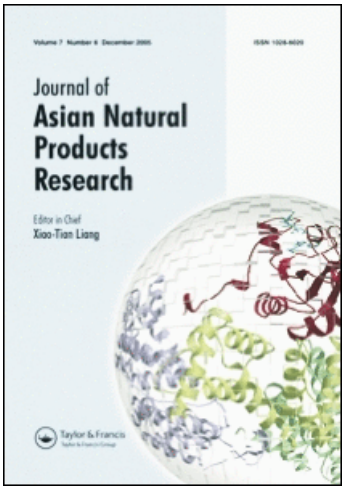

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Labdane-type diterpenes as new cell cycle inhibitors and apoptosis inducers from Vitex trifolia L.

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Labdane-type diterpenes as new cell cycle inhibitors and apoptosis inducers from *Vitex trifolia* L.

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Five labdane-type diterpenes, vitexilactone (**1**), (*rel* 5*S*,6*R*,8*R*,9*R*,10*S*)-6-acetoxy-9-hydroxy-13(14)-labden-16,15-olide (**2**), rotundifuran (**3**), vitetrifolin D (**4**), and vitetrifolin E (**5**), have been isolated from *Vitex trifolia* L., a Chinese folk medicine used to treat cancers, as new cell cycle inhibitors and apoptosis inducers through a bioassay-guided separation procedure and were identified by spectroscopic methods. Compounds **1**–**5** dramatically induced apoptosis both on tsFT210 and K562 cells at higher concentrations while at lower concentrations they inhibited the cell cycle progression of both tsFT210 and K562 cells at the G_0/G_1 phase. MIC values for **1**–**5** for inducing apoptosis and concentration regions for **1**–**5** for inhibiting cell cycle both on tsFT210 and K562 cells have also been determined. Furthermore, the inhibitory effects of **1**–**5** on the proliferation of tsFT210 and K562 cells have been evaluated by MTT assay to obtain IC_{50} values to confirm that **1**–**5** are anticancer components of *Vitex trifolia* L., which exert their anti-proliferative effect on cancer cells through inducing apoptosis and inhibiting the cell cycle. The present results provide labdane-type diterpenes, **1**–**5**, as a new class of cell cycle inhibitors and compounds **1**, **2**, **4**, and **5** as new apoptosis inducers, which also explains, for the first time, the usage of *Vitex trifolia* L. by Chinese people to treat cancers.

Keywords: *Vitex trifolia*; Diterpene; Labdane-type diterpene; Cell cycle inhibitor; Apoptosis inducer; Anticancer; *Verbenaceae*

1. Introduction

At the cellular level, cancers are the unlimited and undesired proliferation of cancerous cells with dysfunction of the control process of cell cycle and/or apoptosis. Thus it is reasonable to consider that chemical agents inhibiting the cell cycle or inducing apoptosis might be useful chemotherapeutic agents. In fact, cancer chemotherapeutic as well as chemopreventive agents have been suggested to exert parts of their pharmacological effects by regulating the control process of cell cycle or apoptotic cell death [1]. Also, many anticancer drugs

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clinically used nowadays have been demonstrated to exert their therapeutic effect through inhibiting cell cycle or inducing apoptosis even though the discovery and development of these drugs did not lie in their activities on apoptosis and cell cycle. From this viewpoint, we have undertaken the screening of new cell cycle inhibitors and apoptosis inducers from natural resources using mammalian tsFT210 cells [2–6].

During the screening, we have examined several thousand Chinese medicinal herbs [5,6] and found that the crude extract of the fruit of *Vitex trifolia* L. (family *Verbenaceae*) possesses strong activity in inducing apoptosis and inhibiting the cell cycle at the G_0/G_1 and G_2/M phases, which coincided with antineoplastic property of the title plant. The fruit of *V. trifolia* L. has long been used as a traditional Chinese medicine, Fructus Viticis (Manjingzi in Chinese), for curing colds, headache, *etc.* [7], and in parts of China it is also used as a folk medicine to treat certain cancers. Although some active components, such as antibacterial and anti-inflammatory compounds, have been hitherto reported for several species of the genus *Vitex* [8,9], no report has so far been seen on the anticancer components of the same genus and as yet no report on the anticancer principles of the title plant. Thus, chemical and biological investigations were undertaken, with particular attention focused on the active components of the title plant, and have led to the isolation of five labdane-type diterpenes, **1**–**5**, from the fruit of *V. trifolia* L. as its anticancer components, by a bioassay-guided separation procedure using mammalian tsFT210 cells. The isolation, identification and biological properties of **1**–**5** are described here.

