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Lignans and phenylpropanoids from the roots of *Ficus hirta* and their cytotoxic activities

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ABSTRACT

One undescribed lignan, one new natural product, along with fourteen known compounds, were isolated from the roots of *Ficus hirta*. The structures of the isolates were elucidated by comprehensive spectroscopic technologies, including UV, IR, HRESIMS, and NMR. The absolute configuration of **1** was determined by comparison of experimental and calculated ECD data. The cytotoxicity of all the compounds against HeLa and HepG2 cell lines was evaluated and compound **7** showed considerable cytotoxic effect towards HepG2 cells. Also, the apoptotic effect of **7** on HepG2 cells and the effect of **7** on the key proteins (p-JNK and p-p38) in MAPK (Mitogen-activated protein kinases) pathways were studied by flow cytometry and western blotting experiment. As a result, compound **7** induced the apoptosis of HepG2 cells, and dose-dependently increased the phosphorylation of JNK and p38. Thus, **7** might trigger HepG2 cells apoptosis via JNK/p38 MAPK signaling pathway.

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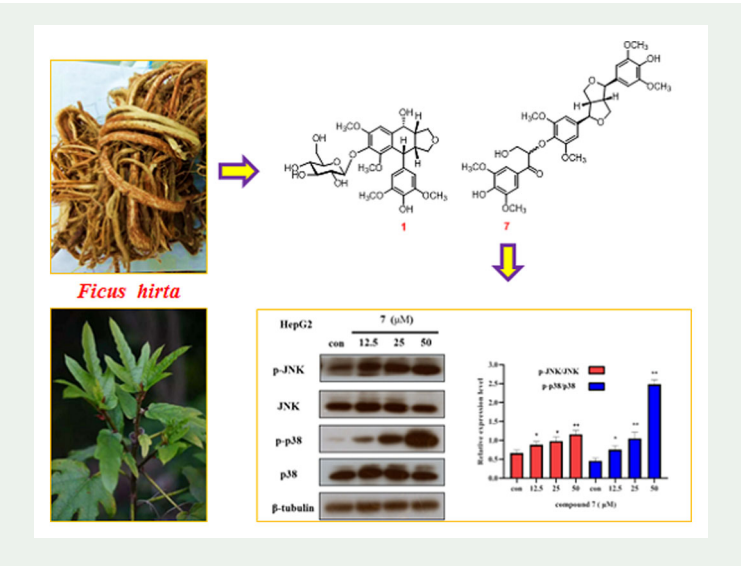

1. Introduction

In recent years, cancer remains one of the most dangerous diseases with a high fatality rate. Finding new chemotherapeutic agents inducing apoptosis of tumor cells is considered one of the most effective strategies for the treatment of cancer. Natural products play an essential role in the development of anticancer drugs. Therefore, it is of great significance to find potential anticancer agents from traditional Chinese medicine.

The pathological progress of tumor cell apoptosis is regulated by several signaling pathways. JNK (c-Jun N-terminal kinase) and p38 are the key proteins in MAPK (Mitogen-activated protein kinases) signaling pathways, which play crucial roles in the development of cancer, including cell growth, proliferation, and apoptosis (Sui et al. 2014; Kim et al. 2019; He et al. 2020).

Ficus hirta Vahl. (Wuzhimaotao), belonging to the Moraceae family is widely distributed in the south of China, such as Yunnan, Guangdong, and Fujian provinces. The roots of *F. hirta* were folk medicine for the treatment of cough, hepatitis, rheumatism, edema, and fatigue. Recent pharmacological studies revealed that *F. hirta* possessed antitumor, hepatoprotective, antibacterial, and anti-inflammatory effects (Qu et al. 2003; Chen et al. 2012; Zeng et al. 2012; Wan et al. 2016; Cai et al. 2017; Yang et al. 2018; Ye et al. 2020). Chemical studies have reported that a series of phenylpropanoids, flavonoids, coumarins, triterpenoids, and phenolic acids have been isolated from the root of *F. hirta* (Ya et al. 2010; Cheng et al. 2017a; 2017b; Ye et al. 2020). Also, lignans are the important source of natural products and exhibit a variety of biological activities (Tomassini et al. 2018; Zálešák et al. 2019; Frezza et al. 2020). In this paper, the phytochemical study on *F. hirta* led to the isolation of 16 lignans and simple phenylpropanoids (Figure 1), including one undescribed lignan (**1**) and one new natural product (**7**). All compounds were tested for their cytotoxicity against Hela and

