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•Research article•

Discovery of alkaloids from the leaves of *Isatis indigotica* Fortune with neuroprotective activity

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[ABSTRACT] Seven alkaloids including five undescribed ones (**1a**/**1b**, **2**, **3** and **5**) were obtained from the leaves of *Isatis indigotica* Fortune. Their structures were established by extensive spectroscopic analyses. The absolute configurations of compounds **1a**, **1b**, **3** and **5** were determined by comparison of the experimental and calculated electronic circular dichroism (ECD) spectra. Subsequently, the neuroprotective effects of all the isolates against H_2O_2-induced injury in SH-SY5Y cells were evaluated *in vitro* by MTT assay. Moreover, Annexin V-FITC/PI double staining was performed, while the activities of antioxidant enzymes (SOD, CAT and GSH-Px) for compounds **1a** and **1b** were measured.

[KEY WORDS] *Isatis indigotica*; Alkaloids; Neuroprotective effects; Structure elucidation
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Introduction

Isatis indigotica Fortune, a biennial herbaceous plant belonging to the Cruciferae family, is widely distributed and cultivated in China. Its leaves and dried roots are called "*Da-Qing-Ye*" and "*Ban-Lan-Gen*" in Chinese, respectively. "*Da-Qing-Ye*" is also an important raw material of "*Qing-Dai*" (*Indigo naturalis*), a dark blue powder [1]. The leaves of *I. indigotica* have been widely used to treat influenza, cold, fever and other infections for hundreds of years in China [2]. Previous investigations showed the pharmacological effects of the leaves of *I. indigotica* including anti-inflammatory effects, cytotoxicity against leukemia cells, antipyretic effects, antioxidant and neuroprotective activities [3-5]. Different types of chemical constituents, such as alkaloids, lignans, flavonoids, organic acids have been isolated from *I. indigotica* [6-13].

Neurodegenerative diseases, such as Alzheimer's disease (AD), are a class of progressive illnesses [14]. Previous studies show that hydrogen peroxide (H_2O_2) is closely related to the development of neurodegenerative diseases, which is commonly applied for modeling in neuronal cells [15, 16]. Consequently, in order to search for new bioactive compounds with potent neuroprotective activity from *I. indigotica*, seven alkaloids including five undescribed ones (**1a/1b**, **2**, **3** and **5**) (Fig. 1) were isolated. Herein, the isolation, structure elucidation together with their neuroprotective effects against human neuroblastoma SH-SY5Y cells damaged by H_2O_2 were described.

Results and Discussion

Isatinoline A (**1**) was isolated as yellow oil and its molecular formula of $C_{14}H_{14}N_2O$ was analyzed by the HRESIMS at *m/z* 227.1176 $[M + H]^+$ (Calcd. for $C_{14}H_{15}N_2O$, 227.1179), accounting for nine double bond equivalents (DBEs). The ^{1}H NMR spectrum and HSQC NMR spectroscopic data (Table 1) suggested the presence of an *ortho*-disubstituted benzene ring at δ_H 7.73 (1H, br d, J = 8.0 Hz, H-5), 7.12 (1H, t, J = 8.0 Hz, H-6), 7.44 (1H, t, J = 8.0 Hz, H-7), 7.30 (1H, br d, J = 8.0 Hz, H-8), a nitrogen-bearing proton at δ_H 11.13 (1H, s, H-1), four sets of methylenes at δ_H 3.75 (1H,

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These authors have no conflict of interest to declare.
Dedicated to the 90th Anniversary of the Founding of Shenyang Pharmaceutical University

