Fitoterapia 137 (2019) 104240

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Neuroprotective effect of catechins derivatives isolated from Anhua dark tea on NMDA-induced excitotoxicity in SH-SY5Y cells

Jianyu Liu[a,c,1], Yanhua Fan[b,1], Donghwa Kim[d], Ting Zhong[b], Ping Yi[b], Chengcheng Fan[a,c], Andong Wang[a,c], Xiaosheng Yang[b], Sangkook Lee[d], Xuhong Ren[a,c,*], Yongnan Xu[a,c,*]

[a] Key Laboratory of Structure-Based Drug Design and Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China
[b] State Key Laboratory for Functions and Applications of Medicinal Plants, Guizhou Medical University, Guiyang 550014, China
[c] School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang 110016, China
[d] College of Pharmacy, Natural Products Research Institute, Seoul National University, Seoul 08826, Republic of Korea

ARTICLE INFO

Keywords:
Anhua dark tea
Catechin derivatives
NMDA receptors
Neuroprotective effects

ABSTRACT

Anhua dark tea known as the earliest produced Chinese dark tea, has been commercially available and famous for its unique flavor and health care effect. NMDA receptors are glutamate-coupled ion channels that critically involved in survival of neuronal cells and neurodegenerative diseases. Thus, it is considered a promising target for the therapy of neurodegenerative disease. In this study, four catechins including two new catechins derivatives (**1**–**2**), together with thirteen known flavonoids were isolated from Anhua dark tea. The structures of compounds **1**–**2** [2*S*,3*R*-6-methoxycarbonylgallocatechin (**1**) and 2*R*,3*R*-6-methoxycarbonylgallocatechin (**2**)] were determined on the basis of their spectroscopic data. The preliminary bioassay indicated that compound **1** showed the best neuroprotective effects via *N*-methyl-D-aspartate (NMDA) receptors inhibition. Compound **1** protected SH-SY5Y cells against NMDA-induced injury and cell apoptosis via the modulation of NR2B expression, the activation of PI3K/Akt signaling and caspase-dependent pathway. The results suggested compound **1** would be a potent dietary therapy reagent for prevention of excitable brain injury.

1. Introduction

Chinese dark tea (CDT) is a kind of full-fermented tea product based on mature tea leaves treated by microbial fermentation [1]. CDTs are famous for its unique flavor and characteristic reddish-black colour. According to the different producing areas and the processing technologies, CDTs is divided into Pu-erh tea (Yunnan), Anhua dark tea (Hunan), Qing-zhuan tea (Hubei), Bian-xiao tea (Sichuan) and Liu-bao tea (Guangxi) [2]. Anhua dark tea, known as the earliest produced CDT dating back to the Ming Dynasty in Hunan province of China, is very popular for its unique flavor and health care effects [3]. In addition, it also has a wide range of bioactivities such as antioxidant, anticancer, prevention of cardiovascular diseases, neuroprotective and anti-hypertensive effects, etc. [3]. The main components of tea such as catechin and its derivatives were usually altered owing to the microbial fermentation. Because of different manufacturing processes, catechins might degrade or polymerize into different compounds in different kinds of tea. For example, catechins formed theaflavins during full fermentation in black tea. Dimeric flavan-3-ol oolonghomobisflavans were isolated from oolong tea based on semi-fermentation [4]. In contrast, owing to the unique complex estrogenic microbial fermentation process after being dried and rolled, it has been reported that there are unique constituents in CDT. During the microbial post-fermentation in Fuzhuan brick tea, catechin derivatives were degraded to several kind of B-ring fusion derivatives [5–7], while in Pu-erh tea, some A-ring derivatives were isolated [1,8,9] and this indicated that different components were produced during post-fermentation of different kind of dark tea.

N-methyl-D-aspartate (NMDA) receptors are glutamate-coupled ion channels that critically involved in learning and memory and are promising targets of rapid acting antidepressants [10]. NMDA receptors are mainly composed of two subunits including GluN1 and GluN2. Dysfunction of NMDA receptors are related to various neurological diseases and disorders such as stroke, Alzheimer's disease and depression [11]. Therefore, NMDA receptor has been considered as an important pharmacological target of neurological diseases and disorders.

* Corresponding authors at: Key Laboratory of Structure-Based Drug Design and Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China.
E-mail addresses: renxuhong2008@163.com (X. Ren), ynxu@syphu.edu.cn (Y. Xu).
[1] These authors contributed equally to this work and shared first authorship.

https://doi.org/10.1016/j.fitote.2019.104240
Received 8 May 2019; Received in revised form 7 June 2019
Available online 12 June 2019
0367-326X/

N-methyl-D-aspartate (NMDA) can increase calcium influx and activate NMDA receptors, which is similar to pathologic events in cerebral ischemia [12]. Therefore, the NMDA-induced SH-SY5Y injury model was widely accepted as activity screening model for the neuroprotective effects of NMDA receptors inhibitor. The crystal structures of GluN1-GluN2B NMDA receptors bound to an allosteric inhibitor, ifenprodil (selective for GluN2B-containing receptors), has been identified recently [13,14]. Ifenprodil decreased NMDA receptor GluN2B levels and showed neuroprotective effects [15].

The activation of NMDA receptors requires the glutamate binding subunit of NR2. This activation requires opening of the bi-lobed architecture of the GluN2B ATD (an amino-terminal domain) and rotated GluN1B-GluN2B heterodimeric causing dilation of the gating ring (the ligand binding domain, LDB) [16]. As GluN2B plays critical role in the activation of NMDA, several NMDA antagonists targeting NR2B was developed as neuroprotective agents such as ifenprodil, haloperidol and eliprodil [17–19].

In this study, two new carboxyl-catechins named 2*S*,3*R*-6-methoxycarbonylgallocatechin (**1**), 2*R*,3*R*-6-methoxycarbonylgallocatechin (**2**) together with thirteen known catechins and flavonoids were isolated from Anhua dark tea (Fig. 1). The neuroprotective activity of these compounds in NMDA induced excitotoxic trauma model was tested and the results indicated most of the compounds showed considerable neuroprotective activity, thus the underlying mechanism of the most active compound **1**was further investigated.

2. Results

Compound **1** was obtained as a yellow amorphous powder. The molecular formula $C_{17}H_{16}O_9$ was established on the basis of HR-ESI-MS at *m/z* 363.0751 $[M-H]^-$ (calcd for $C_{17}H_{15}O_9$, 363.0716). The ^1H NMR (CD_3OD, 400 MHz) showed the present of two symmetric aromatic protons at δ_H 6.35 (2H, s, H-2′, H-6′) and their associated ^{13}C resonances assigned from HSQC experiment were at 105.4, along with the other four aromatic carbons at δ_C 129.4, 132.7, and 145.6 × 2, respectively, indicated that there was a part of galloyl group (no carbonyl group in it). Another aromatic proton was at δ_H 5.96 (1H, s) and three groups of aliphatic protons at δ_H 2.55 (1H, dd, J = 6.8, 16.0 Hz), 2.75 (1H, dd, J = 4.8, 16.4 Hz), 4.03 (1H, m), and 4.72 (1H, d, J = 6.4 Hz). The above evidences indicated the presence of a C-pyran ring of catechin characteristic structural fragment (flavan-3-ol) in **1**. The ^{13}C NMR (CD_3OD, 100 MHz) also showed an ester carbonyl carbon signal at

