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Chemical Characterization of the Main Bioactive Constituents from Fruits of Ziziphus jujuba

Lu Bai,a Hai Zhang,b,∗ Qingchao Liu,a Yong Zhao,b Xueqin Cui,a Sen Guo,a Li Zhang,a Chi-Tang Ho,c and Naisheng Bai,a ∗

a College of Chemical Engineering, Department of Pharmaceutical Engineering, Northwest University, 229 Taibai North Road, Xi’an, Shaanxi, 710069, China

b Laboratory Animal Center, Fourth Military Medical University, 169 Changle West Road, Xi’an, Shaanxi, 710032, China

c Department of Food Science, Rutgers University, 65 Dudley Road, New Brunswick, New Jersey 08901, USA

Corresponding Author
∗(N.B.) To whom correspondence should be addressed.

Telephone: 0086-29-88305682
Fax: 0086-29-88302223
E-mail: nsbai@nwu.edu.cn

Corresponding Author
Telephone: 0086-29-84774789
Fax: 0086-29-83242045
E-mail: hzhang@fmmu.edu.cn

Abstract
The fruit of *Ziziphus jujuba* Mill., also called hongzao in Chinese, has long history of
cultivation in China. From the fruit of *Z. jujuba*, twenty-seven known compounds
were isolated and identified as the main constituents of the fruits. They were
3-O-(trans-p-coumaroyl)-alphtolic acid (1), 3-O-(cis-p-coumaroyl)-alphtolic acid (2),
3β-O-(trans-p-coumaroyl) maslinic acid (3), pomonic acid (4), 2-oxo-pomolic acid
(5), benthamic acid (6), terminic acid (7), oleanic acid (8), betulinic acid (9), quercetin
3-O-rutinoside (10), quercetin 3-O-robinobioside (11), apigenin (12), traumatic acid
(13), (Z)-4-oxotetradec-5-enoic acid (14), 7(E)-9-keto-hexadec-7-enoic acid (15),
9(E)-11-oxo-octadecenoic acid, (9Cl) (16), and magnoflorine (27), etc. The HPLC
fingerprint of *Z. jujuba* fruits was established at the same time. The compounds 4, 5, 7,
11, 14, 15 and 16 were isolated from *Z. jujuba* for the first time. Compound 14 was
isolated from the nature for the first time. Furthermore, cytotoxicity against four
human tumor cell lines (MCF-7, A549, HepG2 and HT-29) of the isolated compounds
(1-17 and 27) was evaluated. Among these compounds, compounds 1, 2, 3, 6, 7, 9 and
12 had strong growth inhibitory effects on cancer cell lines. These results indicated
that jujube extracts exhibited cytotoxicity on these cancer cell lines.

**KEYWORDS:** *Ziziphus jujuba* Mill., triterpenoids, cytotoxicity

1. Introduction
The fruit of *Ziziphus jujuba* Mill., also called hongzao in Chinese, has long history of cultivation in China. *Ziziphus jujuba* Mill., belonging to the Rhamnaceae family, was widely distributed in northern China. Hongzao is commonly used as food and in Chinese folk medicine for the treatment of fatigue, anorexia, and other health problems for thousands of years.¹

Phytochemical studies have revealed that *Z. jujuba* contains various chemical constituents, including triterpenic acids,²⁻⁴ flavonoids,⁵ saponins,⁶ alkaloids,⁷ amino acids,⁸ phenolic acids,⁹,¹⁰ polysaccharides¹¹ and other constituents. The fruits of *Z. jujuba* were reported to have a variety of biological activities such as anti-tumor,¹²⁻¹⁴ antioxidant,⁸,¹⁵,¹⁶ anti-inflammatory,¹⁷,¹⁸ hepatoprotective property,¹⁹ gastrointestinal protective property,²⁰ and sedative effects.²¹

It has been reported that *Z. jujuba* fruit possesses hundreds of nutrients. Polysaccharides, cAMP and triterpenoid acids are the most characteristic and functional constituents.²² The structure of triterpenoids is diversified, and it has a wide range of biological activities.²³,²⁴ Much effort has been devoted to verify the effectiveness of *Z. jujuba* against cancer and the result shows that *Z. jujuba* exerts anticancer activities on several tumor cell lines.²⁻⁴

As part of our continuing research for bioactive components from nutritional fruits, we studied the jujube plant cultivated in Jiaxian, Shaan’xi province of China and isolated 27 compounds including nine triterpenoids (1⁻⁹) and 18 other compounds (10⁻27) as major constituents in the fruits of *Z. jujuba*. The HPLC fingerprint of *Z. jujuba* fruits was established at the same time. Structures of compounds 1⁻9, 11 and
14-17 are shown in Figure 1. Cytotoxicities against several human tumor cell lines (MCF-7, A549, HepG2, HT-29) of 18 isolated compounds are reported.

