Chemical constituents from *Lonicera japonica* flower buds and their anti-hepatoma and anti-HBV activities

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Three new naturally occurring monoterpenoids, japopenoid A (1), japopenoid B (23) japopenoid C (24), and one new caffeoylquinic acid derivative (28), together with thirty-one known compounds (2–22, 25–27, 29–35), were isolated and identified from the flower buds of *Lonicera japonica* Thunb. Their structures were determined by extensive 1D and 2D NMR spectroscopic methods, high-resolution mass spectrometry, and the absolute configurations of 1, 23, 24 were determined by comparison of their electronic circular dichroism (ECD) spectrum with literature and theoretical calculation. Structurally, compound 1 is a monoterpenoid featured with an unusual tricyclic skeleton. All compounds (1–35) were evaluated for their cytotoxicities against human liver cancer cell lines (HepG 2 and SMMC-7721). Compound 12 exhibited the most potent activity with IC<sub>50</sub> values of 26.54 ± 1.95 and 8.72 ± 1.57 μg/ml against HepG 2 and SMMC-7721, and the IC<sub>50</sub> values of compound 13 were 26.54 ± 1.95 and 12.35 ± 1.43 μg/ml, respectively. Western blot results further proved that compound 13 induces hepatoma cell apoptosis via the intrinsic apoptosis pathway. In addition, most terpenoids showed inhibitory activity against HBsAg and HBeAg secretion, and HBV DNA replication. In particular, 25 μg/ml of compound 11 inhibits HBsAg and HBeAg secretion, and HBV DNA replication by 39.39 ± 5.25, 15.64 ± 1.25, and 16.13 ± 4.10% compared to the control (p < 0.05). These results indicated that *L. japonica* flower buds could be served as functional food for anti-hepatoma and anti-HBV activities.

1. Introduction

Liver cancer is comprised of a malignant tumor that occurs in the liver and represents a major source of morbidity and mortality worldwide [1]. Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological type and likely accounts for 70–85% of cases [2]. Cirrhosis precedes most cases of HCC, compared to other causes of cirrhosis, and hepatitis B virus (HBV) infection is associated with a higher risk of developing HCC [3,4]. According to the statistics, HBV infection accounts for up to 54% of HCC cases globally [5]. Since HBV infection is closely related to the occurrence of liver cancer [6], the treatments for HBV and HCC are equally important.
mechanism of action of the active compounds with anti-hepatoma activity.

2. Experiment

2.1. General experimental procedures

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX-400 spectrometer (Bruker, Karlsruhe, Germany). Chemical shifts are shown as δ-values with reference to tetramethylsilane (TMS) as an internal standard. High-resolution ESI-MS (HR-ESIMS) was measured on a Bruker with reference to tetramethylsilane (TMS) as an internal standard. Chemical shifts are shown as δ-values with reference to tetramethylsilane (TMS) as an internal standard. Chemical shifts are shown as δ-values with reference to tetramethylsilane (TMS) as an internal standard.

2.2. Plant materials

The *L. japonica* flower buds were collected in May 2017 from Pingyi County, Linyi City, Shandong Province, China (Fukangwanjia Pharmaceuticals). The plants were identified by Dr. XB Zeng of County, Linyi City, Shandong Province, China (Fukangwanjia Pharmaceuticals). The plants were identified by Dr. XB Zeng of County, Linyi City, Shandong Province, China (Fukangwanjia Pharmaceuticals). The plants were identified by Dr. XB Zeng of County, Linyi City, Shandong Province, China (Fukangwanjia Pharmaceuticals). The plants were identified by Dr. XB Zeng of County, Linyi City, Shandong Province, China (Fukangwanjia Pharmaceuticals). The plants were identified by Dr. XB Zeng of County, Linyi City, Shandong Province, China (Fukangwanjia Pharmaceuticals).

2.3. Extraction and isolation

*L. japonica* flower buds EtOH extract was prepared according to our previous method [9,10]. The EtOH extract was partitioned from with cyclohexane, EtOAc, and n-butOH, respectively. The EtOAc fraction yield was 1.3% relative to the dry raw material. Then the EtOAc fraction was divided into 20 fractions (Fractions 1–20) with a silica gel column (5 × 45 cm, 100–200 mesh, CH2Cl2–MeOH, 100: 1 → 1: 0, v/v).

