Phenylpropanoid and dibenzofuran derivatives from *Crataegus pinnatifida* with antiproliferative activities on hepatoma cells

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**ABSTRACT**

Eleven aromatic compounds, including four pairs of undescribed phenylpropanoids and two undescribed dibenzofurans (1a/1b-4a/4b and 5-6), were isolated from the fruits of *C. pinnatifida*. Their structures were established by extensive spectroscopic analyses. Their relative and absolute configurations were determined by the assistance of quantum chemical calculations of NMR chemical shifts and electronic circular dichroism (ECD). All isolates were screened for the cytotoxicity against two human hepatoma cell lines, HepG2 and Hep3B. It was found that compound 7 exhibited noticeable cytotoxicity against both cells with the IC50 values of 12.24 (HepG2) and 24.90 (Hep3B) μM. Further Annexin V-FITC/PI staining assay suggested that 7 could induce apoptosis in a concentration-dependent manner to exert antiproliferative activities on hepatoma cells.

1. Introduction

Aromatic compounds are a significant class of organic compounds which are widely distributed in nature [1,2]. Phenylpropanoids and dibenzofurans represent two large groups of naturally occurring aromatic compounds, which have a diverse range of medicinal properties including anti-cancer, anti-inflammatory and antiviral activities [3,4]. Moreover, a large percentage of enantiomers in phenylpropanoids characterized by C6-C3 unit have also been reported [5]. Considering the free rotation of the flexible chain and the presence of chiral carbons, accurately relative configuration and optically pure compounds were required for the detailed investigation [6,7].

*Crataegus pinnatifida*, belonging to the Rosaceae family, is widely distributed in Korea, Siberia and China [8]. In the process of our continuous search for biologically active and structurally diverse metabolites from the fruits of *C. pinnatifida*, eleven aromatic compounds including four pairs of undescribed phenylpropanoids and two undescribed dibenzofurans (1a/1b-4a/4b and 5-7) were isolated. The relative configurations were determined through quantum chemical calculations of the NMR data in combination with the NOESY data. In light of the absence of any Cotton effects in the ECD spectrum and optical rotations, four pairs of optically pure compounds were separated with Daicel Chiral IG column. Their absolute configurations were determined by comparison of the experimental and calculated ECD spectra. All the isolates were screened in vitro for their cytotoxicity against two human hepatoma cell lines (HepG2 and Hep3B). Compound 7 exhibited promising activity with IC50 values of 12.24 and 24.90 μM, respectively. Furthermore, Annexin V-FITC/PI staining assay indicated that 7 might induce apoptosis in HepG2 and Hep3B cells.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were recorded by using a JASCO DIP-370 digital polarimeter. The UV spectra were measured on a Shimadzu UV-1700 spectrometer (Shimadzu, Japan). HRESIMS data were acquired using a Micro Q-TOF spectrometer (Bruker Daltonics, Billerica, USA). ECD spectra were performed on Bio-Logic MOS 450 spectrometer (Bio-Logic Science Instruments, Seyssinet-Pariset, France). NMR experiments were obtained on Bruker ARX-400 (Bruker Corporation, Bremen, Germany) spectrometers with TMS as an internal standard. RP-HPLC separations were conducted using a Shimadzu LC-20AR series pumping system equipped with an SPD-20A UV detector and performed with an YMC reversed-phase column (250 × 10 mm, 5 μm, YMC Company, Kyoto, Japan). Chiral HPLC separation was performed by Daicel Chiral...
columns (IG) (4.6 × 250 mm, 5 μm, Daicel Polymer Ltd., Tokyo, Japan). All solvents for extraction and chromatography were commercially purchased and routinely distilled prior to use. Annexin V-FITC and propidium iodide was purchased from Bimake (Houston, USA). The apoptotic analysis was carried out using an Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). The cells were analyzed with a BD FACS CantoTM flow cytometer (BD Biosciences, New Jersey, USA).

2.2. Plant material

The fruits of Crataegus pinnatifida were collected in August 2017 from Hebei province, China. Professor Jin-Cai Lu identified this plant (School of Traditional Chinese Materia Medical, Shenyang Pharmaceutical University). A voucher specimen (No. 20180604) has been deposited in the Herbarium of Shenyang Pharmaceutical University.

