Active constituents of Zanthoxylum nitidum from Yunnan Province against leukemia

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Abstract

Zanthoxylum nitidium (Roxb.) DC (Rutaceae) is well known for inhibiting the proliferation of human gastric, liver, kidney and lung cancer cells, whereas research on its potential use in treatments of leukaemia targeting Fli-1 gene is relatively rare. 26 compounds were isolated from the chloroform and petroleum ether extracts of the roots and leaves of Z. nitidium . The structures of four compounds (4-6 and 16) were confirmed and attributed for the first time by UV-visible spectroscopy, 1D and 2D NMR and HR-ESI-MS. Compounds 1-2 and 11 were isolated from Z. nitidium for the first time. Of the assayed compounds, compounds 14 and 24 showed inhibitory activities against leukaemia HEL cells with IC 50 values of 3.59 and 15.95 μM. In addition, to further investigate the possible mechanism, the cell cycle and apoptosis assays were investigated for the first time, which indicated that compound 14 caused obvious S phase arrest in HEL cells and induced cell apoptosis, but compound 24 induced only cell apoptosis of HEL cells. These results suggested that compounds 14 and 24 were the potential candidates for anti-leukaemia drug for the first time.

1. Introduction

Leukaemia is closely related to the haematopoietic system, which includes bone marrow [1], and malignant tumours of the haematopoietic system pose a serious threat to human health and life. Although early high-dose combination chemotherapies can achieve complete remission in many patients, the 5-year survival rate of these patients is still unsatisfactory [2], and the discovery of new anti-leukaemia drugs is still important.

Identifying candidate drug molecules in natural products is an important pathway for discovering innovative drugs. *Zanthoxylum nitidium* (Roxb.) DC, locally called “liangmianzhen”, belongs to the genus *Zanthoxylum* of the family Rutaceae [3]. And the plant is distributed in Guangdong, Fujian, Hunan, Yunnan, and Taiwan. The chemical components of *Z. nitidium* are diverse and complex, and most of the constituents are alkaloids, flavonoids, lignans and coumarins. Research on the active substances from *Z. nitidium* had mainly focused on the alkaloids, especially benzophenanthridine, furanquinoline, quinolones, amides, and aporphine, and a much smaller number of non-alkaloids have been reported [3]. Studies on the biological activity of *Z. nitidium* have focused on its inhibition of the proliferation of human gastric, liver, kidney, lung and nasopharyngeal carcinoma cells [3], whereas researches on its ability to against leukaemia is comparatively rare. It has been reported that Fli-1 has been found to have varying degrees of high expression in leukaemia cells [6]. More studies have also confirmed that Fli-1 gene not only played an important regulatory role in the process of vascular endothelial cell generation and tumor cell proliferation, but also had a role in promoting tumorigenesis and development [7]. Fli-1 gene had be proved to be a new targets for drug screening.

In our previous work, ethanol extracts and other extracts of *Z. nitidium* had significant inhibitory effects on the proliferation of HEL cells, and evaluation of in vitro toxicity tests showed that they had no significant toxicity. Significantly, Fli-1 genes had a high expression of in HEL cells against leukaemia from

previous work in 2016. In order to find a lead compound with a good effect on Fli-1 gene from extracts of *Z. nitidium,* 26 compounds were isolated, purified and identified from the roots and leaves of *Z. nitidium*, and their antitumor activities against HEL cells were studied. We collected the dry roots and leaves of *Z. nitidium* on Mengla County of Xishuangbanna from Yunnan province. Twenty-two alkaloids were isolated and identified, including three previously undescribed alkaloids isolated from natural sources for the first time. During the isolation of these alkaloids, we found that four compounds gave false-positive results with the modified potassium caesium iodide colouring agent. These compounds were judged to be non-alkaloids by ^{1}H NMR and ^{13}C NMR spectra and included three coumarins and one furanose, one of these is an undescribed compound from natural sources for the first time. The chemical structures of compounds **4**, **5**, **6** and **16** were characterized through extensive spectroscopic analyses based on UV, IR, 1D and 2D NMR, and HR-ESI-MS spectra. The antitumor activities of 26 compounds against t HEL cells were evaluated for the first time. In addition, the possible mechanism of two active compounds was also investigated for the first time.

2. Results

2.1 Isolation and Structural Elucidation

The dried roots and leaves (20 kg) of *Z. nitidium* were heated and refluxed in 95% EtOH. The resulting extract was concentrated and then partitioned between petroleum ether and chloroform. The extracts were further separated by solvent fractionation and various forms of column chromatography (CC) to afford compounds **1–26** (Figure 1).

*2.1.1 Chemical Structure of Compound **4***

Compound **4** was isolated as a yellow solid and gave a positive result with the improved caesium potassium iodide test. Its molecular formula was determined to be $C_{16}H_{18}O_5$ based on its positive HR-ESI-MS data (*m/z* 291.1585 $[M + H]^+$). The UV profile of **4** displayed the λ max values of 206, 263 and 323 nm, and its IR spectrum showed absorptions representing a lactone ring (1726 cm^{-1}) and an aromatic ring (1502 and 1432 cm^{-1}). The above data indicated that compound **4** contains a lactone ring. The ^{1}H NMR data (Table 1) showed three aromatic proton signals at δ_H 7.96 (m, 1H), 6.16 (s, 1H), and 6.33 (d, J = 1.5 Hz, 1H); two methoxyl proton signals at δ_H 3.94 (s, 3H) and 3.90 (s, 3H); two methyl proton signals at δ_H 1.68 (s, 3H) and 1.73 (s, 3H); and one methylene signal at δ_H 4.54 (dd, J =7.5, 1.5 Hz, 2H). In addition, the ^{13}C NMR and DEPT spectra of compound **4** showed the following groups: C × 7, CH × 4, CH_2 × 1, OCH_3 × 2, and CH_3 × 2. The above nuclear magnetic resonance data are similar to the reported compound **4'** in the literature [8-9].

The 1D NMR signals of compound **4'** and compound **4** were compared in Table 1. The proton signal at C-8 of compound **4'** was the same as that of **4**. As shown in Figure 2, the HMBC correlations of the protons at δ_H 4.54 (dd, J = 7.5, 1.5 Hz 2H) with C-2’ (δ_C 120.17), C-3’ (δ_C 139.03), and C-5 (δ_C 128.79) suggested that the 3', 3'-dimethyl-2'-butenyloxy group of compound **4** is attached at the C-5 position. The HMBC

correlations of δ_H 7.96 with C-5a (δ_C 149.04), C-2 (δ_C 160.89), and C-5 (δ_C 128.79) and of δ_H 6.16 (s, 1H) with C-8a (δ_C 103.85) and C-2 (δ_C 160.89) indicate that the lactone ring is close to C-8. Finally, the proton signal for 7-OCH_3 (δ_H 3.94, s), based on the HMBC data, is correlated with the signal for C-7 (δ_C 156.56), and the signal for 8-OCH_3 (δ_H 3.90, s) is correlated with the signal for C-8 (δ_C 152.31). The two -OCH_3 groups are at C-7 and C-8. The above nuclear magnetic resonance data indicated that compound **4** is consistent with *5*-(3', 3'-dimethyl-*2'*-butenyloxy)-*7*, *8*-methoxy-coumarin, which has been previously reported in the literature [10]. Due to describing compound **4** did not assign the NMR data, we assigned the NMR data of compound **4** for the first time.