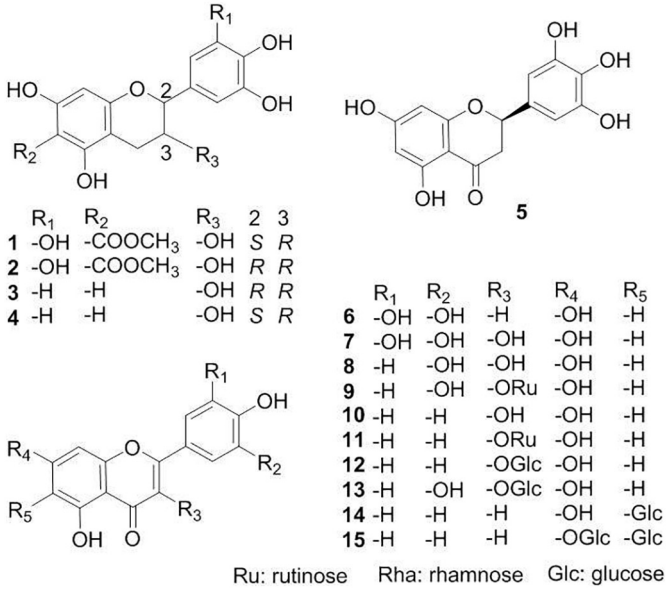

Fig. 1. Structures of compound **1–15.**

δ_C 170.3 along with methyl signal at δ_C 51.5, which should be substituted at C-6 or C-8 of the A-ring.

The position of substitution was further supported by HMBC experiments (Fig. 2A). The signals of C-5 and C-9 can be assigned based on the correlations from δ_H 4.72 (1H, d, J = 6.4 Hz, H-2) to δ_C 160.2 (C-9), and from δ_H 2.55 (1H,dd, J = 6.8, 16.0 Hz, H-4a), and 2.75 (1H, dd, J = 4.8, 16.4 Hz, H-4b) to δ_C 159.7 (C-5) and 160.2 (C-9). Thus, an unresolved signal at δ_C 160.7 must be arisen from C-7. The correlations from δ_H 5.96 (1H, s) to δ_C 160.2 (C-9), indicated the substitution was located on the C-6 position.

The large coupling constant (J = 6.4 Hz) between H-2 and H-3 indicated a 2,3-*trans* configuration [9]. Further comparison the [α]20 Dof **1** [−29°, (*c* 1.0, MeOH)] with those of (+)-gallocatechin [[α]20 D + 150°, (*c* 0.1, MeOH)] and (−)-gallocatechin [[α]24 D − 11.2°, (c 0.1, MeOH)] revealed the 2*S*, 3*R* absolute configurations in **1**. The absolute configuration can be further confirmed by the CD spectrum, in which compound **1** gave a high-amplitude negative Cotton effect (CE) near 240 nm, corresponding to the ^1L_a electronic transition of the flavan-3-ols [20], consistent with 3*R*-configuration. Thus, the absolute configuration was determined to be 2*S*, 3*R*-configuration, which was further confirmed by comparison of the experimental ECD spectrum with those calculated (Fig. 3A). Compared with the literature [9], the structure of **1** was considered to be methyl ester of 6-carboxylgallocatechin. Based on the above evidence, the structure of **1** was elucidated as 2*S*, 3*R*-6-methoxycarbonylgallocatechin.

Compound **2** was obtained as a yellow amorphous powder, possessing a molecular formula of $C_{17}H_{16}O_9$, as deduced from the HR-ESI-MS measurement at *m/z* 363.0830 $[M-H]^-$ (calcd for $C_{17}H_{15}O_9$, 363.0716). The ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) spectra of **2** (Table 1) were closely resembled to those of **1**, and only slightly differences present at the region of saturated carbon (sp^3) and their associated ^1H resonances. Remarkably, in the ^1H NMR spectrum, the signal of H-2 located at δ_H 4.89 which was partly covered by the residual water signal (δ_H 4.90). To check out the coupling constant of H-2 and H-3, a drop of deuterium water (D_2O) was added, and the residual water signal shift to high field at δ_H 4.79 and the signal of H-2 can be recognized easily (Fig. 2B). The small coupling constant (much like singlet) between H-2 and H-3 in **2** indicated 2,3-*cis* configuration. Further comparison the [α]20 Dof **2** [−30.5°, (*c* 1.0, MeOH)] with (−)-epigallocatechin [[α]24 D − 50°, (c 0.1, EtOH)] revealed the 2*R*,3*R* absolute configurations in **2**. Furthermore, **2** gave a high-amplitude negative CE near 240 nm, consistent with 3R-configuration. Thus, the absolute configuration was determined to be 2*R*, 3*R*-configuration, and it was further confirmed by comparing with calculated ECD spectrum (Fig. 3B). Compared with the structure of **1**, compound **2** was confirmed as its optical isomer, and the structure of **2** was elucidated as 2*R*, 3*R*-6-methoxycarbonylgallocatechin.

Compounds **1–2** have additional methoxycarbonyl group in comparison with gallocatechin and epigallocatechin, respectively. And these compounds were presumed to be formed through enzymatic Kolbe-Schmitt reaction [21] and methylation [22] from catechin derivatives which are considered as main components of green tea (Fig. 4). It has been reported that during the microbial post-fermentation in Fuzhuan brick tea, catechin derivatives were degraded to several kind of B-ring fission derivatives [5–7], while in Pu-erh tea, some A-ring derivatives were isolated [9,23] and this indicated that different components were produced during post-fermentation of different kind of dark tea. In our study, two A-ring methoxycarbonyl group substitute derivatives of catechins were isolated and this evidence indicated that similar process might also occur in post-fermentation of Anhua dark tea and Pu-erh tea.

To determine the proper concentration of NMDA to establish the excitatory injury model, SH-SY5Y cells were exposed to various concentrations of NMDA. As shown in Fig. 5B, the NMDA treatment for 30 min led to significant decrease of cell viability compared to the control group. The cell viability of SH-SY5Y cells treated with 2 mM