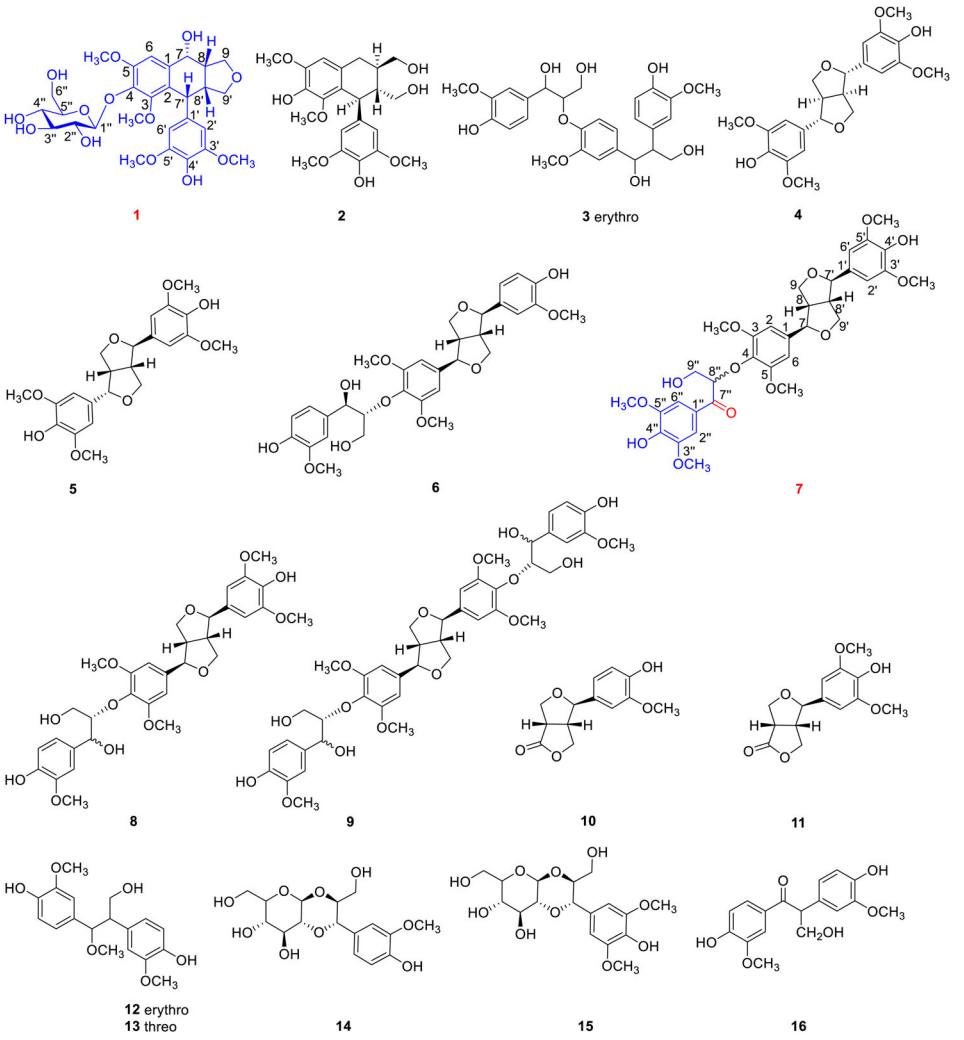

Figure 1. Structures of compounds **1** − **16** isolated from the roots of *F. hirta*.

HepG2 cell lines *in vitro*. Besides, the apoptotic effect of **7** on HepG2 cells and the effect of **7** on the crucial proteins (p-JNK and p-p38) in the MAPK signaling pathways were evaluated.

2. Results and discussion

Compound **1**, obtained as a white amorphous powder. Its molecular formula of $C_{28}H_{36}O_{13}$ was determined by the positive HRESIMS data (m/z 603.2043 $[M+Na]^+$, calcd 603.2048). The ^{1}H NMR spectrum (Table S1) of **1** showed three aromatic proton signals at δ_H 6.35 (2H, s) and 6.79 (1H, s). Moreover, four methines groups [δ_H 2.41 (1H, m), δ_H 2.94 (1H, m), 4.16 (1H, overlaps), and 4.87 (1H, overlaps)] and four methoxy groups [(δ_H 3.69 (3H, s), 3.77 (6H, s) and 3.94 (3H, s)] were observed. The ^{13}C NMR spectra (Table S1) exhibited 28 carbon resonances due to two methylenes, four