2.3. Extraction and isolation

The air-dried fruits of C. pinnatifida (50 kg) were extracted with 70% EtOH under reflux three times. The EtOH extract (3600 g) was partitioned sequentially with ethyl acetate and n-BuOH. The EtOAc extract (500 g) was subjected to a silica gel column and eluted with CH2Cl2-H2O. This mixture was distributed in four fractions (Fr.1–3) on the basis of silica gel TLC detection sequentially with ethyl acetate and CH3OH to provide three fractions (Fr.A1, Fr.A2 and Fr.A3). Fr.A1 (23 g), Fr.A2 (78 g) and Fr.A3 (48 g) were separated by ODS column chromatography, eluted with a gradient of MeOH-H2O from 10% to 90%, respectively, and then re-distributed in four fractions (Fr.1–3) on the basis of silica gel gel TLC analysis. Fr.2 (31 g) was further purified by a silica gel CC using CH2Cl2-MeOH (10:1–1:1, v/v) of increasing polarity to produce 4 fractions (Fr.A-D). Fr.C was subjected to column chromatography on HP-20 macroporous resin with H2O, 20%, 50% and 90% EtOH to provide three fractions (Fr.A1, Fr.A2 and Fr.A3). Fr.A1 (23 g), Fr.A2 (78 g) and Fr.A3 (48 g) were separated by ODS column chromatography, eluted with a gradient of MeOH-H2O from 10% to 90%, respectively, and then re-distributed in four fractions (Fr.1–3) on the basis of silica gel gel TLC analysis. Fr.2 (31 g) was further purified by a silica gel CC using CH2Cl2-MeOH (30:1 to 1:1, v/v) to yield Fr.2.1–2.8. Fr.2.3–2.3.8 was obtained from Fr.2.3 by preparative HPLC (MeOH/H2O, 45:55, v/v). Compound 1 (6.0 mg, tR 39.4 min) was obtained from fraction 2.3.1 by semi-preparative HPLC eluted with CH3CN-H2O (38:62, v/v). Fraction 2.3.2 was purified in the same way as Fr.2.3.1 to obtain 2 (2.9 mg, tR 37.4 min) and 3 (3.1 mg, tR 45.7 min). In a similar manner, compound 4 (5.4 mg, tR 48.2 min) was isolated from Fr. 2.3.4 by RP-HPLC (MeOH/H2O, 51:49, v/v). Fr. 2.3.6 was fractionated by HPLC (CH3CN-H2O, 39:61, v/v) to give 6 (3.4 mg, tR 32.7 min) and 7 (3.7 mg, tR 30.6 min). Similarly, HPLC purification was performed on Fr. 2.3.7 to afford 7 (4.6 mg, tR 35.4 min).

Compounds 1a (2.1 mg) and 1b (2.3 mg) were obtained on a Daicel Chiral AD-H column, with n-hexane/isopropanol (1:1, v/v) at the flow rate of 0.5 mL/min. Compounds 2a (1.1 mg) and 2b (1.0 mg), 3a (1.4 mg) and 3b (1.5 mg) were obtained by using a Daicel Chiral AD-H column and an elution mixture of n-hexane/isopropanol (2:1, v/v) at the flow rate of 0.5 mL/min. Compounds 4a (2.3 mg) and 4b (1.9 mg) were obtained by using a Daicel Chiral AD column with an elution mixture of n-hexane/isopropanol (1:1, v/v) at the flow rate of 0.4 mL/min.

4-oxethyl-4-(3-methoxy-4-hydroxy-benzene)-2,2-dimethyl-1,3-dioxolane (1): Colorless oil, [α]D20 + 20.0 (0.10, MeOH); UV (MeOH) εmax (log ε) 280 (0.40) nm; [α]D20 + 15.5 (0.10, MeOH); ECD (MeOH) λmax (Δε) 209 (+1.27), 223 (+0.49) nm.

2,3-(1-methoxy-2-propene-1,2-diyl)-bis(2-methoxy-phenol) (2): Colorless oil, [α]D20 −2.0 (0.10, MeOH); UV (MeOH) εmax (log ε) 229 (0.21), 283 (0.10) nm; 1H and 13C NMR data see Tables 1 and 2; HRESIMS: m/z 355.1158 [M + Na]+ (calcd for C18H20O5Na 355.1152).

