Arch Pharm Res Vol 26, No 11, 925-928, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Cytotoxic Effects of Sesquiterpene Lactones from the Flowers of *Hemisteptia lyrata* B.

Tae Joung Ha, Dae Sik Jang[1], Jong Rok Lee[2], Kyung Dong Lee, Jun Lee[2], Seon Woo Hwang[2], Hwa Jin Jung[2], Sang Hae Nam[3], Ki Hun Park[2], and Min Suk Yang[1]

Institute of Agriculture and Life Science, Gyeongsang National University, Chinju 660-701, Korea, [1]Graduate School of Molecular Life Sciences, Ewha Womans University, Seoul 120-750, Korea, [2]Department of Agricultural Chemistry, Division of Applied Life Science, Gyeongsang National University, Chinju 660-701, Korea, and [3]Department of Food Processing, Chinju National University, Chinju 660-758, Korea

(Received January 23, 2003)

Four guaia-12,6-olide type sesquiterpene lactones, aguerin B (**1**), 8α-acetoxyzaluzanin C (**2**), cynaropicrin (**3**), and deacylcynaropicrin (**4**), were isolated from the flowers of *Hemisteptia lyrata* Bunge. It is the first report on the isolation of compounds **1-4** from Hemisteptia species. All the isolates (**1-4**) were examined for their cytotoxic activity against SK-OV-3, LOX-IMVI, A549, MCF-7, PC-3, and HCT-15 human cancer cell lines.

Key words: *Hemisteptia lyrata* Bunge, Sesquiterpene lactone, Aguerin B, 8α-Acetoxyzaluzanin C, Cynaropicrin, Deacylcynaropicrin, Cytotoxic activity

INTRODUCTION

Sesquiterpene lactones (SQLs) have a distinctive functional group like α,β-unsaturated lactone moiety, which represent a reactive receptor site for biological nucleophile, in particular, thiol and amino groups. Therefore, SQLs can cause irreversible alkylations of L-cysteine or thiol-containing enzyme (Hoffmann and Rabe, 1985). Consequently, a wide spectrum of biological activities involving such actones has been reported. Structure activity studies have demonstrated that thiol-containing enzymes such as phospho fructokinase (Hall *et al.*, 1978), glycogen synthase (Grippo *et al.*, 1992), DNA polymerase (Hall *et al.*, 1978; Lee *et al.*, 1977), and thymidylate synthase (Hall *et al.*, 1987) are inhibited by lactones.

The whole plant of *Hemisteptia lyrata* B. (Compositae) has been used as an anti-febrile, anti-bleeding, anti-tumor, anti-bacterial, and anti-inflammatory remedy (People press, 1977) and also has been consumed as young plants in early spring in the form of herb salads. Several flavonoids (Ha *et al.*, 2002a), polyacetylenes (Ha *et al.*, 2002b), and sesquiterpene lactones (Jang *et al.*, 1998; Jang *et al.*, 1999; Ha *et al.*, 2002c; Ha *et al.*, 2003) have been reported from this plant. In the present paper, we will describe the isolation and the total assignments for the proton and carbon NMR signals of four anti-tumoral guaianolide sesquiterpene lactones (**1-4**). Also we compared their *in vitro* cytotoxic activity against human cancer cell lines.

Correspondence to: Min Suk Yang, Department of Agricultural Chemistry, Division of Applied Life Science, Gyeongsang National University, Chinju 660-701, Korea
Tel: 82-55-751-5467, Fax: 82-55-757-0178
E-mail: msyang@nongae.gsnu.ac.kr

MATERIALS AND METHODS

General procedure

Optical rotations were recorded on a PERKIN-ELMER polarimeter. IR spectra were recorded on a Bruker IFS66 infrared Fourier transform spectrophotometer (KBr) and UV spectra were measured on a Beckman DU650 spectrophotometer. Low-resolution EIMS and HREIMS were obtained on a JEOL JMS-700 spectrometer. ^{1}H- and ^{13}C-NMR spectra along with 2D-NMR data were obtained on a Bruker AM 500 (^{1}H-NMR at 500 MHz, ^{13}C-NMR at 125 MHz) spectrometer in $CDCl_3$ solution.

