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Sesquiterpene Lactones Isolated from *Carpesium Abrotanoides* L. by LC-MS Combined with HSCCC Inhibit Liver Cancer Through Suppression of the JAK2/STAT3 Signaling Pathway

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Abstract

Carpesium abrotanoides L. is rich in sesquiterpene lactones, the experiments of anti-tumor activity in vitro showed that total sesquiterpene lactone extracts exhibited the most cytotoxicity activities against HepG-2 cells (IC_{50} = 4.2 μg/mL). In order to isolated the active compounds quickly, the strategy of HPLC-ESI-Q-TOF-MS/MS combined with high-speed counter-current chromatography (HSCCC) was implemented. Three sesquiterpene lactone compounds, 2*α*,5*α*-dihydroxy-11*α*H-eudesma-4(15)-en-12,8*β*-olide (**1**), telekin (**2**), oxoeudesm-11(13)-eno-12,8*α*-lactone (**3**) were obtained. Their structures were elucidated by detailed analysis of 1D, 2D NMR and HRMS data. In addition, compounds **1**-**3** were evaluated for their cytotoxic activities against HepG-2 cell line with IC_{50} value of 9.83, 2.95, and 4.15 μM, respectively. Moreover, all compounds not only can reduce the expression of JAK2 and STAT3 mRNA but also inhibit the *p*-JAK2 and *p*-STAT3 protein expression levels in a dose-dependent manner in the HepG-2 cells. In conclusion, three sesquiterpene lactone compounds inhibited the proliferation of HepG-2 cells via suppressing the JAK2/STAT3 signaling pathway.

Introduction

Carpesium L., a genus of Compositae plants including about 21 species is mainly distributed in southern Europe and Asia. *Carpesium abrotanoides* L., a famous ethnomedicine “Tianmingjing”, which was widely used for treating bronchitis, bruises, hepatitis for a long time in ethnic minority areas of Southwest China, especially for Tujia, and Miao minorities. Phytochemical research had demonstrated that it is enriched in sesquiterpene lactones with the *α*-methylene-*γ*-butyrolactone skeleton [1–4]. Some of them possessed a wide of pharmacological activities, including anti-cancer [5], anti-inflammatory [6], anti-bacterial [7–8].

Liver cancer, is the sixth most commonly diagnosed cancer and the third leading cause of cancer death worldwide in 2020, accounts for 906,000 new cases and 830,000 deaths annually [9]. Among them, more than 75% of primary liver cancers are belonging to the hepatocellular carcinoma (HCC), which the incidence is increasing year by year [10]. So far, Sorafenib and lenvatinib are the only two first-line agents for treating advanced HCC. However, their median survival times are several months, and had side effects [11]. So it is of great significance to find new safe and effective drugs for the treatment of liver cancer. *Carpesium abrotanoides* L., is rich in sesquiterpene lactones with significant cytotoxicity effects on the HepG-2 cell line. Hence, the present study aimed to find the main active components of this plant against liver cancer and clarify its mechanism of action.

The JAK2/STAT3 signaling pathway had drawn increasingly research interests in the development of HCC. Abnormal activation of this pathway is closely related to the occurrence, development, invasion, metastasis, and prognosis of HCC [12]. Modern clinical and pathological studies found evidence that more than 60% of HCC patients have high expression of STAT3 in tissue samples [13]. Besides, phosphorylation of STAT3 is characteristic of STAT3 activation, and its transcription factors relies on the activation of JAK receptors, especially JAK2/STAT3 [14]. The abnormal activation of STAT3 is associated with abnormal regulation of the upstream kinase JAK2, and JAK2 mutations are also involved in the

occurrence and development of tumor [15]. On the other hand, whole-genome sequencing revealed that the JAK/STAT signaling pathway is one of the two main oncogenic pathways in HCC [16].

It is well known that the universal-traditional methods of extraction, separation and analysis spent more time and laborious getting compounds [17–18]. Therefore, the present work is focus on isolating target sesquiterpenes rapidly via the strategy of high-pressure liquid chromatography coupled with electrospray ionization-quadrupole time-of-flight mass spectrometry (HPLC-ESI-Q-TOF-MS/MS) combined with high-speed counter-current chromatography (HSCCC). Herein, the isolation, structure elucidation, bioassay, and three compounds anti-liver cancer activity through the JAK2/STAT3 signaling pathway were described.

Results And Discussion

Anti-tumor bioassay of TEC and SLEC by the CCK-8 method

The cytotoxicity of TEC and SLEC *in vitro* were evaluated against seven tumor cell lines, including Hela, HepG-2, SW620, A549, HL-60, SMMC-7721, and PC-3 using the CCK-8 assay, taxol was used as the positive control. The results (Table 1) showed that the TEC and SLEC exhibited potent effect on different tumor cells. In particular, the SLEC extracts showed good cytotoxicity against HepG-2 and SMMC-7721 cells with the IC_{50} values of 4.2 and 23.6 μg/ml, indicating that the sesquiterpene lactones might be the active constituents of this plant against HCC.

Table 1
Anti-tumor activities data of three different parts of *Carpesium abrotanoides* L.

Extracts	Cell lines IC_{50} (μg/ml)						
	Hela	**HepG-2**	**SW620**	**A549**	**HL-60**	**Smmc-7721**	**PC-3**
TEC	255.4	28.3	176.7	-	145.9	109.1	-
SLEC	58.9	4.2	92.4	281.0	-	23.6	272.9
The rest	>300	>300	>300	-	-	>300	-

LC-MS analysis

According to the results of anti-tumor *in vitro*, we attempted to analyse the chemical constituents of SLEC fraction. By optimizing the liquid phase and mass spectrometry conditions, mass spectrometry analysis in positive and negative modes respectively, are shown in Fig. 1. The results revealed that sesquiterpene lactones with a higher response in the positive mode, and mainly exhibited in the form of $[M + H]^+$ or $[M + Na]^+$. The retention time, molecular formula, and fragment ions of 18 potential sesquiterpene lactones were summarized in Table 2. Further study of molecular ion peaks and fragmentation pattern of multi-level mass spectrometry revealed that the lactone ring of sesquiterpene lactones will continuously lose one molecule of H_2O and CO units which are the characteristic cleavage law in positive mode. However, there are still a large number of isomers that need to be further determined by other methods.

Table 2
Eighteen potential sesquiterpene lactone compounds from the SLEC fraction of *Carpesium abrotanoides* L.

