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Discovery of cycloneolignan enantiomers from *Isatis indigotica* Fortune with neuroprotective effects against MPP$^+$-induced SH-SY5Y cell injury

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ABSTRACT

A pair of new cycloneolignan enantiomers (**1a** and **1b**) were isolated from the leaves of *Isatis indigotica* Fortune. Their structures were elucidated by extensive spectroscopic data analysis, including 1D and 2D NMR, HRESIMS, MS/MS analysis, together with theoretical electronic circular dichroism (ECD) calculations. Compounds **1a** and **1b** were then evaluated for their neuroprotective effects against MPP$^+$-induced SH-SY5Y cell injury. As a result, compounds **1a** (77.64%) and **1b** (78.62%) exhibited moderate neuroprotective activity at the concentration of 12.5 μM compared with that of MPP$^+$ treated group (62.00% at 1 mM) by MTT assay. Furthermore, Annexin V-FITC/PI analysis showed that apoptosis ratios of **1a** and **1b** were reduced to 10.99% and 9.31%, respectively.

1. Introduction

Isatis indigotica Fortune is a biennial herbaceous plant from Cruciferae family, which is widely distributed and cultivated in China. Its dried roots and leaves were named "Ban-Lan-Gen" and "Da-Qing-Ye" in Chinese, respectively. Notably, the latter one is the important raw material of "Qing-Dai" (*Indigo naturalis*), which is a dark blue powder [1]. In China, the leaves of *I. indigotica* have been widely used as the traditional Chinese medicine (TCM) for the treatment of fever, cold, influenza, and other infections for hundreds of years [2]. Clinical efficacy of *I. indigotica* has long attracted the attention of chemists and pharmacologists to search their constituents and bioactivities. Previous investigations showed that extracts of *I. indigotica* had extensive pharmacological effects, including cytotoxicity against leukemia cells, anti-inflammatory, antipyretic effects [3,4], antioxidant [5] and neuroprotective effects [6]. Around 100 chemical constituents with different structural types, such as alkaloids [7–10], lignans [11], flavonoids [12], organic acids [13,14] were isolated from *I. indigotica*.

Cycloneolignan is a small group of lignans and consists of two C6-C3 units. The reason why it is called cycloneolignan is that the two C6-C3 moieties are connected with a ring, which is commonly formed by a C3 part with another benzene ring or two benzene rings [15]. To the best of our knowledge, it was the first time that cycloneolignan enantiomers were obtained from *I. indigotica* and it was the only cycloneolignan that we have isolated. Previous investigations have reported that cycloneolignans have neuroprotective, anti-inflammatory, and anti-tumor effects [16–18]. Because of their structural diversities and wide range of biological activities, cycloneolignans have attracted more and more interests in recent years.

As part of our continuing efforts to discover compounds with potent neuroprotective activity from *I. indigotica*, a pair of new cycloneolignan enantiomers (**1a** and **1b**, Fig. 1A) were obtained for the first time. Herein, the isolation, structure elucidation along with their neuroprotective effects on the human neuroblastoma SH-SY5Y cells damaged by MPP$^+$ were described.

2. Materials and methods

2.1. General experimental procedures

Specific optical rotation values were measured on a JASCO DIP-370 digital polarimeter (Jasco, Tokyo, Japan). UV spectra were performed on a Shimadzu UV-1700 spectrometer (Shimadzu, Tokyo, Japan). HRESIMS were performed on an Agilent G6520 Q-TOF spectrometer