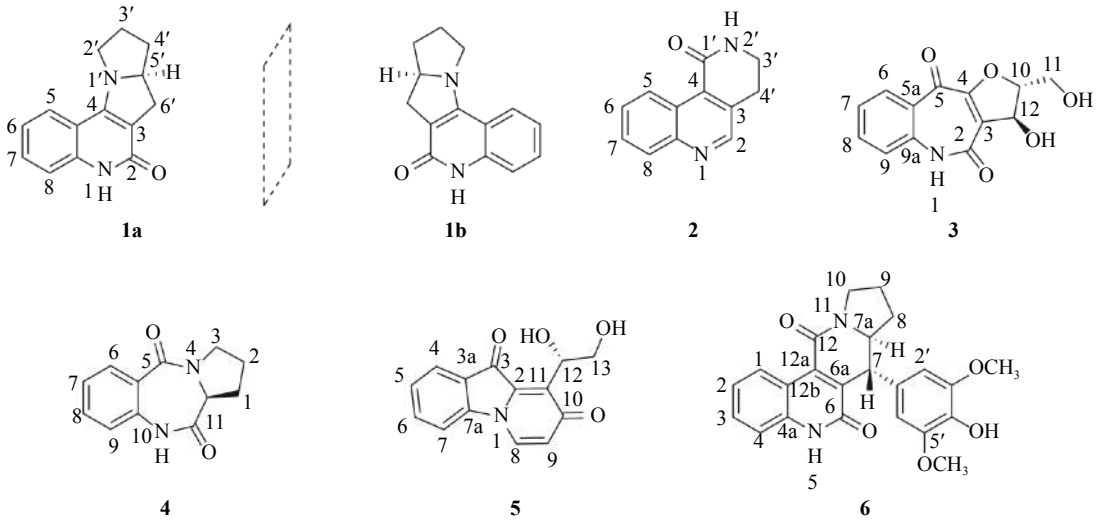

Fig. 1 Alkaloids isolated from the leaves of *Isatis indigotica*

Table 1 ^{1}H NMR (400 MHz) spectroscopic data of compounds 1–3 and 5 in DMSO-d_6

Position	1	2	3	5
1	11.13 s	–	11.13 s	–
2	–	8.95 s	–	–
4	–	–	–	7.80 overlapped
5	7.73 br d (8.0)	9.27 br d (8.2)	–	7.38 t (7.8)
6	7.12 t (8.0)	7.65 t (8.2)	8.11 br d (8.2)	7.83 overlapped
7	7.44 t (8.0)	7.74 t (8.2)	7.24 t (8.2)	7.92 d (7.8)
8	7.30 br d (8.0)	8.04 br d (8.2)	7.67 t (8.2)	8.64 d (7.6)
9	–	–	7.49 d (8.2)	6.35 d (7.6)
10	–	–	4.43 m	–
11	–	–	3.56 m	–
12	–	–	5.19 m	5.48 dt (6.0, 10.3)
13	–	–	–	3.58 m
2′	3.75 t (8.0); 3.27 m	8.42 br s	–	–
3′	1.93 m; 1.86 m	3.43 m	–	–
4′	1.99 m; 1.35 m	3.07 t (6.6)	–	–
5′	4.09 m	–	–	–
6′	2.88 dd (16.1, 10.7); 2.75 dd (16.1, 4.8)	–	–	–
11-OH	–	–	5.09 t (5.6)	–
12-OH	–	–	5.84 d (6.5)	5.79 d (10.3)
13-OH	–	–	–	4.78 t (5.9)

t, J = 8.0 Hz, H-2′) and 3.27 (1H, m, H-2′), 1.93 (1H, m, H-3′) and 1.86 (1H, m, H-3′), 1.99 (1H, m, H-4′) and 1.35 (1H, m, H-4′), 2.88 (1H, dd, J = 16.1, 10.7 Hz, H-6′) and 2.75 (1H, dd, J = 16.1, 4.8 Hz, H-6′) along with a methine proton at δ_H 4.09 (1H, m, H-5′). The down-field chemical shifts of H-2′ and H-5′ indicated that they were bound to a nitrogen atom, respectively. The ^{13}C NMR of **1** (Table 2) exhibited 14 carbon signals corresponding to six aromatic carbons (δ_C 140.1, 129.6, 123.6, 120.7, 115.7 and 112.7), one carbonyl carbons (δ_C 160.6), two olefinic carbons (δ_C 157.9 and 110.1), four methylene carbons (δ_C 51.0, 31.5, 31.0 and 26.2) and a methine carbon (δ_C 65.5). As 6 of the 9 indices of hydrogen deficiency dictated by the molecular formula were accounted for by one benzene ring, one carbonyl carbon and one olefinic group, the presence of three additional rings was suggested in **1**.