NO.	t_R(min)	Ion mode (m/z)	Formula	Error(ppm)	Fragment ions (m/z)
1	17.2	445.2059$[M+H]^+$	$C_{21}H_{32}O_{10}$	-3.37	427, 265, 247, 219, 191
2	25.5	331.1510$[M+H]^+$	$C_{19}H_{22}O_5$	-8.57	291, 273, 241
3	29.3	271.1321$[M+Na]^+$	$C_{15}H_{20}O_3$	4.06	231, 203, 185, 175, 159
4	30.9	265.1431$[M+H]^+$	$C_{15}H_{20}O_4$	-3.40	247, 229, 219, 201, 159
5	47.5	249.1476$[M+H]^+$	$C_{15}H_{20}O_3$	-6.02	231, 213, 203, 185, 157
6	49.6	271.1298$[M+Na]^+$	$C_{15}H_{20}O_3$	-4.43	231, 203, 185, 175, 159
7	50.4	267.1689$[M+H]^+$	$C_{15}H_{22}O_4$	-2.62	249, 231, 203
8	51.4	249.1477$[M+H]^+$	$C_{15}H_{20}O_3$	-5.62	231, 213, 203, 185, 175, 157
9	52.0	273.1472$[M+Na]^+$	$C_{15}H_{22}O_3$	1.83	233, 215, 197, 187, 159
10	53.2	249.1479$[M+H]^+$	$C_{15}H_{20}O_3$	-4.82	231, 213, 203, 185, 173, 159
11	53.8	247.1323$[M+H]^+$	$C_{15}H_{18}O_3$	-4.45	229, 211, 201, 183, 173, 159
12	55.5	251.1634$[M+H]^+$	$C_{15}H_{22}O_3$	-5.18	233, 215, 197, 187, 161
13	57.1	271.1298$[M+Na]^+$	$C_{15}H_{20}O_3$	-4.43	231, 213, 203, 185, 175, 161
14	58.6	273.1479$[M+Na]^+$	$C_{15}H_{22}O_3$	4.40	233, 205, 187
15	60.4	249.1472$[M+H]^+$	$C_{15}H_{20}O_3$	-7.63	231, 203, 185, 171, 157
16	67.9	349.1675$[M+H]^+$	$C_{19}H_{24}O_6$	6.88	331, 303, 262, 244
17	69.0	233.1524$[M+H]^+$	$C_{15}H_{20}O_2$	-7.73	215, 197, 187
18	70.5	295.1593$[M+H]^+$	$C_{16}H_{22}O_5$	9.27	277, 249, 231, 185, 179, 161

HSCCC separation

The selection of an appropriate two-phase solvent system is essential for efficient isolation in HSCCC, which usually required a suitable *K-value* for the target ingredients by LC-MS tests [19]. Generally, the *K value* should be in the range from 0.5 to 2.0 that is suitable for isolation. However, the chemical type we studied is sesquiterpene lactone, which is rarely reported [20–21]. The *K*-values of three target compounds were tested by HPLC analysis as shown in Table 3. Peak A and Peak B are suitable for *n*-

hexane-ethyl acetate-methanol-water (1:9:9:1, v/v/v/v). So when the lower phase ran for about 150 min, the pump was suspended and the solvent system was replaced as chloroform-methanol-water (4:3:2, v/v/v). After 320 min, Peak A (60-66 min, step 1), B (74-82 min, step 1), C (80-92 min, step 2) were obtained according to the peak shapes as shown in Fig. 2. The purities of these three compounds were 95.8 %, 98.2 %, and 97.1 %, respectively.

Table 3
K-values of compounds from SLEC in different solvent systems

NO.	Solvent System (v/v)	*K*-value of target A	*K*-value of target B	*K*-value of target C
1	*n*-hexane-ethyl acetate-methanol-water 1:1:1:1	0.25	0.43	4.65
2	*n*-hexane-ethyl acetate-methanol-water 1:9:9:1	1.30	0.62	3.32
3	*n*-hexane-ethyl acetate-methanol-water 3:1:1:1	0.68	0.46	0.50
4	*n*-hexane-methanol-water 5:4:1	2.12	0.29	3.20
5	chloroform-methanol-water 4:3:2	0.38	4.20	1.56

Besides the solvent system, other factors were also investigated, such as the flow rate of mobile phase, revolution speed and the loading amount of sample. Different flow rates (1.5, 2.0, 2.5 and 3.0 mL/min), different revolution speed (700, 800, 835 and 850 rpm) and sample loads (50, 100, 150 and 200 mg) were all examined. The results revealed that when the flow rate, revolution speed and sample loads were 3.0 mL/min, 835 rpm and 200 mg respectively, satisfactory separation efficiency and peak resolutions could be achieved, which are shown in Fig. 3.

Structural elucidation

Three sesquiterpene lactones were determined as 2α,5α-dihydroxy-11αH-eudesma-4(15)-en-12,8*β*-olide (**1**) [22], telekin (**2**) [23], oxoeudesm-11(13)-eno-12,8α-lactone (**3**) [24] (Fig. 3), respectively. Their structures were identified by HRMS, ^{1}H-NMR, ^{13}C-NMR, and compared with relevant references.

2*α*,5*α*-dihydroxy-11*α*H-eudesma-4(15)-en-12,8*β*-olide (**1**): white power; HRMS: *m/z* 311.1497 $[M + COOH]^-$ (calcd. for 311.1495, $C_{16}H_{23}O_6$), ^{1}H NMR (600 MHz, CD_3OD) δ_H: 0.89 (s, 3H, H-14), 1.17 (d, *J* = 7.2 Hz, 3H,

H-13), 1.41 (td, J = 6.2, 5.5, 1.9 Hz, 1H, H-1β), 1.43 (m, 1H, H-6β), 1.69 (dd, J = 13.8, 6.1 Hz, 1H, H-6α), 1.80 (m, 1H, H-1α), 1.83 (m, 1H, H-9β), 2.03 (dd, J = 15.4, 4.7 Hz, 1H, H-9α), 2.40 (ddd, J = 12.2, 5.4, 1.9 Hz, 1H, H-3β), 2.61 (m, 1H, H-3α), 2.83 (dtd, J = 12.7, 6.4, 4.3 Hz, 1H, H-7), 2.97 (p, J = 7.1 Hz, 1H, H-11), 3.80 (tt, J = 11.3, 5.1 Hz, 1H, H-2), 4.59 (td, J = 4.5, 1.7 Hz, 1H, H-8), 4.80 (t, J = 1.5 Hz, 1H, H-15), 4.94 (t, J = 1.5 Hz, 1H, H-15). ^{13}C NMR (151 MHz, CD_3OD) δ_C: 180.8 (C-12), 149.7 (C-4), 108.8 (C-15), 78.5 (C-8), 72.1 (C-5), 66.1 (C-2), 43.7 (C-1), 40.9 (C-3), 40.7 (C-11), 37.1 (C-7), 36.5 (C-10), 35.4 (C-9), 26.6 (C-6), 21.5 (C-14), 8.2 (C-13).

telekin (**2**): white power; HRMS: *m/z* 249.1480 $[M + H]^+$ (calcd. for 249.1491, $C_{15}H_{21}O_3$). ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 0.98 (s, 3H, H-14), 3.37 (m,1H, H-7), 4.58 (td, J = 5.2, 1.5 Hz, 1H, H-8), 4.71(t, 1H, H-15), 4.88 (t, 1H, H-15), 5.61 (d, 1H, J = 1.1 Hz, H-13), 6.16 (d, 1H, J = 1.2 Hz, H-13). ^{13}C NMR (151 MHz, $CDCl_3$) δ_C: 170.8 (C-12), 150.1 (C-4), 142.1 (C-11), 120.3 (C-13), 109.0 (C-15), 77.0 (C-8), 74.3 (C-5), 37.6 (C-7), 36.5 (C-10), 35.6 (C-1), 35.4 (C-2), 33.8 (C-3), 31.8 (C-9), 21.8 (C-14), 21.6 (C-6).