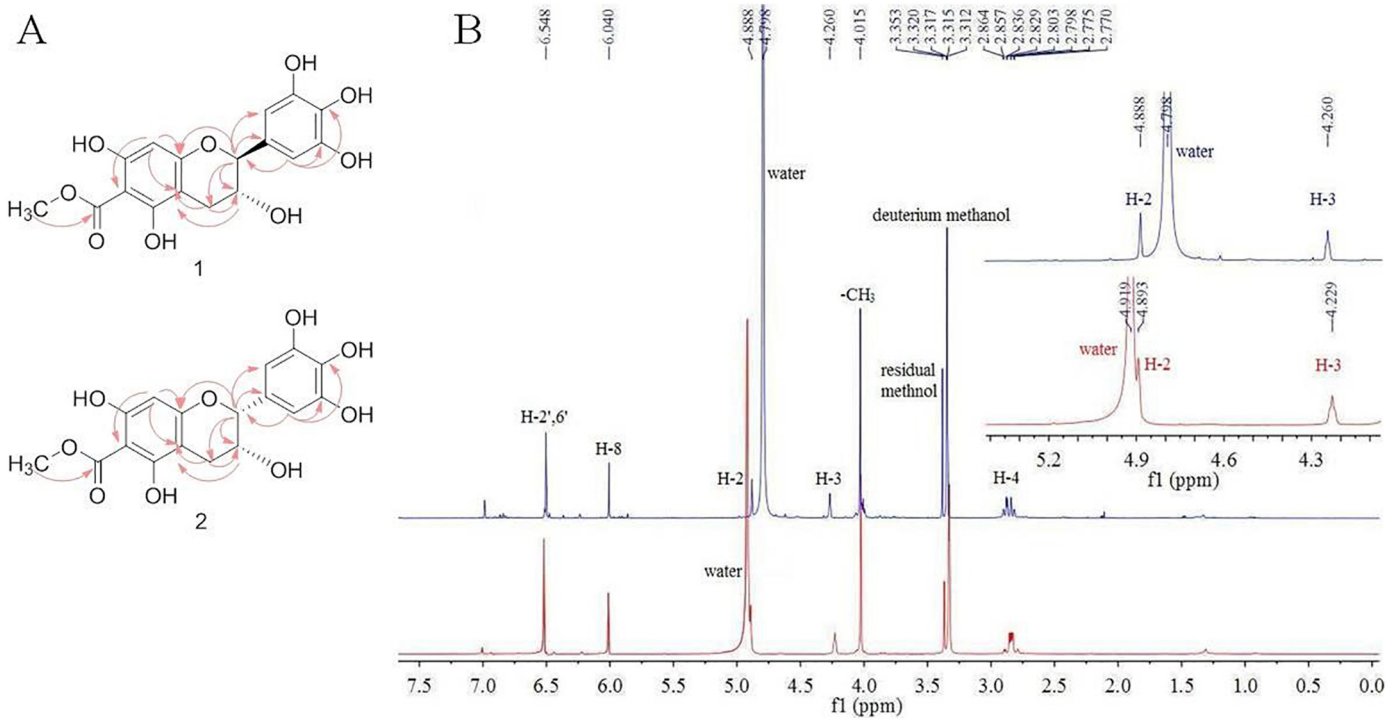

Fig. 2. Related spectrum data of compound **1** and **2**. (A) Key HMBC correlations of compound **1** and **2**. (B) Overlaid ^{1}H NMR of compound **2.** The NMR measurement was performed with CD_3OD and the signal of H-2 was partly covered by residual water signal (lower one in red), while after added one drop of D_2O, the signal can be easily detected (upper one in blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NMDA for 0.5 h decreases to 49.51%. Hence, 2 mM NMDA was chosen for subsequent experiments. We further tested the activity of neuroprotective effects of compound **1–15** using NMDA induced cell injury model (Table 2). Among the isolated compounds, pretreatment with 20 μM compound **1** and **2** can effectively protect the SH-SY5Y cells against neurotoxicity induced by NMDA with neuroprotective rate of 85.26% and 77.10% compared to NMDA treated group (Table 2 **and** Fig. 5D). Compound **1** showed no obvious cytotoxicity at the concentration below 20 μM (Fig. 5C). Lactate dehydrogenase (LDH) is a cytosolic enzyme that is an indicator of cellular toxicity. Pretreatment with compound **1** remarkably reduced the LDH release compared with the NMDA-treated group (Fig. 5E). Based on these results, we presumed that compound **1** might show neuroprotective activity via counteracting the NMDA-induced excitatory damage.

NR2B have received considerable attention in recent years because of its critical role in the activation of NMDA. Numerous neuroprotective agents including ifenprodil, haloperidol and eliprodil selectively targeting NR2B have been developed. As shown in Fig. 6A**,** NMDA treatment for 30 min significantly increase NR2B, while pretreatment with compound **1** inhibited the expression of NR2B (Fig. 6B). We further performed molecular docking study to investigate the potent interaction model of compound **1** and NMDARs (Fig. 7). Like ifenprodil, an NR2B specific inhibitor, compound **1** formed two hydrogen bonds with the amino acid residues Gln110 and Glu236, which was proposed to be the key interaction to the anchoring of the binding pocket. There is an additional hydrogen-bond interaction with Glu106 between NR2B and compound **1**. In addition, there are also hydrophobic interactions with the hydrophobic residues including Ala107, Thr174, Tyr175, Phe176,

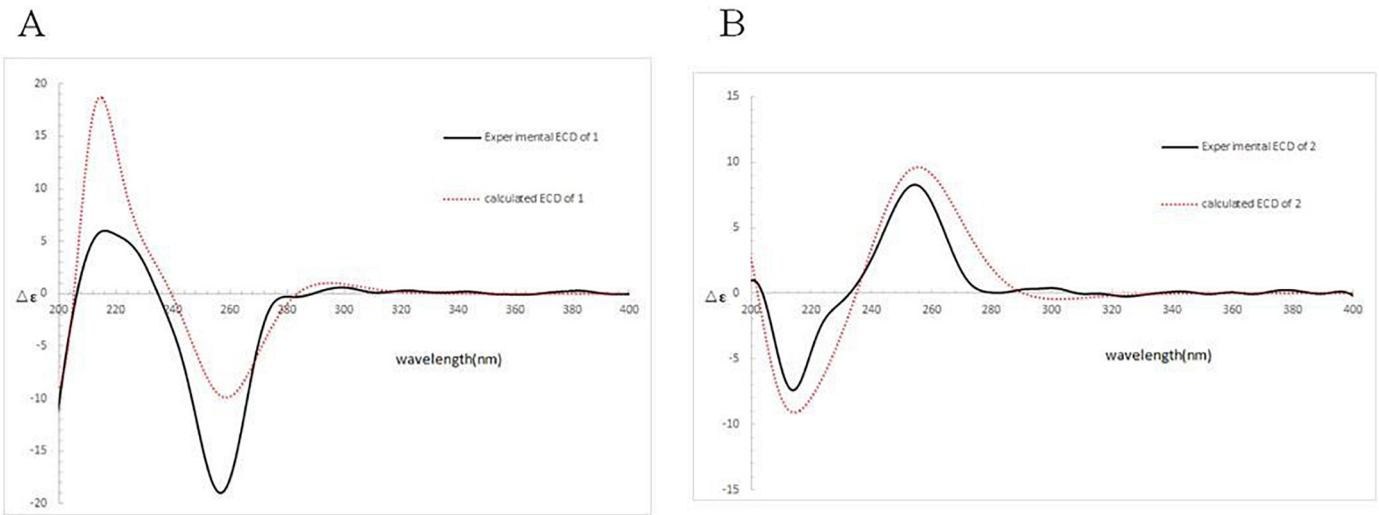

Fig. 3. Experimental ECD and calculated ECD of compounds **1** and **2.**

Table 1
NMR data of compound **1** and **2** (CD_3OD, 400 MHz).

1			**2**	
No.	δ_H	δ_C	δ_H*	δ_C
1	–	–	–	–
2	4.72(1H,d,J = 6.4 Hz)	81.9	4.89(1H,s)	79.2
3	4.03(1H,m)	66.6	4.26(1H,m)	65.4
4	2.55(1H,dd,J = 6.8,16.0 Hz) 2.75(1H,dd,J = 4.8,16.4 Hz)	25.5	2.79(1H,dd,J = 2.8,11.2 Hz) 2.85(1H,dd,J = 2.0,11.2 Hz)	27.3
5	–	159.7	–	159.6
6	–	93.6	–	93.6
7	–	160.7	–	161.4
8	5.96(1H,s)	95.1	6.04(1H,s)	95.4
9	–	160.2	–	160.6
10	–	99.9	–	99.4
1′	–	129.4	–	129.2
2′,6′	6.35(2H,s)	105.4	6.55(2H,s)	105.5
3′,5′	–	145.6	–	145.4
4′	–	132.7	–	132.7
-OCH_3	3.99(3H,s)	51.5	4.02(3H,s)	51.5
-C=O	–	170.3	–	170.4

* ^1H NMR data of compound **2** was detected with CD_3OD added one drop of D_2O.