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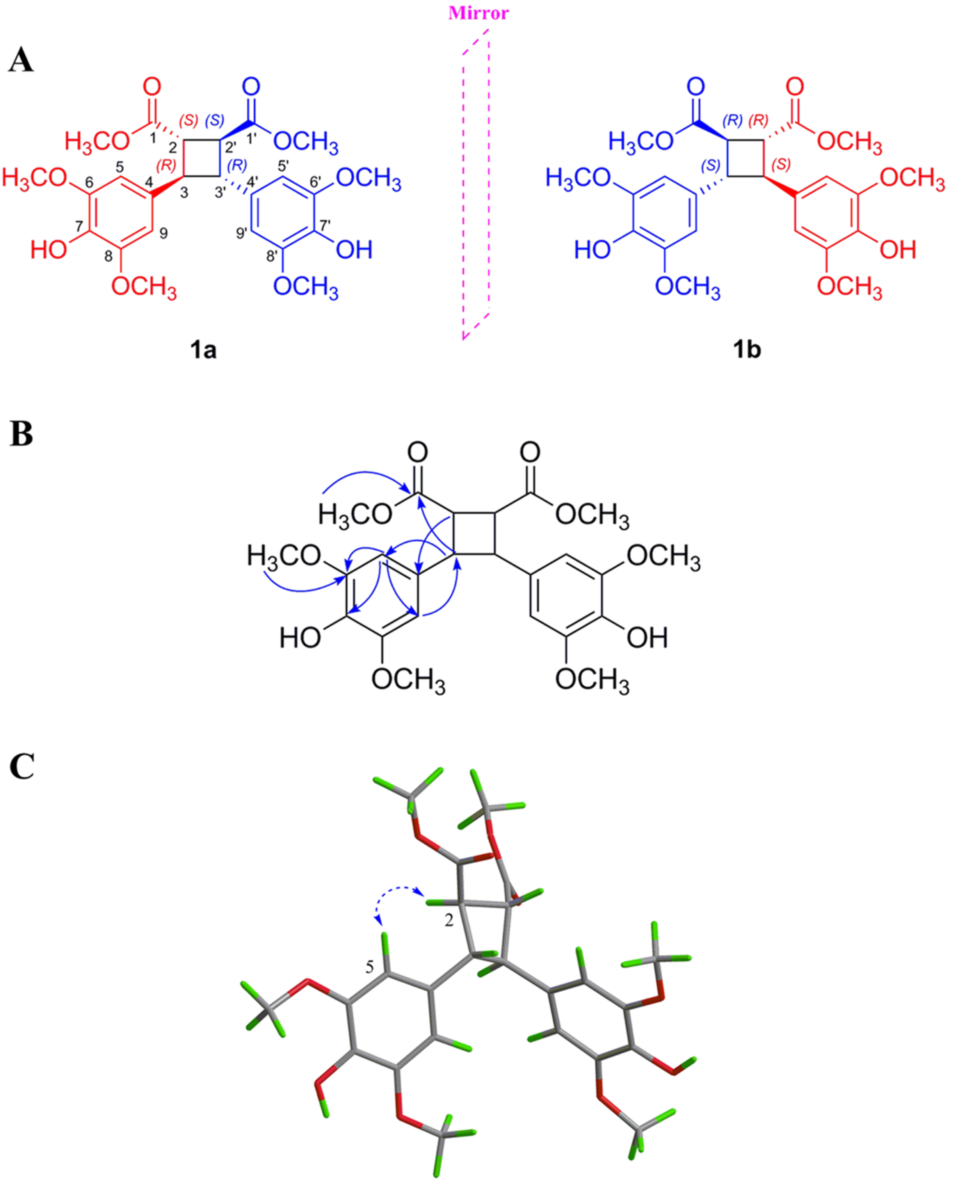

Fig. 1. (A) The chemical structures of compounds **1a** and **1b**. (B) The key HMBC correlations of compound **1**. (C) The key NOESY correlations of compound **1**.

(Agilent, Palo Alto, CA, USA). The NMR spectra were recorded by Bruker ARX-400 and AV-600 spectrometers (Bruker Corporation, Bremen, Germany) with trimethylsilane (TMS) as the internal standard and the chemical shifts were indicated as δ values (ppm). ECD spectra were recorded on a Bio-Logic MOS 450 detector (Bio-Logic Science Instruments, Seyssinet-Pariset, France). Semi-preparative RP-HPLC separation was performed on an instrument equipped with a LC-6AD pump and an SPD-20A ultraviolet–visible light absorbance detector using an YMC C_{18} semi-preparative column (250 mm × 10 mm, 5 µm, Shimadzu, Tokyo, Japan). The Chiralpak AD-H column (250 mm × 4.6 mm, 5 µm, Daicel Polymer Ltd., Tokyo, Japan) was used in the HPLC system. The organic solvents were distilled prior to the separation process. Column chromatography was carried out on silica gel (100–200 or 200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), polyamide column (60–90 mesh, Chemical Inc., Taizhou, China), Dianion HP-20 macroporous resin (Mitsubishi, Chemical Co., Japan), ODS gel (60–80 µm, Merck, Germany). Silica gel GF254 (Qingdao Marine Chemical, Ltd., Qingdao, China) was used for thin-layer chromatography (TLC). MTT assays were performed on a Varioskan Flash Multimode Reader (Thermo Scientific Co. Ltd, Massachusetts, USA). Annexin V-FITC and propidium iodide (PI) detection kit was purchased from Bimake (Houston, TX, USA). Apoptotic cells were tested by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

