

Full Length Research Paper

# Assignments of $^1\text{H}$ and $^{13}\text{C}$ NMR signals of *Euphorbia* factor L1 and investigation of its anticancer activity *in vitro*

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**In this article, we reported the isolation and characterization of *Euphorbia* factor L1 from Caper *Euphorbia* seed. The complete  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals were assigned with the help of 1D, 2D NMR techniques. In addition, the anticancer activities of *Euphorbia* factor L1 were investigated. It exhibited potent cytotoxicity to KB, KBv200, MCF-7 and MCF-7/ADR cells with the  $\text{IC}_{50}$  values of  $30.83 \pm 2.93$ ,  $28.11 \pm 3.08$ ,  $39.47 \pm 4.03$  and  $42.69 \pm 4.27$   $\mu\text{g/ml}$ , respectively.**

**Key words:** Caper *Euphorbia* seed, NMR signals, anticancer activity.

## INTRODUCTION

The genus *Euphorbia* is the largest in the spurge family, comprising more than 2000 species. Some species of the genus *Euphorbia* have been used as medicinal plants for treatment of skin diseases, gonorrhoea, migraine, intestinal parasites and cancer (Shi et al., 2008). *Euphorbia lathyris* L. has received worldwide attention as a renewable source of industrial raw materials (hydrocarbons and oleic acid) (Appendino, et al., 1999). In China, seeds of *E. lathyris* L. (Caper *Euphorbia* seed) were used as medication for cancer (Hohmann et al., 2008). A series of diterpenes based on the lathyrene skeleton (L1-L8) have been isolated from the seeds (Adolf et al, 1970; Adolf et al, 1984; Narayanan et al., 1984; Itokawa et al., 1990; Shi et al., 2008). Biological activity of these lathyrene-type diterpenes have been carried out showing cytotoxicity to cancer cells and ability of reversing MDR (Appendino et al., 2003). Recently, we isolated *Euphorbia* factor L1 from Caper *Euphorbia* seed. To obtain the detailed structure information of this compound we carried out the NMR tests

including 1D  $^1\text{H}$ , 1D  $^{13}\text{C}$ , Heteronuclear Multiple-Bond Correlation (HMBC), Heteronuclear Multiple Quantum Coherence (HMQC), H-H Correlation spectroscopy, (H-H COSY), Distortionless Enhancement by Polarization Transfer (DEPT) and High Resolution Electrospray ionization Mass Spectrometry (HREIMS). In this article, we reported the complete assignments of  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals. In addition, the anticancer activity of this compound was firstly investigated by MTT assay for cytotoxicity and cell viability.

## MATERIALS AND METHODS

### General

Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. NMR data were recorded on a Varian Inova-500 NB spectrometer,  $\text{CDCl}_3$  as solvent and TMS as internal standard. Chemical shifts ( $\delta$ ) are expressed in ppm with reference to the TMS. Mass spectra were acquired on a VG-ZAB mass spectrometer. IR spectra were obtained on a Nicolet 5DX-FTIR spectrophotometer. Column chromatography was performed either on silica gel (200-300 mesh, Qingdao Marine Chemical, Qingdao, People's Republic of China) or silica gel H (10-40  $\mu\text{m}$ , Qingdao Marine Chemical). Fractions were monitored by TLC, visualized by

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heating silica gel plates sprayed with 5% H<sub>2</sub>SO<sub>4</sub> in EtOH. Melting points were detected on a Fisher–Johns hot-stage apparatus and were uncorrected. Cell viability was measured by Model 550 Microplate reader (BIO-RAD, USA).

### Chemicals and reagents

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), was purchased from Sigma Chemical Co. Adriamycin (ADR) and vincristine (VCR) was from ZhuHai MingZhi Pharmaceuticals Inc. (Guangdong province, China). Other routine laboratory reagents were obtained from commercial sources of analytical or HPLC grade.

