Anticancer activity of sesquiterpenoids extracted from *Solanum lyratum* via the induction of mitochondria-mediated apoptosis

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Abstract. Sesquiterpenoids are a major type of compound found in *Solanum lyratum (S. lyratum)*. The present study aimed to investigate whether sesquiterpenoids from *S. lyratum* demonstrated cytotoxicity against the MCF-7, HCT-8, A-549, SGC-7901 and BEL-7402 cell lines, and the mechanism of solajiangxin H and lyratol D, which exhibited high cytotoxicity against SGC-7901 cells (half maximal inhibitory concentration, IC_{50}=4.8 and 5.9 μg/ml), was associated with mitochondria-mediated apoptosis. The results of the Cell Counting Kit-8 assay indicated that 15 sesquiterpenoids had cytotoxicity against the aforementioned cultured cells. The results of DAPI staining and western blot analysis, used to study the anticancer mechanisms of solajiangxin H and lyratol D in SGC-7901 cells, suggested that solajiangxin H and lyratol D induced the apoptosis of SGC-7901 cells significantly (P<0.01), downregulated the expression of the antiapoptotic proteins B-cell lymphoma (Bcl)-2 and survivin, and upregulated the expression of the proapoptotic proteins Bcl-2-like protein 4, second mitochondria-derived activator of caspase, cleaved (c)-caspase-3 and c-caspase-9. The present study therefore demonstrated that 15 sesquiterpenoids from *S. lyratum* exhibited anticancer activity in MCF-7, HCT-8, A-549, SGC-7901 and BEL-7402 cells, and that the anticancer mechanisms of solajiangxin H and lyratol D may be associated with mitochondria-mediated apoptosis. Additionally, the present study provides evidence in support of the hypothesis that *S. lyratum* may be a promising candidate for the development of novel cancer therapies.

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Introduction

Solanum lyratum (S. lyratum), a common herbal medicine, is widely used in China for the treatment of malaria, icterus, cholecystitis, gonorrhea, rheumatoid arthritis, leucorrhea and cancer (1). Various compounds including saponins, organic acids, terpenoids, polyphenols, flavonoids, sterols, coumarins and polysaccharides have been identified in *S. lyratum* since the 20th century (2,3). The anticancer activity of *S. lyratum* has received attention, with numerous studies investigating the phenomenon (4-8). Previous studies indicated that water extract from *S. lyratum* induces apoptosis in the human gastric cancer SGC-7901 cell line and HeLa cells, and the mechanism may be associated with changes in the expression level and activity of the apoptotic proteins B-cell lymphoma-extra-large (Bcl-xl), BH3 interacting domain death agonist (Bid), p53, caspase-3 and caspase-9 (4,5). An additional study reported that extracts of *S. lyratum* induced cytotoxicity and apoptosis of the human colon adenocarcinoma Colo 205 cell line, which may be associated with cyclin-dependent kinase 1 (Cdk1), p27, p53, cyclin B1, cyclin E, caspase-3, caspase-8, procaspase-9, B-cell lymphoma-2 (Bcl-2), Bcl-2-like protein 4 (Bax) and cytochrome c activity (6). Additionally, there are studies regarding the cytotoxicity activity of *S. lyratum* against different cancer cell lines (7,8). However, the aforementioned studies mainly focus on the anticancer activity of the crude extracts of *S. lyratum*, thus the results cannot explain the active anticancer constituent of *S. lyratum*. Thus, an investigation into the anticancer activity and mechanism of the monomeric compounds isolated from *S. lyratum* is required.

Therefore, the presented study explored the anticancer activity and mechanism of the sesquiterpenoids isolated from *S. lyratum*, which may be useful for the interpretation of the anticancer effect of *S. lyratum* and the development of novel therapies from this plant.

Materials and methods

Plant material. S. lyratum Thunb was obtained from Tongrentang Pharmaceutical Group (Beijing, China) in 2013 and a voucher specimen (voucher no. YP20130913) was stored in Nanjing University of Chinese Medicine (Nanjing, China) for future reference.

Chemicals and reagents. Analytical grade chloroform, ethyl acetate, n-butyl alcohol, cyclohexane, acetone, methanol and silica-gel were obtained from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China) and Sephadex LH-20 and preparative thin layer chromatography (TLC) were obtained from H&E Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) and RPMI-1640 media were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The Cell Counting Kit (CCK)-8 and DAPI were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Primary antibodies against Bcl-2 (cat. no. AB112; 1:1,000), survivin (cat. no. AS792; 1:1,000), Bax (cat. no. AB026; 1:500), cleaved-caspase (c-caspase)-3 (cat. no. AC033; 1:1,000), β-actin (cat. no. AF0003; 1:1,000) and horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. A0192; 1:1,000) were purchased from Beyotime Institute of Biotechnology (Shanghai, China), while antibodies against second mitochondria-derived activator of caspase (Smac; cat. no. 15108; 1:1,000) and c-caspase-9 (cat. no. 9501; 1:1,000) were purchased from Cell Signaling Technology (Beverly, MA, USA). BeyoECL Plus reagent was purchased from Beyotime Institute of Biotechnology.