**Fig. 1** Structure of compounds 1-9, 11 and 14-17 from *Ziziphus jujuba*.

2. Materials and methods

2.1 General experimental procedures.

Optical rotations were determined with a Perkin-Elmer Model 241 MC polarimeter.

UV spectra were recorded on a Shimadzu UV-2401A. $^1$H and $^{13}$C nuclear magnetic
resonance (NMR) spectra were measured on a Bruker Avance 400 spectrometer (Bruker BioSpin GmbH, Beijing, China) with tetramethylsilane (TMS) as the internal standard, and chemical shifts were recorded in $\delta$ values. 2D NMR spectra include homonuclear $^1$H correlation spectroscopy (COSY), through-space $^1$H correlation spectroscopy (ROESY), one-bond heteronuclear $^1$H-$^{13}$C correlation spectroscopy (HSQC), heteronuclear multiple-bond correlation spectroscopy (HMBC). Low- and High-resolution ESIMS were recorded on a Q-TOF Global mass spectrometer. Analytical HPLC was performed on an Agilent 1260 separation module connected to a G1315D DAD detector using a Phenomenex Luna C$_{18}$ HPLC column (150 × 4.6 mm, 5 µm, 100 Å). Preparative HPLC was performed on a Shimadzu LC-20AD preparative chromatography system connected to an SPD-M20A DAD detector using Phenomenex Luna Silica and C$_{18}$ HPLC columns (250 × 21.2 mm, 5 µm, 100 Å). LC-MS analysis was carried out on a Agilent 1200 series LC, equipped with G1367B Hip-ALS, G1315D DAD detector and 6110 Quadrupole MS. HPLC analysis was performed on an Agilent 1260 LC Series using Luna C-18 column (5 micron, 4.6 mm I.D. × 250, Phenomenex, Inc., Torrance, CA, USA) with a flow rate of 1.0 mL/min, and the column temperature was maintained at 30 °C. The mobile phase was composed of A (0.2% (v/v) phosphoric acid-water solution) and B (acetonitrile) with a gradient elution: 0 min, 100% A; 0-25 min, 100-85% A; 25-35 min, 85-80% A; 35-38 min, 80-75% A; 38-45 min, 75-70% A; 45-55 min, 70-45% A; 55-60 min, 45-30% A; 60-62 min, 30-95% A; 62-65 min, 95% A.

2.2 Chemicals and reagents.
CD$_3$OD, CDCl$_3$, and CF$_3$COOH (HPLC grade) were obtained from Merck (Darmstadt, Germany). CH$_3$OH (HPLC grade), CH$_3$CH$_2$OH (HPLC grade), and CH$_3$CN (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other organic solvents used in the current study, such as CH$_3$OH, ethyl acetate (EtOAc), acetone, and chloroform (CHCl$_3$) were of analytical grade. They are commercially available from Hengxing Chemical Reagent Co., Ltd. (Tianjin, China). CCK-8 and Annexin V/PI apoptosis detection kit were purchased from Qi hai Biological Technology Ltd. (Shanghai, China).

2.3 Cell culture.

Human breast cancer cell line MCF-7, human alveolar basal epithelial cell line A549, human liver carcinoma cell line HepG2 and human colorectal adenocarcinoma cell line HT-29 were purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO$_2$ at 37 °C.

2.4 Materials.

The fruits of hongzao (Z. jujuba) were collected in October 2013 from Jiaxian, Shaan’xi Province, China. Their botanical origins were identified by the corresponding author, and voucher specimens were deposited in the herbarium of Northwest University, under number ZJ-01. Thin-layer chromatography (TLC) analysis was performed on precoated E. Merck Silica Gel 60 GF254 plates. Flash column chromatography (CC) was performed on silica gel (200-300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), reversed phase C18 (octadecylsilyl, ODS)
silica gel (Silicycle, 50 µm, Canada), or Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA). CCK-8 and PI detection kit were purchased from Qihai Biological Technology Ltd. (Shanghai, China).