Fraction 3 (6.9 g, yellow colloidal) was further chromatographed on the Sephadex LH-20 column (2.3 cm × 75 cm) eluted with CH2Cl2–MeOH (8: 2) to afford subfractions 3A–3G via thin layer chromatography (TLC). Fr. 3F (2.4 g) was chromatographed on the Sephadex LH-20 column (2.3 cm × 75 cm) eluted with CH2Cl2–MeOH (8: 2) again. Fr. 3F-4 (0.2 g) was subjected to preparative HPLC (Cosmosil 5C18-MS-II, 5 μm, 20 × 250 mm, flow rate: 8 mL/min, wave length: 254 nm, MeOH: H2O, 53: 47, v/v) yielding compound 1 (2.94 mg, tR: 21.88 min) and compound 2 (3.64 mg, tR: 24.80 min). Fr. 3F-5 (0.1 g) was first subjected to the Sephadex LH-20 column chromatography (1.4 × 123.5 cm) isocratic eluted with CH2Cl2–MeOH (80: 20, v/v) and 6 subfractions (Fr. 3G-1 to Fr. 3G-6). Fr. 3G-3 (0.3 g) was subjected to the Sephadex LH-20 column chromatography (1.4 × 123.5 cm) isocratic eluted with CH2Cl2–MeOH (80: 20, v/v) to afford 6 subfractions (Fr. 3G-3-1 to Fr. 3G-3-6), Fr. 3G-3-2 was separated by preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 52: 48, v/v) producing compounds 5 (1.03 mg, tR: 11.02 min), 6 (5.01 mg, tR: 11.68 min), 7 (9.58 mg, tR: 23.07 min) and 8 (5.60 mg, tR: 30.28 min). Next, the Fr. 3G-6 (0.1 g) was subjected to the Sephadex LH-20 column chromatography (1.4 × 123.5 cm, CH2Cl2–MeOH, 20: 80, v/v) again. The resulting Fr. 3G-6-4 was subjected to preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 53: 47, v/v) yielding compounds 9 (7.98 mg, tR: 11.23 min) and 10 (7.52 mg, tR: 15.63 min).

Fr. 4 (2.9 g, yellow colloidal) was subjected to the Sephadex LH-20 column chromatography (2.3 cm × 75 cm) isocratic eluted with CH2Cl2–MeOH (80: 20, v/v) to afford 6 subfractions (Fr. 4-1 to Fr. 4-6). The Fr. 4-2 (1.6 g) was subjected to preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 58: 42, v/v) yielding compounds 12 (0.5 mg, tR: 20.95 min) and 13 (0.5 mg, tR: 35.50 min).

Fr. 6 (yellow colloidal, 2.0 g) was separated by Sephadex LH-20 column chromatography (2.3 cm × 75 cm) isocratic eluted with CH2Cl2–MeOH (80: 20, v/v) to yield 12 subfractions (Fr. 6-1 to Fr. 6-12). Fr. 6-11 was subjected to preparative HPLC (8 mL/min, 330 nm, MeOH: H2O, 58: 42, v/v) yielding compounds 12 (0.5 mg, tR: 20.95 min) and 13 (0.5 mg, tR: 35.50 min).

Fr. 8 (yellow colloidal, 1.0 g) was submitted to Sephadex LH-20 column chromatography (2.3 cm × 75 cm) isocratic eluted with CH2Cl2–MeOH (80: 20, v/v) to afford 4 subfractions (Fr. 8-1 to Fr. 8-9). Fr. 8-3 was subjected to preparative HPLC (5 mL/min, 254 nm, MeOH: H2O, 60: 40, v/v) yielding compound 14 (3.37 mg, tR: 20.30 min). Similarly, subfraction Fr. 8-5 was purified with preparative HPLC (8 mL/min, 205 nm, MeOH: H2O, 55: 45, v/v) to yield compound 15 (3.12 mg, tR: 20.40 min).
(7.05 mg, tR: 25.54 min). And compound 16 (19.97 mg, tR: 20.48 min) and compound 17 (12.0 mg, tR: 31.99 min) were also obtained from Fr. 8-8 by the same method (5 mL/min, 254 nm, MeOH: H2O, 45: 55, v/v) as outlined above.

Fraction 9 (4.5 g) was chromatographed on the Sephadex LH-20 column (2.3 cm × 75 cm) eluted with CH2Cl2–MeOH (8:2), and then Fr. 9-3 was separated by preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 52: 48, v/v) yielding compound 18 (6.63 mg, tR: 9.44 min).