2.2. *Plant material*

The dried leaves of *I. indigotica* were collected from Anhui Province in China in June 2015, and then authenticated by Professor Jin-Cai Lu (Department of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, PR China). A voucher specimen (No. 20160707) has been deposited in the Department of Natural Products Chemistry, Shenyang Pharmaceutical University.

2.3. *Extraction and isolation*

The air-dried leaves of *I. indigotica* (50 kg) were extracted with 80% ethanol for twice under reflux for 4 h, resulting in 4280 g of crude extract. The residual extract was suspended in H_2O and partitioned successively with CH_2Cl_2 and *n*-BuOH. The CH_2Cl_2 extract (864 g) was performed on a silica gel column under reduced pressure, eluting with a gradient system of CH_2Cl_2/MeOH (100:1–3:1) to obtain four fractions

Table 1
^1H (600 MHz) and ^{13}C NMR (100 MHz) spectroscopic data of **1** in DMSO-d_6.

No.	**1**	
	δ_H (multi, J in Hz)	δ_C
1/1′	–	172.4
2/2′	3.32, dd (3.7, 6.0)	44.0
3/3′	3.48, dd (3.7, 6.0)	47.6
4/4′	–	131.2
5/5′, 9/9′	6.58, s	104.5
6/6′, 8/8′	–	147.9
7/7′	–	134.6
7/7′-OH	8.29, br. s	–
1/1′-OCH_3	3.66, s	51.9
6/6′/8/8′-OCH_3	3.73, s	56.0

(Fr. 1–4). Then Fr. 3 (289.3 g) was chromatographed on a polyamide column eluted with H_2O, 30%, 60% and 90% EtOH to afford Fr. A − C. The Fr. A (88.9 g) was further performed on a HP-20 macroporous resin eluting with a mixture gradient of EtOH-H_2O (from 0:100 to 90:10) to yield 3 fractions (Fr. A1 − Fr. A3). Fr. A3 (15.5 g) was then processed on an ODS column with EtOH-H_2O (from 10:90 to 90:0) to yield fractions Fr. A3.1 − Fr. A3.2, respectively. Then Fr. A3.1 (6.0 g) was processed on a silica gel column with the mobile phase PE/EtOAc (from 50:1 to 1:1) to obtain fractions purified by preparative and semi-preparative HPLC (CH_3CN/H_2O 27:73, flow rate 2.5 mL/min, detection wavelength UV 210 nm) to afford compound **1** (13.2 mg, t_R = 61 min). Chiral resolution of **1** was performed on a Daicel Chiralpak AD-H column (eluted with *n*-hexane/isopropanol, v/v, 1:1, flow rate 0.3 mL/min, detection wavelength UV 254 nm) to obtain **1a** (4.4 mg, t_R = 30.7 min) and **1b** (8.0 mg, t_R = 36.7 min).

2.3.1. Isatiscycloneolignan A (1)

Yellow solid, $[\alpha]_D^{20} - 3.6$ (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 229.0 (4.67), 255.0 (4.19) nm; ^1H and ^{13}C NMR data see Table 1; HRESIMS: m/z 499.1575 $[M + Na]^+$, (calcd for $C_{24}H_{28}O_{10}Na$, 499.1580).

*(+)-(2S,3R,2′S,3′R)-Isatiscycloneolignan A (**1a**).* $[\alpha]_D^{20} + 30.3$ (c 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 237 (−1.66), 254 (+1.43) nm.