Extraction and isolation. The air-dried whole plant (60 kg) of *S. lyratum* was finely cut using scissors and extracted by refluxing 4 times with 120 l ethyl alcohol. After filtration, the residues were used for the next extraction. The ethyl alcohol solvent was evaporated under reduced pressure to produce a crude extract (4.0 l), which was then suspended in water and successfully partitioned into chloroform, ethyl acetate and n-butyl alcohol. The chloroform fraction (238.6 g) was subject to column chromatography (CC) over silica gel (100-200 mesh) and eluted with cyclohexane-acetone (95:5-50:50) to produce 8 fractions. Fraction 3 (8.2 g) was separated using CC over silica gel (cyclohexane-ethyl acetate) to produce compound 1 (58 mg) and a mixture, this was additionally isolated by preparative thin layer chromatography (TLC; chloroform-ethyl acetate) and purified on Sephadex LH-20 (chloroform-methanol) producing compounds 2 (123 mg) and 3 (73 mg). Fraction 4 (25.4 g) was isolated by CC over silica gel (cyclohexane-ethyl acetate) and preparative TLC (chloroform-acetone) to provide compounds 4 (186 mg), 5 (92 mg) and 6 (57 mg). Fractions 5 (9.1 g) and 6 (8.4 g) were separated by CC over silica gel (petroleum ether-acetone) and preparative TLC (cyclohexane-acetone), and purified on Sephadex LH-20 (chloroform-methanol) to provide compounds 7 (93 mg), 8 (51 mg), 9 (105 mg), 10 (108 mg), 11 (61 mg) and 12 (72 mg). Fractions 2 (3.8 g) and 8 (19.2 g) were isolated by reversed-phase CC over silica gel (methanol-water) and purified on Sephadex LH-20 (chloroform-methanol) to produce compounds 13 (87 mg), 14 (73 mg) and 15 (68 mg). A total of 15 compounds were identified by nuclear magnetic resonance (NMR) data and compared with the existing literature. The purity of the compounds was verified by high performance liquid chromatography (HPLC). The HPLC analysis was carried out on LC-20AT (Shimadzu, Kyoto, Japan), equipped with an auto sampler, a binary solvent delivery pump and a diode array detector, using Empower 2 software (Waters, Milford, MA, USA). The chromatographic separation was carried out on an Ultimate AQ C_{18} (4.6x250 mm, 5 μm; Welch, Shanghai, China) at 35˚C. The mobile phase was composed of acetonitrile and water. The injection volume and the flow rate were 10 μl and 1.0 ml/min, respectively.

Cell culture. The MCF-7, HCT-8, A-549, SGC-7901 and BEL-7402 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). In total, 5 types of cell were separately cultured in RPMI-1640 medium including 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37˚C in 5% CO_2/95% air.

CCK-8 assay. A CCK-8 assay was used to investigate the cytotoxicity of the 15 sesquiterpenoids against cultured MCF-7, HCT-8, A-549, SGC-7901 and BEL-7402 cells and the IC_{50} value, defined as the concentration that led to a 50% decrease in the number of cells, was used to evaluate their cytotoxicity. Cells were seeded at a density of $2x10^4$/well into 96-well plates with RPMI-1640 medium. Subsequent to 24 h incubation, the cells were treated with different concentrations of fractions and compounds. A total of 48 h following treatment, CCK-8 solution was added and the cells were cultured at 37˚C in 5% CO_2/95% air for 3 h. The optical density was then determined at 450 nm with a microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis assay. According to the CCK-8 assay results of the 15 sesquiterpenoids, solajiangxin H and lyratol D, which had high cytotoxicity against SGC-7901 cells, IC_{50}=4.8 and 5.9 μg/ml, respectively, were selected to study their anticancer activities and mechanisms. Following treatment with solajiangxin H and lyratol D at doses of 0 (control), 2 or 4 μg/ml for 48 h, the SGC-7901 cells were washed 3 times with PBS. The washed SGC-7901 cells were resuspended in staining buffer and stained with DAPI according to the manufacturer's protocol. Images were then captured of the SGC-7901 cells using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Western blot assay. Subsequent to treatment with solajiangxin H and lyratol D at concentrations of 0 (control), 2, 4 and 8 μg/ml for 48 h, respectively, the SGC-7901 cells were collected and their total proteins were extracted using cell lysis buffer, ultrasound and centrifugation at 12,000 x *g* for 15 min at 4˚C. The concentration of total proteins was determined using an enhanced Bicinchoninic Acid assay kit (Beyotime, Haimen, China) according to the manufacturer's protocol. Subsequently, ~35 μg total protein was separated by SDS-PAGE, transferred onto polyvinylidene fluoride membranes and incubated with the primary antibodies. The protein was then incubated with HRP-conjugated secondary antibody, and the anti- and proapoptotic proteins were detected immediately subsequent to incubation using BeyoECL Plus reagent. In addition,

antibody directed against β-actin was used to assess the level of protein loading.

Statistical analysis. All data were presented as the mean ± standard deviation. A one-way analysis of variance using SPSS 21.0 (IBM SPSS, Armonk, NY, USA) determined whether the results showed statistical significance between groups, and P<0.05 was considered to indicate a statistically significant difference.

Results

Sesquiterpenoids isolated from S. lyratum. A total of 15 sesquiterpenoids, including compounds 1 (solajiangxin I, $C_{18}H_{28}O_3$), 2 (7-hydroxylsolajiangxin I, $C_{18}H_{28}O_4$), 3 (solajiangxin H, $C_{18}H_{28}O_4$), 4 (lyratol D, $C_{15}H_{20}O_3$), 5 (dehydrovomifoliol, $C_{13}H_{18}O_3$), 6 (blumenol A, $C_{13}H_{20}O_3$), 7 (solajiangxin A, $C_{15}H_{24}O_4$), 8 (solajiangxin B, $C_{15}H_{18}O_4$), 9 (solajiangxin C, $C_{15}H_{18}O_3$), 10 (solajiangxin D, $C_{15}H_{24}O_4$), 11 (solajiangxin E, $C_{18}H_{28}O_3$), 12 (2-hydroxysolajiangxin E, $C_{18}H_{28}O_4$), 13 (lyratol A, $C_{15}H_{24}O_3$), 14 (lyratol B, $C_{15}H_{24}O_3$) and 15 (lyratol C, $C_{15}H_{26}O_4$) were successfully isolated from *S. lyratum*, and their chemical structures are shown in Fig. 1. In addition, the NMR data are as follows:

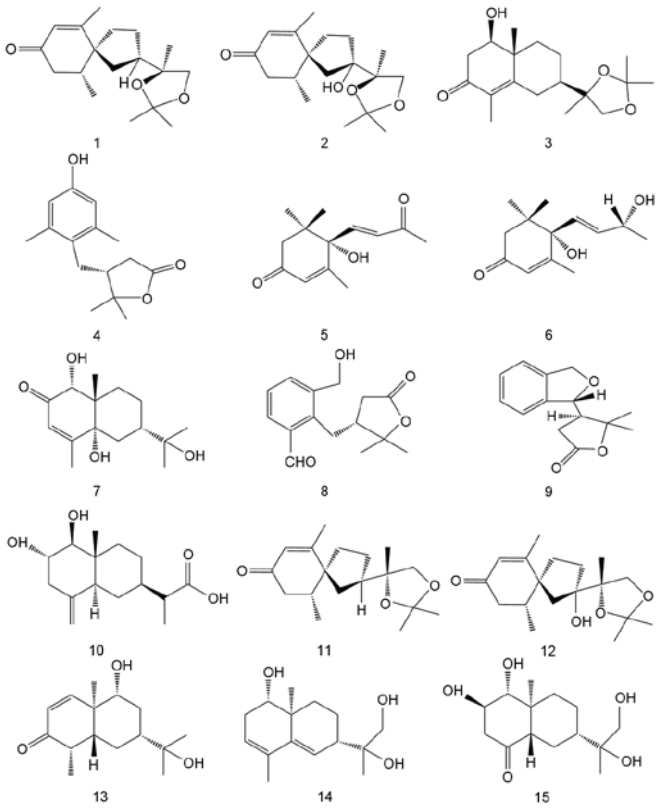

Figure 1. The chemical structure of 15 the sesquiterpenoids isolated from *S. lyratum* (compounds 1-15).

Compound 1 (solajiangxin I, $C_{18}H_{28}O_3$) presented as a colorless viscous oil; purity: 97.3%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.67 (1H, dd, *J*=13.0, 7.3 Hz, Ha-1), 2.05 (1H, dd, *J*=13.0, 11.6 Hz, Hb-1), 2.17 (1H, m, H-2), 1.80 (1H, m, Ha-3), 2.02 (1H, m, Hb-3), 1.86 (1H, m, Ha-4), 1.97 (1H, m, Hb-4), 5.98 (1H, s, H-7), 2.68 (1H, dd, *J*=16.8, 4.8 Hz, Ha-9), 2.61 (1H, dd, *J*=16.8, 4.3 Hz, Hb-9), 2.28 (1H, m, H-10), 3.61 (1H, d, *J*=8.7, Ha-12), 3.74 (1H, d, *J*=8.7, Hb-12), 1.31 (3H, s, H-13), 1.90 (3H, s, H-14), 1.13 (3H, d, *J*=7.1, H-15), 1.50 (3H, s, H-2'), 1.49 (3H, s, H-3'); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 35.5 (C-1), 46.3 (C-2), 28.1 (C-3), 33.7 (C-4), 50.6 (C-5), 165.6 (C-6), 124.6 (C-7), 197.6 (C-8), 43.6 (C-9), 39.8 (C-10), 82.1 (C-11), 72.9 (C-12), 24.7 (C-13), 21.6 (C-14), 16.5 (C-15), 109.6 (C-1'), 26.2 (C-2'), 27.7 (C-3') (9).

Compound 2 (7-hydroxylsolajiangxin I, $C_{18}H_{28}O_4$) presented as a colorless viscous oil; purity: 98.1%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 1.88 (1H, d, *J*=13.9 Hz, Ha-1), 1.79 (1H, d, *J*=13.9 Hz, Hb-1), 1.56 (1H, m, Hb-3), 1.73 (1H, m, Ha-3), 1.84 (1H, m, Ha-4), 1.98 (1H, m, Hb-4), 5.66 (1H, s, H-7), 2.78 (1H, dd, *J*=16.2, 5.0 Hz, Ha-9), 2.21 (1H, dd, *J*=16.2, 4.1 Hz, Hb-9), 2.46 (1H, m, H-10), 3.81 (1H, d, *J*=8.5, Ha-12), 4.13 (1H, d, *J*=8.5, Hb-12), 1.51 (3H, s, H-13), 1.86 (3H, s, H-14), 1.13 (3H, d, *J*=7.2, H-15), 1.20 (3H, s, H-2'), 1.39 (3H, s, H-3'), 4.67 (1H, s, C2-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 43.2 (C-1), 84.1 (C-2), 36.6 (C-3), 33.8 (C-4), 50.6 (C-5), 166.4 (C-6), 124.8 (C-7), 198.9 (C-8), 43.1 (C-9), 40.3 (C-10), 85.5 (C-11), 72.7 (C-12), 22.3 (C-13), 20.1 (C-14), 16.5 (C-15), 109.6 (C-1'), 26.4 (C-2'), 27.3 (C-3') (9).

Compound 3 (solajiangxin H, $C_{18}H_{28}O_4$) presented as a colorless viscous oil; purity: 95.1%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 3.78 (1H, dd, *J*=12.4, 5.1 Hz, H-1), 2.47 (1H, dd, *J*=16.1, 12.3 Hz, Ha-2), 2.57 (1H, dd, *J*=16.1, 5.2 Hz, Hb-2), 1.77 (1H, br t, *J*=14.5 Hz, Ha-6), 2.97 (1H, br d, *J*=14.5 Hz, Hb-6), 1.52 (1H, m, H-7), 1.91 (1H, m, Ha-8), 1.43 (1H, m, Hb-8), 1.31 (1H, m, Ha-9), 2.07 (1H, m, Hb-9), 3.70 (1H, d, *J*=8.3, Ha-12), 3.63 (1H, d, *J*=8.3, Hb-12), 1.31 (3H, s, H-13), 1.10 (3H, s, H-14), 1.68 (3H, s, H-15), 1.41 (3H, s, H-2'), 1.46 (3H, s, H-3'); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 74.1 (C-1), 42.0 (C-2), 197.5 (C-3), 129.4 (C-4), 161.5 (C-5), 29.3 (C-6), 46.4 (C-7), 22.7 (C-8), 37.1 (C-9), 41.9 (C-10), 82.7 (C-11), 72.4 (C-12), 22.6 (C-13), 16.1 (C-14), 11.3 (C-15), 26.6 (C-2'), 27.3 (C-3') (9).