- oxoeudesm-11(13)-eno-12,8α-lactone (**3**): white power; HRMS: *m/z* 249.1480 $[M + H]^+$ (calcd. for 249.1491, $C_{15}H_{21}O_3$). ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 0.96 (s, 3H, H-14), 1.02 (d, J = 5.8 Hz, 3H, H-15), 1.35 (q, J = 11.9 Hz, 1H, H-9), 1.45 (dd, J = 14.8, 11.8 Hz, 1H, H-6), 1.55 (m, 1H, H-2), 1.83 (m, 1H, H-5), 1.87 (m, 1H, H-4), 2.04 (m, 1H, H-6), 2.10 (m, 1H, H-2), 2.43 (m, 1H, H-3), 2.39 (dd, J = 9.4, 4.2 Hz, 1H, H-9), 2.70 (m, 1H, H-7), 4.21 (ddd, J = 12.0, 9.2, 3.1 Hz, 1H, H-8), 5.44 (d, J = 3.2 Hz, 1H, H-13), 6.11 (d, J = 3.5 Hz, 1H, H-13).^{13}C NMR (151 MHz, $CDCl_3$) δ_C: 169.8 (C-12), 140.2 (C-11), 120.1 (C-13), 80.8 (C-8), 50.0 (C-10), 48.7 (C-5), 44.8 (C-7), 44.1 (C-3), 35.2 (C-2), 34.5 (C-9), 29.6 (C-4), 24.1 (C-6), 22.0 (C-14), 20.0 (C-15).

Cytotoxicity of isolated compounds

Compounds **1**-**3** from the SLEC fraction were evaluated for their cytotoxic activities against HepG-2 cell line with IC_{50} values of 9.83, 2.95, and 4.15 μM, respectively.

The effect of compounds 1-3 on the mRNA expression of JAK2 and STAT3 in HepG-2 cells

The HepG-2 cells were treated with compounds **1**-**3** at three different concentrations after 24 hours, respectively. As shown in Fig. 4A and 4B, compounds **1** and **2** at high and medium concentrations treatment significantly reduced the mRNA expression level of JAK2 in a dose-dependent manner compared with the control group ($p < 0.01$). Moreover, treatment of compound **3** was also significantly decreased the mRNA expression level of JAK2 in a dose-dependent manner compared with the control group (Fig. 4C, $p < 0.05$). In addition, compounds **1**-**3** also significantly down-regulated the mRNA expression of STAT3 in a dose-dependent manner compared with the control group ($p < 0.01$). The results

indicated that transcription of the mRNA of JAK2 and STAT3 in JAK2/STAT3 signaling pathway could be more sensitively regulated by compounds **1**-**3**.

Compounds 1-3 inhibited the JAK2/STAT3 signaling pathway

The growing evidence suggests that the JAK2/STAT3 signaling involved in many types of cancers. Therefore, the protein expression of JAK2, p-JAK2, STAT3, and p-STAT3 in HepG-2 cells were detected by Western blot. The results showed that compounds **1**-**3** treatment with the high and medium concentrations can significantly reduce the protein expression levels of *p*-JAK2 and *p*-STAT3 compared with the control group in a dose-dependent manner ($p < 0.05$ or $p < 0.01$). This suggests that compounds **1**-**3** could significantly inhibit the expression of *p*-JAK2 and *p*-STAT3 proteins. In addition, The JAK2 and STAT3 protein expression levels of compounds **1**-**3** were no statistical significance (Fig. 5).

Discussion

Eucalyptane-type sesquiterpenoids are the predominant constituents within the genus *Carpesium*, especially sesquiterpenoid lactones with an α-methylene-γ-lactone region, which showed significant cytotoxic to human cancer cells [25]. The present study has reported that the total ethanol extracts and total sesquiterpene lactone extracts exhibited the most cytotoxicity activities against HepG-2 cells with IC_{50} values of 28.3 and 4.2 μg/ml (Table 1), indicated that the sesquiterpenoid lactones should be the active ingredient. Three active eucalyptane-type sesquiterpenoids (Fig. 3), 2α,5α-dihydroxy-11αH-eudesma-4(15)-en-12,8β-olide (**1**), telekin (**2**), oxoeudesm-11(13)-eno-12,8α-lactone (**3**), rapidly isolated and purified from the whole herb of *Carpesium abrotanoides* L. by the strategy of HPLC-ESI-Q-TOF-MS/MS combined with HSCCC. Compared with traditional separation methods, this strategy will be more quickly and accurately to obtain three target compounds. Among them, compounds **1** and **3** were isolated from this specie for the first time, and compounds **2** and **3** possess an α-methylene-γ-lactone group showed more cytotoxicity against HepG-2 better than compound **1**, suggesting that an α-methylene-γ-lactone group should be an active group. The present study also was to investigate the anti-liver cancer mechanism of action of compounds **1**-**3** in HepG-2 cells.

JAK2/STAT3 signaling pathway is found commonly involved in many types of cancers, which is an important therapeutic target in cancer treatment. The JAK2/STAT3 signaling pathway was activated in the form of phosphorylation [26]. After the phosphorylation of JAK2 caused by cancer-promoting pathological factors, p-JAK2 can cause phosphorylation of STAT3, and p-STAT3 forms a dimer that enters the nucleus and regulates various proliferation and invasion [27]. Our qRT-PCR results (Fig. 4) have showed that compounds **1**-**3** can significantly down-regulated the mRNA expression of JAK2 and STAT3 in a dose-dependent manner compared with the control group. In addition, the Western blot results (Fig. 5) demonstrated that compounds **1**-**3** also can significantly decreased the expressions of p-JAK2 and p-STAT3 in HepG-2 cells, suggesting that compounds **1**-**3** can inhibit the cell proliferation via suppressed the activation of the JAK2/STAT3 pathway.

