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 treating leukaemia 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 firstly by UV-visible spectroscopy, 1D and 2D NMR and HR-ESI-MS. Compounds 1-2 and11 were isolated from Z. nitidium for the first time. Of the assayed compounds, compounds 14 and24 showed inhibitory activities against leukaemia HEL cells with IC50 values of 3.59 and 15.95 μM. In addition, to further investigate the possible mechanism, the cell cycle and apoptosis assays were investigated, which indicated that compound14 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 isvery 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 provinces. The chemical components of *Z. nitidium* are diverse and complex, and most of the constituents include 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 against leukaemia is comparatively rare. It was reported that Fli-1 showed 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 been proved to be a new target for drug screening. So investigating the active compounds against leukaemia from *Z. nitidium* is very significant in order to look for the Fli-1 inhibitor.

In our previous work, ethanol extracts of *Z. nitidium* had significant inhibitory effects on the proliferation of HEL cells, and toxicity tests in vitro showed that they had no significant toxicity. It is worth mentioning thatFli-1 genes had a high expression of in HEL cells against leukaemia. 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. 22 compounds (compounds **5-26**) of them were alkaloids, and the other four compounds (compounds **1-4**) were judged to be be false-positive non-alkaloids when coloured with the modified potassium caesium iodide. non-alkaloids which were proved by ^{1}H NMR and ^{13}C NMR spectra. 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 HEL cells were evaluated firstly. In addition, the possible mechanism of two active compounds was also investigated.

2. Results

- *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 recrystallization and various forms of column chromatography (CC) to afford compounds **1–26** (Figure 1).

< Figure 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].

< Table 1 >

The reported [8] suggested the ^{1}H-NMR signals of the C-8 of compound **4'** was the same as that of compound **4**, but other proton signals was slightly distinct. The ^{13}C-NMR data of compound **4**was not assigned in the literature, so its 1D and 2D NMR data were detected and analysed as the following. 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]..

< Figure 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].

< Table 2 >

Just like compound **4**, the 13C-NMR data of compound **5** was missing in the previous literature, and the 1D and 2D NMR data were also detected and analyzed. As shown in 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.

< Figure 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].

< Table 3 >

The NMR data of compound **6**was assigned firstly according to its 2D-NMR. 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.

< Figure 4 >

- *Chemical Structure of Compound **16***

Compound **16** was isolated as a tawny solid, which 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 ^1H 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].

< Table 4 >

To clarify the structure of **16**, we assigned the NMR data of compound **16** for the first time. Based on the ^1H 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. The above nuclear magnetic resonance data showed compound **16** was consistent with 4-hydroxy-7, 8-demethy-furoquinoline, which has been previously reported in the literature [14], but no its 1D and 2D NMR data attribution was performed on it. Herein, its NMR data of compound **16** was also assigned.

< Figure 5 >

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], 6*β*-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].

- *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), their IC_{50} values against HEL proliferation were tested by the CTG method, and Adriamycin was chosen as positive control (IC_{50}: 0.021 μM). As shown in Table 5, compound **14** (IC_{50}: 3.59 μM) and compound **9** (IC_{50}: 7.65 μM) showed themost potent inhibitory activity with the positive control, while compounds **15** (IC_{50}: 15.52 μM) and **24** (IC_{50}: 15.95 μM) exhibited moderate inhibitory activities against HEL cells. Structure type of compound **14** and compound **24** is different, which suggested the inhibitory activity against HEL cell lines of *Z. nitidium* may derived from different compounds.

< Table 5 >

- *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.

< Figure 6 >

- *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. Compaired with untreated cell, cells treated with compounds **14** and **24** displayed significant dose-denpendent increases in Figure 7. At the same time, compared with the control group (DMSO), compound **24** at 7.5 μM and 15.0 μM displayed significant increases. Compound **24** at the concentration of 7.5, 15, 30 μM can promote the apoptosis rate from 6.11%, 17.34% to 25.81% in a dose-dependent manner. Hence, these observations demonstrated that compounds **14** and **24** induced obvious apoptosis in HEL cells in a concentration-dependent manner firstly.

< Figure 7 >

3. Discussion

Looking for candidate drug molecules from natural products is still an important pathway for discovering innovative anti-leukaemia drugs. 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, cell cycle and apoptosis against HEL of compounds **14** and **24** with different structure types were tested. 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, which indicated that benzophenanthrene alkaloids may be the main active compounds of *Z. nitidium* against leukemia.

4. Materials And Methods

- *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.).

- *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).

- *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.

- *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).

- *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 $C_{13}H_{12}O_4N$.

- *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.

- *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.

- *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.

- *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.