2. Results and discussion

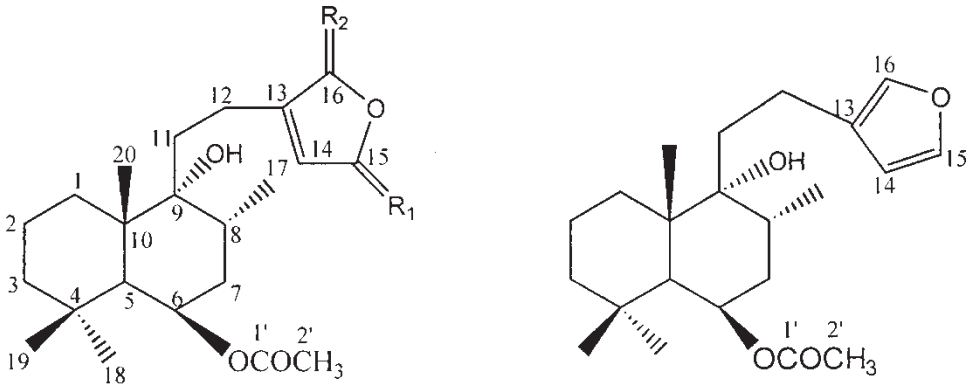
2.1 Isolation and identification of diterpenes 1–5

The air-dried fruits of *Vitex trifolia* L. were extracted with 95% ethanol to give a crude extract that possessed strong activity in terms of inducing apoptosis and inhibiting cell cycle at the G_0/G_1 and G_2/M phases. By monitoring the separation procedure for the same activity, assayed by flow cytometry with the combined use of morphological observation, compounds **1**–**5** were then isolated as the active components of the title plant.

Compound **1**, colorless needles (acetone), mp 146.5–148.0°C, $[\alpha]_D^{28}$ − 15.4 (*c* 1.1, $CHCl_3$) and compound **2**, a colorless syrup, $[\alpha]_D^{28}$ − 9.1 (*c* 3.3, acetone), both gave the same pseudo-molecular ion peak at *m/z* 401 $[M + Na]^+$ in the ESI-MS measurements, corresponding to their same molecular composition of $C_{22}H_{34}O_5$. They were thus identified as vitexilactone (**1**) and (*rel* 5*S*,6*R*,8*R*,9*R*,10*S*)-6-acetoxy-9-hydroxy-13(14)-labden-16,15-olide (**2**) [10] (figure 1) respectively according to their UV, IR and NMR data.

Compound **3**, a colorless syrup, $C_{22}H_{34}O_4$ (*m/z*: 385 $[M + Na]^+$), $[\alpha]_D^{28}$ − 19.9 (*c* 1.4, acetone), was identified as rotundifuran [11] (figure 1) by means of 2D NMR spectroscopy.

Compound **4**, a colorless syrup, $[\alpha]_D^{28}$ − 110.6 (*c* 0.9, acetone) and compound **5**, colorless needles, mp 144.0–145.0°C, $[\alpha]_D^{28}$ + 122.1 (*c* 1.4, acetone), had the molecular formula $C_{24}H_{38}O_5$ (**4**) and $C_{22}H_{36}O_4$ (**5**). The composition for **4** contains one more C_2H_3O unit than that for **5**. Correspondingly, the ^1H and ^{13}C NMR spectra of **4** and **5** in $CDCl_3$ solution showed very similar signal patterns except for the disappearance of an acetyl group in **5**. Eventually, the compounds were identified as vitetrifolins D (**4**) and E (**5**) [12], respectively, on the basis of their 2D NMR data.



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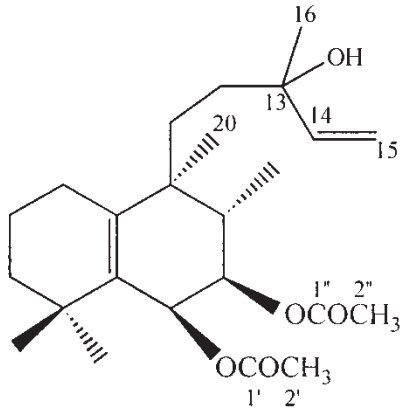
2: R_1=H_2, R_2=O

3



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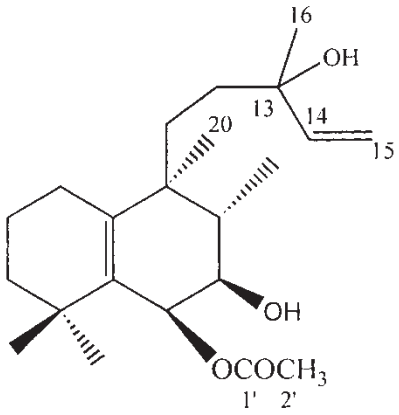

5

Figure 1. Structures of compounds **1**–**5**.