Plant materials

The flowers of *Hemisteptia lyrata* Bunge were collected at Parkjeon, Hamyang, Korea in June 1998, and identified by prof. Myong Gi Chung. A voucher specimen (Park, K. H. 103) of this raw material has been deposited at the

Herbarium of the Gyeongsang National University (GNUC).

Isolation

The dried flowers (1 kg) were extracted with $CHCl_3$ (30 L) at room temperature for 72 h. The extract was washed with brine, dried over anhydrous Na_2SO_4, and then concentrated to give a thickish residue (120 g). The residue was chromatographed on a silica gel (1.2 kg) column eluted with a gradient of 100% hexane to 100% EtOAc and then to 20% MeOH to afford twenty fractions (F1-F20). Fraction F13 (1.8 g) was eluted with $CHCl_3$-EtOAc mixtures of increasing polarity (49:1→1:1) by using silica gel column chromatography. Altogether, 120 fractions (100 mL each) were collected and combined to give nine major subfractions (F13-1 through F13-9), based on the comparison of TLC profiles after examination by short-wave UV light (254 nm) and by spraying with 10% v/v sulfuric acid in water. Continually, subfraction F13-6 (200 mg) was purified by repeated chromatography on silica gel [1. *n*-hexane-ether (9:1), 2. *n*-hexane-EtOAc (1:1)] to give compound **1** (30 mg). Subfraction F13-7 (140 mg) was also purified by repeated chromatography on silica gel [1. *n*-hexane-EtOAc (9:1), 2. CH_2Cl_2-EtOAc (19:1)] to give compound **2** (16 mg). Compounds **3** (62 mg) and **4** (23 mg) were isolated from fraction F16 (4.2 g) using repeatedly preparative TLC [**3**: 1. CH_2Cl_2-EtOAc (49:1), 2. $CHCl_3$-MeOH (19:1); **4**: 1. $CHCl_3$-EtOH (49:1), 2. *n*-hexane-Et_2O (1:4)].

Aguerin B (1)

Colorless oil; $[\alpha]_D^{25}$ +113.6° (*c* 1.58, $CHCl_3$); UV ($CHCl_3$) λ_{max} : 235 nm; MS *m/z* (EI, 70 eV, rel. int.): 330 (M^+, 23), 261 (6), 244 (75), 226 (54), 216 (34), 198 (27), 148 (38), 119 (32), 69 (100), 55 (13); IR ν_{max} 3510, 3080, 2930, 1770, 1725 and 1650 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.73 (1H, ddd, *J* = 13.2, 11.0, 7.6 Hz, H-2b), 1.99 (3H, d, *J* = 1.0 Hz, H-4'), 2.22 (1H, m, H-2a), 2.38 (1H, dd, *J* = 14.5, 3.9 Hz, H-9a), 2.71 (1H, dd, *J* = 14.5, 5.2 Hz, H-9b), 2.84 (1H, dd, *J* = 10.6, 8.9 Hz, H-5), 2.97 (1H, m, H-1), 3.19 (1H, m, H-7), 4.25 (1H, dd, *J* = 10.6, 9.0 Hz, H-6), 4.55 (1H, m, H-3), 4.94 (1H, d, *J* = 1.5 Hz, H-14b), 5.08 (1H, m, H-8), 5.14 (1H, d, *J* = 1.1 Hz, H-14a), 5.36 (1H, dd, *J* = 1.7, 1.7 Hz, H-15b), 5.49 (1H, dd, *J* = 1.9, 1.9 Hz, H-15a), 5.60 (1H, dd, *J* = 3.0, 1.3 Hz, H-13b), 5.67 (1H, d, *J* = 1.5 Hz, H-3'b), 6.18 (1H, d, *J* = 1.3 Hz, H-3'a), 6.21 (1H, dd, *J* = 3.4, 2.0 Hz, H-13a); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: see Table I.