Conclusion

In summary, we investigated the cytotoxic activities of the total extracts and total sesquiterpene lactone extracts against seven tumor cell lines (Hela, HepG-2, SW620, A549, HL-60, PC-3, Smmc-7721), the results demonstrated that these two extracts exhibited the most cytotoxicity activities against HepG-2 cell line. After that, 18 sesquiterpene lactones in the total sesquiterpene lactone extracts were determined by HPLC-ESI-Q-TOF-MS/MS method, in addition, three sesquiterpene lactone compounds have been quickly isolated via the HSCCC technology. But more interestingly, these three sesquiterpene lactones exhibited significantly the inhibitory effects on HepG-2 cells by regulating the JAK2/STAT3 signal pathway.

Materials And Methods

Apparatus

HPLC-ESI-Q-TOF-MS/MS were performed on an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA), equipped with an online vacuum degasser, a QuatPump, a manual injection valve with a 20 μL sample loop, a thermostated column compartment, a diode assay detector (DAD), and an Agilent ChemStation, has been selected to analysis of samples. Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization (ESI) interface was coupled in parallel by splitting the mobile phase 1:3 using an adjustable high-pressure stream splitter (Valco Instrument Company, Houston, TX, USA). MS data were acquired across the range *m/z* 100~1500 in positive and negative ion modes (dry gas: N2, 325°C, 5.0 L/min; sheath gas: N2, 350°C 12 L/min; fragmentor voltage, 135 V; capillary voltage, 3500 V). All operations, acquisition and analysis of data were monitored by Agilent MassHunter Acquisition Software Version A.01.00 and operated under MassHunter Acquisition Software Version B.05.00.

HSCCC was performed on a model TBE-300C HSCCC instrument (Shanghai Tauto Biotechnique Co.Ltd., China). The instrument was equipped with three multilayered preparation coils (diameter of PTFE tube, 1.6 mm, total volume 300 mL) and a 20 mL sample loop. The revolution speed was adjustable from 0 to 900 rpm. The HSCCC system consists of a TBP-5002 pump, a model of TBD-2000UV detector, a DC-0506 constant temperature circulating implement, and an AV2.2.0 B chromatography workstation. During the separation process, the temperature of separation columns was controlled at 25°C, and the effluents were monitored at 214 nm. ^{1}H and ^{13}C NMR spectra were measured on Bruker Avance 600 AV (^{1}H at 600 MHz, ^{13}C at 151 MHz) NMR spectrometers. Using tetramethylsilane (TMS) as internal standard, and all chemical shifts were reported in parts per million (ppm, δ).

Chemicals and plant material

Ethanol, methanol, *n*-hexane, ethyl acetate, chloroform, hydrochloric acid and Sodium hydroxide, which were used for the preparation of the fractions and the separation of HSCCC are purchased from Hunan Huihong Reagent Co., Ltd. (Changsha, China). Ultrapure water was purified and filtered by a MilliQ water (18.2 MΩ) system (Millipore, Bedford, MA, USA). Formic acid and acetonitrile used for LC-MS were of

chromatographic grade (Merk, Germany). *Carpesium abrotanoides* L. were obtained from Dao County of Hunan Province in China in 2019. A voucher specimen (No.2019-TMJ) was deposited in the Department of Pharmaceutical Chemistry, Research Institute of Chinese Medicine.

Preparation of plant extracts and fractionation

The whole dry crushing plant of *Carpesium abrotanoides* L. (2.0 kg) were extracted by 95% ethanol (20 L) with third times and soaked for 24 h. After combining and drying, the total ethanol extracts (TEC) (458.1 g) was obtained. TEC fraction (400.0 g) were dissolved in a solution of 2% NaOH to *p*H=9, and then adjust the pH to 2 with 1% concentrated hydrochloric acid to get precipitate, and finally yielded the total sesquiterpene lactone extracts (SLEC) (42.0 g).

Anti-tumor activity screening by CCK-8 method

Take the cells (Hela, HepG-2, SW620, A549, HL-60, PC-3, SMMC-7721) in the logarithmic growth phase, the cells were inoculated into 96-well plates at a density of 1×10^5/mL (100 μL/well). Two extracts of TEC and SLEC (50, 100, 150, 200, and 250 μg/mL) or compounds **1**-**3** (2.5, 5, 7.5, 10, 15, and 20 μM) was added to adherent cells with 96 wells for each group and repeated 3 times. After the adhesion is complete, add the samples, continue to incubate for 48 hours, and add 10 μL CCK-8 solutions to each well. At 37°C, 5% CO_2 was incubated for 4 hours, and the absorbance (OD) value at 450 nm was analyzed with a Bio-Tek microplate reader.

Cell proliferation (%) = (mean OD of treated cells / mean OD of untreated cells])×100

LC-MS analysis

HPLC-ESI-Q-TOF-MS/MS was used to analyze the chemical constituents of SLEC. The final conditions of the instrument are as follows: Agilent HPLC column (Eclipse XDB C_{18}, 4.6×250 mm, 5 μm), mobile phase A is 0.1% formic acid water, mobile phase B is acetonitrile, elution procedure (0-8 min, 2% B; 8-10 min, 2%-20% B; 10-50 min, 20%-52% B; 50-60 min, 52% B; 60-65 min, 52%-80% B; 65-68 min, 80-98% B; 68-75 min, 98% B). The column temperature was 25°C, the volume flow rate was 1.0 mL/min, and the injection volume was 5 μL. Spectra were recorded from 190 to 400 nm (peak width 0.2 min and data rate 1.25 s^{-1}) while the chromatogram was acquired at 214 nm.

Measurement of Partition Coefficients

The selection of solvent system is mainly based on the distribution coefficient of the targets. The specific operation of HPLC method is as follows: the upper and lower phase solvents of a certain volume (20 mL) are prepared according to the proportion of the solvent system, and after the phase separation is balanced, the same volume of upper and lower phase solvents are placed in different containers (10 mL). Add a certain amount of sample (200 mg), shake violently to dissolve and rest. The same volume of upper and lower phase solvent was injected into HPLC to determine the peak area of the target

components in the upper and lower phase (AS and AM), to calculate the partition coefficient (K) (K = AS/AM).

HSCCC Separation

Five solvent systems consisting of *n*-hexane-ethyl acetate-methanol-water (1:1:1:1, v/v/v/v), *n*-hexane-ethyl acetate-methanol-water (1:9:9:1, v/v/v/v), *n*-hexane-ethyl acetate-methanol-water (3:1:1:1, v/v/v/v), *n*-hexane-methanol-water (5:4:1, v/v/v), and chloroform-methanol-water (4:3:2, v/v/v) were used for the separation of SLEC. Sample solution was prepared by dissolving 0.1 g of SELC in 20 ml mobile phase of the solvent system for separation.

