in the aerial part and roots, is distributed mainly in Qinghai and Gansu provinces of China (Liu, 1999). Its chemical constituents have never been reported so far. To determine the anticancer activity of the chemical constituents, ethyl acetate fraction containing 16 known compounds including a phorbol diterpene previously synthesised and obtained from a natural source for the first time, were investigated. Compounds **1–16** were isolated from the plant for the first time. This article reports the isolation and structure elucidation of phorbol-13-actate (**1**) using spectroscopic methods including 1D-, 2D-NMR, as well as

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cytotoxic activity against HL-60, SMMC-7721, A-549, MCF-7, SW-480 cell lines of two known compounds, ergosterol (**6**) and 3β,5α-dihydroxy-15β-cinnamoy-loxy-14-oxo-lathyra-6 *Z*,12 *E*-diene (**7**).

2. Results and discussion

Compound **1** was obtained as a white amorphous powder with molecular formula $C_{22}H_{30}O_7$, determined from ESI-MS *m*/*z* at 405 $[M-H]^-$. The ^{13}C NMR and DEPT spectra exhibited 22 carbon signals, including five methyls (one of which was belonging to acetyl group), two methylenes, seven methines and eight quaternary carbons. The ^1H NMR signals at δ_H 1.25 (6 H, s, overlapped), 1.77 (3 H, s), 1.09 (3 H, s) assigned to the methyl groups at C-16, C-17, C-18 and C-19, δ_H 2.15 (3 H, s) belonging to acetyl group, and those at δ 7.59 (1 H, br s) and δ 5.69 (1 H, m) assigned to the methine group at C-1 and C-7, respectively, as well as 3.37 (1 H, d, $J = 2.2$ Hz) and 3.93 (1 H, d, $J = 2.2$ Hz) ascribed to a hydroxymethyl group were characteristic of a tigliane-type diterpenoid (Wu, Sorg, & Hecker, 1994). The ^{13}C NMR spectra exhibited signals for four methyl groups at δ_C 10.2, 15.5, 17.4 and 24.2, a quaternary carbon at δ_C 148.3, a methine at δ_C 107.9 and an α,β-unsaturated ketone at δ_C 134.4, 161.0 and 210.6; in addition, there were four rings deduced from eight unsaturated degrees calculated from the molecular formula, which supported that **1** was a tigliane-type diterpenoid. The complete assignment of ^1H and ^{13}C NMR data were unambiguously made by HSQC and HMBC experiments.

The tigliane diterpene skeleton was further confirmed both by the ^1H–^1H COSY correlations (from H-1 through H-2 to H-3) and HMBC correlations (Figure 1). The presence of α,β-unsaturated ketone was supported by correlations between δ_H 7.59 (H-1) and δ_C 210.6, δ_H 1.77 (H_3-20) and δ_C 134.4, 161.0, 210.6 in the HMBC spectrum. In the HMBC spectrum, the methyl group (belonging to acetyl group) signal at δ_H 2.15 (3 H, s) correlated with the signals of the hydroxylated methine group at δ 77.5 (C-12) and the oxymethine group at δ 69.3 (C-13), which suggested that the acetyl group was attached to C-13. The ROSEY correlations between the methyl group signal at δ 1.25 (H-17) and δ 2.15 (3 H, s) affirmed the deduction (Figure 1). Thus, compound **1** was elucidated as

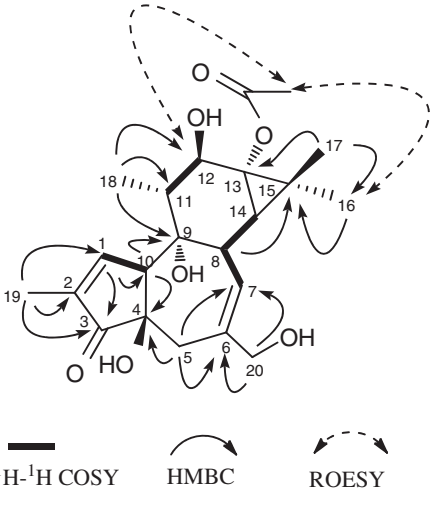

Figure 1. Key 2D NMR correlations of **1.**

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phorbol-13-actate (Figure 2). This compound was ever reported with its bioactivity (Goel, Makkar, Francis, & Becker, 2007; Sahar et al., 2002).