8α-Acetoxyzaluzanin C (2)

Colorless oil; $[\alpha]_D^{25}$ +44.4° (*c* 0.40, $CHCl_3$); UV ($CHCl_3$) λ_{max} : 242 nm; MS *m/z* (EI, 70 eV, rel. int.): 304 (M^+, 2), 279 (6), 262 (4), 244 (7), 226 (7), 197 (9), 166 (35), 149 (43), 124 (100), 95 (24), 69 (21); IR ν_{max} : 3500, 2900, 1760, 1715 and 1650 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.73 (1H, ddd, *J* = 13.2, 10.7, 7.4 Hz, H-2b), 2.15 (3H, s, H-2'), 2.23 (1H, ddd, *J* = 13.3, 7.4, 7.4 Hz, H-2a), 2.35 (1H, dd, *J* = 14.3, 4.2 Hz, H-9a), 2.69 (1H, dd, *J* = 14.3, 5.2 Hz, H-9b), 2.83 (1H, m, H-5), 2.96 (1H, ddd, *J* = 10.5, 8.3, 8.3 Hz, H-1), 3.10 (1H, dddd, *J* = 9.4, 9.4, 3.2, 3.2 Hz, H-7), 4.20 (1H, dd, *J* = 10.3, 9.2 Hz, H-6), 4.55 (1H, dd, *J* = 7.2, 7.2 Hz, H-3), 4.95 (1H, s, H-14b), 5.00 (1H, m, H-8), 5.14 (1H, s, H-14a), 5.36 (1H, s, H-15b), 5.49 (1H, s, H-15a), 5.63 (1H, d, *J* = 3.0 Hz, H-13b), 6.24 (1H, d, *J* = 3.4 Hz, H-13a); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: see Table I.

Table I. ^{13}C-NMR data of sesquiterpene lactones (**1-4**) from *H. lyrata*

C	1[a]	2[a]	3[a]	4[a]
1	45.3 *d*	45.3 *d*	45.3 *d*	45.3 *d*
2	39.0 *t*	39.1 *t*	39.1 *t*	39.4 *t*
3	73.1 *d*	78.6 *d*	73.7 *d*	73.8 *d*
4	152.3 *s*	152.3 *s*	152.2 *s*	152.6 *s*
5	51.3 *d*	51.3 *d*	51.4 *d*	51.5 *d*
6	78.6 *d*	73.8 *d*	78.6 *d*	78.7 *d*
7	47.4 *d*	47.4 *d*	47.6 *d*	51.1 *d*
8	73.7 *d*	74.0 *d*	74.3 *d*	72.1 *d*
9	37.1 *t*	37.5 *t*	37.0 *t*	41.4 *t*
10	141.9 *s*	141.8 *s*	141.8 *s*	142.8 *s*
11	137.4 *s*	137.4 *s*	137.4 *s*	138.1 *s*
12	169.2 *s*	169.1 *s*	169.2 *s*	169.7 *s*
13	122.6 *t*	122.5 *t*	122.7 *t*	123.1 *t*
14	118.1 *t*	118.1 *t*	118.2 *t*	117.1 *t*
15	113.5 *t*	113.5 *t*	113.5 *t*	113.2 *t*
1'	166.4 *s*	170.1 *s*	165.4 *s*	
2'	136.0 *s*	21.1 *q*	139.4 *s*	
3'	126.6 *t*		126.6 *t*	
4'	18.3 *q*		62.1 *t*	

[a]Recorded at 500 MHz in $CDCl_3$. Multiplicities were obtained by the DEPT spectrum.

Cynaropicrin (3)

Colorless oil; $[\alpha]_D^{25}$ +134.6° (*c* 1.15, EtOH); UV ($CHCl_3$) λ_{max} : 241 nm; MS *m/z* (EI, 70 eV, rel. int.): 346 (M^+, 5), 262 (10), 244 (37), 226 (22), 216 (13), 198 (15), 148 (24), 91 (49), 85 (100); IR ν_{max} : 3420, 3080, 2930, 1770, 1725 and 1650 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.74 (1H, ddd, *J* = 13.1, 11.1, 7.6 Hz, H-2b), 2.24 (1H, ddd, *J* = 13.2, 7.3, 7.3 Hz, H-2a), 2.40 (1H, dd, *J* = 14.6 3.8 Hz, H-9a), 2.72 (1H, dd, *J* = 14.6, 5.2 Hz, H-9b), 2.84 (1H, dd, *J* = 10.4, 9.0 Hz, H-5), 2.97 (1H, ddd, *J* = 10.8, 7.9, 7.9 Hz, H-1), 3.19 (1H, dddd, *J* = 9.4, 9.4, 3.2, 3.2 Hz, H-7), 4.27 (1H, dd, *J* = 10.6, 9.1 Hz, H-6), 4.38 (2H, s, H-4'), 4.55 (1H, dddd, *J* = 7.4, 7.4, 1.9, 1.9 Hz, H-3), 4.95 (1H, d, *J* =