In each HSCCC separation, the separation column was initiated by filling with the upper phase at 30.0 mL/min, and then the apparatus was rotated 835 rpm. The mobile phase was pumped into the column at 3.0 mL/min. After the equilibration reached, samples were injected into the injection valve. The solvent was continuously monitored at 214 nm and the peak fractions were collected under monitor by with a UV detector.

Quantitative real-time PCR analysis

Total RNA was extracted from HepG-2 using Trizol reagent (Ambion, Austin, USA). About 500 ng of total RNA for each sample was reverse-transcribed to prepare cDNA using PrimeScript II RTase reverse transcriptase (Takara, Beijing, China). The quantitative real-time polymerase chain reaction (qRT-PCR) was performed using an CFX-Connect 96 (Applied Biosystems, Foster City, CA) with the SYBR FAST qPCR Master Mix (Solarbio, Beijing, China). The primer sequences used for PCR were as follows: JAK2, forward: ACTAAATGCTGTCCCCC, reverse: TTCATGCAGTTGACCGT; STAT3, forward: TGAGGGAGCAGAGATGTG, reverse: TGGGGGCTTGGTAAAA; GAPDH, forward: CCACTCCTCCACCTTTG, reverse: CACCACCCTGTTGCTGT. The relative expression of each target gene compared with GAPDH was calculated using the $2^{-\Delta\Delta Ct}$ method [28].

Western blotting

The total protein was extracted from HepG-2 cells using RIPA lysis buffer (solarbio, Beijing, China), and the protein concentration was detected by BCA Protein Assay Kit (solarbio, Beijing, China). The loading protein (20 μg) was electrophoresed and separated on a 12 % SDS-polyacrylamide gel (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (0.22μm, Millipore, USA). The PVDF membrane was blocked in 5% skimmed milk at room temperature for 1 hour, the membranes were incubated with primary antibodies (JAK2, p-JAK2, STAT3, p-STAT3, and GAPDH) (Bioswamp) overnight at 4°C.Then, the membranes followed by secondary antibodies goat anti-rabbit IgG (1:10000)) incubation for 1 h at 37°C and detected by ECL.

Statistical analysis

The results were expressed as means standard deviation (SD). One-way analysis of variance (ANOVA) and t-tests were used for comparisons among and between groups, respectively. All statistical analysis was carried out using SPSS 21.0 with statistical significance set at $p < 0.05$ or $p < 0.01$.

Declarations

Funding

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References

1. Zhang JP, Wang GW, Tian XH, Yang YX, Liu QX, Chen LP, **et al**. **The genus Carpesium: a review of its ethnopharmacology, phytochemistry and pharmacology**. *J Ethnopharmacol*. *2015*;*163*:*173-91*.
2. Wang L, Qin W, Tian L, Zhang XX, Lin F, Cheng F (2018) **et al**. **Caroguaianolide A-E, five new cytotoxic sesquiterpene lactones from** *Carpesium abrotanoides L*. Fitoterapia 127:349–355
3. Wu JW, Tang CP, Chen L, Qiao Y, Geng MY, Ye Y (2015) Dicarabrones A and B, a pair of new epimers dimerized from sesquiterpene lactones via a [3+2] cycloaddition from *Carpesium abrotanoides*. Org Lett 17:1656–1659
4. Hu QL, Wu PQ, Liu YH, Qi FM, Yu CX, Zhao Y et al (2018) Three new sesquiterpene lactones from *Carpesium abrotanoides*. Phytochem Lett 27:154–159
5. Lee JS, Min BS, Lee SM, Na MK, Kwon BM, Lee CO et al (2002) Cytotoxic sesquiterpene lactones from *Carpesium abrotanoides*. Planta Med 68:745–747
6. Lee JH, Hwang KH, Kim GH (2013) In Vitro evaluation of anti-inflammatory activity for salad-food material *Carpesium Abrotanoides*. J Food Biochem 37:18–25
7. Wu HB, Wu HB, Wang WS, Liu TT, Qi MG, Feng JC et al (2016) Insecticidal activity of sesquiterpene lactones and monoterpenoid from the fruits of *Carpesium abrotanoides*. Ind Crops Prod 92:77–83
8. Mayur B, Sandesh S, Shruti S, Sung-Yum S (2010) Antioxidant and α-glucosidase inhibitory properties of *Carpesium abrotanoides L*. J Med Plants Res 4:1547–1553
9. Sung H, Ferlay J, Siegel R, Laversannr M, Soerjomataram I, Jemal A et al (2021) Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 71:209–249
10. Lokau J, Schoeder V, Haybaeck J, Garbers C (2019) Jak-stat signaling induced by interleukin-6 family cytokines in Hepatocellular Carcinoma. Cancers 11:1704–1722
11. Møller NB, Budolfsen C, Grimm D, Krüger M, Infanger M, Wehland M et al (2019) Drug-Induced Hypertension Caused by Multikinase Inhibitors (Sorafenib, Sunitinib, Lenvatinib and Axitinib) in Renal Cell Carcinoma Treatment. Int J Mol Sci 20:4712–4734

12. Wu XF, Zhu H, Yan JZ, Khan M, Yu XY. Santamarine inhibits NF-κB activation and induces mitochondrial apoptosis in A549 Lung adenocarcinoma cells via oxidative stress. Biomed Res Int. 2017; 2017:1-11
13. Lee C, Cheung ST (2019) STAT3: An Emerging Therapeutic Target for Hepatocellular Carcinoma. Cancers 11:1646–1665
14. Zhang Y, Yang C, Cheng H, Fan Z, Huang Q, Lu Y et al (2018) Novel agents for pancreatic ductal adenocarcinoma: emerging therapeutics and future directions. J Hematol Oncol 11:14–30
15. Wu P, Wu D, Zhao LF, Huang LJ, Shen G, Huang J et al (2016) Prognostic role of STAT3 in solid tumors: a systematic review and meta-analysis. Oncotarget 7:19863–19883
16. Fujimoto A, Furuta M, Totoki Y, Tsunoda T, Kato M, Shiraishi Y (2016) **et al**. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. Nat Genet 48:500–509
17. Shen BB, Zhou RR, Yang YP, Li JY, Liang XJ, Chen L et al (2019) Antimicrobial activity-guided identification of compounds from the deciduous leaves of *Malus doumeri* by HPLC-ESI-Q TOF-MS/MS. Nat Prod Res 33:2515–2520
18. Liu XH, Zhou RR, Shen BB, Chen L, Zhou QYJ, Wan D et al (2017) Determination and isolation of four anti-tumour saponins from *Lonicera macranthoides* by HPLC-ESI-QTOF/MS and HSCCC. Curr Pharm Biotechnol 18:1106–1114
19. Zhao CX, He CH (2007) Sample capacity in preparative high-speed counter-current chromatography. J Chromatogr A 1146:186–192
20. Wu H, Su Z, Yang Y, Ba H, Aisa HA (2007) Isolation of three sesquiterpene lactones from the roots of *Cichorium glandulosum* Boiss. et Huet. by high-speed counter-current chromatography. J Chromatogr A 1176:217–222
21. Cao K, Qian W, Xu Y, Zhou Z (2019) Purification of sesquiterpenes from *Saussurea Lappa* roots by high speed counter current chromatography. Iran J Pharm Res 18:1499–1507
22. Yang C, Shi YP, Jia ZJ (2002) Sesquiterpene lactone glycosides, eudesmanolides, and other constituents from *Carpesium macrocephalum*. Planta Med 68:626–630
23. Wang XR, Shen T (2012) Chemical constituents in *Carpesium divaricatum*. Chin Tradit Herbal Drugs 43:661–663
24. Xie WD, Weng CW, Niu YF, Lai PX, Row KH (2010) Eudesmane sesquiterpenes and other constituents from *Aster himalaicus*. Chem Biodivers 7:221–224
25. Quintana J, Estévez F (2018) Recent Advances on Cytotoxic Sesquiterpene Lactones. Curr Med Chem 24:4355–4361
26. Shan H, Yao S, Ye Y, Yu Q (2019) 3-Deoxy-2β,16-dihydroxynagilactone E, a natural compound from *Podocarpus nagi*, preferentially inhibits JAK2/STAT3 signaling by allosterically interacting with the regulatory domain of JAK2 and induces apoptosis of cancer cells. Acta Pharmacol Sin 40:1578–1586