Declarations

Funding

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Supplementary

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**.

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

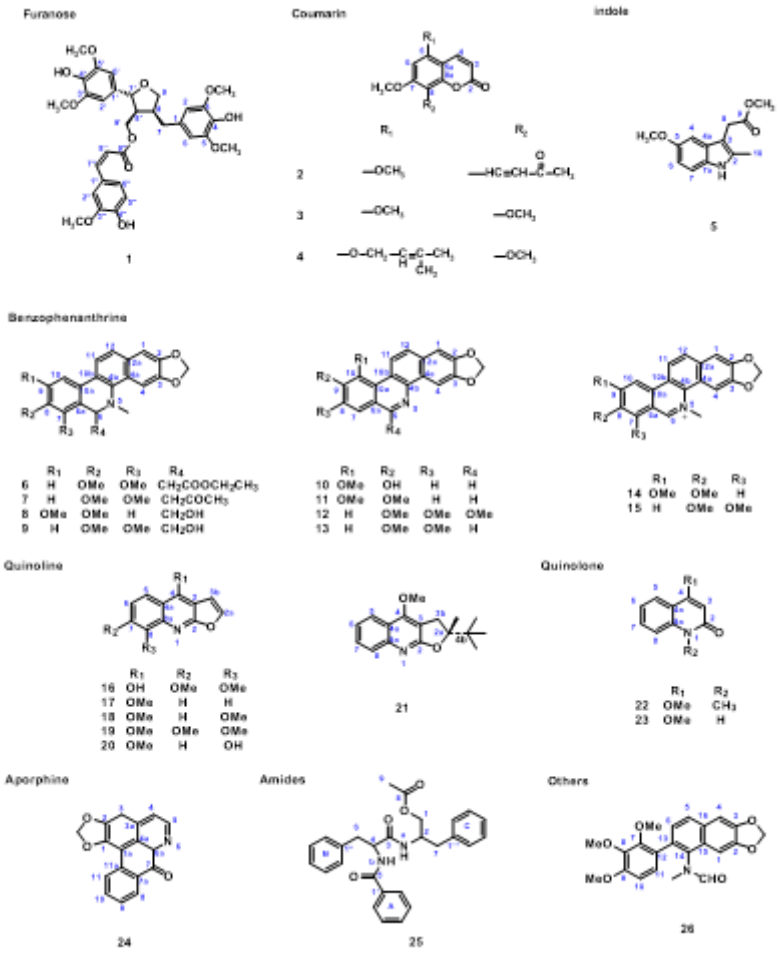
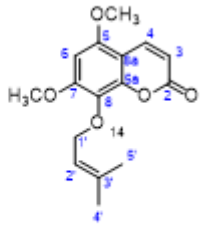
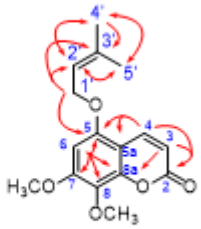


Figure 1

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

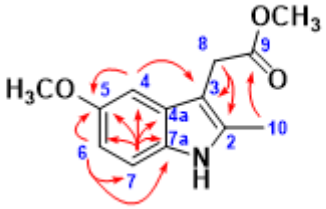


Figure 3

HMBC correlations of compound 5

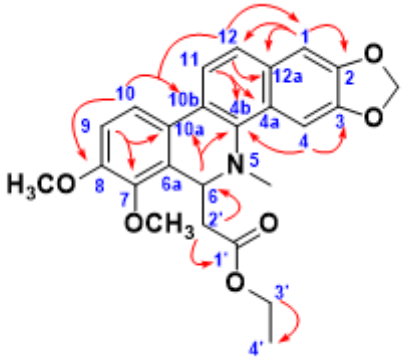


Figure 4

HMBC correlations of compound 6.