### Plant

Caper *Euphorbia* seed was purchased from Anguo, Hebei province and identified by professor Hu-biao Chen (School of Chinese Medicine, Hong Kong Baptist University). A voucher specimen of the plant is deposited at Herbarium of Department of Pharmaceutical Sciences, School of Basic Science, Guangzhou Medical College.

### Extraction and isolation

Powder of Caper *Euphorbia* seed (14 kg) was refluxed with 95 % EtOH to collect an ethanolic extract. The extract was concentrated and suspended in H<sub>2</sub>O and partitioned successively with EtOAc and n-BuOH to afford corresponding extracts. The EtOAc extract was separated by silical gel and Sephadex LH-20 column chromatography to afford *Euphorbia* factor L1 (2.0 g).

### Cell lines and cell culture

KB and KBv200 are human epidermoid carcinoma cell lines obtained from Chinese Academy of Medical Sciences (Beijing, China). KBv200 is a classic multidrug resistant cell line expressing high levels of P-gp cloned from drug-sensitive parental KB cells by stepwise exposure to increasing doses of VCR and ethylmethane sulfonate (EMS) mutagenesis. Comparing with KB cell line, KBv200 cell line was resistant to VCR about 100-fold. MCF-7 and MCF-7/ADR are human breast carcinoma cell lines obtained from Professor Zhe-Sheng Chen (St. John's University, USA). MCF-7/ADR cells overexpressing P-gp are derivative from MCF-7 by stepwise exposure to increasing doses of ADR. Cells were maintained in RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). All cells were cultured in a humidified atmosphere incubator of 5% CO<sub>2</sub> and 95% air at 37°C (Zhang et al., 2007).

### MTT assay

Cells were harvested during logarithmic growth phase and seeded in 96-well plates at a density of  $4 \times 10^4$  cells/ml in a final volume of 190 µl/well. After incubation of 24 h, 10 µl of tested compound of full range concentrations was added. After 68 h treatment, 10 µl MTT (10 mM stock solution of saline) was added to each well for 4 h. Subsequently, the plates were centrifuged at 1,500 rpm for 10 min and the supernatant was removed, and MTT crystals of each well were solubilized with 100 µl anhydrous DMSO. Thereafter, cell viability was measured by Model 550 Microplate reader (BIO-RAD, USA) at 540 nm with 655 nm as reference filter. Experiments were performed at least three times. The 50% inhibitory concentration (IC<sub>50</sub>) was determined as the compound concentration causing

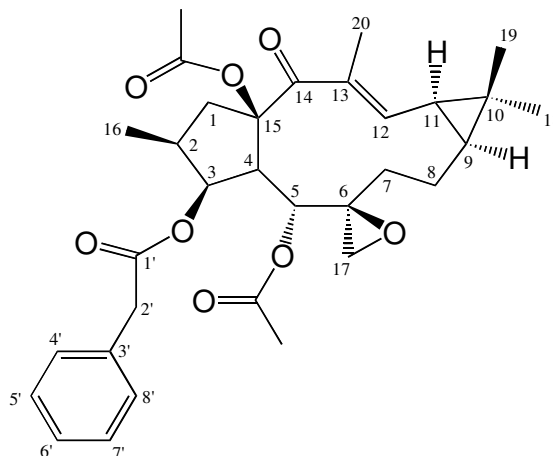


Figure 1. Structure of *Euphorbia* factor L1.

50% reduction in cell viability and calculated from the cytotoxicity curves (Bliss's software). Cell survival was calculated using the following formula:

$$\text{Survival (\%)} = (\text{mean experimental absorbance} / \text{mean control absorbance}) \times 100\% \text{ (Zhang et al., 2009).}$$

### Statistical analysis

Results were performed by t-test or one-way ANOVA with SPSS 13.0 software (SPSS Inc., USA). Data were presented as means  $\pm$  SD of at least triplicate determinations.  $P < 0.05$  was indicative of significant difference.