Compound 4 (lyratol D, $C_{15}H_{20}O_3$) presented as a pale yellow lamellar crystal; purity: 98.2%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.31 (1H, dd, *J*=18.3, 3.6, Ha-3), 2.43 (1H, dd, *J*=18.3, 10.9, Hb-3), 2.54 (1H, m, H-4), 2.43 (1H, dd, *J*=14.1, 5.0, Ha-6), 2.79 (1H, dd, *J*=14.1, 11.3, Hb-6), 6.43 (1H, s, H-9), 6.43 (1H, s, H-11), 1.46 (3H, s, H-13), 1.59 (3H, s, H-14), 2.56 (3H, s, H-15), 2.26 (3H, s, H-16); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 175.5 (C-2), 34.1 (C-3), 46.6 (C-4), 86.6 (C-5), 27.3 (C-6), 127.2 (C-7), 137.9 (C-8), 115.4 (C-9), 153.5 (C-10), 115.8 (C-11), 137.8 (C-12), 21.7 (C-13), 27.7 (C-14), 20.3 (C-15), 20.3 (C-16) (10).

Compound 5 (dehydrovomifoliol, $C_{13}H_{18}O_3$) presented as a pale yellow oil; purity: 93.0%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.37 (1H, d, *J*=17.0 Hz, H-2a), 2.51 (1H, d, *J*=17.0 Hz, H-2b), 5.98 (1H, s, H-4), 6.94 (1H, d, *J*=15.4 Hz, H-7), 6.44 (1H, d, *J*=15.4 Hz, H-8), 2.33 (3H, s, H-10), 1.89 (3H, s, H-11), 1.08 (3H, s, H-12), 1.01 (3H, s, H-13); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 41.6 (C-1), 49.3 (C-2), 197.3 (C-3), 127.8 (C-4), 160.1 (C-5), 79.5 (C-6), 144.8 (C-7), 130.5 (C-8), 196.8 (C-9), 28.2 (C-10), 18.7 (C-11), 24.5 (C-12), 22.9 (C-13) (11).

Compound 6 (blumenol A, $C_{13}H_{20}O_3$) presented as a pale yellow oil; purity: 91.2%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.36 (1H, d, *J*=17.3 Hz, H-2a), 2.57 (1H, d, *J*=17.3 Hz, H-2b), 5.98 (1H, s, H-4), 5.80 (1H, d, *J*=15.5 Hz, H-7), 5.81 (1H, dd, *J*=15.5, 5.3 Hz, H-8), 4.58 (1H, m, H-9), 1.32 (3H, d, *J*=6.7, H-10), 1.08

(3H, s, H-11), 1.05 (3H, s, H-12), 1.85 (3H, s, H-13); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 41.6 (C-1), 49.5 (C-2), 198.3 (C-3), 127.4 (C-4), 162.1 (C-5), 78.5 (C-6), 128.6 (C-7), 135.4 (C-8), 68.8 (C-9), 23.2 (C-10), 22.3 (C-11), 23.8 (C-12), 18.9 (C-13) (11).

Compound 7 (solajiangxin A, $C_{15}H_{24}O_4$) presented as a white powder; purity: 917.5%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.30 (1H, br s, H-1), 5.58 (1H, s, H-3), 1.67 (1H, dd, *J*=13.1, 3.7 Hz, Ha-6), 1.14 (1H, dd, *J*=13.1, 6.2 Hz, Hb-6), 1.78 (1H, m, H-7), 1.54 (1H, br d, *J*=12.6 Hz, Ha-8), 1.46 (1H, m, Hb-8), 2.17 (1H, br t, *J*=13.4 Hz, Ha-9), 1.18 (1H, m, Hb-9), 1.17 (3H, s, H-12), 1.19 (3H, s, H-13), 0.86 (3H, s, H-14), 1.90 (3H, s, H-15), 6.61 (1H, br s, C1-OH), 5.32 (1H, s, C5-OH), 4.11 (1H, s, C11-OH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 79.9 (C-1), 197.5 (C-2), 122.7 (C-3), 164.5 (C-4), 73.3 (C-5), 29.1 (C-6), 42.3 (C-7), 20.1 (C-8), 28.5 (C-9), 40.7 (C-10), 70.8 (C-11), 27.1 (C-12), 27.5 (C-13), 20.2 (C-14), 19.0 (C-15) (12).

Compound 8 (solajiangxin B, $C_{15}H_{18}O_4$) presented as a colorless viscous oil; purity: 90.6%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.56 (1H, dd, *J*=15.6, 11.0 Hz, Ha-3), 2.01 (1H, dd, *J*=15.6, 6.1 Hz, Hb-3), 2.41 (1H, m, H-4), 3.12 (2H, br d, *J*=6.6 Hz, H-6), 7.51 (1H, br d, *J*=7.7 Hz, H-9), 7.31 (1H, br t, *J*=7.7 Hz, H-10), 7.63 (1H, br d, *J*=7.6 Hz, H-11), 1.39 (3H, s, H-13), 1.54 (3H, s, H-14), 4.70 (2H, s, H-15), 10.19 (1H, s, H-16); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 175.6 (C-2), 33.6 (C-3), 46.7 (C-4), 87.4 (C-5), 26.3 (C-6), 139.2 (C-7), 134.1 (C-8), 136.5 (C-9), 127.9 (C-10), 134.1 (C-11), 140.3 (C-12), 21.7 (C-13), 26.5 (C-14), 62.0 (C-15), 193.6 (C-16) (12).

Compound 9 (solajiangxin C, $C_{15}H_{18}O_3$) presented as a colorless viscous oil; purity: 91.4%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.58 (1H, dd, *J*=17.5, 10.1 Hz, Ha-3), 2.67 (1H, dd, *J*=17.5, 9.0 Hz, Hb-3), 3.17 (1H, m, H-4), 5.88 (1H, d, *J*=9.2 Hz, H-6), 7.06 (1H, br d, *J*=7.6 Hz, H-9), 7.13 (1H, br t, *J*=7.6 Hz, H-10), 7.05 (1H, br d, *J*=7.6 Hz, H-11), 1.10 (3H, s, H-13), 1.35 (3H, s, H-14), 4.69 (1H, d, *J*=12.1 Hz, Ha-15), 4.82 (1H, d, *J*=12.1 Hz, Hb-15), 2.47 (3H, s, H-16); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 176.3 (C-2), 32.5 (C-3), 51.4 (C-4), 70.4 (C-5), 79.4 (C-6), 135.0 (C-7), 137.6 (C-8), 132.8 (C-9), 128.7 (C-10), 129.3 (C-11), 138.4 (C-12), 26.8 (C-13), 29.0 (C-14), 64.3 (C-15), 21.0 (C-16) (12).