The diagnostic HMBC cross-peaks (Fig. 2) of H-5/C-7, C-8a and C-4, H-6/C-4a and C-8, H-8/C-4a and H-1/C-3 and C-4a were used to establish the molecular core of **1**, which indicated that it was a typical quinolin-2(1*H*)-one. The ^{1}H-^{1}H COSY correlations of H-2′/H-3′/H-4′/H-5′/H-6′ together with the HMBC correlations of H-2′/C-4′ and C-5′, H-3′/C-5′ and H-6′/C-4′ showed that there was a pyrrolizidine moiety. Furthermore, the pyrrolizidine moiety was deduced as being attached to C-3 and C-4 by the HMBC correlations of H-2′/C-4, H-6′/C-3 and C-4. The aforementioned analysis allowed the determination of the gross structure of **1**.

The chiral resolution led to the isolation of the enantiomers **1a** and **1b**, whose absolute configurations were proposed by comparing the experimental ECD spectrum with the

Table 2 ^{13}C NMR (100 MHz) spectroscopic data of compounds 1–3 and 5 in DMSO-d_6

Position	1	2	3	5
2	160.6	150.8	160.9	134.8
3	110.1	133.3	119.7	185.5
3a	–	–	–	122.9
4	157.9	130.1	157.7	125.1
4a	112.7	124.5	–	–
5	123.6	126.2	177.8	126.4
5a	–	–	122.6	–
6	120.7	127.7	130.0	137.4
7	129.6	128.7	123.1	112.1
7a	–	–	–	146.6
8	115.7	129.4	135.1	133.3
8a	140.1	147.8	–	–
9	–	–	120.9	115.9
9a	–	–	138.7	–
10	–	–	89.3	181.1
11	–	–	60.8	131.5
12	–	–	74.4	68.2
13	–	–	–	64.6
1′	–	164.0	–	–
2′	51.0	–	–	–
3′	26.2	38.5	–	–
4′	31.5	25.9	–	–
5′	65.5	–	–	–
6′	31.0	–	–	–

ECD spectrum predicted from quantum mechanical time-dependent density functional theory (TDDFT) calculations. As shown in Fig. 3, the calculated ECD of 5′*R*-**1** matched with the experimental ECD of **1b**. Thus, **1a** and **1b** were assigned as (−)-(5′*S*)-isatinoline A and (+)-(5′*R*)-isatinoline A, respectively.

Isatinoline B (**2**) was isolated as yellow oil and its molecular formula of $C_{12}H_{10}N_2O$ was analyzed by the HR-ESI-MS at *m*/*z* 221.0836 $[M + Na]^+$ (Calcd. for $C_{12}H_{10}N_2ONa$, 221.0840), accounting for nine double bond equivalents (DBEs). The ^{1}H NMR spectrum and HSQC NMR spectroscopic data (Table 1) suggested the presence of an *ortho*-disubstituted benzene ring at δ_H 9.27 (1H, br d, J = 8.2 Hz, H-5), 7.65 (1H, t, J = 8.2 Hz, H-6), 7.74 (1H, t, J = 8.2 Hz, H-7), 8.04 (1H, br d, J = 8.2 Hz, H-8), a olefinic proton at δ_H 8.95 (1H, br d, J = 8.2 Hz, H-2), a nitrogen-bearing proton at δ_H 8.42 (1H, br s, H-2′) along with two sets of methylenes at δ_H 3.43 (2H, m, H-3′) and 3.07 (2H, t, J = 6.6 Hz, H-4′). Analysis of the ^{13}C NMR data (Table 2) revealed 12 carbon signals including an *ortho*-disubstituted aromatic moiety (δ_C 147.8, 129.4, 128.7, 127.7, 126.2, 124.5), three olefinic carbons (δ_C 150.8, 133.3, 130.1), a carbonyl carbon (δ_C 164.0) as well as two methylenes (δ_C 38.5, 25.9). The downfield chemical shift of C-2 indicated that it was bound to a nitrogen atom. These functional groups accounted for seven out of nine DBEs, and the remaining DBEs required the presence of two cyclic systems in **2**.