Pro177 and Thr233. The results indicated that compound **1** exhibited neuroprotective effects associated with NR2B inhibition.

Since ERK and Akt played important roles in the cell survive and proliferation, we further tested the effects of compound **1** on the activation of ERK1/2 and Akt in response to the NMDA treatment. As shown in Fig. 8, treatment with 2 mM NMDA led to significant decrease in the protein levels of p-ERK1/2 and p-Akt (Ser473). While, pretreatment with compound **1** for 12 h remarkably increased the p-Akt and p-ERK1/2 after NMDA treatment compared to the untreated group.

When tested the cytotoxicity of NMDA in SH-SY5Y cells, we found cell apoptosis occurred in 40–50% cells treated with 2 mM NMDA for 30 min. Therefore, we wonder whether compound **1** exhibited the neuroprotective activity involvement in anti-apoptosis. We evaluated the effects of compound **1** on cell apoptosis induced by NMDA treatment using Hoechst 33342 staining and Annexin V-PI double staining. Pretreatment with compound **1** decreased the cell apoptosis induced by NMDA (Fig. 9). Pretreatment with compound **1** significantly decreased DNA condensation and nuclear fragmentation caused by NMDA

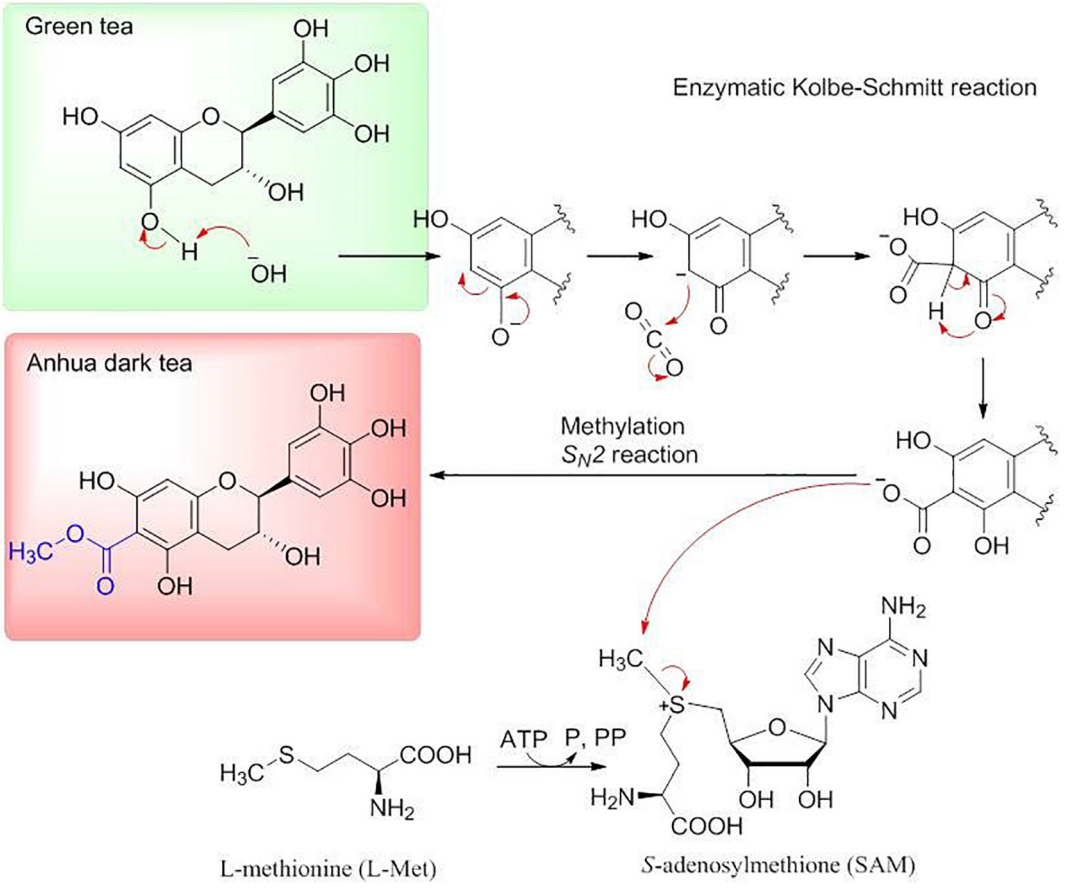

Fig. 4. Presumed mechanism of **1** formed through enzymatic Kolbe-Schmitt reaction and methylation from catechin by the microorganisms.