methines, four methoxy, twelve aromatic carbons, and a glucose group. Careful analysis of the NMR data of **1** suggested that it was similar to cissuside (Kumar et al. 2019) except for the differences of substituent groups in phenyl ring, the substituent location of glucose group, and the chemical shift of C-7 (**1**: δ_C 80.4; cissuside: δ_C 33.8). After acid hydrolysis, the sugar unit of **1** was identified as D-glucose (18.03 min) by HPLC analysis. The HMBC correlations (Figure S2) from δ_H 6.79 to C-7/C-4/C-5, δ_H 3.69 to C-5, and δ_H 3.94 to C-3 confirmed the presence of the 3, 5-dimethoxyphenyl ring. Furthermore, the HMBC correlation (Figure S2) from H-1‴ to C-4 indicated that the sugar moiety was located at C-4. The chemical shift of C-7 and the molecular formula of $C_{28}H_{36}O_{13}$ suggested that a hydroxyl was connected to C-7. The coupling constant of $J_{7',8'}$ (1.8 Hz) implied that H-7′ was *cis*-oriented with H-8′ (Liu et al. 2015), which was further confirmed by the NOESY correlation of H-7′ and H-8′. Also, The NOESY cross-peaks (Figure S3) of H-7′/H-7 and H-7′/H-8 implied that H-7, H-8, H-7′, and H-8′ had the same orientation. Accordingly, the planar structure of **1** was established. The absolute configuration of **1** was determined by comparison of experimental and calculated ECD data. As shown in figure S4, the calculated ECD spectrum of (7 R, 8S, 7′R, 8′R)-**1a** was agreed with the experimental spectrum of **1**. Thus, the absolute configuration of **1** was determined and compound **1** was named ficuslignin A.

Compound **7**, obtained as a white amorphous powder. Its molecular formula of $C_{33}H_{38}O_{13}$ was determined by the positive HRESIMS data (m/z 643.2385 $[M+H]^+$, calcd 643.2378). The ^{1}H NMR spectrum (Table S1) of **7** showed six aromatic proton signals at δ_H 7.36 (1H, s), 7.35 (1H, s), 6.68 (2H, s) and 6.65 (2H, s), together with six methoxy groups at δ_H 3.85 (6H, s), 3.84 (6H, s), and 3.72 (6H, s). The ^{13}C NMR spectra (Table S1) exhibited 33 carbon resonances, including three aromatic rings. According to 1 D NMR and HSQC spectra, two sets of similar signals at 4.71 (87.6), 3.13 (55.5), 3.95/4.27 (72.9), and 4.75 (87.2), 3.12 (55.7), 3.90/4.27 (72.8) indicated the presence of a typical skeleton of ditetrahydrofuran. A further detailed analysis of NMR spectra showed that compound **7** had the similar planar structure to (-)-(7 R,7′R,7″S,8S,8′S,8″S)-4′,4″-dihydroxy-3,3′,3″,5,5′, 5″-hexamethoxy-7,9′:7′,9-diepoxy-4,8″-oxy-8,8′-sesquineolignan-7″,9″-diol except for the significant downshift of C-7″ (δ_C 197.3) (Xiong et al. 2011). Furthermore, the HMBC correlations from H-8″/9″ to C-7″ indicated that C-7″ was substituted by a carbonyl (Figure S2). The coupling constants of $J_{7,8}$ (4.0 Hz) and $J_{7',8'}$ (4.0 Hz), together with the chemical shifts of H-7/H-7′ (4.71/4.75), H-8/H-8′ (3.13/3.12), C-7/C-7′ (87.6/87.2), and C-8/C-8′ (55.5/55.7), proved the *cis*-relationship of two aryl groups with H-8 and H-8′, respectively (Kamaya et al. 1983; Kim et al. 2010). The H-7/H-8 trans and H-7′/H-8′ trans configurations of **7** were further confirmed by the values of $\Delta\delta_{H\text{-}9}$ (0.39 ppm) and $\Delta\delta_{H\text{-}9'}$ (0.37 ppm) (Shao et al. 2018). The absolute configuration of ditetrahydrofuran moiety in **7** was characterized as 7 R,8S,7′R,8′S by comparison of experiment ECD spectrum and that of known compound (Kim et al. 2010) (a strong positive cotton effect at 208 nm (Figure S28) matched well with colocasinol A). Compound **7** was previously described as a synthetic product without any NMR and MS data (Kamaya et al. 1983). We herein reported it as a natural product for the first time and it was named ficuslignin B.