*2.1.2 Chemical Structure of Compound **5***

Compound **5** was isolated as a tawny oil and gave a positive result in the improved caesium potassium iodide test, and it was therefore presumed to be an alkaloid. Its molecular formula was determined to be $C_{13}H_{15}O_3N$ based on its positive HR-ESI-MS data (*m/z* 234.1124 $[M + H]^+$). The UV profile of **5** displayed the λ max values at 218 and 279 nm. The IR spectrum showed absorptions for an *α*, *β*-unsaturated ester carbonyl (1731 cm^{-1}) and an aromatic ring (1593 and 1430 cm^{-1}). The ^1H NMR data in Table 2 showed that there are three aromatic protons with signals at δ_H 7.04 (m, 1H), 6.75 (dd, *J* = 8.7, 2.4 Hz, 1H), and 6.98 (d, *J* = 8.7 Hz, 1H), a methylene proton with a signal at δ_H 3.65 (s, 3H); and two methoxy protons with signals at δ_H 3.84 (s, 2H) and 3.65 (s, 3H). In addition, the ^{13}C NMR and DEPT spectra of compound **5** indicated the presence of the following groups: C × 6, CH × 3, CH_2 × 1, CH_3 × 1 and OCH_3 × 2. The above nuclear magnetic resonance data indicated that compound **5** is consistent with methyl *2*-(*5*-methoxy-*2*-methyl-*1H*-indol-*3*-yl) acetate, which has been previously reported in the literature [11].

A previous study [11] describing compound **5** did not assign the NMR data. To further determine the structure of **5**, we assigned the NMR data of compound **5** for the first time. As shown in the ^1H NMR spectrum (Table 2), the coupling constant of the proton signals at δ_H 6.75 (dd, *J* = 8.7, 2.4 Hz, 1H) and δ_H 6.98 (d, *J* = 8.7 Hz, 1H) is *J* = 8.7 Hz, suggesting that the two proton signals are ortho-coupled on the benzene ring. The HSQC correlations between H-4 (δ_H 7.04) and C-4 (δ_C 111.14), between H-6 (δ_H 6.04) and C-6 (δ_C 110.83), and between H-7 (δ_H 6.98) and C-7 (δ_C 100.35) revealed that compound **5** contains an aromatic ring. At the same time, the HMBC data shown in Figure 3 show correlations of H-8 (δ_H 3.65) with C-2 (δ_C 172.85), C-3 (δ_C 128.86), and C-4a (δ_C 104.08) and of H-10 (δ_H 2.28) with C-4a (δ_C 104.08) and C-9 (δ_C 133.76), suggesting that the compound contains an indole moiety. Similarly, the HMBC (Figure 3) data showed correlations between H-8 (δ_H 3.65) and C-2 (δ_C 172.85), C-3 (δ_C 128.86), and C-4a (δ_C 104.08) and between H-10 (δ_H 2.28) and C-4a (δ_C 104.08) and C-9 (δ_C 133.76), suggesting the presence of a methyl acetate. Finally, the HMBC data showed correlation of 5-OCH_3 (δ_H 3.84, s) with C-5 (δ_C 154.05) and of 9-OCH_3 (δ_H 3.65, s) with C-9 (δ_C 133.76). These results indicate that the two -OCH_3 groups are at C-5 and C-9. Compound **5** was thus named methyl *2*-(*5*-methoxy-*2*-methyl-*1H*-indol-*3*-yl) acetate.

*2.1.3 Chemical Structure of Compound **6***

Compound **6** was isolated as a yellow oil, gave a positive result in the improved caesium potassium iodide test, and was therefore presumed to be an alkaloid. Its molecular formula was determined to be $C_{25}H_{25}O_6N$ based on its positive HR-ESI-MS data (m/z 436.1752 $[M + H]^+$). The UV profile of **6** revealed λ max values of 201, 283 and 224 nm. The IR spectrum showed absorption bands for an *α*, *β*-unsaturated ester carbonyl (1736 cm^{-1}) and an aromatic ring (1492 and 1463 cm^{-1}). The ^1H NMR data (Table 3) showed that there were two pairs of aromatic protons with signals at δ_H 7.73 (d, *J* = 8.7 Hz, 1H) and 7.50 (d, *J* = 8.7 Hz, 1H) and at 6.99 (d, *J* = 8.5 Hz, 1H) and 7.58 (d, *J* = 8.5 Hz, 1H); two aromatic protons with signals at δ_H 7.57 (s, 1H) and 7.12 (s, 1H); two groups of methyl protons with signals at δ_H 2.68 (s, 3H) and 1.21 (dd, *J* = 7.1 Hz, 3H); three groups of methylene protons with signals at δ_H 6.06 (s, 2H), 2.38 (s, 2H) and 4.17 (d, *J* = 7.1 Hz, 2H); and two groups of methoxy protons with signals at δ_H 3.99 (s, 3H) and 3.95 (s, 3H). In addition, the ^{13}C NMR and DEPT spectra of compound **6** indicated the presence of the following groups: C × 11, CH × 7, CH_2 × 3, CH_3 × 2 and OCH_3 × 2. The above nuclear magnetic resonance data indicated that compound **6** is a benzophenanthrene alkaloid. We found that compound **6** was consistent with ethyl *2'*-(*5*, *6*-dihydrochleletrythrine-*6*-yl) acetate, which has been previously reported in the literature [12].