2.2 Biological activities of diterpenes 1–5

*Diterpenes **1**–**5** Inhibit Proliferation of tsFT210 and K562 Cells* The inhibitory effect of **1**–**5** on the proliferation of tsFT210 and K562 cells was evaluated by the MTT colorimetric assay [13] and the half-inhibitory concentrations (IC_{50}) for **1**–**5** were obtained as mean value ± SD (table 1) by three independent measurements. As shown in table 1, **1**–**5** inhibited the proliferation of tsFT210 and K562 cells and the inhibitory effects of **1**–**4** on tsFT210 cells were stronger than that of the cisplatin (CDDP) used as the positive control. In addition, the minimum inhibitory concentrations (MICs) for **1**–**5** were 10, 10, 1, 1, 50 and 50 $\mu g\,ml^{-1}$ for **1**–**5** and CDDP, respectively, for inhibiting the proliferation of tsFT210 cells and 10, 10, 5, 5 25 and 1 $\mu g\,ml^{-1}$ for **1**–**5** and CDDP, respectively, for inhibiting the K562 cell proliferation.

*Diterpenes **1**–**5** Induce Apoptosis and Inhibit Cell Cycle at G_0/G_1 Phase* The cell cycle inhibitory and apoptosis-inducing activities were assayed for **1**–**5** using tsFT210

Table 1. Inhibitory effect of **1**–**5** on the proliferation of mammalian cancer cells.

	IC_{50} ($\bar{X} \pm SD\ \mu g\ ml^{-1}$, $n = 3$)*	
Compounds	*tsFT210*	*K562*
1	86.9 ± 1.9	57.1 ± 1.4
2	87.1 ± 12.4	54.5 ± 1.6
3	36.2 ± 6.7	26.5 ± 2.1
4	41.3 ± 9.6	35.2 ± 4.3
5	> 100.0	> 100.0
CDDP	> 100.0	33.3 ± 4.5

* IC_{50} is defined as the concentration that results in a 50% decrease of viable cell numbers. Data represent mean values of three independent experiments and were determined by the MTT method.

and K562 cells by flow cytometry, along with morphological observations of the cells under a light microscope. The DNA content of cells tested was measured by flow cytometry and the result obtained as a flow cytometric histogram. Hypodiploid cells generally appear in the sub-G_0/G_1 peak region in the histogram and contain less DNA than the dipoid cells observed as the G_0/G_1 peak because of the apoptosis-induced DNA fragmentation. The proportion of hypodiploid cells in total cell population represents the intensity of apoptosis-inducing activity of the tested sample. Also, the microscopic observations provided morphological evidence for apoptosis. In addition, the distribution of cell nuclei within cell cycle was observed as peaks of multipoid cells in the histogram. By comparison of the results for **1**–**5** from flow cytometric analysis with that of control, we can evaluate the biological effect of **1**–**5**; each experiment was independently repeated at least 3 times to confirm the result.

Typical flow cytometric histograms of tsFT210 cells for **1**–**5** are given in figure 2, and some of the corresponding morphological observations are given in figure 3. Compounds **1**–**5** dramatically induced apoptosis of the tsFT210 cells at higher concentrations as detected as sub-G_0/G_1 peaks by flow cytometry after treatment of the cells at $100\ \mu g\ ml^{-1}$ for 17 h (figure 2 and table 2). This was also confirmed by the morphological characteristics of the corresponding cells as observed under a light microscope as typical cell shrinkage and apoptotic bodies (figure 3). The apoptosis-inducing effect of **1**–**5** weakens with lowering concentrations in parallel to the appearance of their cell cycle inhibitory effect, and at low concentrations **1**–**5** mainly inhibited the cell cycle progression of tsFT210 cells at the G_0/G_1 phase (figure 2 and table 2).

Conclusively, at higher concentrations, the apoptosis-inducing effect of **1**–**5** was their major biological activity, while at lower concentrations the cell cycle G_0/G_1 phase inhibitory activity became the main effect instead of their apoptosis-inducing activity on tsFT210 cells, although a weak apoptosis-inducing activity was still detected (figure 2 and table 2). The MICs of **1**–**5** for inducing apoptosis of tsFT210 cells are 25, 12.5, 6.25, 12.5 and $100\ \mu g\ ml^{-1}$, respectively, while the concentration ranges for the G_0/G_1 phase inhibition of tsFT210 cell cycle were 50–6.25, 50–6.25, 25–6.25, 50–12.5 and 100–50 $\mu g\ ml^{-1}$ for **1**–**5**, respectively.

A similar result was also obtained using human myeloid leukemia K562 cells. At the higher concentrations, **1**–**5** also induced dramatic apoptosis of the K562 cells (data not given), as with tsFT210 cells, while at lower concentrations they mainly inhibited the cell cycle progression of K562 cells at the G_0/G_1 phase. Typical histograms of K562 cells for **1**–**5** (figure 4) and corresponding data from flow cytometric analysis (table 3) are given here.