The presence of quinolone core in **2** was supported by the HMBC correlations (Fig. 2) of H-5/C-4 and C-8a, H-6/C-4a, H-7/C-5 and C-8a, H-8/C-6 and C-4a together with H-2/C-4 and C-8a. In addition, the correlations of H-3′/C-1′ and C-3 together with H-4′/C-2 and C-4 indicated the presence of piperidin-2-one fragment, which was bound to quinolone core at C-3 and C-4. Based on these findings mentioned above, the connectivity of **2** was established and named as isatinoline B.

Isatinoline C (**3**) possessed the molecular formula $C_{13}H_{11}NO_5$ with nine DBEs, established by the (+)-HR-ESI-MS ion at *m*/*z* 284.0533 $[M + Na]^+$ (Calcd. for $C_{13}H_{11}NO_5Na$, 284.0529) and NMR data. Analysis of ^{1}H and HSQC NMR spectroscopic data of **3** (Table 1) revealed the presence of an *ortho*-disubstituted benzene ring at δ_H 8.11 (1H, br d, J = 8.2 Hz, H-6), 7.24 (1H, t, J = 8.2 Hz, H-7), 7.67 (1H, t, J = 8.2 Hz, H-8), 7.49 (1H, d, J = 8.2 Hz, H-9), one nitrogen-bearing proton at δ_H 11.13 (1H, s, H-1), one methylene at δ_H 3.56 (2H, m, H-11), two methines at δ_H 4.43 (1H, m, H-10) and 5.19 (1H, m, H-12) together with two hydroxyl protons at δ_H 5.09 (1H, t, J = 5.6 Hz, 11-OH) and 5.84 (1H, d, J = 6.5 Hz, 12-OH). The downfield chemical shift of H-11 indicated that it was bound to an oxygen atom. Analysis of the ^{13}C NMR data (Table 2) with the aid of the HSQC spectrum revealed 13 carbon signals that were attributable to a 1, 2-disubstituted aromatic moiety, two carbonyls, two olefinic carbons, one oxygenated methylene groups and two methines. These functional groups accounted for seven out of nine DBEs, which required the presence of two cyclic systems in the molecule.

The HMBC (Fig. 2) correlations of H-6/C-5 and C-9a, H-7/C-5a and C-9, H-8/C-9a and C-6, H-9/C-5a, H-1/C-5a, C-9, C-2 and C-3 indicated the presence of a seven-membered ring, which was connected to benzene ring at C-5a and C-9a. The five-membered ring was bound to the seven-membered ring based on the correlations of H-12/C-3 and C-4. The 12-OH was located at C-12 on the basis of δ_H 5.84/C-3, C-10 and C-12. The correlations of δ_H 5.09/C-10 and C-11 showed that the -CH_2OH moiety was located at C-10, which was also

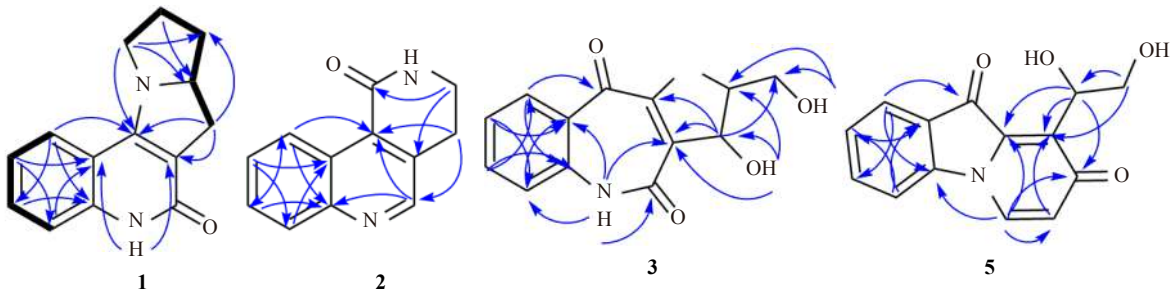

Fig. 2 Key HMBC (arrows, from ^{1}H to ^{13}C) correlations for compounds 1–3 and 5; ^{1}H-^{1}H COSY (thick lines) correlations for compound 1