The previous study [12] of compound **6** did not assign its NMR data. To clarify the structure of **6**, we assigned the NMR data of **6** for the first time. From the ^1H NMR data in Table 3, the coupling constant between the proton signals at δ_H 7.73 (d, *J* = 8.7 Hz, 1H) and 7.50 (d, *J* = 8.7 Hz, 1H) is *J* = 8.7 Hz, and that between δ_H 6.99 (d, *J* = 8.5 Hz, 1H) and 7.58 (d, *J*=8.5 Hz, 1H) is *J* = 8.5 Hz, indicating that the two pairs of proton signals are ortho-coupled on the phenyl ring. As shown in Figure 4, the HMBC data exhibited the correlations of H-1 (δ_H 7.12) with C-2 (δ_C 147.95), C-12 (δ_C 123.99), and C-12a (δ_C 127.53) and of H-4 (δ_H 7.57) with C-3 (δ_C 147.50) and C-4b (δ_C 139.30), indicating that compound **6** is a benzophenanthrene derivative. The direct HSQC (Figure S19, Supplementary Materials) correlations between H-6 (δ_H 4.95) and C-6 (δ_C 55.11) also revealed that compound **6** is a chelerythrine. Similarly, based on the HMBC (Figure 4), the correlations of H-2' (δ_H 2.38) with C-2 (δ_C 172.85), C-1' (δ_C 171.67), and C-6 (δ_C 55.11) and of H-4' (δ_H 1.21) with C-3' (δ_C 60.27) suggest the presence of an ethyl acetate group. Finally, the HMBC correlations of 7-OCH_3 (δ_H 3.99, s) with C-7 (δ_C 145.50) and of 8-OCH_3 (δ_H 3.95, s) with C-8 (δ_C 152.10) suggested that the two -OCH_3 groups were at C-7 and C-8.

*2.1.4 Chemical Structure of Compound **16***

Compound **16** was isolated as a tawny solid, gave a positive result with the improved caesium potassium iodide test, and was therefore presumed to be an alkaloid. Its molecular formula was determined to be $C_{13}H_{11}O_4N$ based on its positive HR-ESI-MS data (m/z 246.0760 $[M + H]^+$). The UV profile of **16** revealed the λ max values of 249, 201 and 316 nm, which are similar to those of quinoline [11]. The IR spectrum showed the absorption bands for an aromatic ring (1516 and 1443 cm^{-1}) and an ether (1151 and 1046

cm^{-1}). The ^{1}H NMR data in Table 4 showed two pairs of aromatic proton signals at δ_H 8.13 (d, J = 9.1 Hz, 1H) and 7.54 (d, J = 9.1 Hz, 1H), and at 7.15 (d, J = 2.7 Hz, 1H) and 7.80 (d, J = 2.7 Hz, 1H), two methoxy proton signals at δ_H 4.23 (s, 3H) and 4.27 (s, 3H), and an active hydrogen signal at δ_H 12.03 (s, 1H). In addition, the ^{13}C NMR and DEPT spectra of compound **16** indicated the presence of the following groups: C × 7, CH × 4 and OCH_3 × 2. Based on the above nuclear magnetic resonance data, compound **16** was consistent with *4*-hydroxy-*7*, *8*-demethy-furoquinoline, which has been previously reported in the literature [14].

To clarify the structure of **16**, we assigned the NMR data of compound **16** for the first time. Based on the ^{1}H NMR data in Table 4, which showed a coupling constant between the proton signals at δ_H 8.13 (d, J = 9.1 Hz, 1H) and 7.54 (d, J = 9.1 Hz, 1H) of J = 9.1 Hz, these two proton signals are ortho-coupled on the phenyl ring. The HMBC data in Figure 5 showed the correlations of H-5 (δ_H 8.13) with C-4 (δ_C 142.30), C-8 (δ_C 151.59), and C-8a (δ_C 157.41) and of H-6 (δ_H 7.54) with C-6 (δ_C 117.32), C-8 (δ_C 151.59), and C-4a (δ_C 114.11), suggesting that compound **16** contains a quinoline ring. Similarly, the coupling constant between the proton signals at δ_H 7.15 (d, J = 2.7 Hz, 1H) and δ_H 7.80 (d, J = 2.7 Hz, 1H) is J = 2.7 Hz, indicating that the protons are ortho-coupled on a furan ring. In addition, from the HMBC data in Figure 5, the correlations of H-3b (δ_H 7.15) with C-2 (δ_C 164.48), C-3 (δ_C 101.61), and C-4 (δ_C 142.30) and of H-2a (δ_H 7.80) with C-2 (δ_C 164.48), C-3 (δ_C 101.61), and C-3b (δ_C 105.34) suggest that this compound is a furan derivative. Finally, HMBC correlations of 7-OCH_3 (δ_H 4.23, s) with C-7 (δ_C 140.17) and of 8-OCH_3 (δ_H 4.27, s) with C-8 (δ_C 151.59) were observed. These results indicated that the two -OCH_3 groups were at C-7 and C-8.

By the comparison of their NMR data with those described in the literature, twenty-six compounds were identified as (+)-*9'*-*O*-transferuloyl-*5*, *5'*-dimethoxylaricriresinol (**1**) [15], *8*-(*3'*-oxobut-*1'*-en-*1'*-yl)-*5*, *7*-trimethoxy-coumarin (**2**) [16], *5*, *7*, *8*-trimethoxy-coumarin (**3**) [14], *5*-(*3'*, *3'*-dimethyl-*2'*-butenyloxy)-*7*, *8*-trimethoxy-coumarin (**4**), methyl *2*-(*5*-methoxy-*2*-methyl-*1H*-indol-*3*-yl) acetate (**5**), ethyl *2'*-(*5*, *6*-dihydrochleletrythrine-*6*-yl) acetate (**6**), *6*-acetonyldi-hydrochelerythrine (**7**) [18], *6β*-hydroxymethyldihydronitidine (**8**) [19], bocconoline (**9**) [20], zanthoxyline (**10**) [21], *O*-methylzanthoxyline (**11**) [21], rhoifoline *B* (**12**) [22], *N*-nornitidine (**13**) [23], nitidine (**14**) [24], chelerythrine (**15**) [25], *4*-hydroxyl-*7*, *8*-demethyfuroquinoline (**16**), dictamnine (**17**) [26], *γ*-fagarine (**18**) [27], skimmianine (**19**) [13], robustine (**20**) [26], *R*-(+)-platydesmine (**21**) [28], *4*-*O*-methyl-*1*-methyl-quinoline-*2*-one (**22**) [27], *4*-methoxy-*2*-quinolone (**23**) [29], liriodenine (**24**) [30], aurantiamide acetate (**25**) [31], and *10*-*O*-demethyl-*12*-*O*-methylarnottianamide (**26**) [32].

2.2 Biological Activities of the Isolated Compounds

To analysis the effects of 26 compounds for isolated from the roots and leaves of *Z. nitidium* against leukaemia cells (HEL cell lines), 26 compounds were tested of IC_{50} value against HEL by the CTG method, and Adriamycin was chosen as positive control (IC_{50}: 0.021 μM). As shown in Table 5, the most potent compound **14** (IC_{50}: 3.59 μM) and compound **9** (IC_{50}: 7.65 μM) showed the similar inhibitory activity with

the positive control (IC_{50}: 0.021 μM), while these compounds **15** (IC_{50}: 15.52 μM) and **24** (IC_{50}: 15.95 μM) exhibited moderate inhibitory activities against HEL cells. In addition, compound **24** whose structure type is different from **14** also exhibited good inhibitory activity against HEL.