*(−)-(2R,3S,2′R,3′S)-Isatiscycloneolignan A (**1b**).* $[\alpha]_D^{20} - 29.0$ (c 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 237 (+1.71), 253 (−1.52) nm.

2.4. ECD calculations

Conformational analysis of compound **1** were carried out with the MMFF94 force field in CONFLEX software. All the conformers obtained were screened based on the energy of optimized structures at the B3LYP/6-31G(d) level with an energy window of 10 kcal/mol on the Gaussian 09 program package [19]. Then, the theoretical ECD calculations of the conformations were performed by the TDDFT method at the B3LYP/6-311++G (2d,p) level with the CPCM model in methanol solution. Finally, the calculated ECD curve was generated by SpecDis 1.51 [20].

2.5. Cell culture

Human neuroblastoma SH-SY5Y cell lines were obtained from American Type Culture Collection (ATCC, Manassas, USA) and were cultured in DMEM medium (Hyclone, Logan, USA), which was supplemented with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C. Logarithmically growing cells were used in all the experiments.

2.6. Cell viability assay

The neuroprotective activities of compounds **1a** and **1b** on SH-SY5Y cell damaged by MPP^+ were measured by MTT assay [21]. Cells were seeded in 96-well culture plates (6×10^3 cells/well) in 100 μL medium for 12 h. Then, cells were pretreated with various concentrations (12.5, 25 and 50 μM) of the test compounds for 1 h and incubated with MPP^+ (1 mM) for another 36 h. The media were replaced by 20 μL phosphate-buffered saline medium containing 0.5 mg/mL MTT and incubated for another 4 h at the temperature of 37 °C, and the supernatant was discarded and DMSO (150 mL/well) was added. Cells treated with 1 mM MPP^+ alone was used as the model group. The absorbance was measured with a microreader at 490 nm (Thermo Scientific Multiskan MK3, Shanghai, China). The cell viability was expressed as a percentage with the control group as 100% and analyzed by GraphPad Prism 6 software.

2.7. Annexin V-FITC/PI analysis

Annexin V-FITC and PI apoptosis assay kit were applied to evaluate apoptotic ratio of the cells [22]. The treated cells were stained with Annexin V-FITC followed by PI at room temperature for 15 min. The samples were then analyzed using flow cytometer (Becton Dickinson, Franklin Lakes, USA) and quantified with Flow Jo 7.6.1 (Oregon, USA).

2.8. Statistical analysis

All results and data were confirmed in at least three separate experiments. Data are expressed as means ± SD. The level of statistical significance was determined by analysis of one-way ANOVA using GraphPad Prism 6 from GraphPad Software (California, USA). $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Phytochemical investigation

Compound **1** was isolated as a yellow solid. It had a molecular formula of $C_{24}H_{28}O_{10}$ with 11 degrees of unsaturation deduced from its HRESIMS ion at m/z 499.1575 $[M + Na]^+$, (calcd for $C_{24}H_{28}O_{10}Na$, 499.1580). Its UV spectrum showed a maximum absorption at 229 and 255 nm, suggesting the existence of benzene moiety. Analysis of ^1H and HSQC NMR spectroscopic data of **1** (Table 1) showed four aromatic protons of a typical 1,3,4,5-disubstituted benzene ring at δ_H 6.58 (4H, s, H-5/5′, 9/9′). Besides, the ^1H NMR also displayed two hydroxyl protons at δ_H 8.29 (2H, br. s, 7/7′-OH), two methylenes at δ_H 3.48 (2H, dd, J = 3.7, 6.0 Hz, H-3/3′), 3.32 (2H, d, J = 3.7, 6.0 Hz, H-2/2′) together with six methoxy groups at δ_H 3.73 (12H, s, 6/6′, 8/8′-OCH_3), 3.66 (6H, s, 1/1′-OCH_3). Combined with the molecular formula, it was assumed that compound **1** might be symmetrical planar structure. The ^{13}C NMR of **1** (Table 1) exhibited 24 carbon resonances divided into twelve aromatic carbons (δ_C 147.9 × 4, 134.6 × 2, 131.2 × 2, 104.5 × 4), two carbonyl carbons (δ_C 172.4 × 2), four methylenes (δ_C 47.6 × 2, 44.0 × 2) along with six methoxyl carbons (δ_C 56.0 × 4, 51.9 × 2).