27. Kim YH, Yoon YJ, Lee YJ, Kim CH, Lee SK, Choung DH et al (2018) Piperlongumine derivative, CG-06, inhibits STAT3 activity by direct binding to STAT3 and regulating the reactive oxygen species in DU145 prostate carcinoma cells. Bioorg Med Chem Lett 28:2566–2572
28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408

Figures

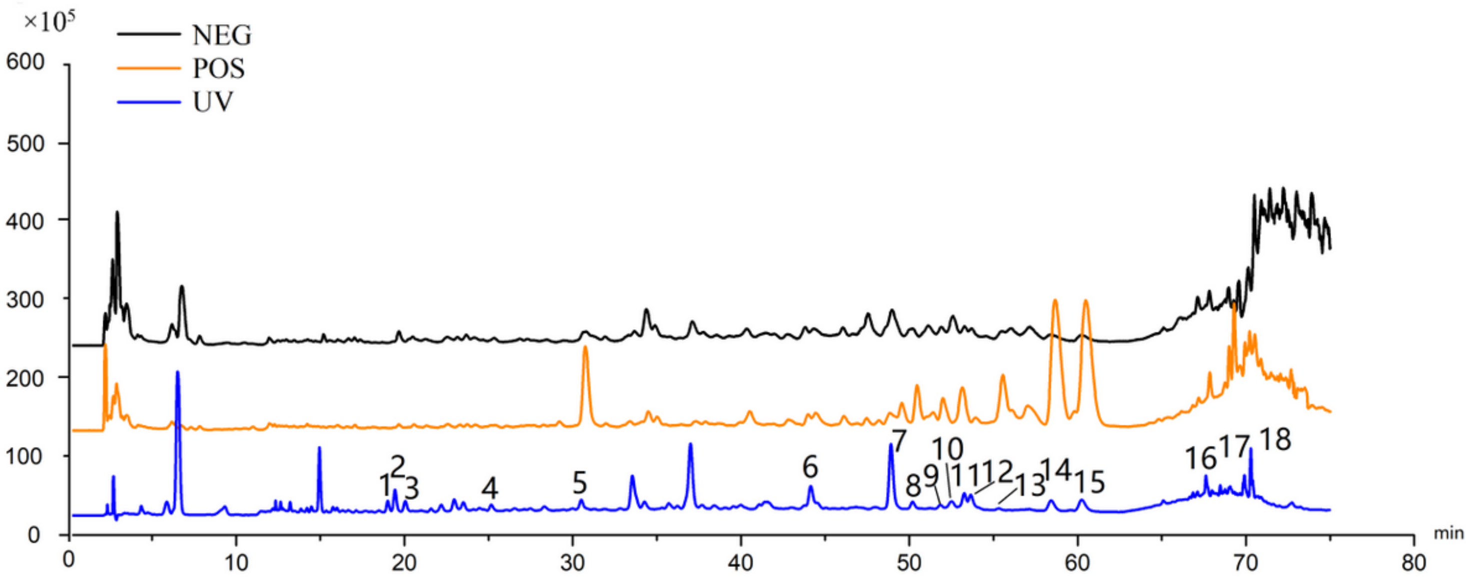

Figure 1

HPLC Chromatogram (214 nm) and MS spectra (negative mode and positive mode) of the SLEC fraction from Carpesium abrotanoides L.