2.4. 13C NMR calculation

Gauge-independent atomic orbital (GIAO) calculations of 13C NMR of the relative conformers of 1a/1b were accomplished by DFT at the B3LYP/6-311+G (d, p) level in chloroform with the PCM model in the Gaussian 09 software package. The 13C NMR chemical shift of tetramethylsilane was calculated at the same level and used as reference. The calculated NMR data of these conformers were averaged according to the Boltzmann distribution theory and their relative Gibbs free energy. The linear correlation coefficient (R2) and DP4+ r probability was calculated for evaluation of the deviations between the experimental and calculated results.

2.5. ECD calculations

Conformational analysis of all the possible conformers of compounds 1-4 were performed by using the MMFF94 force field in CONLEX software. All the conformers obtained were screened based on the energy of optimized structures at the B3LYP/6-31G(d) level in an energy window of 3 kcal/mol in the Gaussian 09 software. Then, the ECD data of all the selected conformers were calculated with the time-dependent density functional theory (TDDFT) method at the B3LYP/6-311 + G (2d, p) levels with the PCM model in methanol solution. Finally, the overall theoretical ECD curves were simulated using SpecDis 1.51 software on the basis of the Boltzmann weighting of each conformer. All these calculations were performed using the Gaussian 09 program package.

2.6. Cell culture

Hep3B, HepG2 cell lines were obtained from the America Type Culture Collection (USA). The cells were cultured in Dulbecco’s modified eagle medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (Biological Industries, Israel) in 37 °C, 5% CO2 incubator.
2.7. Growth inhibition assay

Growth inhibition was detected using the MTT (Amersco, USA) assay with compounds in 96-well plates at a density of $5 \times 10^3$ cells per well in medium and allowed to attach for about 12 h. Cells were treated with a range of concentrations ($12.5, 25, 50, 100 \mu M$). After 48 h, 20 $\mu L$ MTT was added to the wells, and cells were incubated for another 4 h at 37°C. The medium was removed and DMSO (150 $\mu L$/well) was added to each well. Cell viability was measured at 490 nm. All tests needed three separate experiments.

2.8. Annexin V-FITC/PI staining

Annexin V-FITC/PI apoptosis kit (Bimake, Houston, USA) was used to evaluate apoptotic ratio according to the manufacturer's instructions. Cells at a density of $4 \times 10^5$ cells/well were placed into 6-well plate. The treated cells were stained with PI followed Annexin V-FITC at room temperature for 15 min. To quantify apoptotic ratio, cell samples were analyzed by a FACScan flow cytometry (Becton Dickinson, Franklin Lakes, USA).

2.9. Statistical analysis

All results and data were confirmed in at least three separate experiments. SPSS (Statistical Package for the Social Sciences) software was used to determine significant differences via ANOVA followed by Student's t-test. The data were expressed as means ± SD, and values $P < 0.05$ were considered as statistically significant.

3. Results and discussion

The molecular formula of 1 was established as $C_{15}H_{22}O_5$ based on NMR data and its HRESIMS ion at $m/z$ 305.1366 [M+Na]+ (calcd for 305.1359), with 5 degrees of unsaturation. The $^1$H NMR data (Table 1) indicated the presence of a 1,3,4-trisubstituted benzene system [δH 6.83 (1H, d, $J = 1.8$ Hz, H-2), 6.87 (1H, d, $J = 8.0$ Hz, H-5), 6.79 (1H, dd, $J = 8.0, 1.8$ Hz, H-6)], two oxygenated methine groups [δH 4.17 (1H, d, $J = 7.8$ Hz, H-7) and 4.28 (1H, m, H-8)], an oxygenated methylene group [δH 3.50 (1H, dd, $J = 8.6, 7.4$ Hz, H-9a), 3.59 (1H, dd, $J = 8.6, 6.4$ Hz, H-9b)], an oxyethyl group [δH 3.40 (1H, m, H-4′) and 1.18 (1H, t, $J = 7.0$ Hz, H-5′)], and two methyl groups [δH 1.42 (1H, s, H-2′) and 1.38 (1H, s, H-3′)]. The $^{13}$C NMR data displayed 15 carbon signals, corresponding to 6 aromatic carbons, 2 oxygenated methylene carbons, 2 oxygenated methine carbons, a quaternary carbon, 3 methyl...
To discern the two possible diastereoisomers, named as 7 and 8. In order to overcome this obstacle, DFT calculations were utilized to deduce distances from NOESY or H-8/H-4' correlation. However, due to the free rotation of the flexible chain and the lack of correlation between H-8 and H-4', the relative configuration assignment between C-7 and C-8 could not be established definitively when solely based on interproton distances deduced from NOESY or J-based NMR spectroscopic analysis.