## RESULTS AND DISCUSSION

*Euphorbia* factor L1 (Figure 1) was obtained as a colorless crystals from dichloromethane-petroleum ether. The molecular formula C<sub>32</sub>H<sub>40</sub>O<sub>8</sub> was given by high resolution electrospray ionization mass spectrometry (HREIMS) showing  $m/z$  552.272, corresponding to 13 degrees of unsaturation. The <sup>1</sup>H NMR displayed five benzene protons with the chemical shifts between 7.25 and 7.31. In the <sup>13</sup>C NMR, 30 carbon signals were observed. Information supplied by the molecular formula, <sup>1</sup>H NMR and <sup>13</sup>C NMR implied that *Euphorbia* factor L1 posses one mono-substituted benzene ring. The <sup>13</sup>C NMR showed four carbonyls of 196.7, 170.7, 170.6 and 169.4, respectively. The DEPT spectrum indicated that *Euphorbia* factor L1 has six methyl carbons, five methylene carbons, ten methine carbons, and nine quaternary carbons. We compared the 1D NMR data of *Euphorbia* factor L1 with those of (Shi et al., 2008; Appendino, et al., 1999; Itokawa et al., 1990). Here, the NMR signals were completely assigned as listed in Table 1 on the basis of <sup>1</sup>H, <sup>13</sup>C NMR and 2D NMR (HMQC, HMBC and COSY). The main HMBC correlations of *Euphorbia* factor L1 are shown in Figure 2.

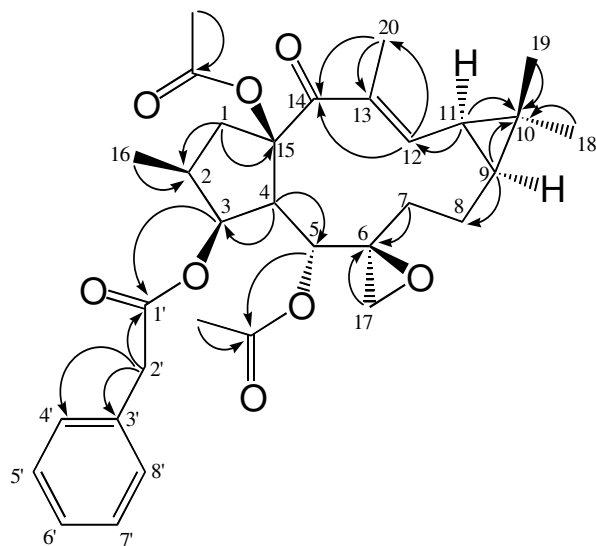
The main chemical data of *Euphorbia* factor L1 were

**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR (500 and 125 MHz,  $\delta$  in ppm, multiplicities, J in Hz) spectral data and HMBC correlations of *Euphorbia* factor L1 (in  $\text{CDCl}_3$ ).

Position	$^{13}\text{C}$	DEPT	$^1\text{H}$	HMBC (H→C)
1	47.8	CH <sub>2</sub>	3.32 (dd, 1H, 8, 14) 1.36 (dd, 1H, 12.5, 14.5)	2, 3, 4, 14, 15,
2	37.7	CH	2.1 (m, 1H)	1, 3, 4, 5, 16
3	80.5	CH	5.48 (t, 1H, 3)	1, 1', 15,
4	49.9	CH	1.87 (m, 1H)	5, 6
5	65.1	CH	6.24 (d, 1H, 9)	4, 6, 7, 15, 5-CO
6	58.8	C		
7	33.5	CH <sub>2</sub>	2.10 (m, 1H) 0.93 (m, 1H)	5, 6, 8, 9, 17,
8	20.0	CH <sub>2</sub>	2.12 (m, 1H) 1.73 (m, 1H)	6, 9, 17,
9	34.7	CH	1.09 (m, 1H)	18, 19
10	25.5	C		
11	28.95	CH	1.48 (dd, 1H, 8, 11.5)	9, 10, 12, 13
12	143.5	CH	6.59 (dd, 1H, 1, 11.5)	14, 9, 20
13	135.9	C		
14	196.7	C		
15	91.7	C		
16	13.4	CH <sub>3</sub>	0.66 (d, 1H, 6.5)	1, 2, 3,
17	55.3	CH <sub>2</sub>	2.48 (d, 1H, 3) 2.30 (dd, 1H, 3, 1)	5, 6, 7
18	28.8	CH <sub>3</sub>	1.21 (s, 3H)	9, 10, 11, 19
19	16.7	CH <sub>3</sub>	1.22 (s, 3H)	9, 10, 11, 18
20	12.2	CH <sub>3</sub>	1.84 (s, 3H)	12, 13, 14
5-COCH <sub>3</sub>	CH <sub>3</sub> 20.9 CO 170.6	CH <sub>3</sub> C	2.01 (s, 3H)	5-CO
15-COCH <sub>3</sub>	CH <sub>3</sub> 21.8 CO 169.4 1' 170.7 2' 41.4 3' 133.7	CH <sub>3</sub> C C CH <sub>2</sub> C	2.12 (s, 3H)	15-CO
3-OAcPh	6' 129.3 4', 8' 128.4 5', 7' 127.1	CH CH CH	7.27 (m, 1H) 7.29 (m, 2H) 7.25 (m, 2H)	 2', 3' 2', 3'