In addition, other known compounds, β-sitosterol (**2**), hydroxyenone (**3**) (Marina, Pietro, & Lucio, 1990), euphol (**4**) (Mohan, Masao, Yasuhiro, Tohru, & Tsuneo, 1990), helioscopinolide B (**5**) (Shizuri et al., 1983), stellasterol (**8**) (Kobayashi & Mitsuhashi, 1974), ent-atisane-14-oxo-3α,13(*R*)-diol-16-ene (**9**) (Wang et al., 2004), 5-hydroxy-7-methoxycoumarin (**10**) (Chen, Huang, Wang, Shao, & Ye, 2010), 4-hydroxy-3-methoxybenzoic acid (**11**) (Yin et al., 2004a), ethyl gallate (**12**) (Zhao, Cui, Cai, & Sun, 2005), ethyl caffeate (**13**) (Zhang, Kong, Li, Gu, & Qin, 1998), 4-O-methyl- ethyl gallate (**14**) (Yin, Cheng, & Lou, 2004 a), quercetin (**15**) (Dong, Li, Liao, Chen, & Sun, 2007) and quercetin-3-O-β-D-glucoside-2″-gallate (**16**) (Yin, Hu, & Lou, 2004 b) were also identified by the NMR spectroscopic analysis and compared with spectral data previously reported.

The cytotoxicity of all compounds against the cancer cell lines HL-60 (human myeloid leukemia), SMMC-7721 (hepatocellular carcinoma), A-549 (human lung cancer), MCF-7 (human breast carcinoma) and SW-480 (colon cancer) was assayed using the MTT method as previously reported (Mosmann, 1983). The results showed that compounds **6** and **7** are moderately active against all the tested human cancer cell lines (Table 1), especially the cytotoxic activity of compound **6** against HL-60 cell line with an IC_{50} value of 3.3 μM while others were inactive ($IC_{50} > 40$ μM). Besides of our studies of anticancer activities of compounds ergosterol (**6**) and 3β,5α-dihydroxy-15β-cinnamoy -loxy-14-oxolathyra-6*Z*,12*E*-diene (**7**), there were other biological activities. The cytotoxicity of ergosterol on LPS-stimulated RAW 264.7 cells with an IC_{50} value of 24.5 μM was examined using the MTT reduction assay and showed that ergosterol acts as an anti-inflammatory substance

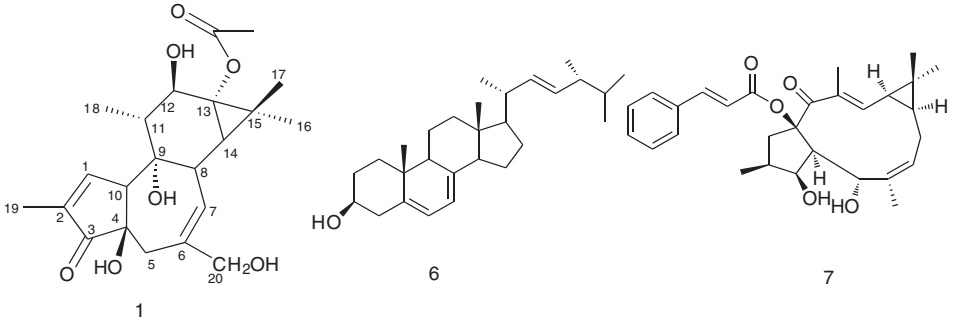

Figure 2. Structures of compounds **1**, **6** and **7.**

Table 1. Cytotoxic activities of compounds **6** and **7** against five human cancer cell lines.