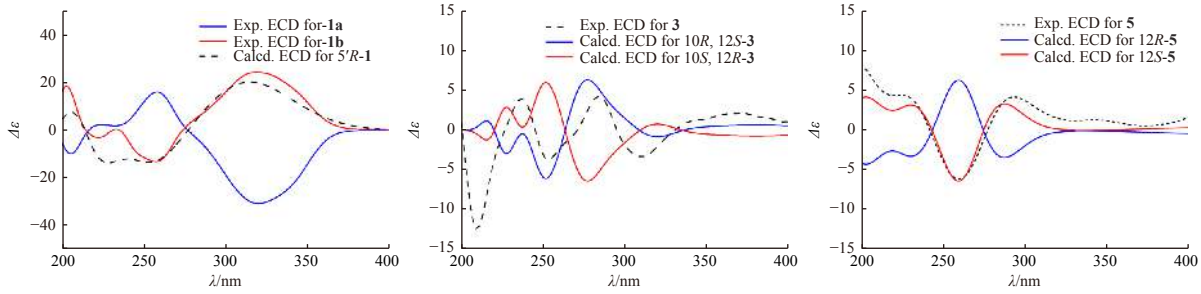

Fig. 3 Experimental and calculated ECD spectra for compounds 1a/1b, 3 and 5 in MeOH

consistent with the remaining index of hydrogen deficiency. The relative configuration of **3** was established by the NOESY spectrum (Fig. 4) of **3**, where the correlations of H-11 with H-12 indicated that H-10 and H-12 were *trans*-orientated.

The absolute configuration of **3** was determined by comparing the experimental ECD spectrum with those predicted from quantum mechanical time-dependent density functional theory (TDDFT) calculations. As shown in Fig. 3, the calculated ECD of 10*R*,12*S*-**3** matched with the experimental ECD of **3**. Thus, **3** was assigned as isatinoline C.

Isatinoline D (**5**) was obtained as orange needle crystal, with the molecular formula $C_{14}H_{11}NO_4$ determined by HR-ESIMS analysis with an ion peak $[M + Na]^+$ at *m/z* 280.0595 (Calcd. for $C_{14}H_{11}NO_4Na$ 280.0580). ^{1}H NMR spectrum (Table 1) indicated the presence of a 1, 2-disubstituted benzene ring at δ_H 7.80 (1H, overlapped, H-4), 7.38 (1H, t, J = 7.8 Hz, H-5), 7.83 (1H, overlapped, H-6), 7.92 (1H, d, J = 7.8 Hz, H-7), a set of *cis*-substituted double bonds (δ_H 8.64, 1H, d, J = 7.6 Hz, H-8; 6.35, 1H, d, J = 7.6 Hz, H-9), one oxygenated methine at δ_H 5.48 (1H, dt, J = 6.0, 10.3 Hz, H-12), one oxygenated methylene at δ_H 3.58 (2H, m, H-13) and two hydroxyl protons at δ_H 5.79 (1H, d, J = 10.3 Hz, 12-OH) and 4.78 (1H, t, J = 5.9 Hz, 13-OH). The ^{13}C NMR spectrum (Table 2) of **5** exhibited 14 carbon resonances, including six aromatic carbons (δ_C 146.6, 137.4, 126.4, 125.1, 122.9 and 112.1), four olefinic carbons (δ_C 134.8, 133.3, 131.5 and 115.9), two carbonyl carbons (δ_C 185.5 and 181.1), one oxygenated methylene carbon at δ_C 64.6 and one oxygenated methine at δ_C 68.2. The remaining two indices of hydrogen deficiency required two cyclic systems combined with the molecular formula. The above information indicated that the core of **5** was an indolin-3-one analogue. In the HMBC spectrum (Fig. 2), the correlations from H-8 to C-2/C-7a/C-9/C-10, H-9 to C-11 confirmed that the pyridin-4-one fragment was linked to C-2 and N-1. The –CHOH–CH_2OH fragment was assigned at C-11 on the basis of the HMBC correlations of H-12 to C-2, C-10 and C-11; H-13 to C-11 and C-12. Therefore, the gross structure was determined.