*2.3 Compounds **14** and **24** Induced cell cycle arrest*

To further confirm the effects of compounds **14** and **24** with different structures on cell cycle, the cell cycle of distribution of HEL cells was examined after treatment with compounds **14** and **24** for 36 h. As shown in Figure 6, significant S transition arrest was observed in HEL cells treated with compound **14**, which was the most significant compound. The fraction of cells in the S phase was dose-dependently increased by the treatment with **14**, and the population of cells in the S phase was markedly increased to 52.04 % in 8 μM **14**-treated cells compared to 37.92 % in untreated cells. However, compound **24** with different structure type has no obvious effect on the cycle experiments against HEL cells.

*2.4 Compounds **14** and **24** induced apoptosis of HEL cells*

To determine whether the antiproliferative activity of **14** and **24** was accompanied by enhanced leukaemia cell apoptosis, cell apoptosis was detected by a flow cytometry assay after staining with an Annexin V-FITC apoptosis detection kit. As shown in Figure 7, Cells treated with compounds **14** and **24** displayed significant dose-denpendent increases in the percentage of Annexin-V-positive cells. Compound **14** from 1.86 % in the DMSO control to 13.99 % for 2.0 μM, 23.96 % for 4.0 μM and 35.98 % for 8.0 μM **14**-treated cells. At the same time, compound **24** at 7.5 μM and 15.0 μM displayed significant increases in the percentage of Annexin-V-positive cells. Compound **24** (7.5, 15, 30 μM) can promote the apoptosis rate from 6.11%, 17.34%, 25.81% in a dose-dependent manner. Hence, these observations for the first time demonstrated that compounds **14** and **24** induced obvious apoptosis in leukaemia cells HEL in a concentration-dependent manner.

3. Discussion

It has been reported in the literature that Fli-1 has been found to have varying degrees of high expression in leukaemia cells. And more studies have also confirmed that Fli-1 gene not only played an important regulatory role in the process of vascular endothelial cell generation and tumor cell proliferation, but also had a role in promoting tumorigenesis and development. Thus identifying candidate drug molecules in natural products is an important pathway for discovering innovative target anti-leukaemia drugs. Thus, 26 compounds were isolated and identified from the roots and leaves of *Z. nitidium.* And it is worth mentioning that the structures of compounds **4**-**6** and **16** were confirmed, and compounds **1**-**2** and **11** were isolated from *Z. nitidium* for the first time. In order to further analyze its new possible mechanism, compounds **14** and **24** with different structure types were tested of cell cycle and apoptosis against HEL. The above studies showed firstly that compound **14** exhibited antiproliferative activity and induced S phase cell cycle arrest and cell apoptosis of HEL cells, but compound **24** induced only cell apoptosis of HEL cells.

4. Materials And Methods

4.1 Chemicals Reagents

INOVA-400 MHz superconducting nuclear magnetic resonance spectrometer (American Varian, TMS internal standard); HPMS5973 mass spectrometer (HP, USA); ZF-2 type three-purpose UV instrument (Shanghai Anting Electronic Instrument Factory); silica gel G (Qingdao Ocean Chemical Plant Branch) and reversed-phase silica gel C-18 (Rp-18, 40-63 m) (Merck, Germany) for column chromatography; silica gel plates GF254 (Qingdao Puke Separation Material Co., Ltd.) for thin-layer chromatography; Sephadex LH-20 (Amersham Biosciences, Sweden); deuterated reagents for NMR spectroscopy (Wuhan Spectrum Company of Chinese Academy of Sciences); 5% (φ) concentrated sulfuric acid ethanol solution, an 8% (ω) phosphomolybdic acid ethanol solution, and a modified caesium iodide potassium test solution for staining TLC plates; 3111 CO_2 incubator (Thermo Fisher Scientific Co., Ltd.); X-15R centrifuge (Backman, USA); Synergy2 multi-function microplate detector (Gene Branch Chengdu Branch); TS100 Nikon binocular inverted microscope (Shanghai Shisen Vision Technology Co., Ltd.); BD AccuriTM C6 flow cytometer (BD Biosciences); 96-well culture plates (Nisi Biotechnology Co., Ltd.); and 6-well culture plates (Nisi Biotechnology Co., Ltd.).

4.2 Biological Reagents

Human leukemic cell lines HEL (ATCC) ; Adriamycin (Solarbio, D8740); dulbecco's modified eagle medium (DMEM, Gibco, C11995500CP); Foetal Bovine Serum (Bio IND, 04-002-1A); antibiotic-antimycotic (LifeTechnologies, 15240-112); bovine serum albumin (LifeTechnologies, 15561012); and Cell Titer Glo CTG, PROMEGA, G7572); Flow Cytometry (ACEN, NovoCyte); Microplate reader (BioTek EPOCH); Annexin V and propidium iodide (PI, DOJINDO, AD10).

4.3 Plant Material

The roots and leaves of *Zanthoxylum nitidum* (Roxb.) DC. were collected in Mengla County, Xishuangbanna of Yunnan province. The plant material was identified as *Zanthoxylum nitidium* (Roxb.) DC. by Dr. Chunfang Xiao, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences.

4.4 Extraction and Isolation

The air-dried roots and leaves of *Z. nitidum* (20.0 kg) were extracted by refluxing in 90% EtOH (100 L) three times (4, 3, and 2 h). After filtration, the combined EtOH extracts were concentrated to remove the alcohol, and the residue was resuspended in an appropriate volume of water. Then, it was extracted three times with equal volumes of petroleum ether and chloroform to afford 180.0 g of petroleum ether extract and 190.2 g of chloroform extract. The chloroform extract (190.2 g) was separated on a silica gel (50-74 μm) column eluted with a gradient of chloroform-MeOH (volume ratio: 100: 1 to 0: 100) to obtain 15 fractions (Fr.1 ~ Fr.15). The Fr.2 fraction was recrystallized from chloroform-methanol to afford compound **10** (1.3 g), and Fr.4 was recrystallized from chloroform-methanol to afford compound **24** (360

mg). Each fraction was repeatedly subjected to normal-phase silica gel column chromatography, reversed-phase silica gel column chromatography and Sephadex LH-20 column chromatography (alternating the use of MeOH and chloroform-MeOH as the eluents) to afford compounds **1** (15 mg), **2** (49 mg), **3** (20 mg), **4** (90 mg), **5** (19 mg), **6** (5 mg), **7** (50 mg), **8** (11 mg), **9** (29 mg), **11** (22 mg), **12** (30 mg), **13** (6 mg), **14** (58 mg), **15** (7 mg), **16** (30 mg), **20** (14 mg), **21** (5 mg), **23** (22 mg), **25** (8 mg), and **26** (20 mg). The petroleum ether extract (180.0 g) was separated on a silica gel (50-74 μm) column eluted with a gradient of petroleum ether-ethyl acetate (volume ratio: 100: 1 to 0: 100) to afford 8 fractions. The same purification method was used to obtain compounds **17** (30 mg), **18** (460 mg), **19** (60 mg), and **22** (31 mg).