Fig. 2. Chemical structure of other known compounds (2–22, 25–27, and 29–35) in L. japonica flower buds.
Fr. 10 (yellow oil, 3.0 g) was submitted to Sephadex LH-20 column chromatography (2.3 × 75 cm) isocratic eluted with CH2Cl2-MeOH (80: 20, v/v) to afford 4 subfractions (Fr. 10-1 to Fr. 10-4). Fr. 10-2 was applied to preparative HPLC (8 mL/min, 205 nm, MeOH: H2O, 30: 70, v/v) and obtain compound 19 (5.83 mg, tR: 38.26 min). Fr. 10-4 was separated by preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 56: 44, v/v) yielding compounds 20 (30.15 mg, tR: 12.81 min) and 21 (3.23 mg, tR: 12.81 min).

Fr. 11 (yellow oil, 3.6 g) was chromatographed on the Sephadex LH-20 column (2.3 × 75 cm) eluted with CH2Cl2-MeOH (80: 20, v/v), and then Fr. 11-2 was separated by preparative HPLC (8 mL/min, 205 nm, MeOH: H2O, 48: 52, v/v) yielding compound 22 (12.14 mg, tR: 29.36 min). Fr. 11-3 was separated by preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 53: 47, v/v), yielding compounds 23 and 24 (10.34 mg, tR: 8.60 min).

Fr. 12 (yellow solid, 1.2 g) was chromatographed on the Sephadex LH-20 column (2.3 × 75 cm) eluted with CH2Cl2-MeOH (82%), and then Fr. 12-3 was separated by preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 60: 40, v/v) yielding compound 25 (6.85 mg, tR: 11.67 min).

Fr. 13 (yellow solid, 5.5 g) was chromatographed on the Sephadex LH-20 column (2.3 × 75 cm, CH2Cl2-MeOH: 80: 20, v/v) to afford subfractions Fr. 13-1 to 13-8 via preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 56: 44, v/v). Compounds 26 (24.20 mg, tR: 11.22 min) were purified from Fr. 13-4 by preparative HPLC (8 mL/min, 330 nm, MeOH: H2O, 45 and 45: 55, v/v) twice.

Fr. 14 (yellow solid, 1.6 g) was chromatographed on the Sephadex LH-20 column (2.3 × 75 cm, CH2Cl2-MeOH: 80: 20, v/v) to afford subfractions Fr. 14-1 to 14-6 via TLC. Compound 27 (22.74 mg, tR: 9.66 min) and 28 (24.20 mg, tR: 11.22 min) were purified from Fr. 13-4 by preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 45 and 45: 55, v/v) twice.

Fr. 15 (yellow oil, 6.0 g) was chromatographed on an ODS column (5.5 × 28 cm) gradually eluted with MeOH-H2O (10: 90 → 100: 0, v/v) to obtain 6 subfractions (Fr. 15-1 to Fr. 15-6). Fr. 15-2, eluted with MeOH-H2O (48: 52, v/v) to yield compound 29 (9.35 mg, tR: 12.20 min) and 30 (9.99 mg, tR: 28.77 min), respectively. And compound 31 (6.46 mg, tR: 47.78 min) was obtained from Fr. 14-6 by the same preparative HPLC (8 mL/min, 254 nm, MeOH: H2O, 55: 45, v/v) twice.

Fr. 16 (yellow colloidal, 37.0 g), was chromatographed on a Sephadex ODS column (5.5 × 28 cm) gradually eluted with MeOH-H2O (10: 90 → 100: 0, v/v) to obtain 9 subfractions (Fr. 16-1 to Fr. 16-9). Fr. 16-3 was subjected to preparative HPLC (Cosmosil 5C18-MS-II, 5 μm, 20 × 250 mm, flow rate: 8 mL/min, wave length: 330 nm, MeOH: H2O, 35: 65, v/v) yielding compound 35 (127.26 mg, tR: 24.50 min).

2.4. Spectroscopic data of new compounds

Japopenoid A (1). Pale yellow oil; [α]D20 +80.3° (c = 0.02, MeOH); UV (MeOH) λmax (log ε) 233.5 nm; HR-ESI-MS m/z 223.1324 [M+H]+; 1H NMR (400 MHz, d6-DMSO) and 13C NMR (100 MHz, d6-DMSO) spectrum information, see Table 1.