The absolute configuration of **5** was determined as 12*S* by comparison of their experimental and calculated ECD spectra at the B3LYP/6-31G (d, p) with the CPCM model in methanol solution. On the basis of the comparison (Fig. 3), **5** was assigned as iatinoline D.

Additionally, two known alkaloids (**4** and **6**) were also isolated from *I. indigotica* and defined as (11a*S*)-2, 3-dihydro-

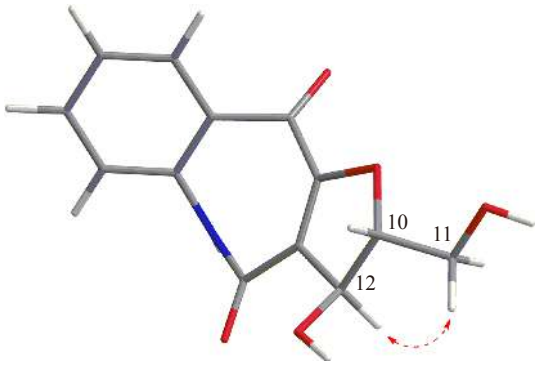

Fig. 4 Key NOESY correlations of compound 3

1*H*-pyrrolo[2, 1-c][1, 4]benzodiazepine-5, 11(10*H*, 11a*H*)-dione (**4**) [17] and isaindigotidione 1 (**6**) [18], respectively.

All the isolates were evaluated for their neuroprotective effects against H_2O_2-induced injury in SH-SY5Y cells by MTT assay. Trolox was used as a positive control. As shown in Fig. S5.1, among the tested compounds, **1a** and **1b** showed better neuroprotective effects *in vitro* and improved cell viability by 27.7% and 14.1%, compared with the H_2O_2 treated group at 25 μmol·L^{-1}, respectively. Meanwhile, the positive control Trolox gave an increase in cell viability by 13.9% at the same concentration. Annexin V-FITC/PI doubling staining was performed to quantify the percentage of apoptotic cells in the total cell population by flow cytometry. As shown in Fig. S5.2, the apoptosis ratio reached to 33.3% compared with the control group after treatment with 200 μmol·L^{-1} H_2O_2. However, after pretreatment with **1a** and **1b**, the percentage of total apoptotic cells was reduced to 20.3% and 31.5% at the concentration of 25 μmol·L^{-1}, respectively.

The H_2DCF-DA staining was carried out to investigate the abilities of **1a** and **1b** to counteract H_2O_2-induced oxidative stress by flow cytometry. Increases in DCF positive ratio caused by H_2O_2 were ameliorated through pretreatment with **1a** and **1b**. As shown in (Fig. S5.3), **1a** and **1b** decreased the DCF positive ratio caused by H_2O_2 injury. The activities of SOD, CAT and GSH-Px were measured to assess the abilities of **1a** and **1b** to increase the antioxidant defenses of SH-SY5Y cells. As shown in Fig. S5.4, **1a** and **1b** pretreatment increased the activities of SOD, CAT and GSH-Px.