1.1 Hz, H-14b), 5.14 (2H, m, H-8 and H-14a), 5.36 (1H, dd, J = 1.7, 1.7 Hz, H-15b), 5.49 (1H, dd, J = 1.7,1.7 Hz, H-15a), 5.62 (1H, d, J = 3.1 Hz, H-13b), 5.96 (1H, d, J = 1.2 Hz, H-3'b), 6.20 (1H, d, J = 3.4 Hz, H-13a), 6.34 (1H, d, J = 0.8 Hz, H-3'a); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: see Table I.

Deacylcynaropicrin (4)

Colorless oils; $[\alpha]_D^{20}$ +40.6° (*c* 1.4, $CHCl_3$); UV (MeOH) λ_{max} : 220 nm; MS *m/z* (EI, 70 eV, rel. int.): 262 (M^+, 12), 244 (M^+-H_2O, 10), 226 (M^+-$2H_2O$, 6), 119 (65), 105 (55), 91 (100), 69 (87), 53 (35); IR ν_{max} : 3411, 2926, 1749 and 1157 cm^{-1}; ^{1}H-NMR, (500 MHz, $CDCl_3$) δ: 1.73 (1H, ddd, J = 13.1, 11.0, 7.6 Hz, H-2b), 2.23 (1H, ddd, J = 13.2, 7.3, 7.3 Hz, H-2a), 2.29 (1H, dd, J = 14.0, 3.9 Hz, H-9a), 2.70 (1H, dd, J = 14.0, 5.1 Hz, H-9b), 2.78 (1H, m, H-7), 2.81 (1H, m, H-5), 2.97 (1H, ddd, J = 10.8, 8.3, 8.3 Hz, H-1), 3.96 (1H, m, H-8), 4.15 (1H, dd, J = 10.5, 9.1 Hz, H-6), 4.55 (1H, m, H-3), 4.98 (1H, s, H-14b), 5.13 (1H, s, H-14a), 5.34 (1H, dd, J = 1.7, 1.6 Hz, H-15b), 5.48 (1H, dd, J = 1.7, 1.6 Hz, H-15a), 6.14 (1H, dd, J = 3.1, 0.7 Hz, H-13b), 6.26 (1H, dd, J = 3.4, 0.7 Hz, H-13a); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: see Table I.

Cytotoxic test

Six kinds of human tumor cell lines were used and all cell lines were obtained from the Korean Cell Line Bank (KCLB). Cell line panels included SK-OV-3 (human ovary adenocarcinoma cell), LOX-IMVI (human melanoma cell), A549 (human non small lung cancer cell), MCF-7 (human breast adenocarcinoma cell), PC-3 (human prostate adenocarcinoma cell), and HCT-15 (human colcrectal adenacarcinoma cell), which were derived from six organs. Human cancer cell lines were cultivated in humidified incubators (37°C, 5% CO_2). The cells were grown in RPMI 1640 with additional glutamine (300 mg/L), 1% penicillin/streptomycin, and 10% fetal calf serum. The cells were free from mycoplasm contamination as routinely tested; cells were seeded in 24-well plates and allowed to grow 24 h before treatment. Cytotoxicity was determined as described previously (Skehan *et al.*, 1990), and calculated as survival of treated cells over control cells×100 [% T/C].

RESULTS AND DISCUSSION

Four guaia-12,6-olide type sesquiterpene lactones, aguerin B (**1**) (Gonzalez *et al.*, 1978), 8α-acetoxyzaluzanin C (**2**) (Asakawa and Takemoto, 1979), cynaropicrin (**3**) (Gonzalez *et al.*, 1978), and deacylcynaropicrin (**4**) (Gonzalez *et al.*, 1978) were isolated from the flowers of *Hemisteptia lyrata* Bunge. All the compounds were identified by physical (mp, $[\alpha]_D$) and spectroscopic data measurement (MS, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR) and by comparison with published values. Compounds **1-4** have not been reported previously from Hemisteptia species.