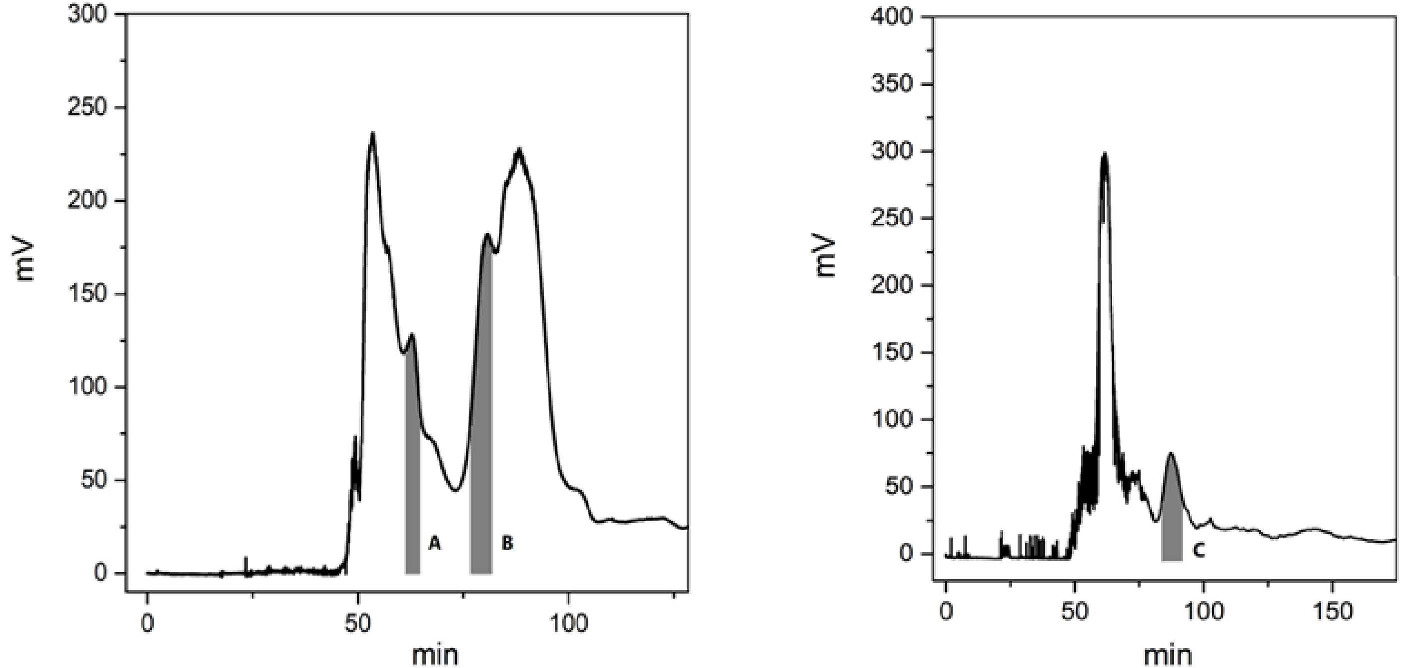

Figure 2

HSCCC chromatograms of Peak A-B (Step 1) and Peak C (Step 2)