Compound 10 (solajiangxin D, $C_{15}H_{24}O_4$) presented as a white powder; purity: 90.2%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.17 (1H, d, *J*=9.2 Hz, H-1), 3.18 (1H, m, H-2), 1.84 (1H, br t, *J*=12.5 Hz, Ha-3), 2.30 (1H, dd, *J*=12.5, 5.1 Hz, Hb-3), 1.53 (1H, br d, *J*=11.4 Hz, H-5), 1.18 (1H, m, Ha-6), 1.51 (1H, m, Hb-6), 2.01 (1H, m, H-7), 1.17 (1H, m, Ha-8), 1.38 (1H, m, Hb-8), 1.74 (1H, m, Ha-9), 1.26 (1H, m, Hb-9), 1.41 (1H, m, H-11), 1.12 (3H, d, *J*=7.1 Hz, H-13), 0.49 (3H, s, H-14), 4.30 (1H, s, Ha-15), 4.70 (1H, s, Hb-15), 5.81 (1H, br s, C1-OH), 4.80 (1H, br s, C2-OH), 11.35 (1H, br s, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 84.3 (C-1), 69.8 (C-2), 43.4 (C-3), 147.5 (C-4), 47.1 (C-5), 27.4 (C-6), 44.2 (C-7), 23.3 (C-8), 36.2 (C-9), 39.1 (C-10), 39.5 (C-11), 177.4 (C-12), 13.9 (C-13), 11.3 (C-14), 107.5 (C-15) (13).

Compound 11 (solajiangxin E, $C_{18}H_{28}O_3$) presented as a colorless viscous oil; purity: 94.0%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.85 (1H, dd, *J*=13.1, 7.7 Hz, Ha-1), 1.57 (1H, dd, *J*=13.1, 11.4 Hz, Hb-1), 2.04 (1H, m, H-2), 1.76 (1H, m, Ha-3), 1.60 (1H, m, Hb-3), 1.61 (1H, m, Ha-4), 1.78 (1H, m, Hb-4), 5.67 (1H, s, H-7), 2.43 (1H, dd, *J*=16.6, 4.8 Hz, Ha-9), 2.08 (1H, dd, *J*=16.6, 4.2 Hz, Hb-9), 1.93 (1H, m, H-10), 3.74 (1H, d, *J*=8.3 Hz, Ha-12), 3.84 (1H, d, *J*=8.3 Hz, Hb-12), 1.32 (3H, s, H-13), 1.94 (3H, s, H-14), 1.00 (3H, d, *J*=7.1 Hz, H-15), 1.41 (3H, s, H-2'), 1.40 (3H, s, H-3'); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 37.1 (C-1), 47.3 (C-2), 28.6 (C-3), 34.1 (C-4), 50.2 (C-5), 166.0 (C-6), 125.8 (C-7), 199.3 (C-8), 43.1 (C-9), 38.6 (C-10), 82.1 (C-11), 73.2 (C-12), 24.8 (C-13), 20.7 (C-14), 15.9 (C-15), 109.1 (C-1'), 26.8 (C-2'), 27.0 (C-3') (13).

Compound 12 (2-hydroxysolajiangxin E, $C_{18}H_{28}O_4$) presented as a colorless viscous oil; purity: 92.3%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.01 (1H, d, *J*=14.1 Hz, Ha-1), 1.65 (1H, d, *J*=14.1 Hz, Hb-1), 1.94 (1H, m, Ha-3), 1.55 (1H, m, Hb-3), 1.80 (1H, m, Ha-4), 1.76 (1H, m, Hb-4), 5.68 (1H, s, H-7), 2.48 (1H, dd, *J*=16.3, 5.0 Hz, Ha-9), 2.13 (1H, dd, *J*=16.3, 4.1 Hz, Hb-9), 2.51 (1H, m, H-10), 3.54 (1H, d, *J*=8.7 Hz, Ha-12), 4.07 (1H, d, *J*=8.7 Hz, Hb-12), 1.18 (3H, s, H-13), 1.83 (3H, s, H-14), 0.81 (3H, d, *J*=7.0 Hz, H-15), 1.23 (3H, s, H-2'), 1.35 (3H, s, H-3'), 4.49 (1H, s, C1-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 44.3 (C-1), 84.2 (C-2), 35.0 (C-3), 33.8 (C-4), 49.2 (C-5), 166.1 (C-6), 124.7 (C-7), 198.5 (C-8), 43.6 (C-9), 39.4 (C-10), 84.6 (C-11), 72.1 (C-12), 22.8 (C-13), 20.6 (C-14), 15.9 (C-15), 108.4 (C-1'), 27.0 (C-2'), 27.3 (C-3') (13).

Compound 13 (lyratol A, $C_{15}H_{24}O_3$) presented as a colorless viscous oil; purity: 95.1%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.17 (1H, d, *J*=9.6 Hz, H-1), 5.70 (1H, d, *J*=9.6, H-2), 2.54 (1H, m, H-4), 1.39 (1H, m, H-5), 1.26 (1H, m, Ha-6), 1.70 (1H, m, Hb-6), 1.45 (1H, m, H-7), 1.91 (1H, m, Ha-8), 1.33 (1H, m, Hb-8), 3.27 (1H, dd, *J*=11.0, 4.5 Hz, H-9), 1.12 (3H, s, H-12), 1.20 (3H, s, H-13), 1.05 (3H, d, *J*=6.8 Hz, H-14), 0.96 (3H, s, H-15); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 156.5 (C-1), 126.4 (C-2), 201.5 (C-3), 41.5 (C-4), 46.8 (C-5), 23.1 (C-6), 46.6 (C-7), 31.0 (C-8), 74.5 (C-9), 41.4 (C-10), 72.1 (C-11), 27.0 (C-12), 26.9 (C-13), 12.5 (C-14), 11.2 (C-15) (14).