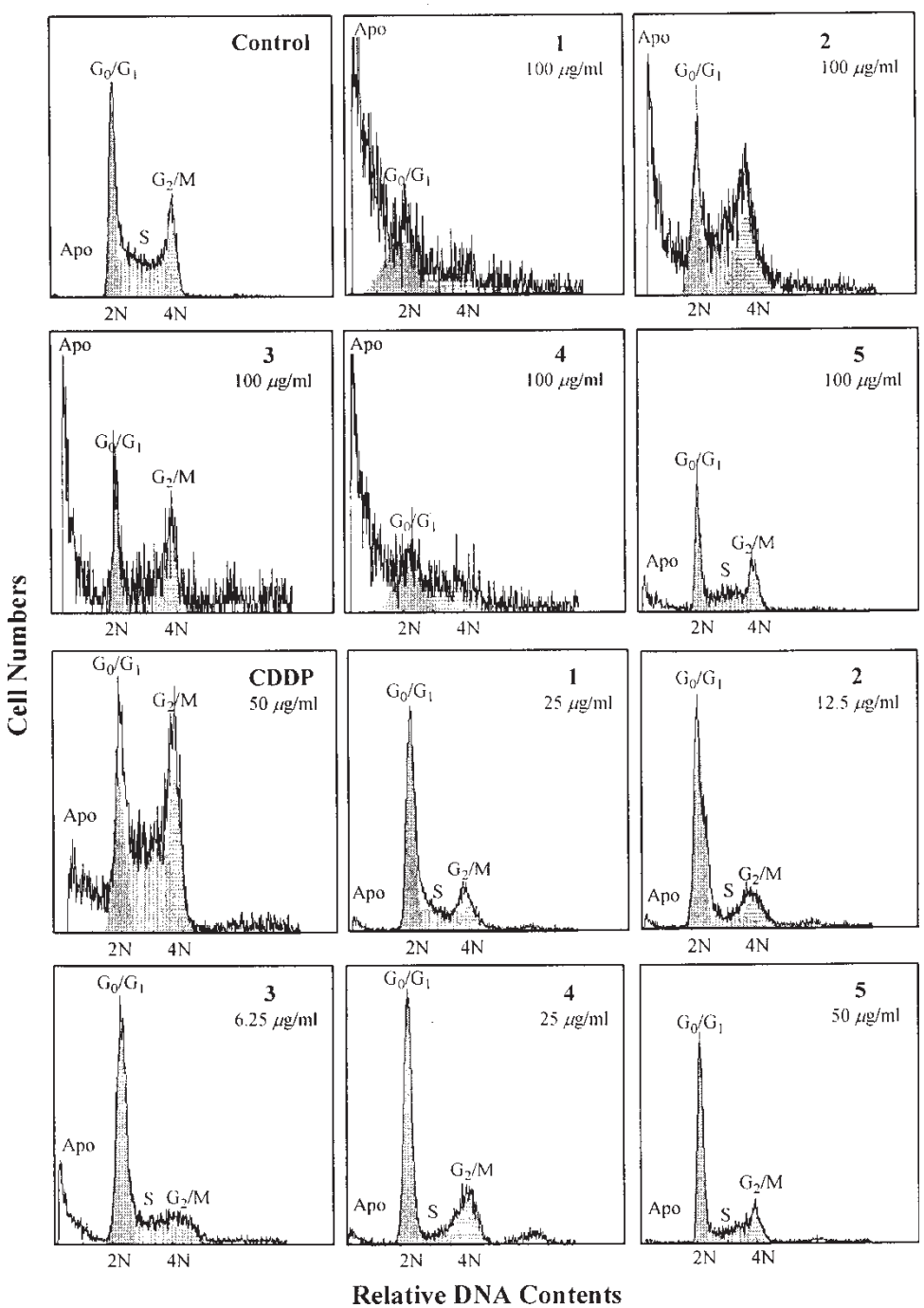

Figure 2. Flow cytometric histograms of tsFT210 cells for compounds **1**–**5**. Exponentially growing tsFT210 cells were exposed for 17 h to CDDP (positive control) and **1**–**5** at the concentrations indicated; the cell nuclei were stained with propidium iodide and then analysed by flow cytometry. Open graphs are the raw data from flow cytometry and filled figures indicate the data calculated by the computer software, WinCycle (Coulter). The Apo region, representing hypodiploid apoptotic cells, was determined from the origin of the abscissa and ended at the beginning of the G_0/G_1 peak. The given result is a representative of three independent experiments.