2.5 Extraction and isolation.

After the cores had been removed, the dried hongzao (20 kg) were pulverized to homogeneous powders. The powders were extracted with EtOAc under cold soak for two times (each for 48 h) and the ratio of material to solvent was 1:3. The resulting solution was then filtered, and the filtrate was combined and concentrated under vacuum at 40 °C to give an extract (157 g). The crude residue was then extracted with 60% EtOH under reflux for 2 hours and extracted twice with EtOAc, finally combined the two EtOAc solutions and concentrated under vacuum to give the EtOAc extract (167 g). The combined water solution was concentrated under vacuum to give the water extract (200 g).

The EtOAc extract was subjected to a normal-phase silica gel column chromatography (600 g of silica gel, 200-300 mesh), eluted with a stepwise gradient of petroleum ether - acetone (from 50:1 to 0:1), to yield four major fractions (A-D). The fractions were monitored using Agilent 1200 LC-MS and thin-layer chromatography (TLC) with the developing solvent petroleum ether-EtOAc (from 20:1 to 0:1).

Fraction B (15 g) was purified by a flash ODS C18 silica gel column, eluted with a gradient of CH3CN-H2O to afford three subfractions, B-1, B-2, and B-3. Fraction B-1 was passed through a Sephadex LH-20 column to obtain 8 (90 mg), and 9 (5 mg).
Fraction B-2 was separated by preparative HPLC to yield 14 (27 mg, $t_R = 52.05$ min), 15 (160 mg, $t_R = 54.12$ min), 16 (33 mg, $t_R = 58.53$ min), and 17 (20 mg, $t_R = 59.67$ min). Fraction B-3 was passed through a Sephadex LH-20 column to give 18 (190 mg, $t_R = 31.82$ min), and 19 (150 mg, $t_R = 44.39$ min).

Fraction C (9.8 g) was separated into four subfractions (C-1, C-2, C-3 and C-4) by a silica gel column chromatography using a stepwise gradient of petroleum ether-acetone (from 5:1 to 1:1) as an eluent. Fraction C-2 was separated by a flash ODS C$_{18}$ silica gel column, eluted with CH$_3$CN-H$_2$O to afford two subfractions, C-2-1, and C-2-2. Fraction C-2-1 was subsequently purified by a Sephadex LH-20 column to give compound 13 (24 mg). C-2-2 was passed through a Sephadex LH-20 column to give 21 (23mg, $t_R = 30.43$ min) and 24 (195 mg, $t_R = 11.75$ min). Fraction C-3 was purified by a Sephadex LH-20 column to obtain fractions C-3-1 and C-3-2. Fraction C-3-1 was subsequently separated by a preparative HPLC to give compound 4 (4 mg), 5 (21 mg), and 7 (9 mg, $t_R = 50.95$ min). Fraction C-3-2 was further purified by a preparative HPLC to obtain 1 (20 mg, $t_R = 61.02$ min), 2 (25.3 mg, $t_R = 47.10$ min), 3 (9.2 mg, $t_R = 51.89$ min) and 6 (11.20 mg).

Fraction D (17 g) was also separated by a flash ODS C$_{18}$ silica gel column, eluted with a gradient of CH$_3$OH-H$_2$O, to obtain three subfractions, D-1, D-2 and D-3. Fraction D-1 was purified by a Sephadex LH-20 column to obtain fractions D-1-1 and D-1-2, and fraction D-1-1 was further purified by a preparative HPLC to yield 10 (5.4 mg, $t_R = 41.36$ min) and 11 (7.0 mg, $t_R = 40.66$ min). Fraction D-2 was subsequently separated by a preparative HPLC to yield 25 (5 mg, $t_R = 39.18$ min) and 26 (6.3 mg, $t_R$.
Fraction D-3 was further purified by a preparative HPLC to yield 20 (10 mg, \( t_R = 18.17 \) min) and 22 (4 mg, \( t_R = 13.22 \) min).