In the NOESY experiment of 1, a significant correlation between H-7/H-9 and C-2 indicated the trans-configuration between H-7 and H-8 (Fig. 3). However, due to the free rotation of the flexible chain (C7-C9), the relative configuration assignment between C-7 and C-8 could not be established definitely when solely based upon interproton distances deduced from NOESY or J-based NMR spectroscopic analysis [9]. In order to overcome this obstacle, DFT calculations were utilized to discern the two possible diastereoisomers, named as (7S,8S)-1 and (7S,8R)-1 [10].

The absence of any Cotton effect in the ECD spectrum and optical rotation indicated compound 1 might be a pair of racemic mixture. The subsequent chiral resolution led to the isolation of the enantiomers 1a and 1b with Daicel Chiral IG column, which showed anticipated mirror-imaged ECD curves (1a: $\Delta \varepsilon = 97.0$, c = 0.300, MeOH; 1b: $\Delta \varepsilon = 86.0$, c = 0.300, MeOH). The absolute configurations of the enantiomers were determined by comparison of the experimental and calculated ECD spectra at the B3LYP/6-311+G (2d, p) level in methanol solution using GPCM solvation model [12–14]. The experimental ECD spectrum of 1a agreed well with the calculated ECD values for the 7S, 8S configuration (Fig. 5). Therefore, the absolute configurations of 1a and 1b were determined as 7S, 8S, and 7R, 8R, respectively.

Compound 2 exhibited a protonated molecular ion peak at m/z 353.1367 [M + Na]+ (calcd for C18H20O5Na, 353.1359) in the HREIMS data, and its molecular formula was determined as C18H20O5. 1H NMR data of 2 indicated the presence of two 1,3,4-trisubstituted benzene systems, which was evidenced by the correlation of C-1 in the HMBC spectrum, as shown in Fig. 4 [11]. Ferrocene C-148.2 (C-2) and 113.7 (C-3) were observed in 13C NMR and HSQC spectrum, which suggested the linkage of the oxymethyl group and C-1. These observations were supported by the HMBC data (Fig. 2). The correlations of H-3/C-1, C-2 and C-1' suggested the establishment of a double bond at C-2 and C-3. The chemical structure of 2 was then identified as (4'S,1'-diethyl-2-propene-1,2-diyl)-bis(2-methoxy-phenol). Moreover, the chiral resolution of compound 2 also led to a pair of enantiomers with Daicel Chiral IG column, which had opposite specific rotations and ECD curves. The absolute configuration of C-1 in 2a/2b was determined by comparing the experimental data of enantiomers 2a/2b with the computed ECD of 15 configuration (Fig. 5). The calculated ECD for the configuration of (1S)-2 matched well with enantiomer 2a. Thus, 2a/2b were assigned as (R)-4,4'-[(1-ethoxy-2-propene-1,2-diyl)]-bis(2-methoxy-phenol) and (S)-4,4'-[(1-ethoxy-2-propene-1,2-diyl)]-bis(2-methoxy-phenol), respectively.

Compound 3 showed a molecular formula C19H22O5Na, as deduced from the HREIMS ion peak at m/z 339.1168 [M + Na]+ (calcd for C18H20O5Na, 339.1203). Analysis of the 1H and 13C NMR spectroscopic data suggested that 2 was an analogue of 1, which had opposite specific rotations and ECD curves. The absolute configuration of C-1 in 3a/3b was determined by comparing the experimental data of enantiomers 3a/3b with the computed ECD of 15 configuration (Fig. 5). The calculated ECD for the configuration of (1S)-2 matched well with enantiomer 3b. Thus, 3a/3b were assigned as (R)-4,4'-[(1-ethoxy-2-propene-1,2-diyl)]-bis(2-methoxy-phenol) and (S)-4,4'-[(1-ethoxy-2-propene-1,2-diyl)]-bis(2-methoxy-phenol), respectively.