In the HMBC spectrum (Fig. 1B), the correlation from methoxy proton (δ_H 3.66) to C-1 showed the presence of a methoxycarbonyl unit. Subsequently, the correlations of H-5/C-6, C-7, and C-9, H-9/C-3, H-3/C-1, H-2/C-4 along with the correlations from methoxyl protons (δ_H 3.73) to C-6 and C-8 indicated the presence of a 4″-hydroxy-3″,5″-dimethoxyphenyl unit. Interestingly, the above data exhibited that compound **1** was a symmetrical cycloneolignan [23–27].

However, there were two possible fragmentation pathways of compound **1**, including [2 + 2] head-to-head cyclization and [2 + 2] head-to-tail cyclization (Fig. 2A). In the head-to-tail arrangement, there might be fragment ions at m/z 238 ($C_{12}H_{14}O_5$, $[COOCH_3\text{-}CHCH\text{-}2,6\text{-dimethoxyphenol}]^{+\bullet}$). Correspondingly, in the head-to-head arrangement, there might be fragment ions at m/z 144 ($C_6H_8O_4$, $[COOCH_3\text{-}CHCH\text{-}COOCH_3]^{+\bullet}$), 238 ($C_{12}H_{14}O_5$, $[COOCH_3\text{-}CHCH\text{-}2,6\text{-dimethoxyphenol}]^{+\bullet}$) and 332 ($C_{18}H_{20}O_6$, $[2,6\text{-dimethoxyphenol-}CHCH\text{-}2,6\text{-dimethoxyphenol}]^{+\bullet}$). To verify the fragmentation pathways of **1**, MS/MS