The water extract (200 g) was subjected to a macroporous resin D101 column chromatography (1 Kg of D101), eluted with a stepwise gradient of EtOH-H\( \text{H}_2\text{O} \), to yield three major fractions (I-III). Fraction II was further purified by a Sephadex LH-20 column to yield 12 (29.5 mg, \( t_R = 15.89 \) min) and 27 (120 mg, \( t_R = 24.92 \) min).

The HPLC chromatograms of compounds and the extracts of *Ziziphus jujuba* is shown in Fig. 2.

**Fig. 2** HPLC chromatograms of compounds and the extracts of *Ziziphus jujuba*. Peaks: 1, 3-O-(trans-p-Coumaroyl)-alphtolic acid (61.02 min); 2, 3-O-(cis-p-Coumaroyl)-alphtolic acid (47.10 min); 3, 3\( \beta \)-O-(trans-p-Coumaroyl) maslinic acid (51.89 min); 10, Quercetin 3-O-rutinoside (41.36 min); 11, Quercetin 3-O-robinobioside (40.66 min); 12, Apigenin (15.89 min); 14, (Z)-4-oxotetradec-5-enoic acid (52.05 min); 15, 7(E)-9-keto-Hexadec-7-enoic acid (54.12 min); 16, 9(E)-11-oxo-octadecenoic acid, (9CI) (58.53 min); 17, (9Z,12Z,15Z)-Octadeca-9,12,15-trienoic acid (59.67 min); 18, Benzoic acid (31.82 min); 19, 2-Hydroxybenzoic acid (44.39 min); 20, 4-Hydroxybenzoic acid (18.17 min); 21, 4-Hydroxybenzlaldehyde (30.43 min); 22, 3,4-Dihydroxybenzoic acid (13.22 min); 23, 4-Hydroxy-3-methoxybenzaldehyde (18.97 min); 24, 1,2,4-Trihydroxybenzene (11.75 min); 25, 4-Hydroxycinnamic acid (39.18 min); 26, p-Hydroxycinnamic acid (35.94 min); 27, Magnoflorine (24.92 min).
2.6 Cell inhibitory assay.

5×10^3 cells were seeded into 96-well plate one day before incubation. The final concentration of 100 µg/mL extracts was added into the cells on second day. After incubation for 24 h, 10 µL of CCK-8 was added and incubated for additional 3 h, and then OD value was measured by spectrophotometer under 450 nm. Cell inhibitory rate was calculated as follows: cell inhibitory rate= (OD\textsubscript{control} - OD\textsubscript{experiment}) / (OD\textsubscript{control} - OD\textsubscript{blank}) ×100%.

2.7 Cell death assay.

3×10^5 cells were seeded onto 6-well plate, 100 µg/mL extracts were added into the wells after the cell adherent. 24 h later, cells were harvested and washed, and then incubated with PI buffer for 15 min under 4 °C in a dark place, samples were subjected to flow cytometry analyses for cell death.

3. Results and discussion

3.1 Extraction and isolation.

The 10 kg air-dried fruit of *Z. jujuba* was extracted. The separation was conducted by using a combination of column chromatography of silica gel, Sephadex LH-20 and a flash ODS C\textsubscript{18} silica gel column together with preparative HPLC to give twenty-seven compounds, namely 3-O-(trans-p-coumaroyl)-alphaltolic acid (1), 3-O-(cis-p-coumaroyl)-alphaltolic acid (2), 3β-O-(trans-p-coumaroyl)-maslinic acid (3), pomonic acid (4), 2-oxo-pomolic acid (5), benthamic acid (6), terminic acid (7), oleanic acid (8), betulnic acid (9), quercetin 3-O-rutinoside (10), quercetin 3-O-robinobioside (11), apigenin (12), traumatic acid (13),
(Z)-4-oxotetradec-5-enoic acid (14),\(^{36}\) 7(E)-9-keto-Hexadec-7-enoic acid (15),\(^{37}\) 9(E)-11-oxo-octadecenoic acid, (9CI) (16),\(^{38}\) (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid (17),\(^{39}\) benzoic acid (18), 2-hydroxybenzoic acid (19), 4-hydroxybenzoic acid (20), 4-hydroxybenzaldehyde (21), 3,4-dihydroxybenzoic acid (22), 4-hydroxy-3-methoxybenzaldehyde (23), 1,2, 4-trihydroxybenzene (24), 4-hydroxycinnamic acid (25), \(p\)-hydroxycinnamic acid (26), and magnoflorine (27).\(^{40}\)

All structures were established by spectroscopic methods, including \(^1\)H, \(^{13}\)C NMR, DEPT, 2D correlation spectroscopy, ESI-MS, and chemical properties. The known compounds were identified by comparing NMR data with those reported in the literature. Compounds 10-27 were identified by direct comparison with commercial standards.