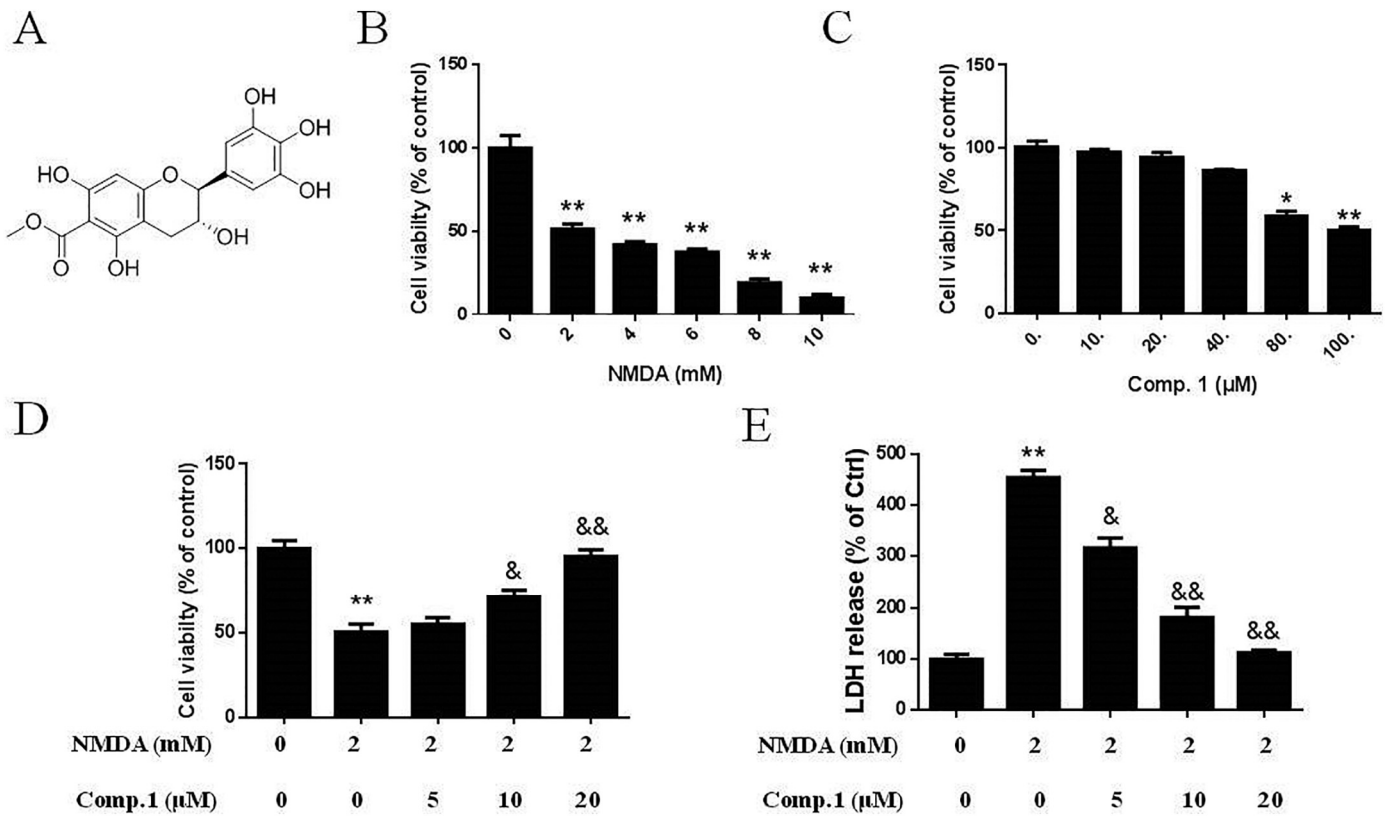

Fig. 5. Protective effects of Compound **1** against NMDA-induced SH-SY5Y cells. (A) Structure of Compound **1**. (B) The effects of Compound **1** on cell viability of SH-SY5Y cell. SH-SY5Y cells were treated with NMDA at the indicated concentrations. (C) Protective activity of Compound **1** in NMDA-treated SH-SY5Y cells. (D) Compound **1** reduced LDH release induced by NMDA treatment. Data were presented as means ± SD ($n = 3$). &$p < .05$ and &&$p < .01$ compared with NMDA-treated. *$p < .05$ and **$p < .01$ compared with control group.

Table 2
Protective effects of compounds 1–15 isolated from Anhua dark tea against NMDA-induced neurotoxicity in SH-SY5Y cells.

Compounds	Neuroprotective activity (%)			Compounds	Neuroprotective activity (%)		
	10	20	40		10	20	40
1	45.68 ± 1.68	85.26 ± 2.57	80.13 ± 3.79	9	2.53 ± 0.09	8.05 ± 1.29	20.68 ± 1.19
2	38.18 ± 0.94	77.10 ± 1.69	72.28 ± 2.96	10	3.59 ± 0.16	19.05 ± 0.28	42.81 ± 1.29
3	29.03 ± 0.75	45.49 ± 1.25	49.34 ± 1.28	11	0.73 ± 0.37	8.04 ± 0.95	10.68 ± 1.08
4	4.57 ± 0.15	18.23 ± 0.97	45.06 ± 1.37	12	−0.81 ± 0.49	5.82 ± 0.53	10.25 ± 1.23
5	3.95 ± 0.16	19.48 ± 0.79	39.50 ± 0.97	13	2.11 ± 0.76	10.45 ± 0.67	16.53 ± 1.37
6	1.35 ± 0.09	15.86 ± 0.73	28.13 ± 0.64	14	−1.37 ± 0.86	0.21 ± 0.08	8.95 ± 0.45
7	23.18 ± 1.07	34.71 ± 1.89	51.18 ± 2.37	15	1.28 ± 0.48	2.38 ± 0.79	3.59 ± 0.42
8	2.56 ± 0.29	15.03 ± 1.39	37.84 ± 2.76				

treatment (Fig. 9A-9B). As shown in Fig. 9C, Annexin V-PI staining showed 20 μM compound **1** reduced the percentage of total apoptotic cells from 27.88% (NMDA treated group) to 2.65% (compound **1** pre-treated group). In addition, NMDA treatment increased ROS generation, while pretreatment with compound **1** could abolish the increase in ROS in NMDA-treated SH-SY5Y cells (Fig. 10A). Western blot analysis indicated that compound **1** significantly decreased the ratio of Bax/Bcl2 (Fig. 10B). In addition, the expression of cleaved-PARP and cleaved-cas3 was also decreased by the pretreatment with compound **1** (Fig. 10C).

In summary, four catechins including two new catechins derivatives (**1–2**) were isolated from Anhua dark tea. We examined the neuroprotective activity of these compounds and found that Compound **1** protected SH-SY5Y cells against NMDA-induced injury and cell apoptosis via modulation of NR2B expression. Compound **1** also increase the cell viability via regulation of ERK and Akt and inhibit the cell apoptosis by regulation of apoptotic related proteins including Bax, Bcl-2, cleaved caspase 3 and 9.

3. Materials and methods

3.1. Regents and chemicals

Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (Logan, UT, USA) MTT and NMDA (*N*-methyl-D-aspartate) were purchased from sigma-aldrich (Saint Louis, MO, USA). The primary antibodies against cleaved-caspase3, p-Akt, Akt, p-ERK1/2 and ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Bax and Bcl-2 protein antibodies were purchased from Abcam (MA, USA). Hoechst33342 were obtained from Sigma-Chemical (St. Louis, MO, USA). All other reagents and chemicals are analytical grade and obtained from Energy Chemicals (Shanghai, China).

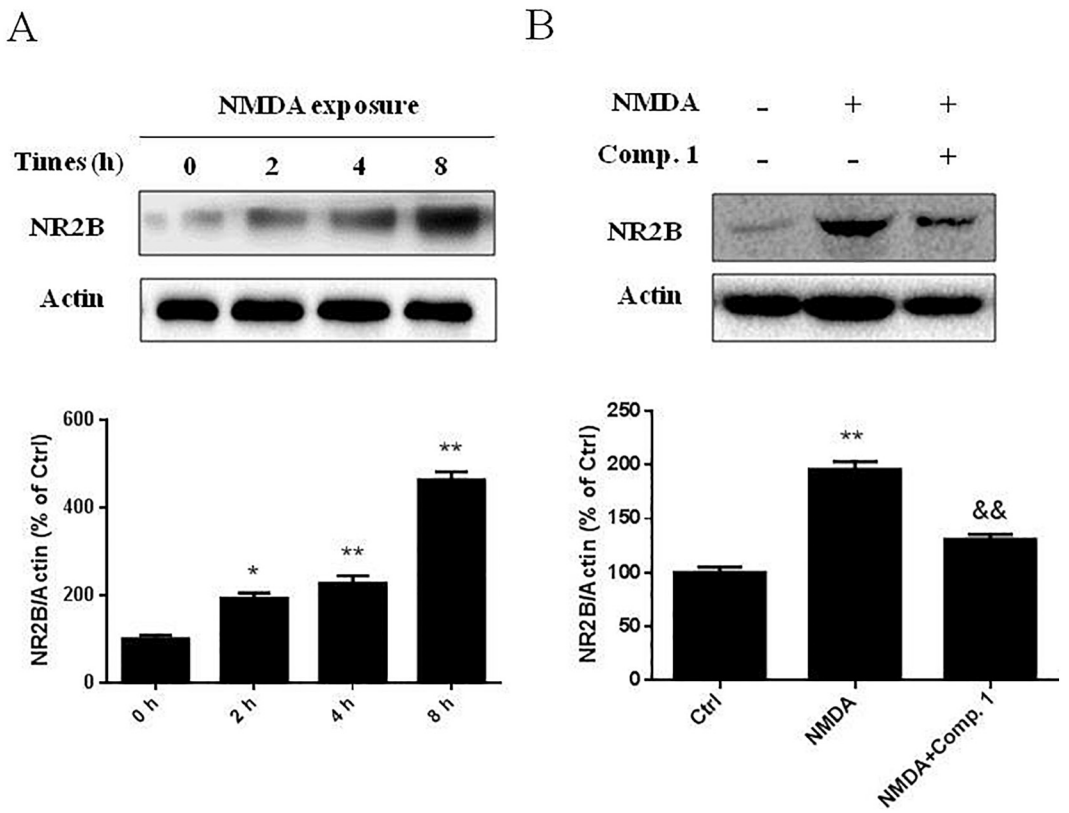

Fig. 6. Effects of Compound **1** on NR2B expression. (A) NMDA treatment time-dependently increased the protein levels of NR2B. (B) Pretreatment with compound 1 reduced the NR2B increased by NMDA treatment. Data are presented as means ± S.D. ($n = 3$). $\#p < .05$ or $\#\#p < .01$ vs. the NMDA-treated group. *p < .05 or **p < .01 vs. the control group.