In addition, 14 known compounds were isolated from the roots of *F. hirta*. These compounds were identified as (+)-lyoniresinol (**2**) (Wang et al. 2013), leptolepisol D (**3**)

(Zhu et al. 2012), (+)-syringaresinol (**4**) (Wang et al. 2009), lirioresinol A (**5**) (Xu et al. 2014), hedyotol C (**6**) (Liao et al. 2014), (-)-(7 R,7′R,7′′R,8S,8′S,8′′S)-4′,4′′-Dihydroxy-3,3′,3′′,5,5′-pentamethoxy-7,9′:7′,9-diepoxy-4,8′′-oxy-8,8′-sesquineolignan-7′′,9′′-diol (**8**) (Xiong et al. 2011), (+)-(7 R,7′R,7′′R,7′′′S,8S,8′S,8′′S,8′′′S)-4′′,4′′′-Dihydroxy-3,3′,3′′,3′′′,5,5′-hexamethoxy-7,9′:7′,9-diepoxy-4,8′′:4′,8′′′-bisoxy-8,8′-dineolignan-7′′,7′′′,9′′,9′′′-tetraol (**9**) (Xiong et al. 2011), salicifoliol (**10**) (Liu et al. 2013), zhepeiresinol (**11**) (Jin et al. 1993), eythero-2,3-bis-(4-hydroxy-3-methoxypheyl)-3-methoxypropanol (**12**) (Yang et al. 2018), thero-2,3-bis-(4-hydroxy-3-methoxypheyl)-3-methoxypropanol (**13**) (Yang et al. 2018), ficuscarpanoside A (**14**) (Wang et al. 2017), ficuscarpanoside B (**15**) (Ouyang and Kuo 2006), evofolin B (**16**) (Duan et al. 2009).

The cytotoxicity of the isolates against Hela and HepG2 cell lines was evaluated by MTT assay. The result showed that compound **7** had distinct cytotoxicity against HepG2 cells at a concentration of 30 μM (Figure S5) and the IC_{50} value is 31.52 μM (Table S2). Subsequently, the apoptotic effect of **7** on HepG2 cells was detected by flow cytometry. As shown in figure S6, **7** induced the cells apoptosis from 6.64% to 12.06% after treated with gradient concentrations of **7** (0, 10, 20, 30 μM). MAPK signaling pathways have been reported to play a vital role in the apoptosis of tumor cells (Tsay et al. 2009; Ju et al. 2013; Hsieh et al. 2017; Xie et al. 2016; Zhu et al. 2016; Lin et al. 2018; Ham et al. 2019; Jiang et al. 2019). Evidences proved that the phosphorylation levels of JNK and p38 were up-regulated when treated with small molecules regulating these signaling pathways (Hsieh et al. 2017; Lin et al. 2018; Ham et al. 2019; Jiang et al. 2019). In this study, the effect of **7** on the MAPK pathway was investigated by western blot assay. As shown in figure S7, compound **7** markedly increased the expression levels of p-JNK and p-p38.

Compound **7** possessed a similar structure with **6**, **8**, and **9**, except for the extra carbonyl group. Compound **7** showed more potent antitumor activity than the others. Preliminary structure – activity relationship (SAR) analysis revealed that the carbonyl group at C-7 was essential for the antitumor activity.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a P-2000 polarimeter at 20 °C (JASCO, Japan). UV spectra were obtained using a Shimadzu UV-2600 spectrometer (Shimadzu, Japan). IR spectra were acquired by a Nicolet iS50 spectrometer (Thermo Scientific, USA). ECD spectra were measured on a MOS-500 circular dichroism spectrometer (Bio-Logic, France). The NMR and HRESIMS experiments were performed on a Bruker Avance III 600 MHz spectrometer (Bruker, Germany) and a Thermo Q Exactive Orbitrap mass spectrometer (Thermo Scientific, USA). Preparative and semi-preparative HPLC were performed on LC-16P instrument equipped with SPD-M20A detector (Shimadzu, Japan). D101 macroporous resin (CangZhou Bon Adsorber Technology Company), silica gel (200-300 mesh, Qingdao Ocean Chemical Company), Sephadex LH-20 (18 – 110 μm, Pharmacia), and YMC ODS-A (50 μm) were applied to column chromatography.