Japopenoid B and C (23 and 24). Orange oil; [α]D20 0.0° (c = 0.02, MeOH); UV (MeOH) λmax (log ε) 201.5, 288.5 nm; HR-ESI-MS m/z 275.1324 [M+H]+; 1H NMR (400 MHz, d6-DMSO) and 13C NMR (100 MHz, d6-DMSO) spectrum information, see Table 1.

Table 1

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2.5. Relative configurations of new compounds 1, 23 and 24

The theoretical calculations of compounds 1, 23, and 24 were performed using Gaussian 09 software (Revision C.01, Gaussian, Inc., Wallingford, CT, 2010) and configured using GaussView 5.0 (Version 5, Semichem Inc., Shawnee Mission, KS, 2009). The predominant conformers were optimized at the B3LYP/6-31G (d, p) level.

2.6. Anti-hepatoma activity assay in vitro

The HepG 2 cells and SMCC 7721 cells were from the American Type Culture Collection (ATCC, Manassas, USA). All the cells were maintained in DMEM containing 10% FBS (foetal bovine serum, HyClone, Logan, UT) and cultured at 37 °C (5% CO2, 95% relative humidity). The cytotoxicity assay was performed, according to the MTT method in 96-well microplates [9]. The protein expressions of BCL-2,
BAX, and BAK in SMCC 7721 cells were determined using Western blot analysis [11].

2.7. Anti-HBV activity assay in vitro

HepG 2.2.15 cells were maintained in DMEM containing 10% FBS (foetal bovine serum, HyClone, Logan, UT) and cultured at 37 °C (5% CO₂, 95% relative humidity). The anti-HBV assay was performed according to previous research [10].

2.8. Statistical analysis

All data is expressed as mean ± SD. At least three independent experiments were performed, each in quintuplicate. The data was analysed using a one-way ANOVA. Statistically significant effects were analysed, and the means were also compared using the least-significant difference (LSD) test. Statistical significance was determined at p < 0.05.

3. Results and discussion

3.1. Structural elucidation of new compounds (1, 23, 24, 28)

Compound 1 was isolated as a pale-yellow viscous oil. The molecular formula was determined to be C₁₃H₁₈O₃ by HR-ESI-MS at m/z 223.1324 [M+H]+ (calculated for C₁₃H₁₉O₃, 223.1327), as well as ¹H NMR and ¹³C NMR spectrum (Table 1). The ¹³C NMR and DEPT spectra indicated 13 carbons, which was consistent with the presence of three methyls, four methylenes (one oxygenated), one methane, and five quaternary carbons (one carbonyl, one olefinic, and two oxygenated carbons). The ¹H NMR and HSQC spectrum displayed 18 directly attached protons, which contained three tertiary methyl groups at δH 1.84 (3H, s, H-11), 1.14 (3H, s, H-12), and 1.43 (3H, s, H-13); three methylene groups at δH 2.43 (2H, m, H-4a, 7a), 1.97 (1H, s, H-4b), 2.02 (1H, s, H-8a), 1.90 (1H, d, J = 4.4 Hz, H-8b), and 1.77 (1H, d, J = 4.7 Hz, H-7b); two oxygenated methylene protons at δH 3.73 (1H, s, H-10a) and 3.35 (1H, s, H-10b); an methine group at δH 5.72 (1H, s, H-2). Careful analyses of the ¹H–¹H COSY experiment revealed three spin systems. The first spin system included the signals of an olefinic hydrogen (H-2) and a methylene hydrogen (H-4). The HMBC correlation (Fig. 3A) from H-2 (δH 5.72) to C-4 and C-5, from Hax-4 (δH 2.43) to C-1 and C-6, and from Heq-4 (δH 1.97) to C-1 and C-5, revealed that a 3,5,6-trisubstituted cyclohexanone ring. The second spin system included the signals of two methylene hydrogens (H-7 and H-8). In the HMBC spectrum, the correlations of H-7 with C-6 and C-8, and of H-8 with C-6 and C-7 clearly positioned an epoxy six-member ring bridge across C-5/C-6. The third spin system only included an oxygenated methylene hydrogen signal (H-10). The HMBC correlation from H-10 to C-9, along with the oxygenated nature of C-9 and C-10, revealed that an oxo-bridge should be positioned between C-9 and C-10. In addition, the HMBC correlation from H-10 to C-9, along with the oxygenated nature of C-9 and C-10, revealed that an oxo-bridge should be positioned between C-9 and C-10. Consequently, a detailed examination of the above information indicated the presence of a tricyclic monoterpenoid skeleton with two epoxy rings. Furthermore, three methoxyl groups were attached to C-3, C-6, and C-9 through the observed HMBC correlation of H-11 (δH 1.84) to C-2, C-3, and C-5. Three methoxyl groups were attached to C-3, C-6, and C-9 through the observed HMBC correlation of H-11 (δH 1.84) to C-2, C-3, and C-5, of H-12 (δH 1.14) to C-4, C-5, C-6, and C-10, and of H-13 (δH 1.43) to C-8 and C-9. Based on the above data and comprehensive 2D NMR experiments (¹H–¹H COSY, HSQC, and HMBC), the structure of compound 1 was established as shown in Fig. 3A. The relative configuration of the stereogenic centers of 1 was elucidated by ¹H–¹H coupling constants. NOESY results showed that the overlap of NOESY peaks was 90% or higher, which indicated that the relative configuration of the stereogenic centers was the same as that in compound 1. Furthermore, the ECD spectrum with strong Cotton effects was only recorded and the wavelength was 200–300 nm. Thus, the ECD spectra with strong Cotton effects were only recorded and the wavelength was 200–300 nm. Furthermore, the electronic circular dichroism (ECD) and UV of the result of the theoretical calculation agrees