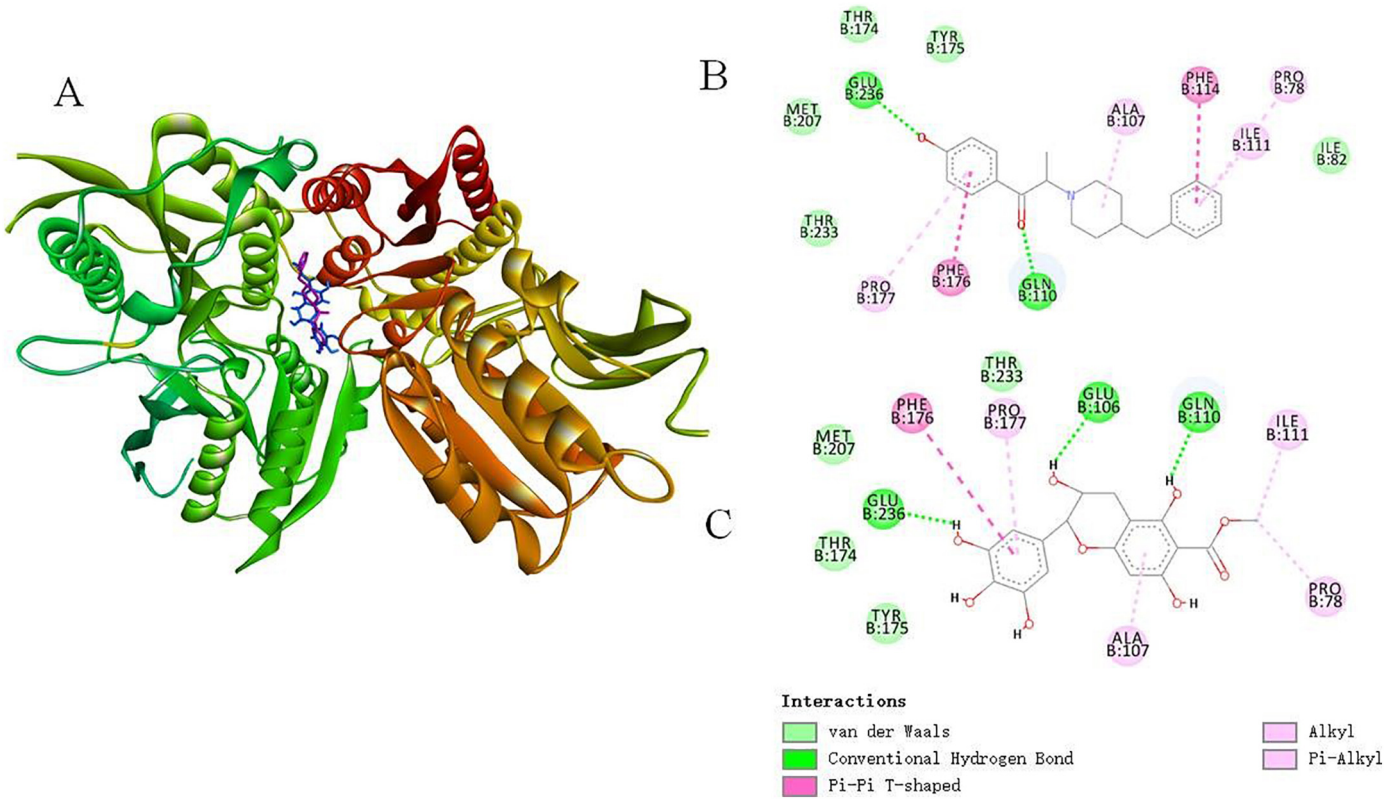

Fig. 7. The binding mode of compound1 and NR2B in the active site of NMDA. (A) Compound 1 and ifendil in the active site of NMDA. (B) Interactions of the cocrystallized ligand of NMDA and NR2B. (C) Interactions between compound 1 and NR2B. Autodock 4.0 was used to perform this docking study.

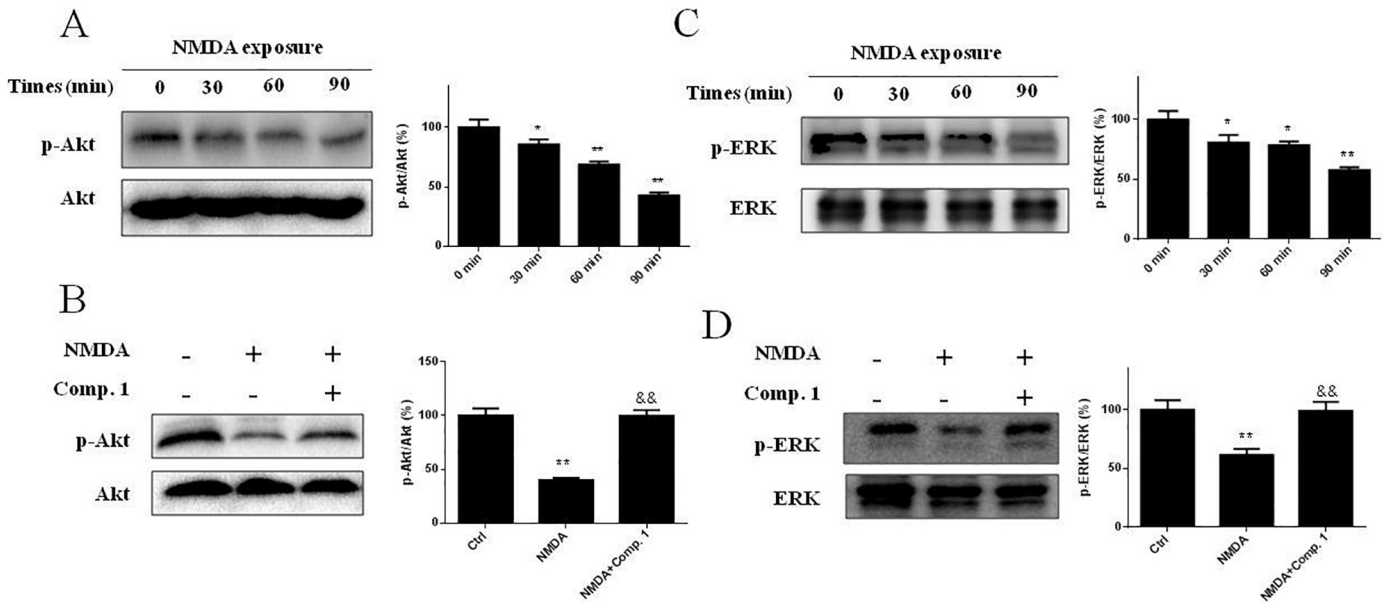

Fig. 8. Effects of compound **1** on the expression of p-ERK1/2 and p-Akt in NMDA treated SH-SY5Y cells. (A) The alteration of p-Akt after exposure to 2 mM NMDA for 0.5 h. (B) Compound **1** pretreatment significantly restored the p-Akt inhibited by NMDA in SH-SY5Y cells. (C) The alteration of p-ERK after exposure to 2 mM NMDA for 0.5 h. (D) Compound **1** pretreatment increased p-ERK in NMDA treated SH-SY5Y cells. Data are presented as means ± S.D. (n = 3). #p < .05 or ##p < .01 vs. the NMDA-treated group. *p < .05 or **p < .01 vs. the control group.