Fig. 1. The structures of compounds 1–7.

Fig. 2. Key HMBC correlations of compounds 1–2 and 4–5.
chemical ECD calculations were applied to determine the absolute configurations. The calculated ECD spectrum of (1R)-3 well matched with the experiment ECD spectrum of enantiomer 3a (Fig. 5). Therefore, 3a and 3b were established as (R)-4,4’-(1-methoxy-2-propene-1,2-diyl)-bis(2-methoxy-phenol) and (S)-4,4’-(1-methoxy-2-propene-1,2-diyl)-bis(2-methoxy-phenol).

Molecular formula of 4 was determined as C_{18}H_{20}O_{6} from the HRESIMS ion peak at \( m/z \) 355.1158 [M + Na]⁺ (calcd for C_{18}H_{20}O_{6}Na, 355.1152), corresponding to 9 degrees of unsaturation. The \(^1\)H NMR data of 4 indicated the presence of two 1,3,4-trisubstituted benzene systems [\( \delta_H \) 6.80 (1H, d, \( J = 1.9 \) Hz, H-2), 6.78 (1H, d, \( J = 8.1 \) Hz, H-5), 6.82 (1H, dd, \( J = 8.1, 1.9 \) Hz, H-6) and 6.83 (1H, d, \( J = 2.1 \) Hz, H-2’), 6.90 (1H, d, \( J = 8.1 \) Hz, H-5’), 6.73 (1H, dd, \( J = 8.1, 2.1 \) Hz, H-6’)]. The \(^{13}\)C NMR data of 4 displayed 18 carbon signals, corresponding to 12 aromatic carbons, an oxygenated methylene carbon, 3 methine carbons and 2 methoxy carbons. A substituted epoxybutyl unit was unambiguously designated by HSQC and HMBC experiments. The correlations from H-2/C-7, H-7/C-6, H-6’/C-7’, and H-7’/C-2’ in the HMBC experiment suggested that two 4-hydroxy-3-methoxybenzyl units were located at C-7 and C-7’, respectively (Fig. 2). Therefore, the gross structure of 7 was established as norlignan. The 7,8-cis and 7,7’-trans orientation were indicated by the \( J_{7,8} \) (4.8 Hz) and \( J_{7,7} \) (10.3 Hz) values, which were confirmed by the NOESY experiment (Fig. 3) [15]. Then, the enantiomers 4a and 4b were successfully separated with the opposite specific rotation and mirror-imaged ECD Cotton effects with Daicel Chiral IG column. The calculated ECD for the configuration of (7’S,7R,8S)-4 matched well with enantiomer 4b. Thus, 4a and 4b were determined as (7’S,7S,8R)-2,3-diguaiacyl-4-hydroxyl tetrahydrofuran and (7’S,7S,8S)-2,3-diguaiacyl-4-hydroxyl tetrahydrofuran.

Compound 5 was assigned as the molecular formula C_{15}H_{14}O_{5} from...
the HRESIMS ion peak at m/z 297.0764 [M+Na]+ (calcd for C15H14O5Na, 297.0733), corresponding to 9 degrees of unsaturation. The 1H NMR spectrum of 5 (Table 2) resembled that of 6-hydroxy-α-pyrufuran [16], except that the transposition of 1-OCH3 and 2-OH occurs in 5. Four mutually coupled aromatic proton signals were observed at δH 7.54 (1H, dd, J = 8.2, 0.9 Hz, H-6), 7.39 (1H, ddd, J = 8.2, 7.3, 1.5 Hz, H-7), 7.32 (1H, dd, J = 7.5, 7.3, 0.9 Hz, H-8) and 8.07 (1H, dd, J = 7.5, 1.5 Hz, H-9). The aromatic proton at δH 8.07, which showed HMBC correlations (Fig. 2) to C-5a, C-7, C-9a and C-9b, was designated as H-9. Similarly, H-7 (δH 7.39) showed HMBC correlations to C-5a, C-9 and H-8 (δH 7.32) showed HMBC correlations to C-6, C-9a. Moreover, long-range HMBC correlations from 1-OH to C-1 and C-9b, from 2-OCH3 to C-3, from 3-OCH3 to C-4, and from 4-OCH3 to C-6 established the 1-hydroxy-2,3,4-trimethoxy substitution pattern of the dibenzofuran, which was further confirmed by the correlations of H-9/OH-1 and H-6/OCH3-4 in the NOESY experiment of 5 (Fig. 3). On the basis of these data, 5 was identified as 1-hydroxy-2,3,4-trimethoxydibenzofuran.