Compound 14 (lyratol B, $C_{15}H_{24}O_3$) presented as a colorless viscous oil; purity: 94.6%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 3.39 (1H, dd, *J*=11.2, 4.3 Hz, H-1), 2.26 (1H, m, Ha-2), 2.27 (1H, m, Hb-2), 5.14 (1H, br d, *J*=4.6 Hz, H-3), 5.34 (1H, br s, H-6), 2.25 (1H, m, H-7), 1.92 (1H, m, Ha-8), 1.44 (1H, m, Hb-8), 1.12 (1H, m, Ha-9), 2.04 (1H, m, Hb-9), 3.49 (1H, d, *J*=11.0 Hz, Ha-12), 3.58 (1H, d, *J*=11.0 Hz, Ha-12), 1.08 (3H, s, H-13), 1.89 (3H, s, H-14), 0.99 (3H, s, H-15); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 75.1 (C-1), 31.5 (C-2), 122.4 (C-3), 131.3 (C-4), 143.4 (C-5), 121.1 (C-6), 43.8 (C-7), 19.0 (C-8), 33.8 (C-9), 37.5 (C-10), 74.6 (C-11), 67.3 (C-12), 21.4 (C-13), 19.6 (C-14), 16.3 (C-15) (14).

Compound 15 (lyratol C, $C_{15}H_{26}O_4$) presented as a colorless prismatic crystal; purity: 92.5%; ^{1}H-NMR (400 MHz, C_5D_5N) δ: 3.44 (1H, d, *J*=9.1 Hz, H-1), 4.00 (1H, m, H-2), 3.04 (1H, dd, *J*=12.4, 5.5 Hz, Ha-3), 2.55 (1H, dd, *J*=12.4, 11.7 Hz, Hb-3), 1.83 (1H, dd, *J*=10.3, 3.6 Hz, H-5), 1.64 (1H, m, Ha-6), 1.88 (1H, m, Hb-6), 2.21 (1H, m, H-7), 1.53 (1H, m, Ha-8), 2.04 (1H, m, Hb-8), 2.73 (1H, m, Ha-9), 1.24 (1H, m, Ha-9), 4.10 (1H, d, *J*=10.8 Hz, Ha-12), 3.83 (1H, d, *J*=10.8 Hz, Hb-12), 1.50 (3H, s, H-13), 1.28 (3H, s, H-14), 4.83 (1H, s, Ha-15), 4.94 (1H, s, Hb-15); ^{13}C-NMR (100 MHz, C_5D_5N) δ: 84.3 (C-1), 71.9 (C-2), 44.0 (C-3), 148.5 (C-4), 48.9 (C-5), 25.0 (C-6), 45.6 (C-7), 21.8 (C-8), 38.7 (C-9), 39.8 (C-10), 74.6 (C-11), 68.7 (C-12), 21.6 (C-13), 11.5 (C-14), 107.6 (C-15) (2).

Cytotoxic potential of sesquiterpenoids from S. lyratum. The anti-proliferation effect of sesquiterpenoids on human breast adenocarcinoma MCF-7, human intestinal adenocarcinoma HCT-8, human lung carcinoma A-549, human gastric cancer

Table I. Cytotoxicity of 15 sesquiterpenoids against cultured MCF-7, HCT-8, A-549, SGC-7901 and BEL-7402 cells.

No.	Compound	IC_{50} (μg/ml)				
		MCF-7	HCT-8	A-549	SGC-7901	BEL-7402
1	Solajiangxin I	18.9±0.8	23.8±1.3	17.6±0.7	18.6±0.7	23.5±3.1
2	7-Hydroxylsolajiangxin I	43.1±1.3	21.3±1.4	16.4±1.0	19.3±1.1	32.1±0.9
3	Solajiangxin H	15.4±0.9	19.6±2.3	28.3±0.5	4.8±0.3	18.4±1.4
4	Lyratol D	33.6±1.8	18.5±0.7	19.3±0.8	5.9±0.2	41.5±0.8
5	Dehydrovomifoliol	19.3±2.0	22.4±1.0	20.3±1.2	10.5±1.4	51.0±1.2
6	Blumenol A	56.4±0.7	16.5±4.1	28.2±1.1	13.5±1.6	25.4±0.5
7	Solajiangxin A	38.9±1.5	40.1±5.4	30.3±1.8	18.9±2.4	41.2±1.3
8	Solajiangxin B	28.6±2.9	23.6±2.6	18.2±0.9	21.3±2.5	32.5±2.4
9	Solajiangxin C	35.1±1.5	27.3±3.8	25.8±2.1	18.4±3.0	12.5±0.4
10	Solajiangxin D	23.4±1.6	18.6±1.1	29.2±0.9	14.1±1.5	31.4±1.7
11	Solajiangxin E	31.2±2.3	25.7±2.4	17.8±1.1	15.3±2.6	28.9±0.7
12	2-Hydroxysolajiangxin E	26.3±0.7	18.4±2.9	16.9±1.6	12.4±1.8	25.4±1.6
13	Lyratol A	31.5±1.4	18.4±0.9	11.3±0.5	9.6±1.4	25.7±2.4
14	Lyratol B	42.8±1.8	15.1±1.2	12.4±0.8	8.7±1.0	18.9±3.1
15	Lyratol C	35.8±2.1	23.4±1.5	13.6±3.2	11.3±2.3	31.2±0.8

Data are presented as the mean ± the standard deviation. IC_{50}, half maximal inhibitory concentration.