3.2. Plant material

The dried roots of *F. hirta* were purchased from Quanzhou city, Fujian province, People's Republic of China, in February 2017. The plant materials were identified by Prof. Zhen-Ji Li (College of the Environment & Ecology, Xiamen University). A voucher specimen (Fh-20170202) was deposited at Fujian Provincial Key Laboratory of Innovative Drug Target, School of Pharmaceutical Sciences, Xiamen University.

3.3. Extraction and isolation

The dried roots of *F. hirta* were extracted three times with 60% ethanol, and then concentrated in a vacuum to yield a residue. The crude extract was suspended in water and then subjected to chromatography on a D101 macroporous resin column eluted with H_2O-ethanol (0, 30%, 55% and 59%). The 30% ethanol residue (fraction B) (650 g) was separated by silica gel chromatography eluted with PE-EtOAc (3:1 to 0:1) and CH_2Cl_2−MeOH (9:1 to 0:1) to obtain 4 fractions (B1 − B4). Fraction B1 (70.0 g) was chromatographed over Sephadex LH-20 column eluted with MeOH to yield 5 subfractions (B1.1-B1.5). Fraction B1.2 (22.0 g) was separated on an ODS CC eluted with MeOH − H_2O (8:92 to 60:40) to give 5 fractions (B1.2.1 − B1.2.5). Fraction B1.2.1 (500 mg) was purified by semi-preparative HPLC (ACN − H_2O, 10:90, 2.0 ml/min) to afford compound **3** (1.5 mg, t_R = 61.0 min). Fraction B1.2.2 (300 mg) was purified by semi-preparative HPLC (MeOH − H_2O, 25:75, 3.0 ml/min) to obtain compounds **2** (6.5 mg, t_R = 32.5 min), **12** (25.0 mg, t_R = 33.0 min), and **13** (25.0 mg, t_R = 33.3 min). Fraction B1.2.3 (5.0 g) was further separated by semi-preparative HPLC (ACN − H_2O, 10:90, 3.0 ml/min) to yield compounds **1** (9.0 mg, t_R = 55.0 min), **14** (8.3 mg, t_R = 13.5 min), and **15** (7.0 mg, t_R = 14.0 min). The 55% ethanol residue (fraction C) (308 g) was separated by silica gel chromatography eluted with PE-EtOAc (3:1 to 0:1) and CH_2Cl_2−MeOH (9:1 to 0:1) to give 8 fractions (C1 − C7). Fraction C1 (20 g) was purified by ODS CC eluted with MeOH − H_2O (20:80 to 100:0) to obtain 6 fractions (C1.1 − C1.6). Fraction C1.1 (200 mg) was further purified by semi-preparative HPLC (ACN − H_2O, 45:55, 3.0 ml/min) to give compounds **10** (2.6 mg, t_R = 24.0 min), **11** (2.6 mg, t_R = 24.3 min), and **16** (20.0 mg, t_R = 33.0 min). Fraction C1.2 (6.0 g) was subjected to ODS CC eluted with MeOH − H_2O (20:80 to 65:40) to afford 4 fractions (C1.2.1 − C1.2.3). Fraction C1.2.2 (1.0 g) was purified by a Sephadex LH-20 column eluted with CH_2Cl_2-MeOH (1:1) to yield 4 subfractions (C1.2.2.1-C1.2.2.4). Fraction C1.2.2.2 (120 mg) was purified by semi-preparative HPLC (ACN − H_2O, 30:70, 3.0 ml/min) to yield compounds **4** (14.0 mg, t_R = 21.7 min), **5** (2.0 mg, t_R = 28.0 min), **6** (1.5 mg, t_R = 41.8 min), **7** (1.0 mg, t_R = 43.8 min), **8** (3.0 mg, t_R = 37.1 min) and **9** (3.0 mg, t_R = 50.0 min).