Experimental

General experimental procedures

Organic solvents were distilled prior to the separation process. Column chromatography was carried out on Dian-

ion HP-20 macroporous resin, silica gel (100−200 or 200−300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), polyamide column (60−90 mesh, Yuwang Chemical Inc. Yucheng, China), and ODS gel (60-80 μm, Merck, Frankfurter, Germany). The NMR spectra were recorded by Bruker ARX-400 and AVIII-600 spectrometers (Bruker Corporation, Bremen, Germany). HR-ESIMS were acquired on a Bruker Micro Q-TOF instrument in a positive-ion mode (Bruker Co., Karlsruhe, Germany). 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). ECD spectra were conducted on a Bio-Logic MOS 450 detector (Bio-Logic Science Instruments, SeyssinetPariset, France). Semi-preparative HPLC was performed on a Shimadzu LC-6AD pump system with a Shimadzu SPD-20A detector, using YMC C_{18} column Qcc (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) were used in the HPLC system. MTT assay was performed using 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). SOD, CAT, and GSH-Px assay kits were obtained from Jiancheng Biochemical Company (Nanjing, China). SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Gibco Company (Grand Island, NY, USA).

Plant material

The dried leaves of *I. indigotica* were obtained from Anhui Province in China in June 2015 and authenticated by Professor LU Jin-Cai (Department of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, China). A voucher specimen (No. 20160707) of the plant is stored at the herbarium of the Department of Pharmacognosy, Shenyang Pharmaceutical University.

Extraction and isolation

The air-dried leaves of *I. indigotica* (50 kg) were extracted twice with 80% EtOH (50 L × 2 h) and filtered to obtain a crude methanol extract (4.28 kg). 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 fractionated by a gradient system of CH_2Cl_2/MeOH (100 : 1−3 : 1, *V/V*) to obtain four fractions (Fr. 1−4). Then, Fr. 3 (289.3 g) was processed on a polyamide column with H_2O, 30% EtOH−H_2O, 60% EtOH−H_2O and 90% EtOH−H_2O to afford Fr. A−C. Fr. A (88.9 g) was processed on a HP-20 macroporous resin eluting with EtOH/H_2O (from 0 : 100 to 90 : 10, *V/V*) to yield three fractions (Fr. A1−A3). Subsequently, Fr. A2 (22.8 g) was processed on an ODS column with EtOH/H_2O (from 10 : 90 to 90 : 0, *V/V*) to yield fractions Fr. A2.1−A2.2, respectively. Fr. A2.1 (17.5 g) was further chromatographed on a silica gel column with PE/EtOAc (from 50 : 1 to 1 : 1, *V/V*) to yield fractions, which were purified by preparative and semi-preparative HPLC (CH_3OH/H_2O 20 : 80, *V/V*, flow rate 2.0 mL·min^{-1}, detection wavelength UV 210 nm) to afford compounds **1** (3.3 mg, t_R 26 min), **2** (2.6 mg, t_R 35 min), **3** (14.5 mg, t_R 46 min), **4** (9.8 mg, t_R 62 min), **5** (34.2 mg, t_R 79 min), and **6** (4.5 mg, t_R 92 min). Chiral resolution of **1** was performed on a Daicel Chiralpak AD-H column (eluted with *n*-hexane/isopropanol, 3 : 1, *V/V*, flow rate 0.3 mL·min^{-1}, detection wavelength UV 210 nm) to obtain **1a** (1.9 mg, t_R 16.5 min) and **1b** (1.4 mg, t_R 21.2 min), respectively.

Isatinoline A (**1**): Yellow oil. $[\alpha]_D^{20}$ −1.5 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 206.0 (3.46), 225.0 (3.82) nm; ^{1}H and ^{13}C NMR data see Tables 1−2; HR-ESIMS: *m/z* 227.1176 $[M + H]^+$ (Calcd. for $C_{14}H_{15}N_2O$, 227.1179).

(−)-(5′s)-Isatinoline A (**1a**) $[\alpha]_D^{20}$ −32.0 (*c* 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 258 (+16.21), 319 (−30.44) nm.

(+)-(5′R)-Isatinoline A (**1b**) $[\alpha]_D^{20}$ +30.3 (*c* 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 257 (−13.05), 318 (+24.40) nm.