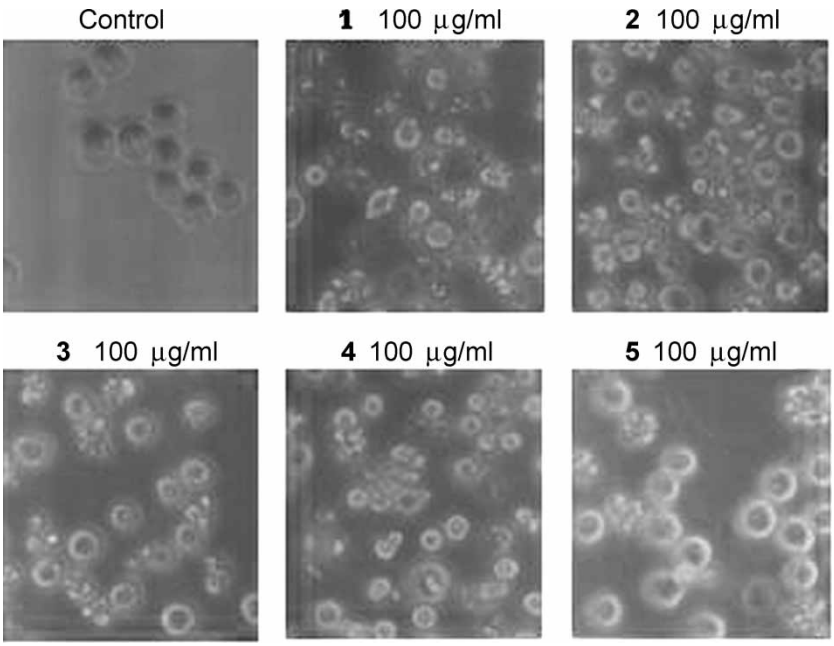

Figure 3. Photographs of tsFT210 cells treated with compounds **1**–**5**. Exponentially growing tsFT210 cells were cultured at 32°C for 17 h in the absence (control) or presence of **1**–**5** at the concentrations indicated, and the morphological characteristics of the cells were observed directly and photographed (× 200) under a light microscope.

The MIC values of **1**–**5** for inducing apoptosis of K562 cells were 25, 25, 12.5, 25 and 100 $\mu g\,ml^{-1}$ for **1**–**5**, respectively, while the concentration ranges for the cell cycle G_0/G_1 phase inhibition of K562 cells were 50–25, 50–25, 25–12.5, 25–6.25 and $100 < -50\,\mu g\,ml^{-1}$ for **1**–**5**, respectively.

In conclusion, **1**–**5** arrested the cell cycle progression of both tsFT210 and K562 cells at the G_0/G_1 phase at lower concentrations, while at higher concentrations they all induced dramatic apoptosis of both tsFT210 and K562 cells.

Table 2. Distribution of the tsFT210 cells within cell cycle and apoptosis*.

		Distribution of the cells (%)			
Groups	*Concentrations ($\mu g\,ml^{-1}$)*	*Apo*	*G_0/G_1*	S	*G_2/M*
Control	–	0.8	32.1	50.9	17.0
1	100.0	69.0	57.9	8.9	33.2
	25.0	4.1	55.5	32.2	12.3
2	100.0	44.6	22.9	42.3	34.8
	12.5	3.6	61.2	22.3	16.5
3	100.0	42.5	27.9	39.9	32.2
	6.25	17.1	58.3	32.6	9.1
4	100.0	60.4	50.6	0.8	48.7
	25.0	4.5	57.3	16.2	26.5
5	100.0	11.4	33.8	44.2	22.0
	50.0	2.0	56.9	27.0	16.1

* Data were obtained from the histograms in Fig. 2. Numbers in the column Apo represent relative percentage of the apoptotic cells counted in the sub-G_0/G_1 peak region to the total cell numbers. The proportion of cells in each phase of the cell cycle (G_0/G_1, S, and G_2/M) was analyzed with the computer software WinCycle (Coulter).