*Ficuslignin A (**1**)*. White, amorphous powder; $[\alpha]_D^{20}$-36 (*c* 0.10, MeOH); ECD (MeOH) λmax (Δε) 205 (+5.98), 217 (-6.15), 242 (+0.59) nm; UV (MeOH) λ_{max} (log ε) 209 (4.40) nm, 242 (3.46) nm; IR (KBr) ν_{max} 3420, 2900, 1609, 1517, 1457, 1215, 1111 cm^{-1}; ^1H and ^{13}C NMR data, see Table S1; HRESIMS *m/z* 603.2043 $[M + Na]^+$ (calcd for $C_{28}H_{36}O_{13}Na$, 603.2048)。

*Ficuslignin B (**7**)*. White, amorphous powder; $[\alpha]_D^{20}$-10 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 203 (-4.17) nm, 208 (+5.29) nm, 214 (-4.72) nm, 219 (+0.51) nm, 225 (+2.40) nm; IR (KBr) ν_{max} 3395, 2938, 2854, 1668, 1600, 1514, 1118 cm^{-1}; ^1H and ^{13}C NMR data, see Table S1; HRESIMS *m/z* 643.2378 $[M+H]^+$ (calcd for $C_{33}H_{39}O_{13}$, 643.2385).

3.4. ECD calculation method

In general, conformational analyses were carried out via random searching in the Sybyl-X 2.0 using the MMFF94S force field with an energy cutoff of 3.0 kcal/mol. The conformers were re-optimized using DFT at the b3lyp/6-311 + g(2d,p) level in the gas phase by the GAUSSIAN 09 program. The energies, oscillator strengths, and rotational strengths (velocity) of the first 60 electronic excitations were calculated using the TDDFT methodology at the b3lyp/6-311++g(2d,p) level in methanol. The ECD spectra were simulated by the overlapping Gaussian function (half the band-width at 1/e peak height, $\sigma = 0.3$). The simulated spectra of the conformers were averaged by the Boltzmann distribution theory to obtain the final calculated ECD spectrum.

3.5. Cell culture

HeLa and HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

3.6. Screening for cytotoxicity

The cytotoxicity of compounds **1**-**16** (30 μM) against HeLa and HepG2 cell lines were screened by MTT test. Briefly, the good growing cells were seeded in 96-well plates and incubated overnight, then treated with positive drug (cisplatin 10 μM) or compounds (30 μM) for 24 h. Cells were further incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h at 37 °C. When the cell supernatants were removed, the crystals were dissolved in DMSO. Finally, the absorbance of the solution was measured at 490 nm on a microplate reader.

3.7. Detection of apoptotic cells by double staining

The apoptotic cells were detected using annexin V-FITC/propidium iodide (PI) double staining apoptosis detection kit (Vazyme, Nanjing, China). Hela cells were seeded in 6-well plates and incubated with various concentrations (0, 10, 20, 30, 40 μM) of compound **7** for 24 h. Then, the cells were collected and washed twice with PBS at 4 °C, resuspended in 100 μL of binding buffer containing 5 μL of annexin V and 5 μL of PI in the dark for 10 min. After that, a 400 μL binding buffer was added. Finally, early or late apoptosis was detected by a flow cytometer (Invitrogen Attune NxT, Thermo Fisher Scientific, USA).

3.8. Western blot analysis

Hela cells were cultured in 6-well plates overnight and then various concentrations of compound **7** (10, 20, 30 μM) were added in for 24 h. After that, cells were treated with RIPA buffer containing protease and phosphatase inhibitors. Proteins were quantified by BCA protein assay kit (Beyotime, China). Subsequently, the equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membranes (Millipore, USA), and blocked with skim milk for 1 h. After washing in TBST three times, the membranes were incubated with primary antibodies: p-JNK1/2 (Thr183/Tyr185), p-p38 MAPK (Thr180/Tyr182), JNK1/2, p38 MAPK, and GADPH (1:2000 dilution) at 4 °C overnight. The membranes were washed by TBST three times again before incubated with secondary antibodies (1:3000 dilution) for 1 h at room temperature. Finally, the immunoreactivity of membranes was detected using an ECL kit (WesternBright ECL, Advansta, USA).

4. Conclusion

In conclusion, one undescribed lignan and one new natural product, together with fourteen known compounds were isolated from the roots of *F. hirta*. Compound **7** showed a considerable cytotoxic effect on HepG2 cells. Besides, the apoptotic effect of **7** on HepG2 cells and the effects of **7** on the key proteins (p-JNK and p-p38) of MAPK pathways were studied by flow cytometry and western blotting experiment. As a result, compound **7** induced the apoptosis of HepG2 cells, and dose-dependently increased the phosphorylation of JNK and p38. Thus, **7** might trigger HepG2 cells apoptosis via JNK/p38 related MAPK signaling pathway. And **7** might be considered as a potential lead for the development of anticancer drugs.

Disclosure statement

No potential conflict of interest was reported by the authors.

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