3.2 Structural elucidations.

Compound 1 was obtained as a white powder, and its molecular formula was C\(_{39}\)H\(_{55}\)O\(_6\) according to the ESI-MS (positive) data: m/z 619 [M + H]\(^+\). The UV spectrum of 1 exhibited maximum absorptions at 230 and 315 nm. The \(^{13}\)C NMR spectrum combined with a DEPT revealed the presence of 39 carbon signals, including a carboxyl carbon (\(\delta\) 180.0), an ester group (\(\delta\) 169.8), 10 aromatic or olefinic (\(\delta\) 110.4 - 169.8), and 2 oxygenated methane (\(\delta\) 68.0, 85.7). All proton resonances were assigned with the aid of \(^1\)H-\(^1\)H COSY and TOCSY spectra. The \(^{13}\)C NMR signals were assigned by HMQC, HMBC, and DEPT spectra. The \(^1\)H-NMR spectrum exhibited six tertiary methyl signals at \(\delta_H\) 1.73, 1.18, 1.00, 0.99, 0.95, 0.90.

Comparison of the \(^1\)H and \(^{13}\)C NMR data of 1 with those of alphitolic acid\(^{41}\) established the presence of the same backbone, except that position 3 was linked with
a \( p \)-hydroxycinnamic acid. The \( p \)-hydroxycinnamic acid group was determined by the signals of C-1´ (\( \delta \) 169.8), C-2´ (\( \delta \) 116.0), C-3´ (\( \delta \) 146.4), C-4´ (\( \delta \) 127.5), C-5´ (\( \delta \) 131.0), C-6´ (\( \delta \) 117.0), C-7´ (\( \delta \) 161.3), C-8´ (\( \delta \) 117.0), C-9´ (\( \delta \) 131.2) and H-2´ at \( \delta_H \) 6.42 (1H, d, \( J = 16.0 \) Hz), H-3´ at \( \delta_H \) 7.631H, d, \( J = 16.0 \) Hz), H-5´, 9´ at \( \delta_H \) 7.49 (2H, d, \( J = 8.8 \) Hz). From the DEPT, we could clearly see that position 3 (\( \delta \) 85.7) was a CH and the \( p \)-hydroxycinnamic acid group was located here due to the correlation between C-1´ (\( \delta \) 169.8) and H-3 (\( \delta_H \) 4.63, 1H, s) in the HMBC. There was also HMBC correlation between C-1´ (\( \delta \) 169.8) and H-2´ (\( \delta \) 6.42, 1H, d, \( J = 16.0 \) Hz), H-3´ (\( \delta \) 7.63, 1H, d, \( J = 16.0 \) Hz) in the spectrum. In addition, H-3 (\( \delta \) 4.63, 1H, s) had correlations with C-2 (\( \delta \) 68.0), C-5 (\( \delta \) 48.6), C-1 (\( \delta \) 42.1), C-4 (\( \delta \) 39.6), C-24 (\( \delta \) 29.3), C-23 (\( \delta \) 18.3) through the HMBC. The relative stereochemistry of 1 was confirmed on the basis of the NOESY correlations. There was no obvious correlation between H-2´ at \( \delta_H \) 6.42 and H-3´ at \( \delta_H \) 7.63 in the NOESY spectrum, and the coupling constant of \( J \) is 16.0 Hz in the methanol-\( d_6 \), suggesting a trans configuration of the double bond. As for compound 2, very similar chemical shifts of all carbon and hydrogen signals to 1 were found, except that H-2´ at \( \delta_H \) 5.88 (1H, d, \( J = 8.8 \) Hz) and H-3´ at \( \delta_H \) 6.90 (1H, d, \( J = 8.8 \) Hz). There was strong cross-peak between H-2´ at \( \delta_H \) 5.88 and H-3´ at \( \delta_H \) 6.90 in the NOESY spectrum, and the coupling constant of \( J \) between 2´ and 3´ is 8.8 Hz in the methanol-\( d_6 \), suggesting that the double bond in 2 is cis configuration. As for compound 3, the \( ^1 \)H and \( ^{13} \)C NMR spectra reveal that it has the same \( p \)-hydroxycinnamic acid group as compound 1, but the skeleton was maslinic acid\(^{42}\) and the \( p \)-hydroxycinnamic acid group was
also located at position 3 due to the correlation between C-1´ (δ 169.2) and H-3 (δH
4.63, 1H, s) in HMBC. There was no obvious correlation between H-2´ at δH 6.39 and
H-3´, at δH 7.63 in the NOESY spectrum, and the coupling constant of J was 16.0 Hz
in the methanol-d6, suggesting a trans configuration of the double bond. Thus, the
structure of compound 1 was determined to be 3-O-(trans-p-coumaroyl)-alphitolic
acid, 2 was identified to be 3-O-(cis-p-coumaroyl)-alphitolic acid, and 3 was
identified to be 3β-O-(trans-p-coumaroyl)-maslinic acid. The complete 1H and 13C
NMR of 1 and 2 assignments are given in Table 1.