Figure 5

HMBC correlations of compound 16

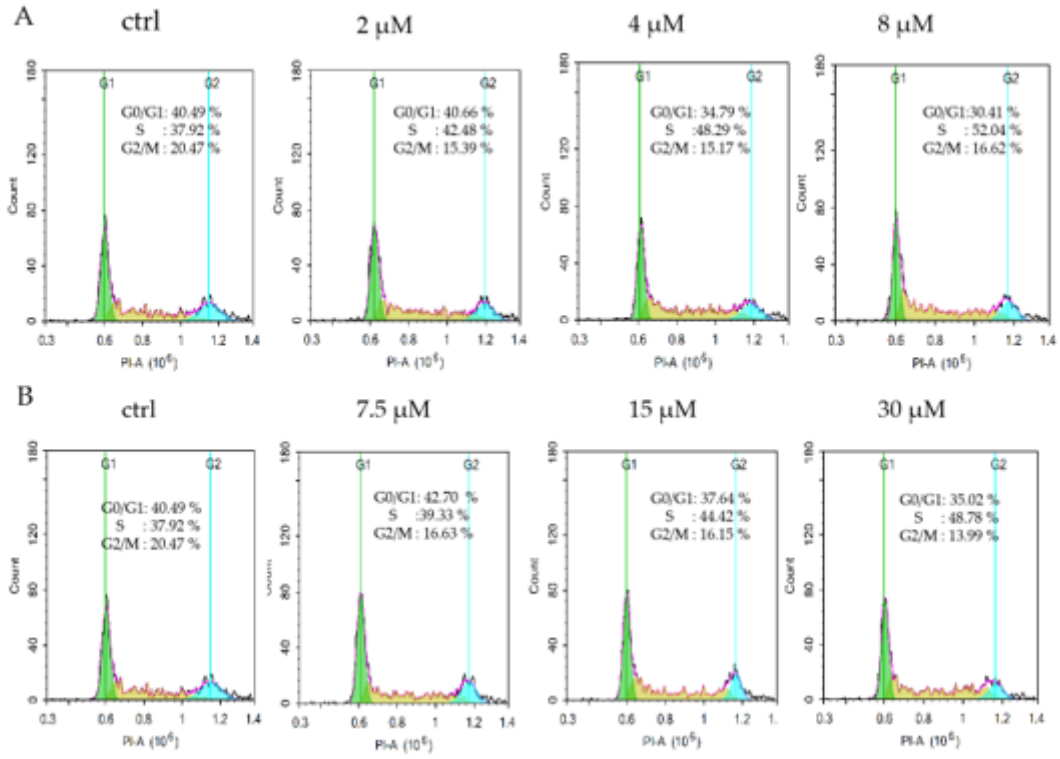


Figure 6

(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.

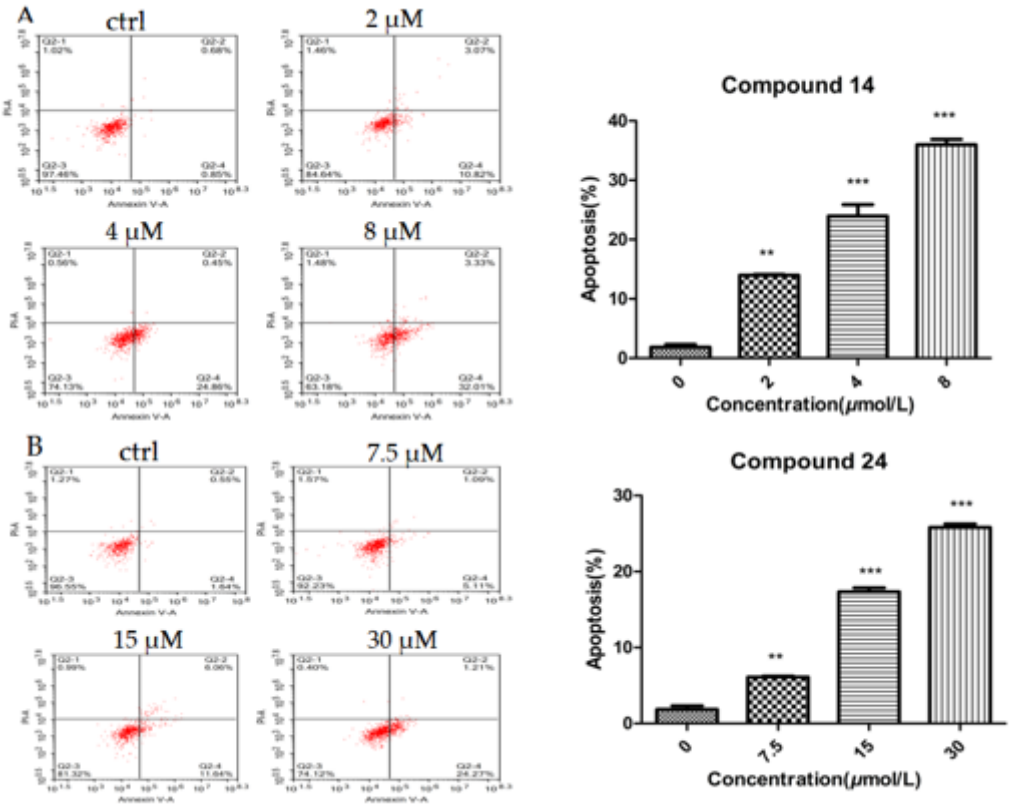


Figure 7

(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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