Isatinoline B (**2**): Yellow oil. UV (MeOH) λ_{max} (log ε) 203.0 (3.71), 244.0 (3.31) nm; ^{1}H and ^{13}C NMR data see Tables 1−2; HR-ESIMS: *m/z* 221.0836 $[M + Na]^+$ (Calcd. for $C_{12}H_{10}N_2ONa$, 221.0840).

Isatinoline C (**3**): Yellow needle crystal. $[\alpha]_D^{20}$ +39.4 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 206.0 (3.82), 236.0 (3.72), 276.0 (3.57) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 253 (−3.67), 284 (+4.32) nm; ^{1}H and ^{13}C NMR data see Tables 1−2; HR-ESIMS: *m/z* 284.0533 $[M + Na]^+$ (Calcd. for $C_{13}H_{11}NO_5Na$, 284.0529).

Isatinoline D (**5**): Orange needle crystal. $[\alpha]_D^{20}$ +55.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 280.0 (3.87) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 250 (−8.72) nm; ^{1}H and ^{13}C NMR data see Tables 1−2; HR-ESIMS: *m/z* 280.0595 $[M + Na]^+$ (Calcd. for $C_{14}H_{11}NO_4Na$, 280.0580).

ECD calculations

Conformational analysis of compounds **1**, **3** and **5** 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/6-31 G(d) level with an energy window of 10 kcal·mol^{-1} on the Gaussian 09 program package [19]. Then, the theoretical ECD calculations of the conformations of compound **3** were performed by the TDDFT method at the B3LYP/6-311++ G(2d, p) level with the CPCM model in methanol solution and the conformations of compounds **1** and **5** were performed by the TDDFT method at the B3LYP/6-31 G(d, p) level with the CPCM model in methanol solution. Finally, the calculated ECD curve was generated by SpecDis 1.51 [20].

Cell culture

Human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and 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.

Effects of these compounds on cell viability

The effects of all isolated compounds toward SH-SY5Y cells injured by H_2O_2 (200 μmol·L^{-1}) were examined according to previoius procedures [21].

Annexin V-FITC/PI staining

Annexin V-FITC and PI apoptosis kits were applied to

evaluate the apoptotic ratio of the cells [19]. The treated cells were stained with Annexin V-FITC followed by PI at room temperature for 15 min. The samples were then analyzed using a flow cytometer (Becton Dickinson, Franklin Lakes, USA) and quantified with Flow Jo 7.6.1 (Oregon, USA).

Reactive oxygen species (ROS) assay

The intracellular ROS levels were evaluated using the ROS-specific fluorescent dye H_2DCF-DA. After indicated treatment, the cells were washed with PBS for three times and stained with H_2DCF-DA, incubated at 37 °C for 30 min in the darkness. Furthermore, the cells were harvested and the generation of ROS was quantified through measurement of the intracellular florescent intensity by flow cytometry. The samples were then analyzed with Flow Jo 7.6.1.

Measurement of intracellular SOD, CAT and GSH-Px activities

The intracellular SOD, CAT and GSH-Px activities of **1a** and **1b** toward SH-SY5Y cells were examined, according to previoius procedures[21].

Statistical analysis

All the data were measured 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.

Conclusions

In conclusion, seven alkaloids including five undescribed ones were isolated from the leaves of *I. indigotica*. Their planar structures and the absolute configurations of compounds **1a**, **1b**, **3** and **5** were determined by extensive NMR, HR-ESIMS analysis as well as the comparison of their experimental and calculated ECD spectra. Subsequently, the neuroprotective effects of all the isolates against H_2O_2-induced injury in SH-SY5Y cells were evaluated *in vitro* by MTT assay. Compounds **1a** and **1b** showed better neuroprotective effects. Moreover, the Annexin V-FITC/PI double staining and the activities of antioxidant enzymes (SOD, CAT and GSH-Px) demonstrated that **1a** and **1b** exhibited neuroprotective activities.

Supporting Information

NMR, HR-ESIMS, UV, ECD spectrum, the chiral HPLC chromatograms and further detailed experimental information are available as Supporting Information, and can be requested by sending E-mail to the corresponding authors.

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