Table 1 1H, 13C, 1H-1H COSY and HMBC Data for 1 and 2 (DMSO-d6)α

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<td>57.6 s</td>
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</tr>
<tr>
<td>18</td>
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</tr>
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<td>18,20,21</td>
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<td>152.1 s</td>
<td></td>
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Carbon multiplicities were determined by DEPT experiments (s = C, d= CH, t = CH₂, q = CH₃); Figures in parentheses denote J values (Hz).

3.3 Jujube extracts inhibit proliferation of different tissue derived cancer cells.

Anti-tumor activities of 18 compounds (1-17 and 27) were evaluated on four human cancer cell lines: MCF-7, A549, HepG2 and HT-29. As shown in Fig. 3, majority of compounds could inhibit different tissues derived cancer proliferation. Compounds 1, 2, 3, 6, 7, 9 and 12 exhibited stronger inhibitory effect on all four cancer cell lines, the highest inhibitory rate was reached to above 99%. Lee et al.³ also showed that compounds 1, 2, 3 and 9 had high cytotoxic activities against K562, B16 (F-10), SK-MEL-2, PC-3, LOX-IMVI and A549 tumor cell lines by the sulforhodamin B (SRB) method. Compound 6 previously isolated from rosemary extract has been reported to have antiproliferative effects on colon cancer cells.⁴³ Some other
compounds, like 5, 8 and 16 possessed a selective inhibitory effect on cancer cells, compound 16 only exhibited stronger inhibitory effect on A549 cells, it could hardly inhibit the proliferation of MCF-7, HT-29 and HepG2 cells. Similarly, compound 1 strongly inhibited the proliferation of MCF-7 and A549 cells, but it barely inhibited HT-29 and HepG2 cells proliferation. Meanwhile, flow cytometry assay revealed that Compounds 1, 2, 3, 6, 7, 9 and 12 significantly promoted cell death on all aforementioned cell lines, but compounds 8 and 16 only induced cell death on one or two cell lines (Fig. 4). Taken together, these data suggested that jujube extracts are potent candidates for cancer prevention.

Fig. 3 Jujube extracts inhibited the proliferation of various cancer cell lines. MCF-7, A549, HepG2 and HT-29 cells were exposed to different Jujube isolates, and cell inhibitory rate was determined with CCK-8 assay.
Fig. 4 Jujube extracts induced cell death on various human cancer cells. MCF-7, A549, HepG2 and HT-29 cells were harvested after exposing to different Jujube isolates, and cell death was determined by flow cytometry assay.

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Chemical Characterization of the Main Bioactive Constituents from Fruits of *Ziziphus jujuba*

Lu Bai,\textsuperscript{a} Hai Zhang,\textsuperscript{b,}\textsuperscript{*} Qingchao Liu,\textsuperscript{a} Yong Zhao,\textsuperscript{b} Xueqin Cui,\textsuperscript{a} Sen Guo,\textsuperscript{a} Li Zhang,\textsuperscript{a}

Chi-Tang Ho,\textsuperscript{c} and Naisheng Bai \textsuperscript{a,}\textsuperscript{*}