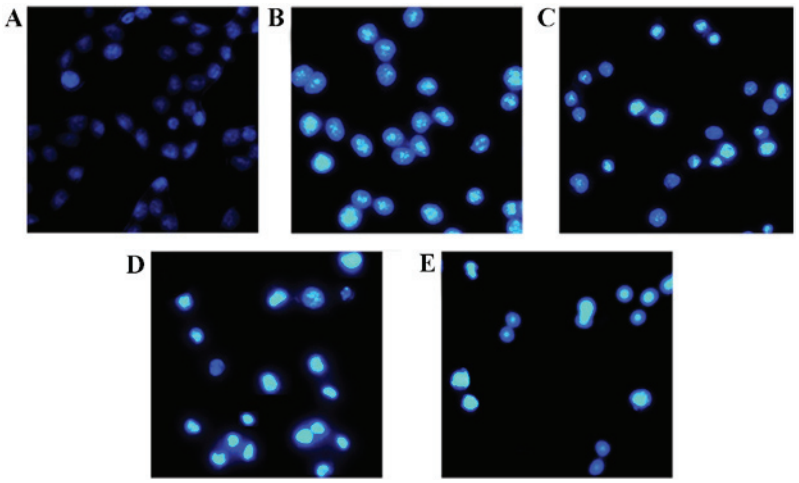

Figure 2. Effects of solajiangxin H and lyratol D on apoptosis in SGC-7901 cells by DAPI staining. SGC-7901 cells were treated with SOG and lyratol D at doses of 0 (control), 2 and 4 μg/ml for 48 h, and then stained by DAPI and images were captured using a fluorescence microscope (magnification, x200). (A) Control; (B) 2 μg/ml SOG; (C) 2 μg/ml lyratol D; (D) 4 μg/ml SOG; and (E) 4 μg/ml lyratol D. SOG, solajiangxin H.

SGC-7901 and human hepatocarcinoma BEL-7402 cells were studied using a CCK-8 assay, which has commonly been used to investigate the cytotoxicity of compounds (15). The results are presented in Table I. The IC_{50} values of the 15 sesquiterpenoids identified were between 4.8 and 56.4 μg/ml. Compounds 3 (solajiangxin H) and 4 (lyratol D) had high cytotoxicity against SGC-7901 cells (IC_{50}=4.8 and 5.9 μg/ml, respectively), so they were selected for additional study into their anticancer mechanisms by DAPI staining and western blot analysis.

Effect of solajiangxin H and lyratol D on apoptosis in SGC-7901 cells. DAPI staining (16) was used to study whether the cytotoxic effects of solajiangxin H and lyratol D on SGC-7901 cells were associated with apoptosis. As depicted in Fig. 2, subsequent to treatment with solajiangxin H and lyratol D at doses of 0 (control), 2 and 4 μg/ml for 48 h, respectively, evident condensation of the nucleus of the SGC-7901 cells was induced, which can be regarded as a characteristic marker of apoptosis. Therefore, the cytotoxic effects of solajiangxin H and lyratol D on SGC-7901 cells were associated with apoptosis.

Effect of solajiangxin H and lyratol D on apoptotic proteins in SGC-7901 cells. A western blot assay was used to study the mechanism of apoptosis induced by solajiangxin H and lyratol D. The present study examined the expression changes

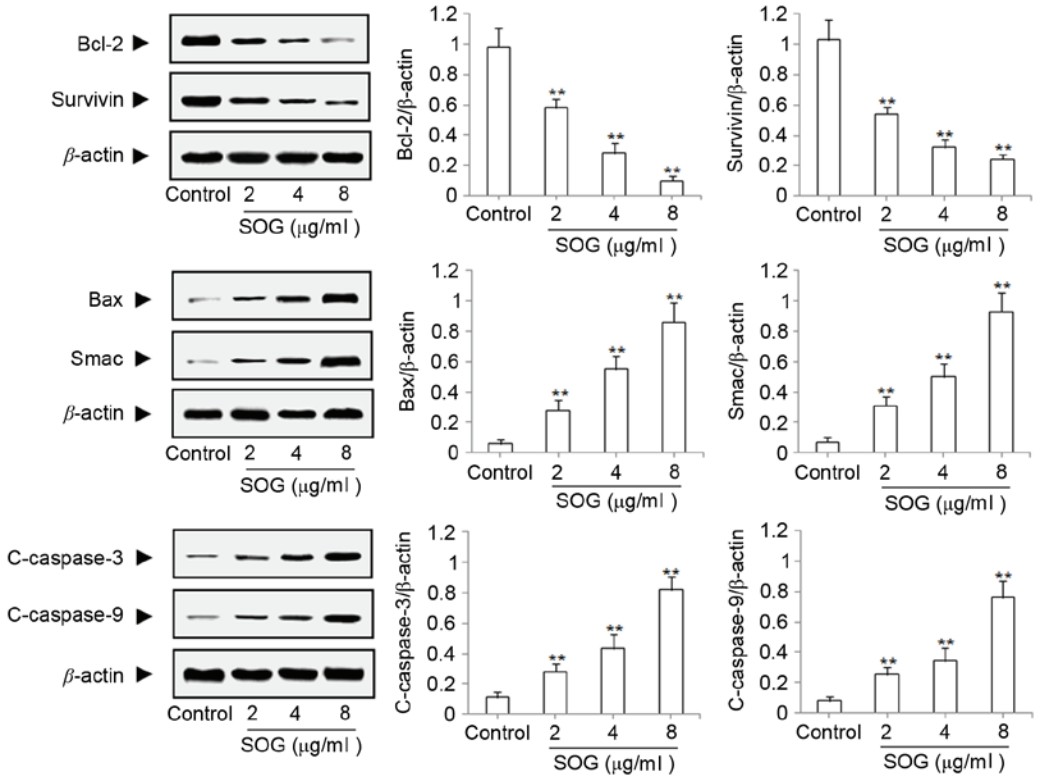

Figure 3. Effects of SOG on the antiapoptotic proteins Bcl-2 and survivin and the proapoptotic proteins Bax, Smac, c-caspase-3 and c-caspase-9 in SGC-7901 cells. SGC-7901 cells were treated with SOG at concentrations of 0 (control), 2, 4 and 8 μg/ml for 48 h, and their total proteins were analyzed by western blot assay. **P<0.01, compared with control. SOG, solajiangxin H; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-like protein 4; Smac, second mitochondria-derived activator of caspase; c-caspase, cleaved caspase.