Fig. 3. Structure identification of compound 1. (A) Key HMBC (H → C, red) correlations; (B) NOESY (H←→H, blue) correlations; (C) Optimized conformation geometry.

Fig. 4. Calculated and experimental results of compound 1. (A) ECD spectrum; (B) UV spectrum.
well with that determined experimentally (Fig. 4, S9 and S10). Thus, compound 1 was assigned as (5R, 6S, 9R)-3,6,9-trimethyl-10,12-dioxa-tricyclo[7.2.1.01,6]dodec-3-en-5-one, which was renamed japopenoid A.

Compounds 23 and 24, isolated as an orange oil, has a molecular formula of C13H16O5 (six unsaturations), which was deduced from the HR-ESI-MS, 13C NMR, and elementary analysis data. The NMR data (Table 1) for 23 and 24 revealed the presence of three methyls, one methylene, one sp3 oxygenated methine, three sp2 methines, three quaternary carbons, and two carbonyl carbons, indicating that 23 and 24 have a lactone ring and a fatty acid. The HMBC correlation (Fig. 5A) from H-2 (δH 7.95) to δC 168.29 (C-1), 148.98 (C-3), and 144.08 (C-4), revealed a disubstituted five-member lactone ring. Considered the quaternary carbon properties of C-3, 4, and 5, a cyclic olefinic bond across C-2/C-3 and an exocyclic double bond across C-4/C-5 were established. In addition, two methoxyl groups were attached to C-3 and C-5 through the observed HMBC correlation of δH 2.23 (H-6, 7) to C-3 and C-5, respectively. The other six carbons formed a 4-hydroxy-2-hexenoic acid group, which was linked to C-5. The HMBC correlations (Fig. 5A) observed from H-2′ (δH 5.86) to C-1′ and C-4′, from H-3′ (δH 6.78) to C-1′ and C-4′, and from H-6′ (δH 0.85) to C-4′ and C-5′, further confirmed the above conclusion. Meanwhile, the JH-2′, H-3′ value of 15.6 Hz revealed that the geometry of the double bond between C-2′ and C-3′ was E. These partial structures were linked on the basis of HMBC data. And the absolute configuration of C-4′ of 23 and 24 were further established to be 4′R and 4′S, since there is no Cotton effect in the ECD spectrum (see supporting information). In addition, the calculation results performed using Gaussian 09 software showed that 4S was the most favored conformation (Fig. 5B). Thus, 23 and 24 are a couple of racemic mixture, determined to be (4R)-4-[1-(3-Methyl-5-oxo-5H-furan-2-ylidene)- ethoxy]-hex-2-enoic acid and (4S)-4-[1-(3-Methyl-5-oxo-5H-furan-2-ylidene)- ethoxy]-hex-2-enoic acid, renamed japopenoid B and japopenoid C, respectively.