listed as the following: (2S\*, 3S\*, 4R\*, 5R\*, 6R\*, 11S\*, 15R\*)-5,15-Diacetoxy-3-phenylacetoxy-14-oxolathyrane 6 (17),12E-diene-6(17)-epoxide; colorless crystal; mp 196-198 °C;  $[\alpha]^{25\text{D}} +108^\circ$  (c 0.10,  $\text{CH}_2\text{Cl}_2$ ); IR (KBr) 1742, 1653, 1624, 1456, 1375, 1265, 1128, 903, 727  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 1; HREIMS m/z 552.272. MTT assay showed that *Euphorbia* factor L1 displayed potent cytotoxicity to KB, KBv200, MCF-7 and MCF-7/ADR cells. *Euphorbia* factor L1 inhibited cell proliferation in a concentration-dependent manner in KB, KBv200, MCF-7 and MCF-7/ADR cells after 72 h treatment. The  $\text{IC}_{50}$  values were  $30.83 \pm 2.93$ ,  $28.11 \pm$

$3.08$ ,  $39.47 \pm 4.13$  and  $45.69 \pm 4.20$   $\mu\text{g}/\text{ml}$ , respectively. The data suggested that *Euphorbia* factor L1 exhibited similar cytotoxicity to drug-sensitive parental KB cells and MDR KBv200 cells ( $P > 0.05$ ). Furthermore, *Euphorbia* factor L1 exhibited similar cytotoxicity to drug-sensitive parental MCF-7 cells and MDR MCF-7/ADR cells ( $P > 0.05$ ). KBv200 and MCF-7/ADR cells are MDR (multidrug resistance) cells overexpressing P-gp. These results implied that *Euphorbia* factor L1 showed potent cytotoxicity to sensitive cells and MDR cells. It has been reported that lathyrane diterpenes have the ability of



**Figure 2.** The main HMBC correlations of *Euphorbia* factor L1 (H→C).

reversing MDR (Jiao et al., 2009; Duarte, et al., 2008; Aiyelaagbe et al., 2007; Duarte et al., 2007). The fact that *Euphorbia* factor L1 was effective to MDR cancer cells (KBv200 and MCF-7/ADR cells) might be related to the reversal of MDR.

## Conclusion

In summary, *Euphorbia* factor L1 was isolated from Caper *Euphorbia* seed and its complete NMR signals were assigned with the aid of  $^1\text{H}$ ,  $^{13}\text{C}$ , DEPT, HMBC, HMQC, H-H COSY and HREIMS. Also, the anticancer activity of *Euphorbia* factor L1 was investigated by MTT assay. The  $\text{IC}_{50}$  values were  $30.83 \pm 2.93$ ,  $28.11 \pm 3.08$ ,  $39.47 \pm 4.13$  and  $45.69 \pm 4.20$   $\mu\text{g/ml}$  to KB, KBv200, MCF-7 and MCF-7/ADR cells, respectively.

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