	Samples		Positive control
Cancer cell lines IC_{50} (μM)	**6**	**7**	Cisplatin
HL-60	3.30 ± 0.15**	11.58 ± 0.32**	1.05 ± 0.01
SMMC-7721	15.48 ± 0.29*	14.51 ± 0.95	16.24 ± 0.09
A-549	14.05 ± 0.58**	14.75 ± 0.14**	9.40 ± 0.23
MCF-7	13.00 ± 0.18**	13.35 ± 0.19**	21.90 ± 0.51
SW-480	14.11 ± 0.12**	17.55 ± 0.50*	19.62 ± 0.53

Notes: Values are expressed as mean ± SEM. The statistical significance between the samples and control is presented as $p < 0.05$ (*) and $p < 0.01$ (**).

that inhibits TNF-α production and COX-2 expression in LPS-stimulated RAW 264.7 macrophages *in vitro* (Kuo, Hsieh, & Lin, 2011). Some lathyrane diterpenoids isolated from *Euphorbia micractina* roots showed moderate activity against HIV-1 replication *in vitro* and significant vascular-relaxing activities against phenylephrine-induced vasoconstriction (Tian et al., 2011 b).

3. Experimental

3.1. *General*

NMR spectra were acquired with Bruker AV-400 and DRX-500 spectrometers with TMS as an internal standard at room temperature (δ in ppm, J in Hz). ESIMS and EIMS were carried out on API QSTAR Pulsar I and AV-3000 mass spectrometers, respectively. Silica gel (100–200 and 200–300 mesh), silica gel H (Qingdao Marine Chemical Ltd., China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for column chromatography (CC). MPLC was performed on a BUCHI Chromatography System including pump module C-605, columns packed with RP-18 silica gel (40-60 μm, Amersham Biosciences, Sweden). Fractions were monitored by TLC, and spots were visualised using sulphuric acid–ethanol (95%) reagent.

3.2. *Plant material*

The whole plants of *E. tangutica* were collected in August 2008 at Datong, Qinghai Province of People's Republic of China. The plant was authenticated by Prof. Liu Shang-Wu (Northwest Institute of Plateau Biology, Chinese Academy of Sciences) and a voucher specimen (E.Y2008081105) was deposited at the Northwest Institute of Plateau Biology, Chinese Academy of Sciences.

3.3. *Extraction and isolation*

Fresh whole plants of *E. tangutica* (11 kg) were grinded and extracted with 90% ethanol (EtOH) at room temperature for 4 times each 5 days, and evaporated by rotary evaporator. The concentrated extract was suspended in H_2O and then partitioned successively with ethyl acetate (EtOAC) and *n*-butanol, respectively.

The ethyl acetate layer was evaporated at 50°C in vacuum to remove solvent, and then subjected to MCI CC eluted with 90% ethanol (EtOH) and acetone, respectively. The 90% EtOH part (85 g) was chromatographed over a silica gel column eluting with chloroform ($CHCl_3$):acetone gradient system (40:1→1:1) to obtain 12 fractions. Fr.3 (1.2 g) was separated by silica gel CC with petroleum ether (PE):actone gradient system (10:1→1:1) to give four subfractions (Fr.3 a, Fr.3 b, Fr.3 c and Fr.3 d). Fr.3 a was further subjected to Sephadex LH-20 eluted with $CHCl_3$:MeOH (1:1) and then to silica gel CC with petroleum ether (PE):ethyl acetate (EtOAC) gradient system (8:1→1:1) to afford **2** (10 mg), **3** (15 mg) and **4** (64 mg). Fr.3 c was subjected to silica gel CC with PE:EtOAC gradient system (10:1→1:1) to afford **6** (8 mg) and **8** (13 mg). Fr.5 (1.9 g) was separated by silica gel CC with petroleum ether (PE):actone gradient system (8:1→1:1) to give three subfractions (Fr.5 a, Fr.5 b and Fr.5 c). Fr.5 b was further subjected to silica gel CC with PE:EtOAC gradient system (5:1→1:1) to give **5** (21 mg). Fr.6 was chromatographed over MPLC eluted with MeOH–H_2O gradient system (5% till 100%) to give four subfractions (Fr.6 a, Fr.6 b, Fr.6 c and Fr.6 d). Fr.6 b was continued to be on Sphadex LH-20 ($CHCl_3$: MeOH, 1:1) to afford **7** (18 mg), **9** (23 mg) and **10** (33 mg). Fr.6 c was subjected to silica gel CC with PE:actone gradient system (6:1→1:1) to give **1** (28 mg). Fr.8 (2.4 g) was chromatographed over an ODS column eluted with MeOH–H_2O gradient system (20% till 100%) to afford **11** (24 mg), **12** (168 mg), **13** (46 mg), **14** (35 mg). Fr.10 (2.3 g) was subjected to MPLC