Compound 28 was isolated as a green oil, with the molecular formula of C24H24O12 as deduced from the [M−H]− peak at m/z 503.1627 (calculated for C24H23O12, 503.1627) via HR-ESI-MS and supported by the 13C NMR spectral data. The 1H NMR and 13C NMR (Table 2) showed the presence of a 4-O-trans-cafeoylquinic acid methyl ester group, which was confirmed to a quinic acid methyl ester.
ester moiety (δH 5.03 (1H, d, J = 3.3 Hz), 3.89 (1H, d, J = 8.8 Hz), 3.59 (1H, s), 3.56 (3H, s, −OCH3), 2.11 (2H, t, J = 10.6 Hz), 1.94 (1H, dd, J = 13.5, 3.0 Hz), 1.78 (1H, dd, J = 12.5, 9.5 Hz); δC 73.07 (C-1), 35.19 (C-2), 69.40 (C-3), 70.95 (C-4), 66.94 (C-5), 37.20 (C-6), 173.55 (C-7), 51.71 (C-OCH3)) and a trans-caffeoyl moiety (δH 7.39 (1H, d, J = 15.9 Hz), 7.03 (1H, s), 6.97 (1H, d, J = 8.0), 6.77 (1H, d, J = 8.0 Hz), 6.11 (1H, d, J = 15.9 Hz); δC 125.35 (C-1′), 115.81 (C-2′), 148.42 (C-3′), 145.58 (C-4′), 114.57 (C-5′), 121.24 (C-6′), 145.04 (C-7′), 113.87 (C-8′), 165.32 (C-9′). HSQC correlations and HMBC correlations analysis also supported the above speculation. In addition, three aromatic proton signals at δH 7.16 (1H, d, J = 2.0 Hz), 6.95 (1H, d, J = 8.0 Hz) indicated the presence of a 2,5-dihydroxybenzoyl moiety, which was confirmed according to the observed HMBC correlations of δH 7.16 (H-6″) to δC 154.04 (C-2″), 123.55 (C-4″), 149.27 (C-5″), 171.63 (C-7″) in Fig. 6. Moreover, the HMBC correlations indicated that the 5″-OH of the 2,5-dihydroxybenzoic acid moiety was linked to the 4′-OH of the caffeoyl moiety through the observed correlations of H-6″ (δH 7.16) to 121.24 (C-6′) (Fig. 6). Therefore, compound 28 is 4-O-[4′-O-5″-(2,5-dihydroxybenzoic acid)-caffeoyl]-quinic acid methyl ester.

The other known compounds were identified as aspertenol B (2) [12], balanophonin B (3) [13], 7-methoxy-1,2-dihydrodocaladene (4) [14], syringic aldehyde (5) [15], scopoletin (6) [16], pinoresinol (7) [17], epipinoresinol (8) [18], p-hydroxy-benzaldehyde (9) [19], 1H-indole-3-carboxaldehyde (10) [20], 3-hydroxy-β-damascone (11) [21], naringenin (12) [22], 3,5-dihydroxy-7-methoxylflavone (13) [23], (3S)-7-dimethyl-3,8-dihydrooctone (14) [24], (2S,2′S)-2,6-dimethyl-6-hydroxyl-2,7-octadienoic acid (15) [25], pyrocatechol (16) [26], p-coumaric acid (17) [27], argaminolic A (18) [28], esculetin (19) [29], protocatechuate (20) [30], taxifolin (21) [31], (−)-(7R,8S)-dihydrodehydrodiconiferyl alcohol (22) [32], 6-hydroxy-benzyl-benzoate-2-O-β-D-glucoside (25) [33], (1′R,3′S,5′R,8′S,2Z,4E)-dihydrophaseic acid (26) [34], 1-O-cafeoyl glicerides (27) [35], benzyl-β-D-glucopyranoside (29) [36], secologanin dimethylacetal (30) [37], 3-O-isorhamnetin 6-O-(p-coumaroyl)-β-D-glucopyranoside (31) [38], 3.2. Anti-hepatoma activities in vitro

*L. japonica* produces many types of secondary metabolites, including flavonoids, terpenoids, phenylpropanoids, caﬀeoylquinic acid derivatives, alkaloids, and so on. All compounds isolated from *L. japonica* in this study were assayed for their anti-hepatoma activities, and the results are listed in Table 3. Among them, compound 12 exhibited the
most potent activity with IC\textsubscript{50} values of 26.54 ± 1.95 and 8.72 ± 1.57 μg/ml against human liver cancer cell lines HepG 2 and SMMC-7721, and compound 13 exhibited significant growth inhibitory effects against HepG 2 and SMMC-7721 with IC\textsubscript{50} values at 26.54 ± 1.95 and 12.35 ± 1.43 μg/ml, respectively. These values were similar in their anti-hepatoma activities to the positive drug, Cisplatin. Compounds 3, 7, 8, and 22 were phenylpropanoids, which consisted of a kind of natural compound composed of benzene ring and three straight chain carbons (C6-C3). Compounds 7, 8, and 22 showed no inhibitory effects against liver cancer cell lines (IC\textsubscript{50} values > 300 μg/ml), but compound 3 exhibited potent activity with IC\textsubscript{50} values of 54.78 ± 0.53 and 73.47 ± 2.16 μg/ml against HepG 2 and SMMC-7721.