3.2. Plant material

Anhua dark tea, produced from the crude green tea prepared with leaves of *Camellia sinensis* var. *assamica*, was provided by Hunan Xiao Hei Shen Development Co., Ltd., in 2015. The plant herbarium specimen was collected, authenticated and deposited in Shenyang Pharmaceutical University.

3.3. Isolation of compounds 1–15

The crude dark tea leaves (4 kg) were extracted three times for 3 h with 60% EtOH under reflux. The extracts were combined and concentrated under a vacuum to yield a residue (1045 g), which was then suspended in water and partitioned sequentially with petroleum ether, CH_2Cl_2, EtOAc and *n*-BuOH. The EtOAc extract (245 g) was chromatographed on a silica gel column and eluted with CH_2Cl_2-MeOH (100:1–0:1 *v*/v) to give thirteen fractions (Fr. A1–13). Fr. A9 was separated on a silica gel column and eluted with CH_2Cl_2-MeOH (100:3) to give two sub fractions (sub Fr. A9a and A9b). A9a was then purified on preparative HPLC (MeOH-H_2O, 10:1 *v*/v) to afford **7** (myricetin, 5 mg). A9b was subjected to preparative HPLC (MeOH-H_2O, 8:1 v/v) to afford **5** (dihydrotricetin, 3 mg) and **6** (tricetin, 6 mg). Fr. A11 was separated on a silica gel column and eluted with CH_2Cl_2-MeOH (50:1) to give two sub fractions (sub Fr. A11a and A11b). A11a was performed on preparative HPLC (MeOH-MeCN-H_2O, 1:1:2 *v*/*v*/v) to afford **1** (2S, 3R-6-methoxycarbonylgallocatechin, 20 mg) and **2** (2R, 3R-6-methoxycarbonylgallocatechin, 2 mg). Fr. A12 was separated on sephadex LH-20 column and eluted with CH_2Cl_2-MeOH (10:1) to give **3** [(−)-epicatechin, 30 mg] and one sub fraction A12a. A12a then was isolated on preparative HPLC (MeOH-MeCN-H_2O, 3:2:1 *v*/v/v) to afford **9** (rutin, 21 mg). Fr. A13 was performed on a silica gel column and eluted with CH_2Cl_2-MeOH (30:1) and further purified on preparative HPLC (MeOH-MeCN-H_2O, 3:2:2 *v*/v/v) to afford **8** (quercetin, 10 mg). The *n*-BuOH extract (219 g) was chromatographed on a silica gel column and eluted with CH_2Cl_2-MeOH-H_2O (100:1:0–0:3:2 *v*/v/v) to give three fractions (Fr. B1–5). Fr. B2 was separated on a silica gel column and eluted with CH_2Cl_2-MeOH-H_2O (9:1:0.05) to give two sub fractions (sub Fr. B2a and B2b). B2a was then separated with preparative HPLC (MeOH-MeCN-H_2O, 3:2:1 v/v/v) to afford **4** [(+)-catechin, 12 mg]. B2b was further purified by preparative HPLC (MeOH-H_2O, 3:2 v/v) to afford **10** (kaempferol, 10 mg). Fr. B3 was separated on a silica gel column and eluted with CH_2Cl_2-MeOH-H_2O (6:1:0.05), and further isolated using preparative HPLC (MeOH-MeCN-H_2O, 3:2:3 v/v/v) to afford **11** (kaempferol 3-O-*a*-L-rhamnopyranosyl(l-6)-*β*-D-glucopyranoside, 12 mg) and **13** (isoquercitrin, 9 mg). Fr. B4 was separated on a silica gel column and eluted with CH_2Cl_2-MeOH-H_2O (6:4:0.9) to give two sub fractions (sub Fr. B4a and B4b). B4a was further purified by preparative HPLC (MeOH-MeCN-H_2O, 3:2:5 v/v/v) to afford **12** (astragalin, 3 mg). B4b was then isolated by preparative HPLC (MeOH-MeCN-H_2O, 3:2:5 v/v/v) to afford **14** (isovitexin, 7 mg) and **15** (saponarin, 9 mg).

3.4. Cell culture

The human SH-SY5Y neuroblastoma cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

3.5. Cell viability assay

MTT assay was employed to test the neuroprotective effect of compound **1–3** on NMDA induced cell injury. Briefly, 1×10^4 cells were plated into 96-well plates and cultured for 12 h before exposure to 2 mM NMDA for 0.5 h to establish the cell injury model. Cells were pretreated with various concentrations of compound **1–3** for 12 h. Treated cells were cultured with fresh culture medium containing 10% FBS for another 24 h and incubated with 0.5 mg/mL MTT solution for 4 h. The crystal formazan was dissolved in 1 mL DMSO and test the OD value at 490 nm and 570 nm.

3.6. Lactate dehydrogenase assay

Before the incubation with MTT solution, the medium was collected to detect LDH leakage according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