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eluted with MeOH–H_2O gradient system (10% till 100%) to obtain three subfractions, and each subfraction was further on Sphadex LH-20 eluted with MeOH to afford **15** (39 mg) and **16** (87 mg).

Phorbol-13-actate (**1**): white amorphous powder, ESI-MS (neg.): *m/z* 405 $[M–H]^-$; ^{1}H-NMR (CD_3OD, 400 MHz): δ 1.09, 1.25, 1.26, 1.77 (3 H each, all s, H_3-18, 16, 17, 19), 2.15 (3 H, s, –OAC), 1.08–1.10 (1 H, overlapped, H-14), 2.04 (1 H, m, H-11), 2.45 (1 H, d, $J = 19.0$ Hz, H-5 a), 2.55 (1 H, d, $J = 19.0$ Hz, H-5 b), 3.14 (1 H, br s, H-10), 3.22 (1 H, m, H-18), 3.28 (1 H, br s, H-8), 3.37 (1 H, d, $J = 2.2$ Hz, H-20 a), 3.93 (1 H, d, $J = 2.2$ Hz, H-20 b), 3.96 (1 H, br s, H-12), 7.59 (1 H, s, H-1); ^{13}C-NMR (CD_3OD, 100 MHz): δ 161.0 (C-1), 134.4 (C-2), 210.6 (C-3), 74.7 (C-4), 38.6 (C-5), 142.4 (C-6), 129.9 (C-7), 40.0 (C-8), 79.7 (C-9), 57.5 (C-10), 46.1 (C-11), 77.5 (C-12), 69.3 (C-13), 36.6 (C-14), 27.0 (C-15), 24.2 (C-16), 17.4 (C-17), 15.5 (C-18), 10.2 (C-19), 68.1 (C-20), 21.1 and 175.9 (–OAc).

Ergosterol (**6**): white amorphous powder, ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.65 (3 H, s, H-24), 0.84 (3 H, d, $J = 6.7$ Hz, H-27), 0.85 (3 H, d, $J = 6.7$ Hz, H-28), 0.93 (3 H, d, $J = 5.6$ Hz, H-26), 0.97 (3 H, s, H-23), 1.06 (3 H, d, $J = 6.8$ Hz, CH_3), 5.59 (1 H, dd, $J = 5.7$, 2.6 Hz, H-6), 5.41 (1 H, dd, $J = 5.7$, 2.5 Hz, H-7), 5.22 (2 H, m, H-19, 20), 3.64 (1 H, m, H-3), 2.47 (1 H, m, H-18), 2.30 (1 H, t, $J = 8.5$ Hz, H-4), 1.67-2.07 (11 H, m), 1.27-1.50 (7 H, m). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 38.3 (C-1), 31.9 (C-2), 70.4 (C-3), 40.7 (C-4), 139.7 (C-5), 119.5 (C-6), 116.2 (C-7), 141.3 (C-8), 46.2 (C-9), 37.0 (C-10), 22.9 (C-11), 39.0 (C-12), 42.8 (C-13), 54.5 (C-14), 31.9 (C-15), 19.6 (C-16), 55.7 (C-17), 40.7 (C-18), 135.5 (C-19), 131.9 (C-20), 42.8 (C-21), 33.0 (C-22), 16.2 (C-23), 12.0 (C-24), 21.1 (C-25), 17.6 (C-26), 19.6 (C-27), 19.9(C-28). ^{1}H-NMR and ^{13}C-NMR data was consistent with the published data (Zhang, Ou, & Lou, 2007).