Table II. *In vitro* cytotoxicity of the compounds (**1-4**) and adriamycin (ADR) toward human cell lines

Compound	Cell lines IC_{50} (μg/mL)					
	SK-OV-3	LOX-IMVI	A549	MCF-7	PC-3	HCT-15
1	8.3±1.1	2.7±1.5	8.7 ±1.3	1.1 ±0.3	3.1±0.7	1.4±0.5
2	12.1±2.1	12.3±1.3	>30	14.3 ±1.4	15.2±3.2	7.3±1.6
3	4.7±1.3	2.3±1.3	5.7 ±0.6	1.1 ±0.5	2.8±0.9	0.9±0.4
4	>30	>30	>30	>30	>30	17.1±2.6
ADR	0.3±0.2	0.1±0.2	0.06±0.1	0.14±0.2	0.5±0.1	0.7±0.2

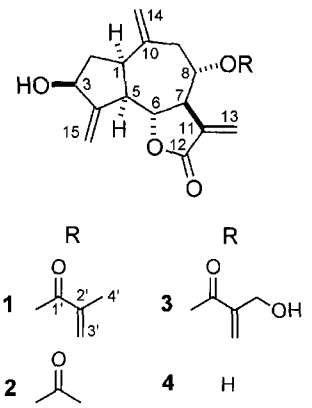

Fig. 1. Structures of compounds **1-4**

Each of compounds **1-4** has an absorption maximum at *ca.* 230 nm in ultraviolet absorption spectra and strong bands at *ca.* 1750 and 1690 cm^{-1} in the infrared absorption spectra, indicating the presence of an α,β-unsaturated lactone group. The infrared spectrum indicated the presence of a hydroxyl group (3500 cm^{-1}) in the all four compounds. Additionally, the infrared (1750 cm^{-1}) and mass (M^+-69 for **1**, M^+-43 for **2**, M^+-85 for **3**) spectra indicated the presence of a methacrylate group, an acetate group, and a 4-hydroxymethacrylate group, respectively. All the four compounds have similar relationships in ^{1}H-^{1}H COSY spectra because of same skeleton. Therefore, the discussion will be paid especially on the compound **3**, which is the most intricate of all. The ^{1}H-NMR spectrum of **3** showed two doublets at δ 5.62 and 6.20, indicating a α-methylene-γ-lactone moiety. These two doublets are due to the two C-13 methylene protons which are both allylic coupled to H-7. Thus, H-7 at δ 3.19 was coupled to H-6 (δ 4.27) and H-8 (δ 5.14). H-8 was coupled to H-9a (δ 2.40) and H-9b (δ 2.72). Moreover, H-6 also coupled with H-5 (δ 2.84), and H-5 showed both vicinal proton with H-1 (δ 2.97) and allyic positions H-15 (δ 5.49 and 5.36). And H-1 exhibited vicinal coupling with H-2a/b at δ 2.24 and δ 1.74,

which exhibited germinal coupling and coupling to H-3 (δ 4.55), and also H-3 was allylic coupled to H-15. All the spectral data of compounds **1-4** showed a very similar pattern. And stereochemistry of **3** was identified with NOESY 2D-NMR experiments. Thus, based on all the above obtained evidence, the structure of **3** was established as 8-*O*-(2-hydroxymethyl-2-propenoyl)-3-hydroxy-4(15),10(14),11(13)guaiatriene-12,6-olide [Cynaropicrin].

All the four compounds were evaluated *in vitro* cytotoxic activity against human cancer cell lines, comprising SK-OV-3, LOX-IMVI, A549, MCF-7, PC-3, and HCT-15 by the sulforhodamine B (SRB) assay method. Compounds **1-3** exhibited significant cytotoxic activity against several cell lines. Compounds **1** and **3** showed the most potent cytotoxic activity against the MCF-7 (IC_{50}: 1.1±0.3 μg/mL and 1.1±0.5 μg/mL, respectively) and HCT-15 cells (IC_{50}: 1.4±0.5 μg/mL and 0.9±0.4 μg/mL, respectively). Whereas compound **4** showed a moderate activity (IC_{50}: 17.1±2.6 μg/mL) only against the HCT-15 cell line.