Fig. 8. Anti-HBV activities of terpenes (1, 2, 4, 11, 14, 15, 23, 30, 32, 33, and 35) and caffeoylquinic acid derivatives (28 and 34). (A) Relative HBsAg levels; (B) Relative HBeAg levels; (C) Relative HBV DNA levels. The figure shows the results of the experiment on the third day. Compounds 1, 2, 4, and 11 were tested for 25 μg/ml, and the other compounds (14, 15, 23, 30, 32–35) were tested for 100 μg/ml. Results are expressed as the mean ± SD (n = 3). *p < 0.05 compared with the control group, **p < 0.01 compared with the control group, ***p < 0.001 compared with the control group.
7721. This could be because the carbonyl carbon from C-9 of compound 3 produced a positive effect on the anti-hepatoma activity assay. Monoterpenoids (1, 2, 4, 11, 14, and 26) showed moderate anti-hepatoma activities with a IC50 value of 100–200 μg/ml, while iridoids (30, 32, 33, and 35) showed no significant cytotoxicity against HepG2 and SMMC-7721 cells. Similarly, caffeoylquinic acid derivatives (28 and 34) also showed no significant anti-hepatoma activities. Some observations could be made according to the above results. The anti-hepatoma activity of flavonoids was optimal, followed by phenylpropanoids and terpenoids.

Next, we choose two representative compounds 3 (phenylpropanoid) and 13 (flavonoid) to investigate the mechanism of cell death. As shown in Fig. 7A, the cell viability was significantly dropped in a time-dependent manner after treatment with compound 3 or 13. The microscopy indicated the massive cell death caused by compound 3 or 13 could be the typical apoptotic cell death characterized by cell shrinkage, pyknosis, massive plasma membrane blebbing and the destruction of cell fragments into apoptotic bodies [42]. The dependence of caspase activation is a major biochemical feature of apoptosis [43]. To validate the mechanism of cell apoptosis caused by compound 3 or 13, we assessed the protein levels of several regulators of apoptosis, including anti-apoptotic protein B-cell lymphoma-2 (BCL-2) and pro-apoptotic proteins BCL-2-associated X protein (BAX) and BCL-2 antagonist killer (BAK). The Western blot analysis revealed that compound 3 or 13 significantly reduced the expression of BCL-2 and promoted the expression of BAX and BAK compared with the control group (Fig. 7B, C, D, E). In conclusion, the above data proved that compounds 3 and 13 induce hepatoma cell apoptosis via the intrinsic apoptosis pathway.

3.3. Anti-HBV activities in vitro

In our previous study, we found that most caffeoylquinic acids from L. japonica flower buds have significant anti-HBV activities [10]. In the anti-HBV study, terpenoids were also active ingredients with inhibiting anti-HBV activity [45], compound 11 exhibits the most potent activity with IC50 values of 26.54 ± 1.95 and 8.72 ± 1.57 μg/ml against HepG2 and SMMC-7721, and the IC50 values of compound 13 were 26.54 ± 1.95 and 12.35 ± 1.43 μg/ml, respectively. The western blot results further proved that compound 13 induces hepatoma cell apoptosis via the intrinsic apoptosis pathway. In addition, most terpenoids showed inhibitory activity against HbsAg and HBeAg secretion, and HBV DNA replication. In particular, 25 μg/ml of compound 11 inhibits HbsAg and HBeAg secretion, and HBV DNA replication by 39.39 ± 5.25, 15.64 ± 1.25 and 16.13 ± 4.10% compared to the control (p < 0.05). These results indicated that L. japonica flower buds could be served as functional food for anti-hepatoma and anti-HBV activities. Further studies on the relevant mechanism of the isolated compounds are needed.

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Declaration of Competing Interest

The authors of the present manuscript have declared that no competing interests exist.

Appendix A. Supplementary material

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References