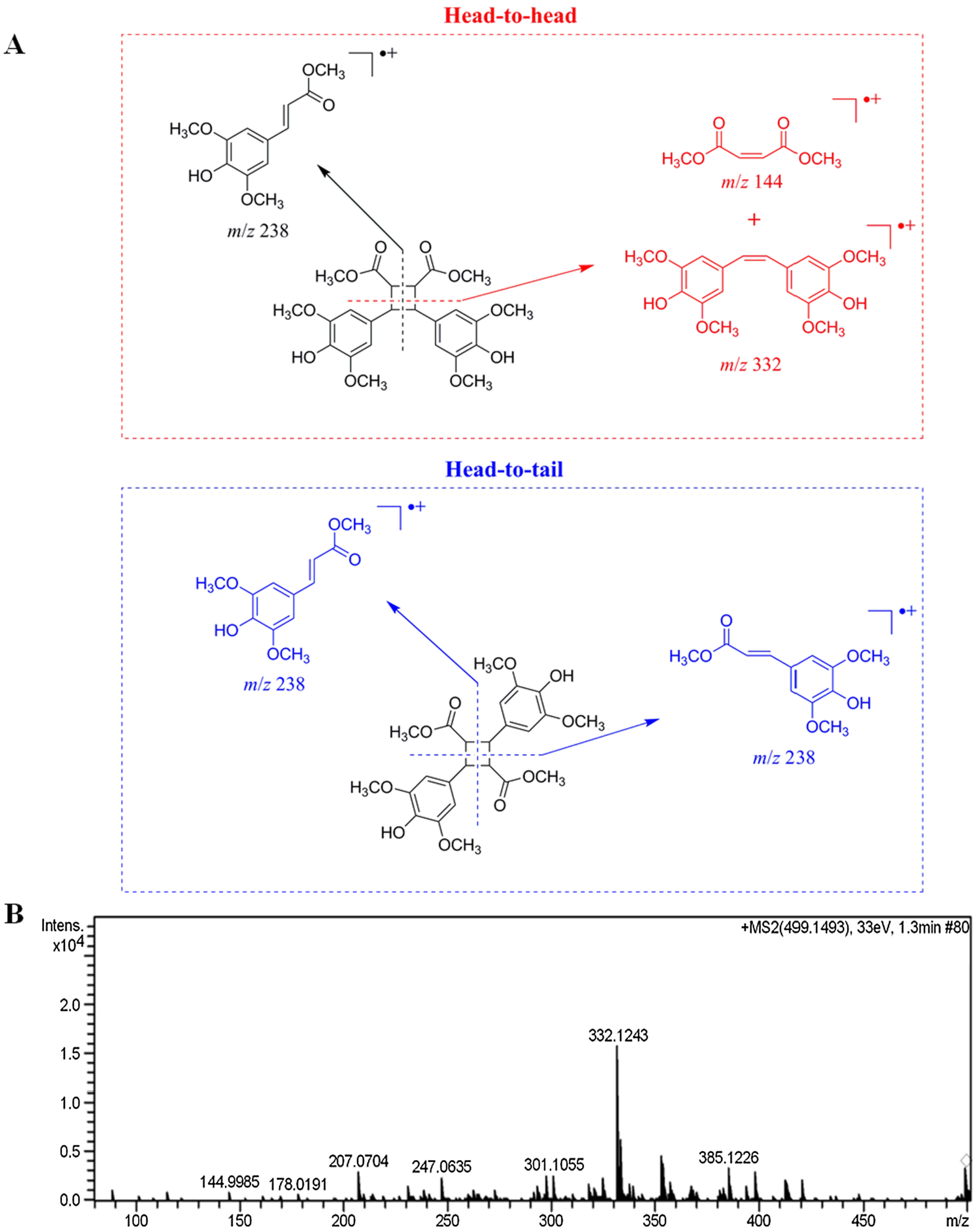

Fig. 2. (A) Possible planar structures and the MS/MS analysis fragmentation analysis of **1**. (B) The MS/MS analysis of compound **1**.

analysis was conducted (Fig. 2B). In the MS/MS analysis, the characteristic fragment ions at *m/z* 144 ($C_6H_8O_4$, $[COOCH_3\text{-}CHCH\text{-}COOCH_3]^{+\bullet}$) and 332 ($C_{18}H_{20}O_6$, $[\text{2,6-dimethoxyphenol-CHCH-2,6-dimethoxyphenol}]^{+\bullet}$) of **1**, which were formed by asymmetrical splitting of the , indicated the two 4-hydroxy-3,5-dimethoxyphenyl groups were vicinal. Thus, it was determined that the fragmentation pathway of **1** was [2 + 2] head-to-head cyclization. Hence, the planar structure of **1** was elucidated and the full assignment of its ^1H and ^{13}C NMR data was permitted based on the 1D, 2D NMR and MS/MS analysis.

For the purpose of determining the relative configuration of compound **1**, the NOESY spectrum was performed (Fig. 1C). In NOESY spectrum, the connection was observed between H-5 and H-2, which suggested that H-5 and H-2 were on the same side. Accordingly, the methoxycarbonyl and the 4″-hydroxy-3″,5″-dimethoxyphenyl fragment were on the opposite side. Nevertheless, there remained two possible relative configurations of compound **1** (Fig. S10, Supporting Information), which were consistent with the 1D and 2D NMR data of symmetrical planar structure. As illustrated in Fig. S10(A) and S10(B), the possible relative configurations of **1** were (2*R**, 3*S**, 2′*S**, 3′*R**)-**1** and (2*R**, 3*S**, 2′*R**, 3′*S**)-**1**, respectively. (2*R**, 3*S**, 2′*S**, 3′*R**)-**1** was a

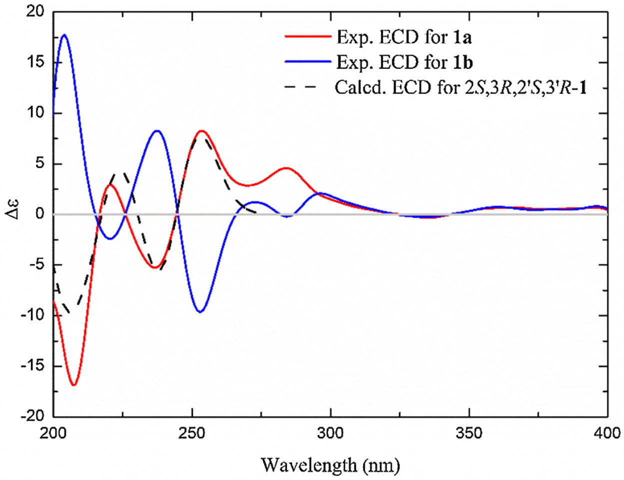

Fig. 3. Calculated and experimental ECD spectra of **1a** and **1b**.