*4.5 Spectroscopic Data of Compounds **4**, **5**, **6** and **16**.*

5-(3', 3'-Dimethyl-2'-butenyloxy)-6, 8-trimethoxy-coumarin (**4**): Yellow solid. UV (CH_3OH) λ max: 249, 201 and 316 nm. ^1H and ^{13}C NMR (Table 4). ESI-MS *m/z* 313 $[M + Na]^+$. HR-ESI-MS $[M]^+$ *m/z* 313.1042 $C_{16}H_{18}O_5$.

Methyl 2-(5-methoxy-2-methyl-1H-indol-3-yl) acetate (**5**): Tawny oil. UV (CH_3OH) λ max: 218 and 279 nm. ^1H and ^{13}C NMR (Table 4). ESI-MS *m/z* 256 $[M + Na]^+$. HR-ESI-MS $[M]^+$ *m/z* 233.1124 $C_{13}H_{15}O_3N$.

Ethyl 2'-(5, 6-dihydrochleletrythrine-6-yl) acetate (**6**): Yellow oil. UV (CH_3OH) λ max: 201, 283 and 224 nm. ^1H and ^{13}C NMR (Table 1). ESI-MS *m/z* 435 $[M + Na]^+$. HR-ESI-MS $[M]^+$ *m/z* 435.1752 $C_{14}H_{13}O_4N$.

4-Hydroxyl-7, 8-demethyfuroquinoline (**16**): Tawny solid. UV (CH_3OH) λ max: 249, 201 and 316 nm. ^1H and ^{13}C NMR (Table 1). ESI-MS *m/z* 268.0 $[M + Na]^+$. HR-ESI-MS $[M]^+$ *m/z* 245.0760 $C_{13}H_{12}O_4N$.

4.6 CTG Assay for the antitumor activity

The human leukaemia cell lines HEL were purchased from the cell bank of the American Type Culture Collection. The HEL cells were cultured in DMEM. All media were supplemented with 10% foetal bovine serum (FBS), 100 units/mL penicillin, and 100 units/mL streptomycin (Invitrogen). The cells were cultured at 37 °C in a humidified environment with 5% CO_2 and passaged once every 2 days, three generations. The cells were incubated in fresh cell culture medium and washed carefully to avoid false-positive results. Briefly, the HEL cells (8×10^3 cells per well) were seeded into 96-well plates at an initial density of 2000 cells/100 μL with 190 μL of medium in each well, and the plates were incubated for 24 h. Then, add 10 μL of serum- free Adriamycin as the positive control, 10 μL of varying concentrations (40, 20, 10, 5, 2.5, 1.25 μM) compounds (5×10^{-6} mol/L) as the test group and 5 well per group. After incubation for 72 h, 10 μL of CTG reagent was added, and the cells were incubated for 10 min. The 96-well plate after centrifugation (1500 r/min, 15 min), pour off the supernatant, add 160 μL of DMSO to each well, and heat and shake for 10 min. Finally, the chemiluminescence of each well was determined by a microplate reader. After the experiment was repeated three times, the IC_{50} value was calculated from the curves generated by plotting

the percentage of viable cells versus the tested concentration on a logarithmic scale using Sigma Plot 10.0 software.

4.7 Cell Apoptosis Analysis

Apoptosis was detected by flow cytometry using Annexin V-FITC according to the manufacturer's protocol (BD Biosciences). Leukaemia cell lines HEL were treated with compounds **14** and **24** for 36 h before Annexin V and propidium iodide staining. Keep the dying cells under dark conditions at room temperature for 15 min before being subjected to flow cytometry analysis.

4.8 Cell Cycle Analysis

Cell-cycle analysis was conducted by propidium iodide (PI) staining. Cell cycle analysis was analyzed after compounds **14** and **24** treatment for 36 h. Briefly, cells were plated in culture dishes and cultured with fresh medium without FBS for 12 h. Then, cells were treated with compounds **14** and **24** for 36 h and remove the supernatant, the treated cells were fixed with 70% ethanol overnight before staining with propidium iodide mixed with RNase. Keep the dying cells under dark conditions at room temperature for 30 min before being subjected to flow cytometry analysis.

4.9 Statistical Analysis

All measurements were made in triplicate, and all data are expressed as means ± SEM of three independent experiments. The significant differences from the respective control for each experimental group were examined by one-way analysis of variance (ANOVA) using GraphPad Prism 5 software. $P < 0.05$ was considered statistically significant.

5. Conclusions

In summary, four compounds with incomplete spectra (**4**-**6** and **16)** and 22 known compounds were isolated and identified from the chloroform and petroleum ether extracts of the roots and leaves of *Z. nitidium*. The chemical structures of compounds **4**-**6** and **16** were elucidated by thorough spectroscopic analyses, and compounds **1**, **2** and **11** have been isolated from *Z. nitidium* for the first time. Meanwhile, among the isolated compounds, **1**, **2**, **9**, **10**, **14**, **15** and **24,** which belong to alkaloids with good inhibitory activity against leukaemia cell lines HEL, and compound **14** (IC_{50}: 3.59 μM) and compound **24** (IC_{50}: 15.95 μM) showed the potent inhibitory activity against HEL. Thus, these results indicated that alkaloids had significant activities against leukaemia cells and had provided a new ideas of the mechanism. Notably, these compounds with benzophenanthrene moieties have more remarkable activities against leukaemia cells. To clear the effect of different structures of compounds on HEL cells. Further cell apoptosis and cell cycle assay showed that compound **14** exhibited antiproliferative activity, and induced S phase cell cycle arrest and cell apoptosis of HEL cells. Compound **24** induced only cell apoptosis of HEL cells. These results firstly suggested two compounds (**14** and **24**) could be the potential lead compounds with a good effect on Fli-1 gene against leukaemia in the further.

Supplemental Information Note

The following are available online. ^{1}H-NMR, ^{13}C-NMR, DEPT, HSQC, HMBC, ^{1}H-^{1}H-COSY, HR-ESI-MS, infrared, and ultraviolet-visible spectra of compounds **4**, **5**, **6** and **16**.

Declarations

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Author Contributions

D.Y. performed part of the chemical experiments and wrote the paper; M.S.Z. and H.X.J conceived and designed the experiments and revised the paper; D.L.L. and D.T.T. performed the biology experiments and revised the paper.

Competing Interest

The authors declare no conflict of interest.