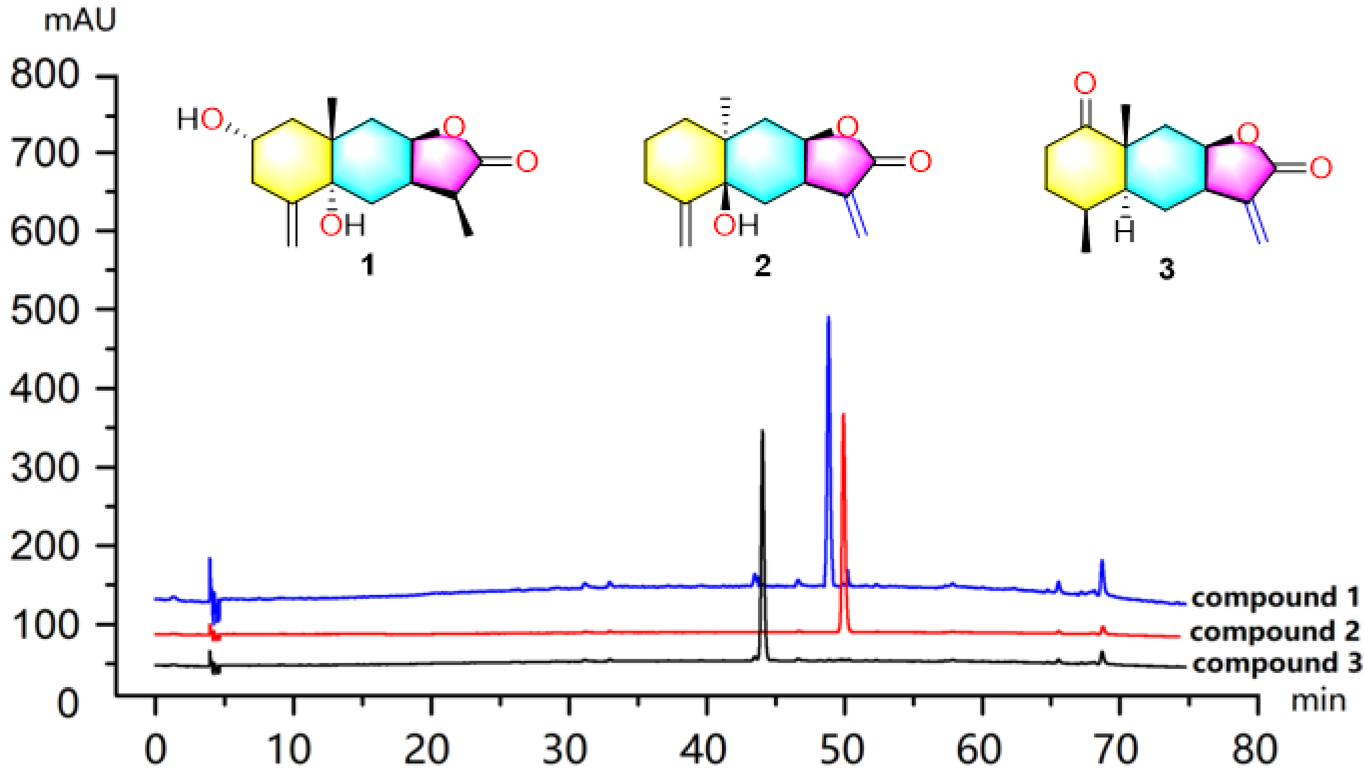

Figure 3

HPLC Chromatogram (214 nm) and structures of the isolated compounds 1-3

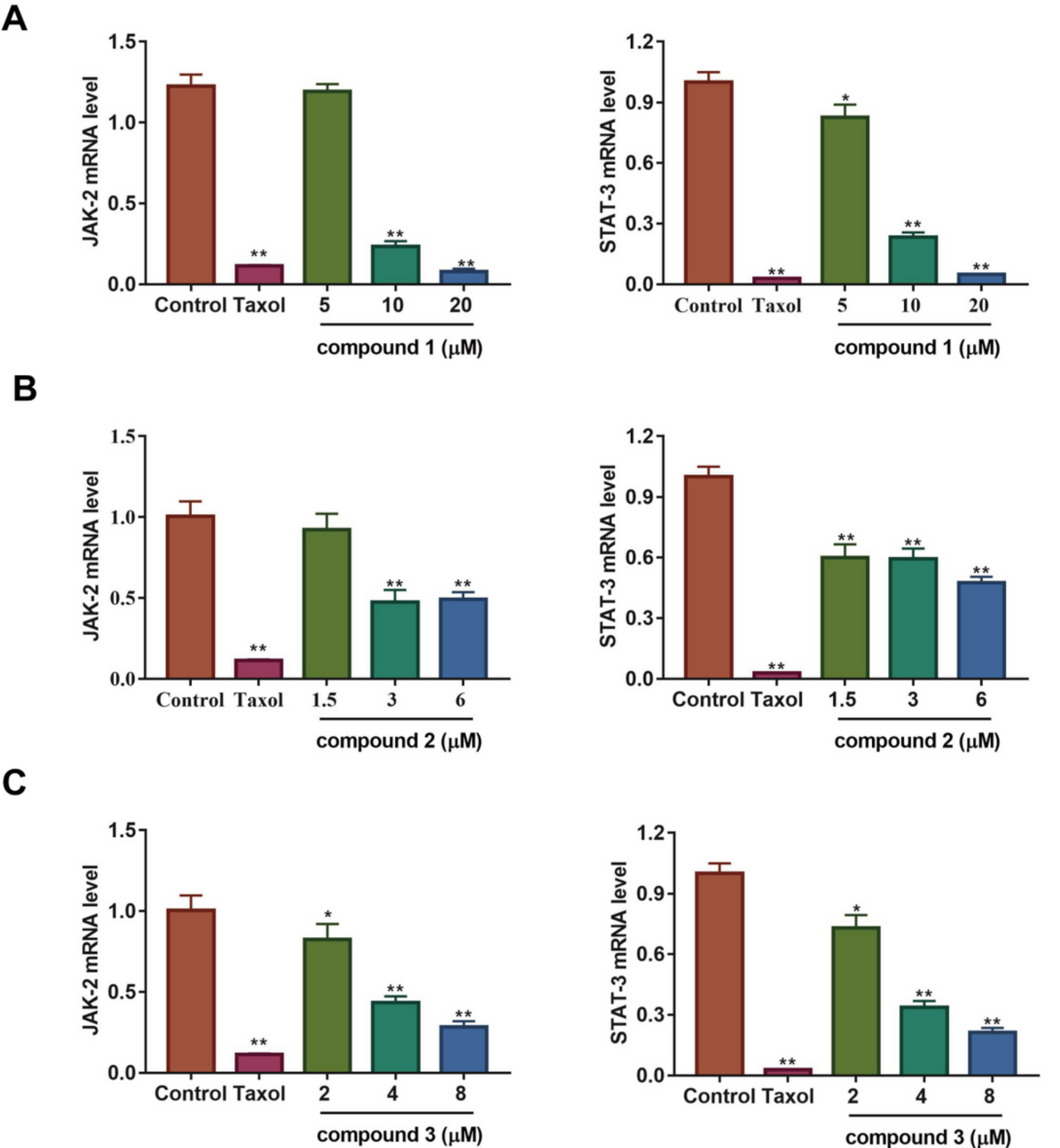

Figure 4

Effect of compounds 1-3 on the mRNA expression of JAK2 and STAT3 in HepG-2 cells. The HepG-2 cells were collected after incubation with different concentrations of compounds 1 (0, 5, 10, and 20 μM), 2 (0, 1.5, 3, and 6 μM), and 3 (0, 2, 4, and 8 μM) for 24 hr for detecting the expression of JAK2 and STAT3. (A-

C) qRT-PCR assay was used to detect the mRNA expressions of JAK2 and STAT3. Data are expressed as the mean ± SD of three independent experiments, * $P < 0.05$, ** $P < 0.01$ compared with control.

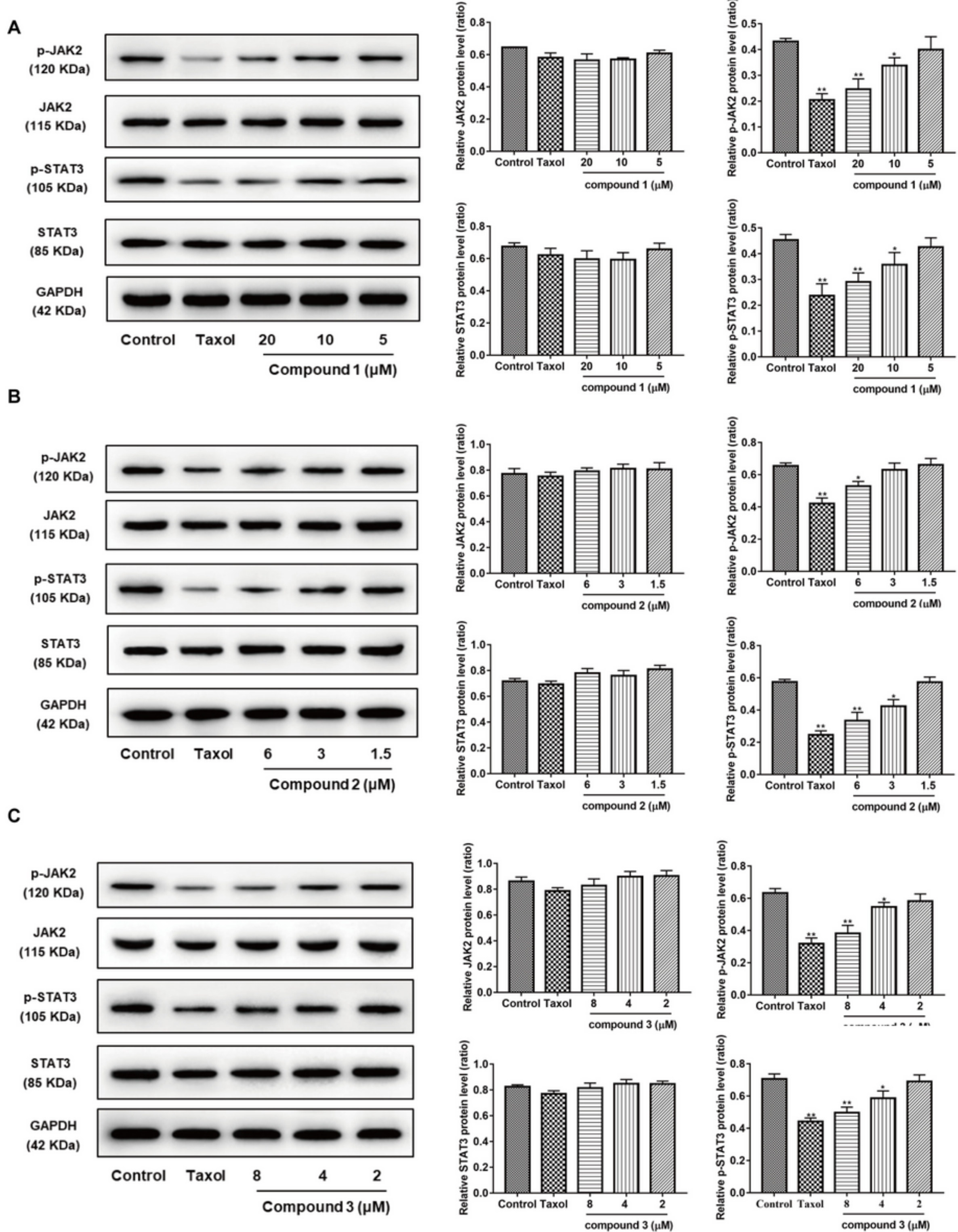

Figure 5

Effect of compounds 1-3 on the JAK2/STAT3 pathway in HepG-2 cells. (A-C) The HepG-2 cells were collected after incubation with different concentrations of compounds 1 (0, 5, 10, and 20 μM), 2 (0, 1.5, 3, and 6 μM), and 3 (0, 2, 4, and 8 μM) for 24 hr, the protein expressions of p-JAK2, JAK2, p-STAT3, and

STAT3 protein levels were detected by Western blot. All values are expressed as the mean ± SD of three independent experiments, * $P < 0.05$, ** $P < 0.01$ compared with control.

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