Compound 6 was determined to have a molecular formula of C15H14O6 from the HRESIMS ion peak at m/z 313.0697 [M+Na]+ (calcd for C15H14O6Na, 313.0683). The 1H NMR spectrum of 6 showed signals for three aromatic protons at δH 6.97 (1H, d, J = 7.9 Hz, H-7), 7.20 (1H, dd, δH 7.9, 7.6 Hz, H-8) and 7.62 (1H, d, J = 7.6 Hz, H-9). In the 13C NMR spectrum of 6, the signal of C-6 was shifted to a lower field by 29.7 ppm, compared with that of 5, indicating the presence of a hydroxy group at C-6. This was further supported by HMBC correlation observed between H-8 (δH 7.20) and C-6 (δC 140.9). The examination of the 1H and 13C NMR spectra showed that 6 were similar to these of 5 (Table 1), except the presence of a hydroxy group at C-6. Therefore, 6

Table 3

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<th>Compound</th>
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a Results are expressed as IC50 means ± SD in μM. The experiments were performed three times.
was established as 1,6-dihydroxy-2,3,4-trimethoxydibenzofuran.

Structure elucidation of the known compound 7 was carried out by a combination of spectroscopic means (HRESIMS, $^1$H and $^{13}$C NMR) and comparison with the published data. Therefore, compound 7 was determined to be crataequinone A [17].

All isolates were screened for the inhibitory effects against two human hepatoma cell lines, HepG2 and Hep3B. As shown in Table 3, Compound 7 had most potent cytotoxic effect against HepG2 and Hep3B cells with IC_{50} of 12.24 and 24.90 μM, respectively. Moreover, 7 inhibited the growth of hepatoma cells in a concentration-dependent

Fig. 6. Apoptosis induced by compound 7 in Hep3B and HepG2 cells. (A) Flow cytometry analysis of Annexin V-FITC/PI-stained in Hep3B cell for 48 h and quantitative analysis of the ratio of apoptotic in Hep3B cells. (B) Flow cytometry analysis of Annexin V-FITC/PI-stained in HepG2 cell for 48 h and quantitative analysis of the ratio of apoptotic in HepG2 cells.
manner, with comparable anti-cancer activity as the positive group (Table 3). Annexin V-FITC/PI double staining was applied to 7 to measure the ratio of apoptotic cells in both cells using flow cytometer analysis (Fig. 6). The results showed that at the dose of 10, 20, 30 μM, compound 7 caused 34.33%, 68.44%, 96.9% apoptosis in Hep3B cells and 15.73%, 68.90%, 87.00% apoptosis in HepG2 cells, respectively. It demonstrated that 7 might have potential antiproliferative activities on hepatoma cells, and the underlying mechanism might involve induction of apoptosis.

4. Conclusion

In the present study, eleven compounds (1a/1b-4a/4b and 5-7) including four pairs of undescribed enantiomers and two undescribed dibenzo furan lignans were isolated from the fruits of C. pinnatifida. The chiral resolution of four pairs of compounds were carried out with Daicel Chiral IG column. Their relative and absolute configurations were determined with the assistance of quantum-mechanical NMR and ECD calculations. In addition, all these compounds were screened for cytotoxic effects against HepG2 and Hep3B cells. 7 displayed noticeable cytotoxic activity against Hep3B and HepG2 cells. Further Annexin V-FITC/PI staining assay revealed that 7 might have antiproliferative activities against hepatoma cells through induction of apoptosis.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by Career Development Support Plan for Young and Middle-aged Teachers in Shenyang Pharmaceutical University (ZQN2018006) and the Project of Innovation Team Foundation (LT2015027).

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