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Tables

Table 1. ^{1}H (600 MHz) and ^{13}C (151 MHz) NMR data for compound **4** in $CDCl_3$

position	δ H（m，*J* in Hz）	δ C	HMBC
2		160.89	
3	6.16, s	110.98	C-8a, C-2
4	7.96, m	138.81	C-5a, C-2, C-5
5		128.79	
6	6.33, d (1.5)	91.33	C-8a, C-5, C-8, C-7
7		156.56	
8		152.31	
8a		103.85	
5a		149.04	
1’	4.54, dd (7.5, 1.5)	70.01	C-2’, C-5, C-3’
2’	5.57, d (1.5)	120.17	C-4’, C-5’
3’		139.03	
4’	1.68, s	17.95	C-5’, C-2’, C-3’
5’	1.73, s	25.79	C-4’, C-2’, C-3’
7-OCH_3	3.94, s	56.43	C-7
8-OCH_3	3.90, s	56.42	C-8

Table 2. ^{1}H (600 MHz) and ^{13}C (151 MHz) NMR data for compound **5** in $CDCl_3$.

position	δ_H (m, J in Hz)	δ_C	HMBC
2		172.80	
3		128.86	
4	7.04, m	111.14	C-5, C-3, C-7
5		154.05	
6	6.75, dd (8.7, 2.4)	110.83	C-7, C-5, C-7a
7	6.98, d (8.7)	100.35	C-7a, C-5, C-6, C-4, C-4a
4a		104.08	
7a		130.24	
8	3.65, s	30.31	C-2, C-3, C-4a
9		133.76	
10	2.28,s	11.69	C-4a, C-9
5-OCH_3	3.84, s	55.95	C-5
9-OCH_3	3.65, s	51.97	C-9

Table 3. ^{1}H (600 MHz) and ^{13}C (151 MHz) NMR data for compound **6** in $CDCl_3$.

position	δ_H (m, J in Hz)	δ_C	HMBC
1	7.12, s	104.29	C-2, C-12a, C-12
2		147.95	
3		147.50	
4	7.57, s	100.98	C-3, C-4b
4a		131.06	
4b		139.30	
6	4.95, m	55.11	C-4b, C-10a
6a		127.96	
7		145.50	
8		152.10	
9	6.99, d (J= 8.5 Hz)	111.61	C-7, C-10a
10	7.58, d (J= 8.5 Hz)	118.79	C-8, C-10b, C-6a
10a		124.90	
10b		123.81	
11	7.73, d (J=8.7 Hz)	119.75	C-4b, C-4a, C-10a
12	7.50, d (J=8.7 Hz)	123.99	C-1, C-10b, C-12a
12a		127.53	
N-CH_3	2.68, s	42.87	C-6
7-OCH_3	3.99, s	61.03	C-7
8-OCH_3	3.95, s	55.81	C-8
-O-CH_2-O-	6.06, s	100.97	
1’		171.67	
2’	2.38, s	39.18	C-1’, C-6
3’	4.17, d (J=7.1 Hz)	60.27	
4’	1.21, d (J=7.1 Hz)	14.18	C-3’

Table 4. ^{1}H (600 MHz) and ^{13}C (151 MHz) NMR data for compound **16** in Pyridine-d_5.

position	δ_{H}（m，J in Hz）	δ_{C}	HMBC
2		164.48	
3		101.61	
4		142.30	
4a		114.11	
5	8.13, d (9.1)	118.76	C-4, C-8, C-8a
6	7.54, d (9.1)	117.32	C-7, C-8, C-4a
7		140.17	
8		151.59	
8a		157.41	
3b	7.15, d (2.7)	105.34	C-2, C-3, C-4
2a	7.80, d (2.7)	142.90	C-2, C-3, C-3b
7-OCH_3	4.23, s	61.07	C-7
8-OCH_3	4.27, s	58.88	C-8
-OH	12.03, s		

Table 5. Inhibitory activity of compounds **1**, **6**, **7**, **8**, **12**, **14**, **15** and **24** against HEL cell lines.

Compounds	IC_{50} (μM) ± SD	Compounds	IC_{50} (μM) ± SD
1	28.84 ±1.53	**14**	3.59 ± 0.82
2	22.43 ± 1.86	**15**	15.52 ± 0.26
3	>30	**16**	>30
4	>30	**17**	>30
5	>30	**18**	>30
6	>30	**19**	>30
7	>30	**20**	>30
8	>30	**21**	>30
9	7.65 ± 0.11	**22**	>30
10	24.94 ± 1.99	**23**	>30
11	>30	**24**	15.95 ± 2.33
12	>30	**25**	>30
13	>30	**26**	>30
DOX	0.021		