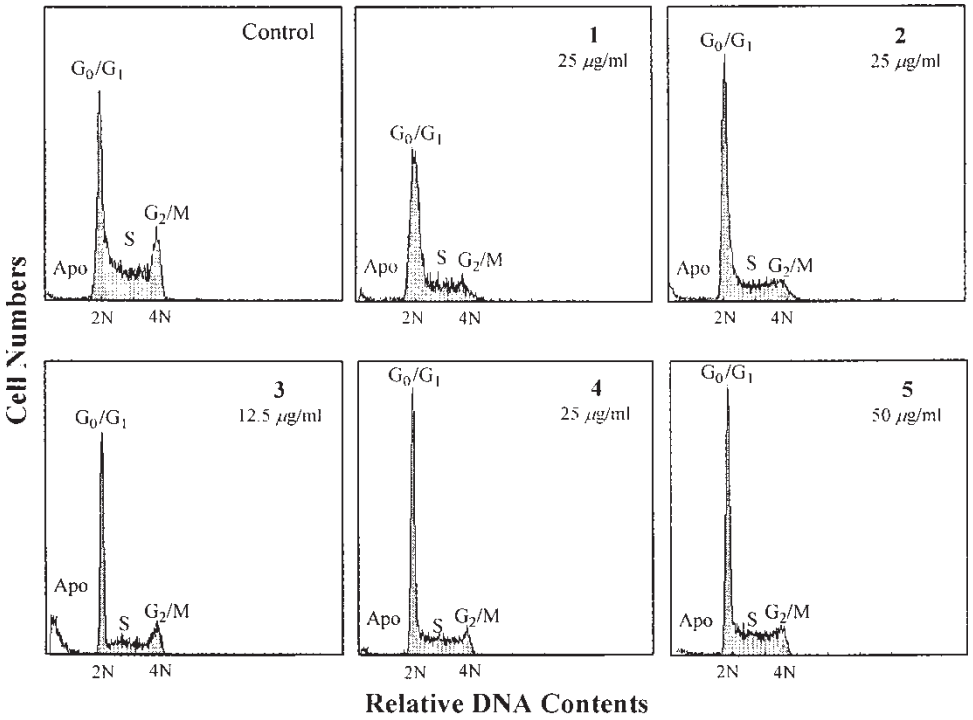

Figure 4. Flow cytometric histograms of K562 cells for compounds **1**–**5**. Exponentially growing K562 cells were exposed for 24 h to **1**–**5** at the concentrations indicated; the cell nuclei were stained with propidium iodide and then analysed by flow cytometry. Open graphs are the raw data from flow cytometry and filled figures indicate the data calculated by the computer software, WinCycle (Coulter). The Apo region, representing hypodiploid apoptotic cells, was determined from the origin of the abscissa and ended at the beginning of the G_0/G_1 peak. The given result is a representative of three independent experiments.

3. Discussion

The fruit of *Vitex trifolia* L. has been used as a folk medicine to cure certain cancers in China for many years, but the anticancer components have not yet been reported. In our attempts to find the active principles of this plant we have now isolated and identified five labdane-type diterpenes, **1**–**5**, for the first time as the anticancer components by a bioassay-guided separation procedure. Compounds **1**–**5** inhibited the cell proliferation of mammalian cancer

Table 3. Distribution of the K562 cells within cell cycle and apoptosis*.

		Distribution of the cells (%)			
Groups	*Concentrations ($\mu g\,ml^{-1}$)*	*Apo*	*G_0/G_1*	*S*	*G_2/M*
Control	–	2.5	36.1	52.1	11.8
1	25.0	3.6	65.2	33.6	1.2
2	25.0	3.0	56.9	38.6	4.5
3	12.5	19.4	53.5	35.4	11.1
4	25.0	2.8	54.8	40.7	4.5
5	50.0	3.2	50.7	45.5	3.8

* Data were obtained from the histograms in Fig. 4. Numbers in the column Apo represent relative percentages of the apoptotic cells counted in the sub-G_0/G_1 peak region to the total cell numbers. The proportion of cells in each phase of the cell cycle (G_0/G_1, S, and G_2/M) was analyzed with the computer software WinCycle (Coulter).

tsFT210 and K562 cells and all of them dramatically induced apoptosis of tsFT210 and K562 cells at higher concentrations and inhibited the cell cycle progression of both the tsFT210 and K562 cells at the G_0/G_1 phase at lower concentrations. These biological data for **1**–**5** suggest that they exert their anti-proliferative effects on tsFT210 and K562 cells through inducing apoptosis and inhibiting cell cycle at the G_0/G_1 phase. Thus, the five labdane-type diterpenes **1**–**5** obtained here should be at least partially responsible for the cancer chemotherapeutic effects of the title plant, and thus explain the use of *V. trifolia* L. to cure certain cancers.

Compounds **1**–**5** are bicyclic diterpenes with a carbon skeleton related to labdane. Although many labdane-type diterpenes, along with their widespread biological activities, have been reported [14,15], no report has so far been seen on the cell cycle inhibitory effect of the labdane-type diterpenes. Thus, the labdane-type diterpenes **1**–**5** are a new chemical class of cell cycle inhibitors. Furthermore, **1**, **2**, **4** and **5** are new apoptosis inducers. This is the second example of apoptosis inducers belonging to labdane-type diterpenes as **3** has been reported by Ko *et al.* to induce apoptosis of HL-60 cells [16], which is consistent with its effect on the tsFT210 and K562 cells at higher concentrations seen here. From our present results and the fact that *V. trifolia* L. has been used clinically to cure cancers in Chinese people, it is reasonable to consider that labdane-type diterpenes may be worth screening for new anticancer agents or even as candidates for new anticancer drugs.