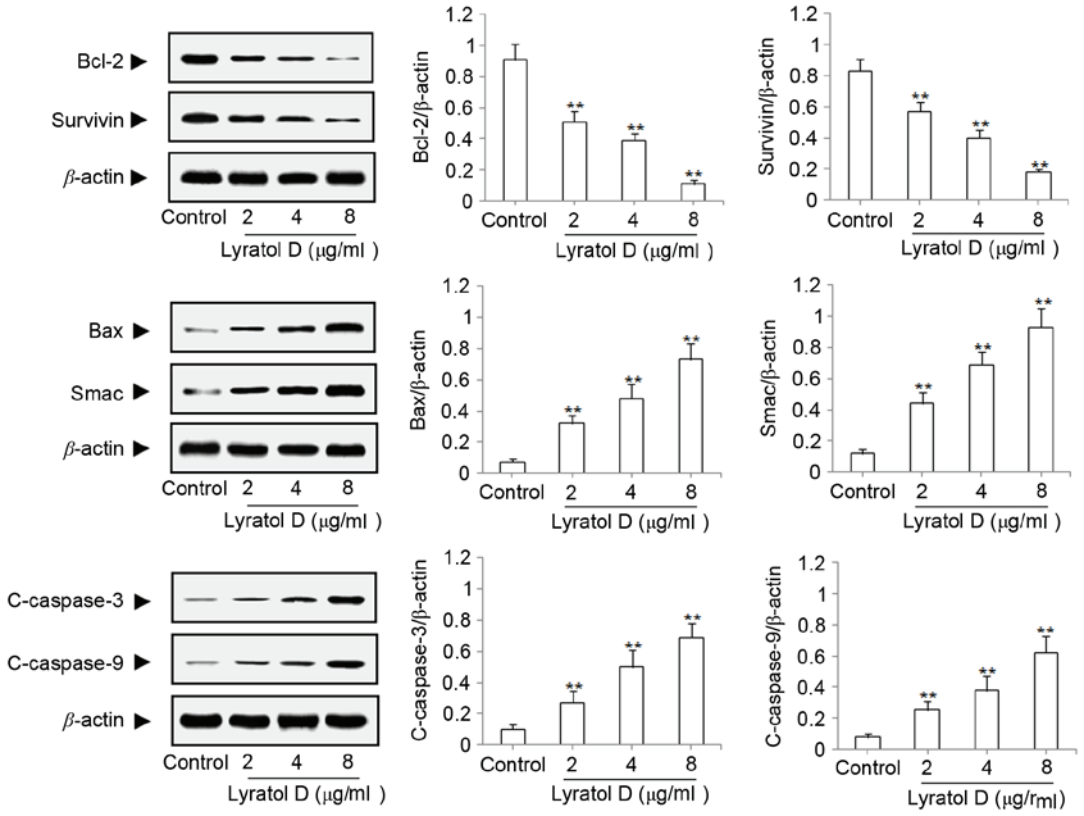

Figure 4. Effects of lyratol D on the antiapoptotic proteins Bcl-2 and survivin, and the proapoptotic proteins Bax, Smac, c-caspase-3 and c-caspase-9 in SGC-7901 cells. The SGC-7901 cells were treated with lyratol D at concentrations of 0 (control), 2, 4 and 8 μg/ml for 48 h, respectively, and total protein content was analyzed by western blot assay. **P<0.01, compared with control. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-like protein 4; Smac, second mitochondria-derived activator of caspase; c-caspase, cleaved caspase.

of apoptotic proteins, including Bcl-2, survivin, Bax, Smac, c-caspase-3 and c-caspase-9 subsequent to treatment with solajiangxin H or lyratol D at concentrations of 2, 4 or 8 μg/ml for 48 h, respectively. As Figs. 3 and 4 demonstrate, the results indicated that solajiangxin H and lyratol D may significantly ($P<0.01$) downregulate the expression of the antiapoptotic proteins Bcl-2 and survivin, and upregulate the expression of the proapoptotic proteins Bax, Smac, c-caspase-3 and c-caspase-9 ($P<0.01$), in a dose-dependent manner (17).

Discussion

In the present study, 15 sesquiterpenoids were isolated from *S. lyratum*, and their antiproliferation effects on MCF-7, HCT-8, A-549, SGC-7901 and BEL-7402 cell lines were investigated using a CCK-8 assay. According to the results of CCK-8 assay, solajiangxin H and lyratol D, which had strong cytotoxicity against SGC-7901 cells (IC_{50}=4.8 and 5.9 μg/ml, respectively), were selected to study their anticancer mechanisms by DAPI staining and western blot assay. These techniques demonstrated that the cytotoxicity of solajiangxin H and lyratol D against SGC-7901 cells was associated with apoptosis.

Previous studies revealed that apoptotic proteins, including Bcl-2, survivin, Bax, Smac, caspase-3 and caspase-9, serve important roles in mitochondria-mediated apoptosis (17,18). The associations between the proteins are complex. For example, the antiapoptotic Bcl-2 protein can suppress the release of the proapoptotic Smac protein from the mitochondria to the cytoplasm, but the proapoptotic Bax protein can inhibit the activity of Bcl-2 protein (19,20). The active form of caspase-3, c-caspase-3, is an important signal for apoptosis, and the activation of caspase-3 is associated with the activation of caspase-9 into c-caspase-9 (21). The antiapoptotic survivin protein can inhibit the c-caspase-9 to activate caspases-3, but Smac can inhibit the activity of survivin (22). Thus, changes in expression level and activity of the apoptotic proteins Bcl-2, survivin, Bax, Smac, caspase-3 and caspase-9 are directly associated with apoptosis. In the present study, changes in the expression levels of apoptotic proteins, including Bcl-2, survivin, Bax, Smac, c-caspase-3 and c-caspase-9, subsequent to treatment with solajiangxin H and lyratol D was investigated. The results indicated that solajiangxin H and lyratol D significantly downregulates the expression of the antiapoptotic proteins Bcl-2 and survivin, and upregulates the expression levels of the proapoptotic proteins Bax, Smac, c-caspase-3 and c-caspase-9 in dose-dependent manners.

In conclusion, the present study demonstrated that 15 sesquiterpenoids isolated from *S. lyratum* exhibit cytotoxicity against MCF-7, HCT-8, A-549, SGC-7901 and BEL-7402 cells. The results of studies on the anticancer mechanisms of solajiangxin H and lyratol D in SGC-7901 cells indicated that the anticancer mechanisms may be associated with mitochondria-mediated apoptosis. However, additional studies on mechanisms of these sesquiterpenoids are required as they may be candidates for treatment of cancer.

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