Figures

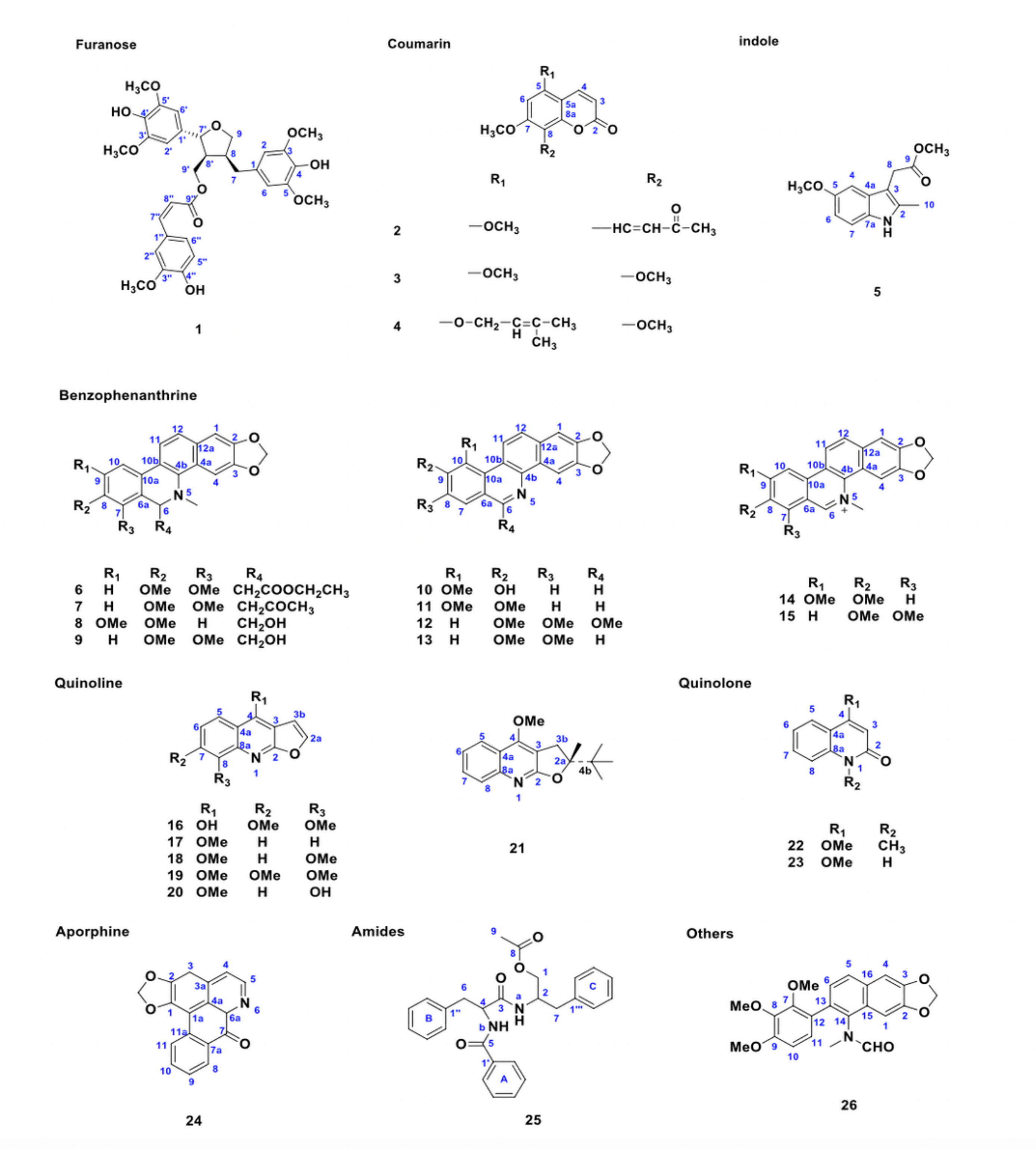

Figure 1

Compounds 1-26 isolated from the roots and leaves Zanthoxylum nitidium

8-(3', 3'-dimethyl-2'-butenyloxy)-5, 7-methoxy-coumarin (4')