3β,5α-Dihydroxy-15β-cinnamoyloxy-14-oxolathyra-6 *Z*,12 *E*-diene (**7**): colourless needles, ESI-MS (neg.): *m/z* 463 $[M–H]^{-1}$; H-NMR ($CDCl_3$, 500 MHz): δ 0.84, 1.10, 1.18, 1.87 (3 H each, all s, H_3-19, 18, 17, 20), 1.04 (3 H, d, $J = 9.0$ Hz, H-16), 3.52 (1 H, dd, $J = 7.2$, 12.8 Hz, H-1 a), 4.38 (1 H, t, $J = 3.2$ Hz, H-3), 5.27 (1 H, dd, $J = 3.5$, 11.6 Hz, H-7), 5.46 (1 H, d, $J = 6.5$ Hz, H-5), 6.47 (1 H, d, $J = 11.5$ Hz, H-12), 6.43 (1 H, d, $J = 16.0$ Hz, H-2′), 7.67 (1 H, d, $J = 16.0$ Hz, H-3′), 7.49 (2 H, m, H-5′, 9′), 7.45 (3 H, m, H-6′, 7′, 8′); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 47.3 (C-1), 37.7 (C-2), 79.7 (C-3), 54.8 (C-4), 65.7 (C-5), 135.3 (C-6), 126.7 (C-7), 23.8 (C-8), 31.8 (C-9), 25.5 (C-10), 27.7 (C-11), 143.2 (C-12), 133.1 (C-13), 197.6 (C-14), 92.6 (C-15), 13.3 (C-16), 17.6 (C-17), 28.6 (C-18), 16.8 (C-19), 11.9 (C-20), 165.8 (C-1′), 117.9 (C-2′), 146.2 (C-3′), 134.1 (C-4′), 128.1 (C-5′, 9′), 128.9 (C-6′, 8′), 130.6 (C-7′). ^{1}H-NMR and ^{13}C-NMR data was consistent with the published data (Wang et al., 2008).

3.4. *Cytotoxic assays*

Cytotoxic assays were performed in 96-well plates at different concentrations by the MTT method. The human cancer cell lines were supplied by the American Type Culture Collection. The cell lines were maintained in RPMI-1640 or in DMEM medium with 10% FBS. The cell lines were cultured at 37°C in an atmosphere of 5% CO_2 in air (100% humidity). 100 µL of adherent cells was seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before the addition of test compounds, while suspended cells were seeded just before drug addition with an initial density of 1×10^5 cell mL^{-1}. Each tumour cell line was exposed to the test compound at concentrations of 0.0625, 0.32, 1.6, 8 and 40 µM in triplicates for 48 h. After incubation for 48 h, an MTT assay was performed at the end of incubation. After compounds were treated, cell viability was detected and a cell growth curve was graphed (Figures S1 and S2). The number of viable cells was proportional to the extent of formazan production. IC_{50} values were calculated by Reed's

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method (Reed & Muench, 1938). Briefly, 20 μL of MTT labelling mixture solution were added to each well, and the cells was incubated for 4 h. The formazan dye formed is soluble in aqueous solutions and the optical density at 595 nm was compared with that of control wells with a screening multiwell spectrophotometer enzyme-linked immunosorbent assay (ELISA) reader.

3.5. *Statistical analysis*

All data presented in this study have been repeated at least thrice from three independent experiments. The results are expressed as a mean ± SEM, and the data were analysed using one-way ANOVA followed by Tukey's test for significant difference. The minimum criterion for statistical significance was set at $p < 0.05$.

4. Conclusion

Sixteen known compounds including diterpenoids, triterpenoids, flavonoids, coumarins and phenolic acids, were isolated from the ethanolic extraction of the whole plants of *Euphorbia tangutica*. Most of the compounds assayed were inactive against the cancer cell lines HL-60, SMMC-7721, A-549, MCF-7 and SW-480 using the MTT method. However, ergosterol displayed significant activity against HL-60 cell line with an IC_{50} value of 3.3 μM. Further studies on the mechanism of anticancer activity of this compound are needed. In addition, the results may also contribute to chemotaxonomic analysis of this complex genus.

Supplementary material

Experimental details, including Figures S1 and S2, are available online.

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