REFERENCES

Asakawa, Y. and Takemoto, T., Sesquiterpene lactones of *Conocephalum conicum*. *Phytochemistry*, 18, 285-288 (1979).

Gonzalez, A. G., Bermejo, J., Cabrera, I., Massanet, G. M., Mansilla, H., and Galindo, A., Two sesquiterpene lactones from *Centaurea canariensis*. *Phytochemistry*, 17, 955-956 (1978).

Grippo, A. A., Hall, I. H., Kiyokawa, H. Muraoka, O., Shen, Y. C., and Lee, K. H., The cytotoxicity of helenalin, its mono and difunctional esters, and related sesquiterpene lactones in murine and human tumor cells. *Drug Design Discovery*, 8, 191-206 (1992)

Ha, T. J., Hwang, S. W., Jung, H. J., Park, K. H., and Yang, M. S., Apigenin, tyrosinase inhibitor from the flowers of *Hemisteptia lyrata* Bunge. *Agric. Chem. Biotechnol.*, 45, 170-172 (2002a).

Ha, T. J., Jang, D. S., Lee, K. D., Lee, J. R., Park, K. H., and Yang, M. S., Studies on the constituents from the flowers of *Hemisteptia lyrata* Bunge II. *Kor. J. Pharmacogn.*, 33, 92-95 (2002b).

Ha, T. J., Yang, M. S., Pak, Y. B., Lee, J. R., Lee, K. D., Kim, H. M., and Park, K. H., New guaiane-type sesquiterpene lactones from *Hemisteptia lyrata* Bunge. *Heterocycles*, 57, 151-155 (2002c).

Ha, T. J., Park, K. H., Jang, D. S., Lee, J. R., Park, K. M., and Yang, M. S., New sesquiterpene lactones from *Hemisteptia lyrata* bunge. *Heterocycles*, 60, 623-629 (2003).

Hall, I. H., Grippo, A. A., Lee, K. H., Chaney, S. G., and Holbrook, D. J., Effect of helenalin and bis(helenalinyl) malonate on nucleic acid protein synthesis in human KB carcinoma cells. *Pharmaceu. Sci.*, 4, 509-514 (1987).

Hall, I. H., Lee, K. H., and Eigebaly, S. A., Antitumor agents XXVII: Effects of helenalin on anaerobic and aerobic metabolism of ehrlich ascites cells. *J. Pharm. Sci.*, 67, 552-554 (1978).

Hall, I. H., Lee, K. H. Starnes, C. O., Eigebaly, S. A., Ibka, T., Wu Y. S., Kimura, T., and Haruna, M., Antitumor agents XXX: Evaluation of alpha-methylene-gamma-lactone containing agents for inhibition of tumor growth, respiration, and nucleic acid synthesis. *J. Pharm. Sci.*, 67, 1235-1239 (1978).

Hoffmann, H. M. R. and Rabe, J., Synthesis and biological activity of α-methylene-γ-butyrolactones. *Angew. Chem. Int. Ed. Engl.*, 24, 94-110 (1985).

Jang, D. S., Yang, M. S., and Park, K. H., Sesquiterpen lactone from *Hemisteptia lyrata*. *Planta Med.*, 64, 289-290 (1998).

Jang, D. S., Yang, M. S., Ha, T. J., and Park, K. H., Hemistepsins with cytotoxic activity from *Hemisteptia lyrata*, *Planta Med.*, 65, 765-766 (1999).

Lee, K. H., Hall, I. H., Mar, E. C., Starnes, C. O., Elgebaly, S. A., Waddell, T. G., Hadgraft, R. I., Ruffner, C. G., and Weidner, I., Sesquiterpene antitumor agents: Inhibitors of cellula metabolism. *Science*, 196, 533-536 (1977).

Merrill, G. B. and Stevens, K. L., Sesquiterpene lactones from *Centaurea solstitialis*. *Phytochemstry*, 24, 2013-2018 (1985).

Peoples Press. *In Encyclopedia of the Traditional Chinese Materia Medica*. p. 1458-1459. Shanghai, P. R. China (1977).

Shehan, P., Storeng, R., Scudiero, D., Monks, A., Mcmahon, J., Vistica, D., Warren, J. T., Bokesh, H., Kenny, S., and Boyd, M. R., New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.*, 82, 1107-1112 (1990).