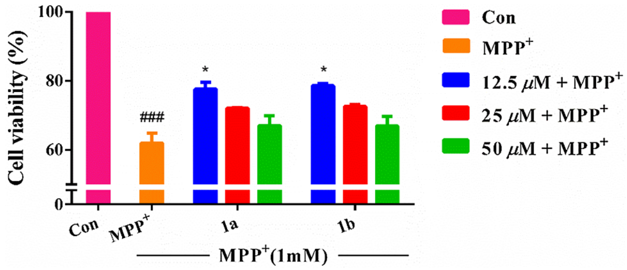

Fig. 4. Neuroprotective effects of compounds **1a** and **1b** against MPP$^+$-induced injury in SH-SY5Y cells by MTT assay at different concentrations (12.5, 25, 50 μM). Data (cell viability) was expressed as means ± SD (n = 3). $^{###}P < 0.001$ versus control; $^{*}P < 0.05$ versus MPP$^+$ treatment cells.

meso compound while (2R^*, 3S^*, 2′R^*, 3′S^*)-**1** was a racemic mixture [28], which was the main difference between the two relative configurations. To confirm the real relative configuration of **1**, the attempt on the chiral resolution was carried out. Surprisingly, the resolution of **1** by chiral-phase HPLC afforded the anticipated enantiomers **1a** and **1b** in a ratio of approximately 1:2, evidenced by their opposite specific rotations (**1a**: $[\alpha]_D^{20}$ + 30.3 and **1b**: $[\alpha]_D^{20}$ − 29.0) and Cotton effects in their experimental electronic circular dichroism (ECD) spectra. The aforementioned data demonstrated that the real relative configuration of compound **1** was 2R^*, 3S^*, 2′R^*, 3′S^* rather than 2R^*, 3S^*, 2′S^*, 3′R^*.

Subsequently, the absolute configurations of **1a** and **1b** were determined by comparison of their experimental and calculated ECD spectra at the B3LYP/6-311++G (2d,p) level with the CPCM model in methanol solution. As shown in Fig. 3, the theoretically calculated ECD of (2*S*,3*R*,2′*S*,3′*R*)-**1** well matched with the experimental ECD of **1a**. Thus, the absolute structures of **1a** and **1b** were given the trivial names as (+)-(2*S*,3*R*,2′*S*,3′*R*)-isatiscycloneolignan A and (−)-(2*R*,3*S*,2′*R*,3′*S*)-isatiscycloneolignan A, respectively.

3.2. Neuroprotective effects against MPP$^+$-induced SH-SY5Y cell injury

Parkinson's disease (PD) is one of the most common forms of neurodegenerative disorders [29]. 1-methyl-4-phenylpyridinium ion (MPP$^+$), a kind of neurotoxin, was implemented to establish the model of PD and then induces apoptosis [30]. Compounds **1a** and **1b** were tested for their neuroprotective effects against MPP$^+$-induced damage in SH-SY5Y cells by MTT assay. The cells were pretreated with compounds (12.5, 25, 50 μM) and then treated with MPP$^+$ (1 mM) (Fig. S11, Supporting Information) for 36 h to measure the potential neuroprotective effects. As shown in Fig. 4, compounds **1a** (77.64%) and **1b** (78.62%) showed moderate protective activities on MPP$^+$-induced damage in SH-SY5Y cells at 12.5 μM in comparison with that of the MPP$^+$ treated group (62.00%).

Next, Annexin V-FITC/PI doubling staining was performed to quantify the percentage of apoptotic cells in the total cell population. As illustrated in Fig. 5, the apoptosis ratio reached to 21.78% compared to the control group when treated with 1 mM MPP$^+$. However, when pretreated with **1a** and **1b** at the concentration of 12.5 μM, the percentage of total apoptotic cells in the treated group was reduced to 10.99% and 9.31%, respectively. The aforementioned results demonstrated that **1a** and **1b** might have potential of anti-apoptosis induced by MPP$^+$ in SH-SY5Y cells.