However, compounds **1**–**5** showed only the cell cycle G_0/G_1 phase inhibitory and apoptosis inducing activities, while the crude extract of *V. trifolia* L. also possesses cell cycle G_2/M phase inhibitory activity in addition to the cell cycle G_0/G_1 phase inhibitory and apoptosis inducing activities. This means that **1**–**5** are not representatives of the G_2/M phase inhibitory components of the crude extract of the title plant. Research on the G_2/M phase inhibitory components and the further studies on action mechanisms of **1**–**5** are being undertaken and the results will be reported elsewhere.

4. Experimental

4.1 General experimental procedures

Thin-layer chromatography (TLC) was performed on silica gel 60 F254 plates (0.25 mm thick, 20 × 20 cm, Merck), silica gel G plates (0.25 mm thick, 20 × 20 cm, Qingdao Haiyang Chemical Group Co., China) or home-made silica gel 60 F254 (Qingdao Haiyang Chemical Group Co., China) plates (0.25 mm thick, 2.5 × 7.5 cm), and the spots were detected under UV lights (254 and 365 nm) or by the use of 10% aqueous sulfuric acid reagent. Vacuum liquid chromatography and open-column chromatography were carried out on SYNTHWARE™ glass vacuum columns (Tianjin Synthware Glass Instruments Co., Tianjin, China) and glass open-columns respectively, and silica gel H (Qingdao Haiyang Chemical group Co., China) and SSC ODS-SS-1020T (Senshu Scientific Co., Ltd., Japan) were used as adsorbents.

Melting points were measured using an XT-type micro melting point apparatus (Beijing Tech Instrument Co. Ltd., China) and are uncorrected. Optical rotations were determined on a JASCO P-1020 polarimeter in $CHCl_3$ or acetone solutions and UV spectra were recorded on a Shimadzu UV-2501PC UV–VIS recording spectrophotometer in MeOH solutions. IR spectra were taken on a Dynamin alignment FTS 175C Fourier-transform infrared spectrophotometer in KBr discs. ESI-MS were measured on an Esquire LC mass

spectrometer. ^{1}H and ^{13}C NMR spectra and 2D NMR spectra were recorded on a JEOL Eclips-600 or Bruker AVANCE DRX-500 FT-NMR spectrometer using TMS as internal standard and chemical shifts are recorded as δ values.

4.2 Plant material

The fruits of *Vitex trifolia* L. were collected in the Chengdu area in Sichuan province, China, in September 1999. The original plant was identified by Professor Q.-S. Sun of Shenyang of the Pharmaceutical University, China, and a voucher specimen has been deposited at the Herbarium of the Shenyang Pharmaceutical University, China.

4.3 Extraction and isolation

Air-dried fruit (3 kg) of *Vitex trifolia* L. was powered and extracted with 95% ethanol (20 l) at room temperature to give a crude extract (188 g), which was then re-extracted in turn with petroleum ether (bp 60–90°C; 2 l) and then chloroform (2 l) to obtain both an active petroleum ether extract, PEE (34.2 g), and a chloroform extract, CE (44.7 g). The PEE was separated by vacuum liquid chromatography over silica gel H using light petroleum–acetone (PA) and chloroform–methanol (CM) as eluting solvent to give six fractions, Fr-I–VI. Subsequently, Fr-III (6.41 g, PA 80:20 eluent) was further chromatographed over silica gel H to give compounds **3** (81.2 mg, CM 99:1 eluent) and **4** (50 mg, CM 99:1 eluent) in order of elution, both as a colorless syrup from chloroform solutions.

The chloroform extract CE (44.7 g) was subjected to vacuum liquid chromatography over silica gel H with PA and then CM to give six fractions, Fr-1–6, of which Fr-3 (6.28 g, PA 80:20 eluent) was further chromatographed over silica gel H by a stepwise elution with cyclohexane–EtOAc (CE) (1:0 → 0:1) solution to afford four fractions, Fr-3-1 to Fr-3-4. Subsequently, Fr-3-2 (0.78 g, CE 80:20 eluent) and Fr-3-3 (1.26 g, CE 80:20 → 70:30 eluent) were further separated by ODS column chromatography to give **2** (5.7 mg, MeOH eluent) and **5** (8 mg, MeOH eluent) in order of elution from Fr-3-2 as a colorless syrup (**2**) and colorless needles (**5**), both from MeOH solutions, and **1** (120 mg, MeOH eluent) from Fr-3-3 as colorless needles from acetone solution.

4.4 Identification of diterpenes 1–5

Vitexilactone (**1**): Colorless needles from acetone solution, mp 146.5–148.0°C, $C_{22}H_{34}O_5$, $[\alpha]_D^{20}$ − 15.4 (*c* 1.1, $CHCl_3$). ESI-MS *m/z*: 401 $[M + Na]^+$. UV, IR, and ^{1}H and ^{13}C NMR data are identical with those for **1** reported in the literature [10].