compound 4

Figure 2

The structure of compound 4’ and HMBC correlations of compound 4

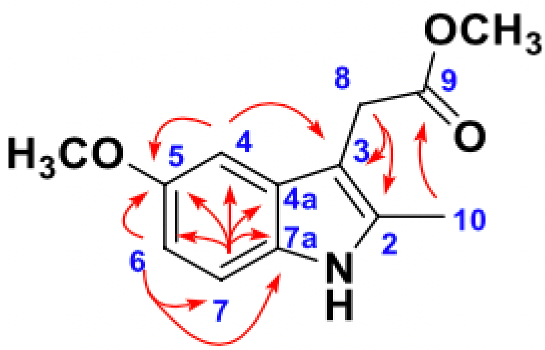

Figure 3

HMBC correlations of compound 5

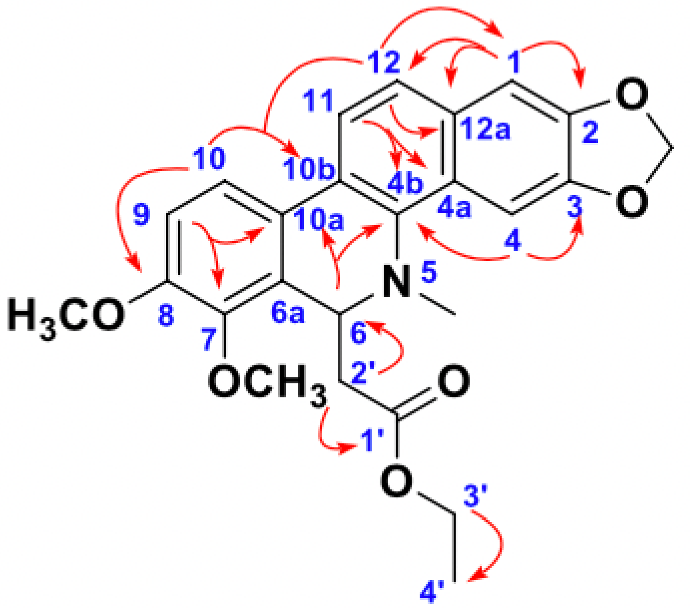

Figure 4

HMBC correlations of compound 6

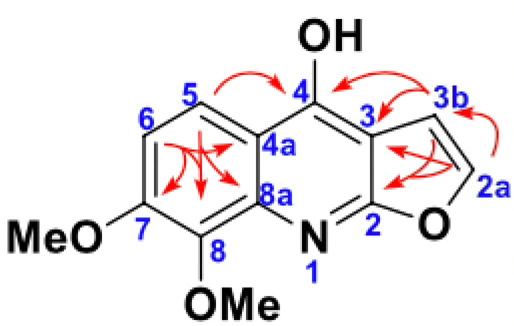

Figure 5

HMBC correlations of compound 16

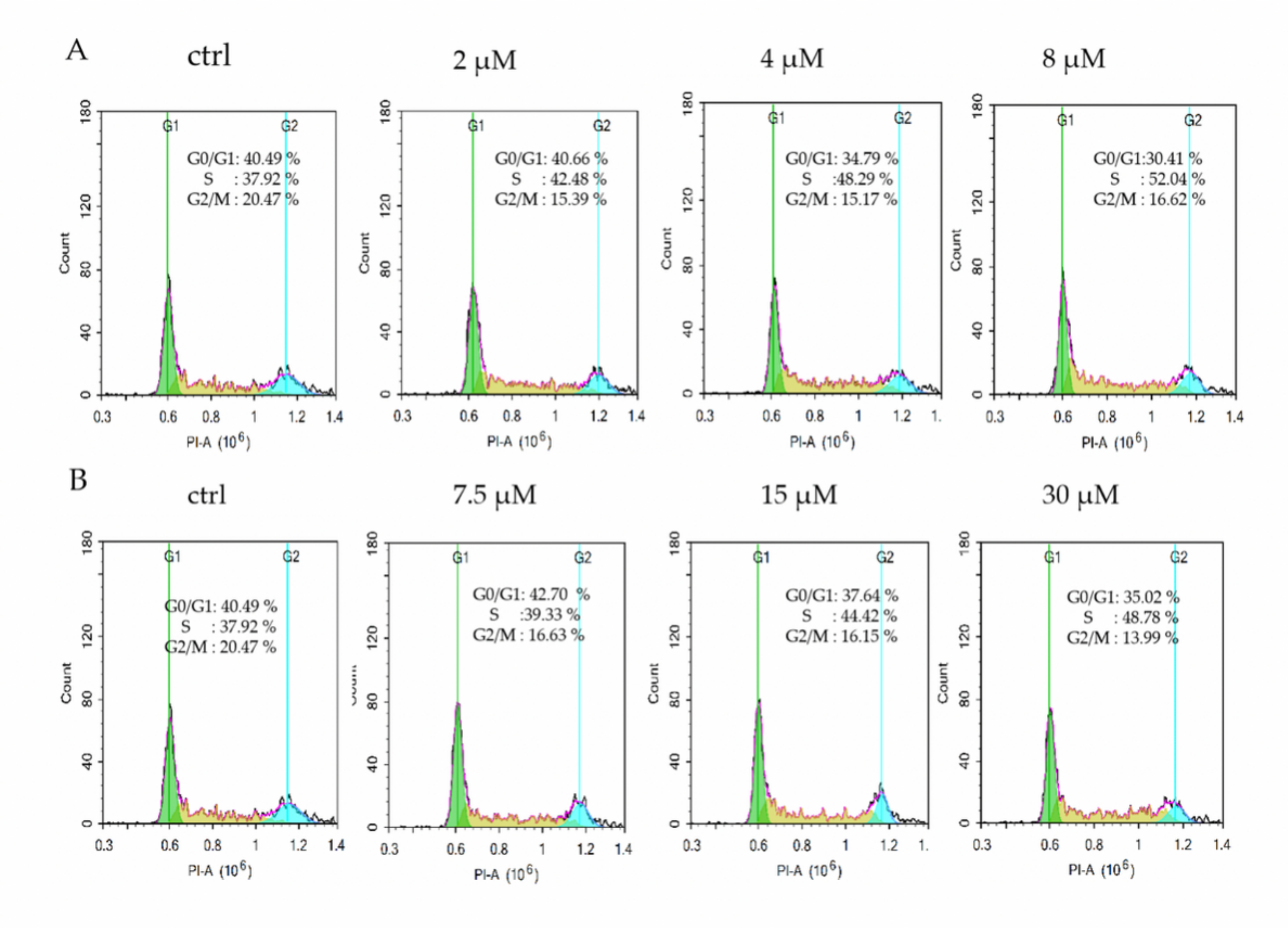

Figure 6

(A) Compound 14 induced cell cycle arrest at the phase. Compound 14 altered cell cycle distribution in HEL cells. Cells were exposed to DMSO or compound 14 at indicated concentrations for 36 h and then were collected for DNA content analysis by flow cytometric analysis as experiment. (B) Compound 24 induced cell cycle arrest at the phase. Compound 24 altered cell cycle distribution in HEL cells. Cells were exposed to DMSO or compound 24 at indicated concentrations for 36 h and then were collected for DNA content analysis by flow cytometric analysis as experiment.

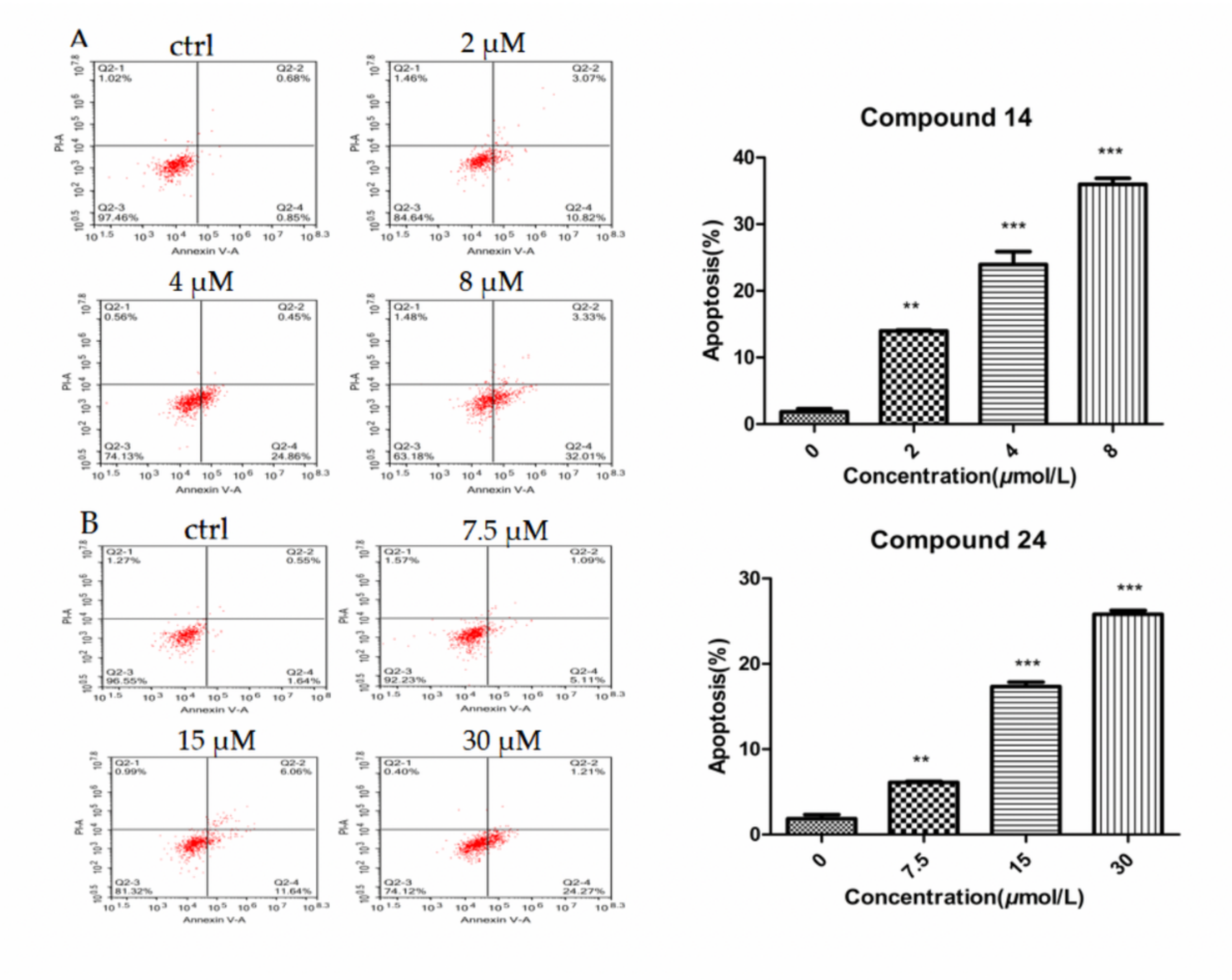

Figure 7

(A) Compound 14 induced apoptosis in HEL cells. Cell apoptosis was analyzed by flow cytometric analysis after Annexin V-FITC/PI staining. Cells were collected and centrifuged at 1500 rpm for 10min after compound 14 treatment at the indicated concentrations for 36 h. (B) Compound 24 induced apoptosis in HEL cells. Cell apoptosis was analyzed by flow cytometric analysis after Annexin V-FITC/PI staining. Cells were collected and centrifuged at 1500 rpm for 10min after compound 24 treatment at the indicated concentrations for 36 h. The changes in corresponding protein expression levels were quantified using Image J. Each bar represents the mean ± SEM (n = 3). $P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ was considered statistically significant compared with the corresponding control values.

Supplementary Files

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