In conclusion, a pair of new cycloneolignan enantiomers (**1a** and

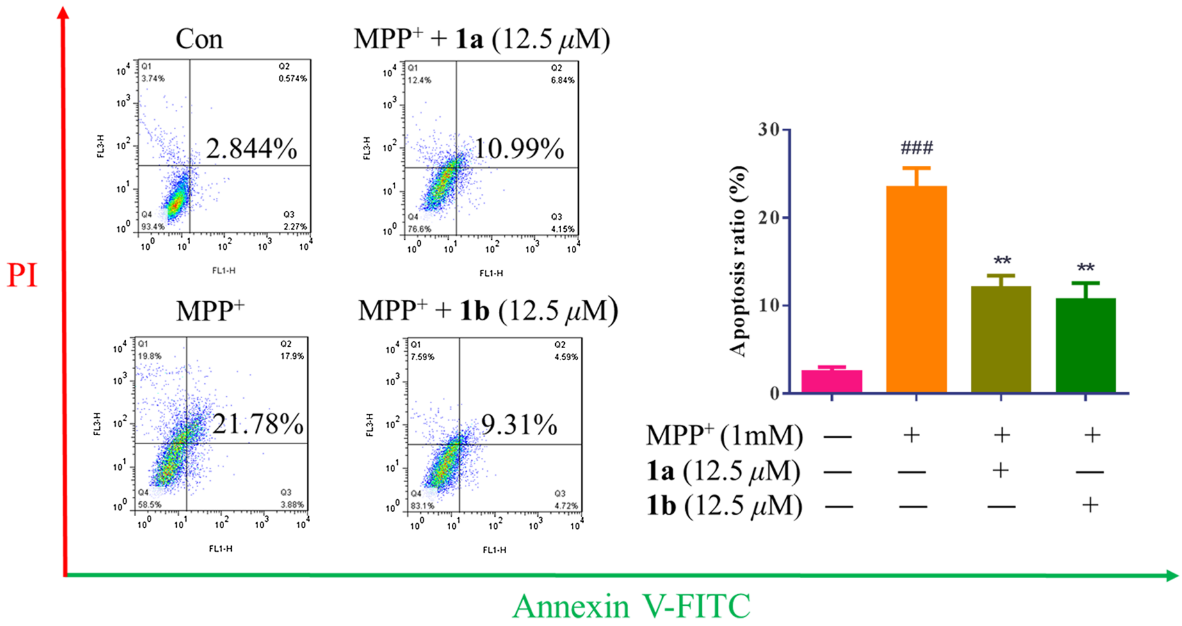

Fig. 5. Analysis of compounds **1a** and **1b** against apoptosis in MPP$^+$-induced SH-SY5Y cells through Annexin V-FITC/PI analysis. Cells were pretreated with **1a** and **1b** (12.5, 25, 50 μM) for 1 h in the presence or absence of MPP$^+$, respectively. Data are presented as means ± SD, and were confirmed at least three independent experiments. $^{###}P < 0.001$ versus control; $^{**}P < 0.01$ versus MPP$^+$ treatment cells.

1b) were isolated from the leaves of *I. indigotica*. Their planar structures and absolute configurations were determined by extensive NMR, HRESIMS, MS/MS analysis, along with the comparison of their experimental and calculated ECD spectra. It was noteworthy that the cycloneolignan enantiomers were found in the leaves of *I. indigotica* for the first time. Correspondingly, the possible fragmentation pathways of compound **1** was discussed. Subsequently, the neuroprotective effects against MPP$^+$-induced SH-SY5Y cell injury of **1a** and **1b** were evaluated *in vitro* by MTT assay and Annexin V-FITC/PI analysis. The results suggested that **1a** and **1b** exhibited neuroprotective activity by reducing the apoptosis ratio. These findings encouraged us to further research the diverse chemical constituents with neuroprotective effects from *I. indigotica*.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.102926.

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