(rel 5S, 6R, 8R, 9R, 10S)-6-Acetoxy-9-hydroxy-13(14)-labden-16,15-olide (**2**): A colorless syrup from MeOH solution, $C_{22}H_{34}O_5$, $[\alpha]_D^{20}$ − 9.1 (*c* 3.3, acetone). ESI-MS *m/z*: 401 $[M + Na]^+$. UV, IR, and ^{1}H and ^{13}C NMR data are identical with those for **2** given in the literature [10].

Rotundifuran (**3**): Colorless syrup from $CHCl_3$ solution, $C_{22}H_{34}O_4$, $[\alpha]_D^{20}$ + 19.9 (*c* 1.4, acetone). ESI-MS *m/z*: 385 $[M + Na]^+$. UV and ^{1}H and ^{13}C NMR data are identical with those reported for **3** in the literature [11].

Vitetrifolins D (**4**): Colorless syrup from $CHCl_3$ solution, $C_{24}H_{38}O_5$, $[\alpha]_D^{20} + 110.6$ (*c* 0.9, acetone). ESI-MS *m/z*: 429 $[M + Na]^+$. UV and ^1H and ^{13}C NMR data are identical with those for **4** given in the literature [12].

Vitetrifolin E (**5**): Colorless needles from MeOH solution, $C_{22}H_{36}O_4$, $[\alpha]_D^{20} + 122.1$ (*c* 1.4, acetone). ESI-MS *m/z*: 387 $[M + Na]^+$. UV and ^1H and ^{13}C NMR data are identical with those for **5** reported in the literature [12].

4.5 Cell culture and bioassay

Cell Lines and Cell Culture. A mouse temperature-sensitive $p34^{cdc2}$ mutant, tsFT210, and human myeloid leukemia, K562, cell lines were used for the bioassay. The cells were routinely maintained in RPMI-1640 medium supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air. The tsFT210 cells were cultured at 32°C, and the K562 cells at 37°C.

Cell Proliferation Assay. The inhibitory effects of compounds **1**–**5** on cancer cell proliferation were assayed by MTT method [13], a colorimetric assay based on the ability of viable cells to reduce yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to blue formazan. Exponentially growing cells were seeded into 96-well flat microtiter plate at a density of 2×10^5 cells ml^{-1} in fresh RPMI-1640 medium and cultured for 17 h at 32°C for tsFT210 cells and for 24 h at 37°C for K562 cells, in the presence or absence of various concentrations of **1**–**5**. Then 10 μl MTT solutions (5 mg ml^{-1} MTT in PBS filter sterilized) were added into each well and further cultured for 4 h under the same conditions. The formazan product was harvested by centrifugation followed by aspirating the supernatant and dissolved in DMSO (100 μl). The optical density (OD) of each well was determined on a SPECTRA MAX Plus plate reader at 570 nm. Data from triple wells were taken for negative control and for each concentration of samples on each plate. Cisplatin (CDDP) was used as a positive control. The inhibition rates (IR%) for compounds **1**–**5** and CDDP at various concentrations were calculated using OD mean values from $IR\% = (OD_{control} - OD_{sample})/OD_{control}$. Then the concentration required for 50% inhibition of the cell growth, IC_{50}, was determined using the Bliss method. The same experiment was repeated independently three times to obtain a mean IC_{50} and its standard deviation.

Flow Cytometry. Exponentially growing tsFT210 and K562 cells were harvested by centrifugation at 4°C for 3 min at 3000 rpm (tsFT210) and for 5 min at 700 rpm (K562). The cells were then suspended in fresh RPMI-1640 medium at a density of 20×10^5 cells ml^{-1} and each 500 μl of the cell suspension was seeded into the 24-well plate. Then, 5 μl of the sample solution was added into each well and the cells were cultured for 17 h at 32°C for tsFT210 and for 24 h at 37°C for K562 cells. The morphological characteristics of the cells treated with the samples were directly observed and photographed under an Olympus CK40 inverted system microscope equipped with an Olympus PM-C35B camera system (Olympus, Japan).

Both the tsFT210 and K562 cells were then treated by the same procedure as described below. The cells were harvested by centrifugation at 3000 rpm for 3 min at 4°C, washed once with cold phosphate-buffered saline, and harvested again under the same conditions. The cells were then stained with 150 μl of propidium iodide (PI) in water solution (PI 50 μg ml^{-1}, sodium citrate 0.1% and Nonidet P-40 0.2%) at 4°C for 30 min under

lightproof conditions. The cells were then diluted with the same volume of phosphate-buffered saline and analyzed by flow cytometry (EPICS XL, Coulter Co., Hialeah, FL, USA). The distribution of the cells within the cell cycle was calculated using the computer software WinCycle (Coulter).

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