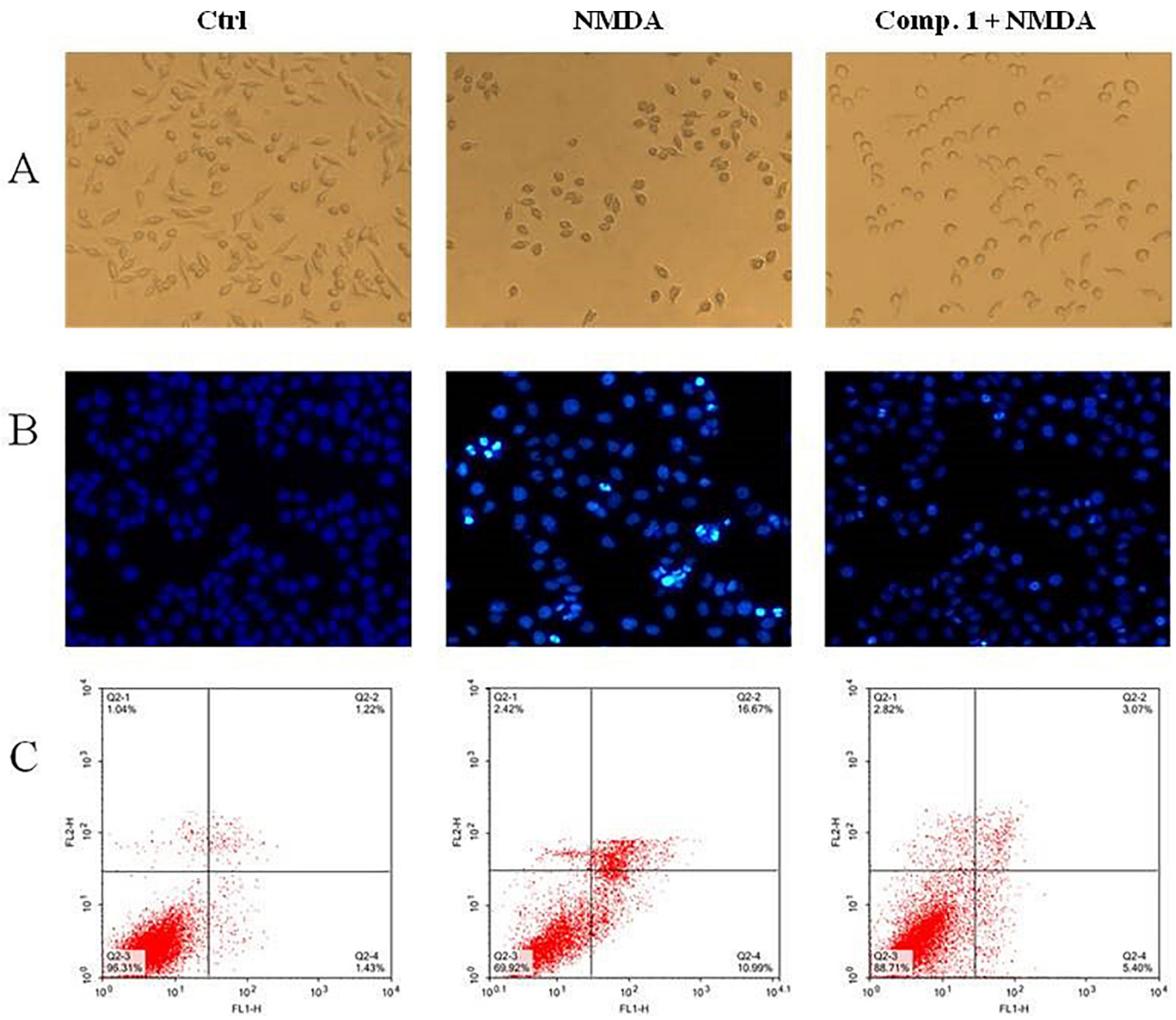

Fig. 9. Effects of Compound **1** on NMDA-induced apoptosis in SH-SY5Y cells. (A) Effects of compound **1** on cell morphologic alterations in NMDA treated SH-SY5Y cells. (B) Effects of pretreatment with Compound **1** on nuclear morphological changes in SH-SY5Y cells treated with NMDA. (C) Compound **1** inhibited the cell apoptosis induced by NMDA in SH-SY5Y cells. Cells were incubated with indicated concentrations of Compound **1** for 12 h before exposure to NMDA for 0.5 h. Remove the supernatant and cells were incubated with complete medium for 24 h.

3.7. Hoechst 33342 staining

Treated cells were stained with 10 μg.mL^{-1} Hoechst 33342 and incubated in dark for 15 min. Then cells were observed using a fluorescence microscope (IX71, Olympus, Japan).

3.8. Flow cytometry analysis for apoptosis

Cell apoptosis was measured by Annexin V-FITC/PI double staining assay kit. Cells were plated into 6-well plates and cultured for 12 h before exposure to compound **1** at indicated concentration for 12 h. Then cells were treated with 2 mM NMDA for 0.5 h and cultured with fresh culture medium containing 10% FBS 24 h before Annexin V-FITC/PI staining.

3.9. Western blot

Western blot analysis was performed as described previously (Fan et al., 2015). Cells were plated overnight into culture dish and pretreated with compound **1** or DMSO for 12 h. Except for the blank control group, cells were subsequently exposure to 2 mM NMDA for 30 min and cultured with complete Medium for 24 h. Treated cells were collected and analyzed by Western blot. Equal amounts of protein (50 μg) were loaded for each sample onto10–15% SDS-PAGE gels and transferred to PVDF membranes. Blots were imaged by ImageQuant LAS 4000 (GE Healthcare Life Sciences, USA).

3.10. Measurement of intracellular ROS

ROS assay was conducted according to ROS/Superoxide Detection Assay Kit (ab139476).the cells were incubated with DCFH-DA (10 μM, 37 °C, 20 min), then the cells were washed twice with PBS and

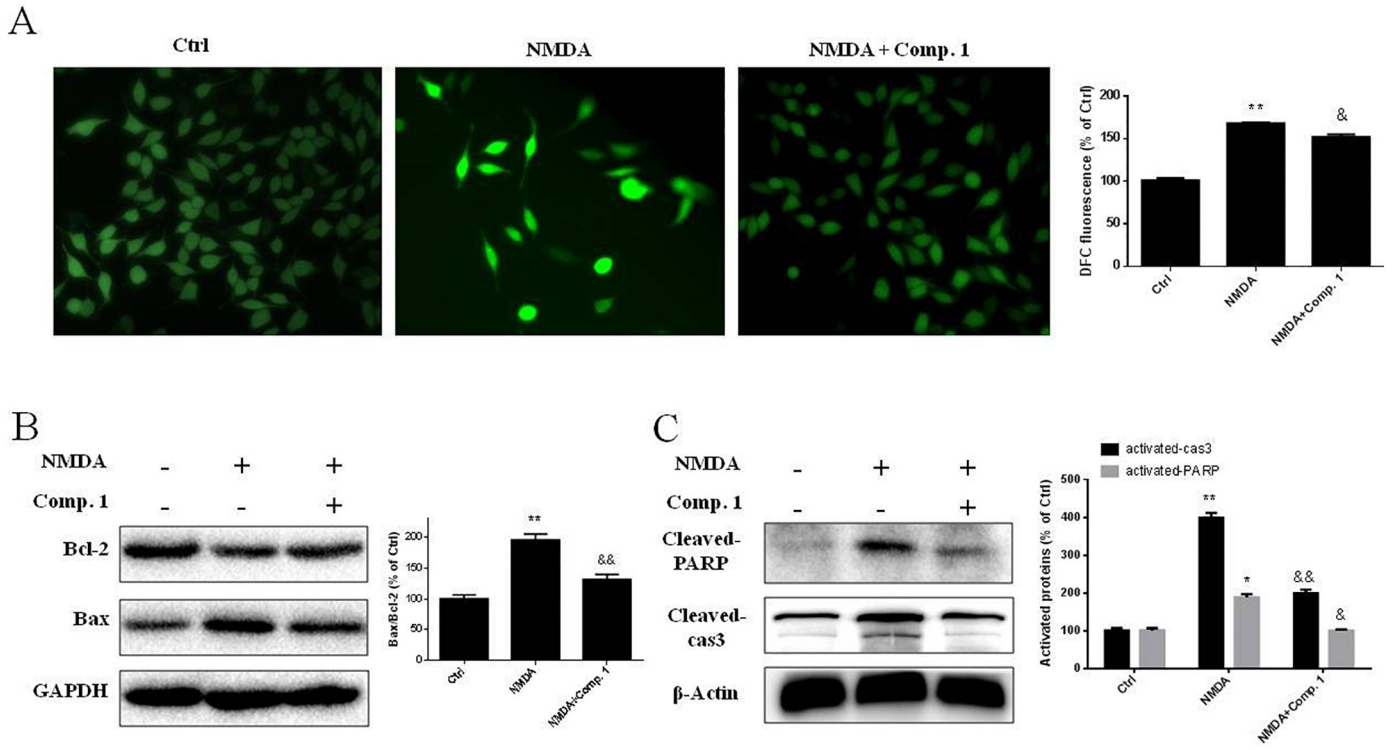

Fig. 10. Effects of Compound **1** on NMDA-induced ROS and cell apoptosis in SH-SY5Y cells. (A) Effects of Compound **1** on production of intracellular ROS. (B) The effects of Compound **1** on protein levels associated with cell apoptosis. Cells were treated with indicated compound **1** or NMDA. Data are presented as means ± S.D. (n = 3). #p < .05 or ##p < .01 vs. the NMDA-treated group. *p < .05 or **p < .01 vs. the control group.

intracellular ROS was detected by flow cytometer (Becton Dickinson, NJ, USA).

3.11. Molecular docking study

The molecular docking simulations were carried out as described previously. Molecular docking study was performed using Autodock 4.0. The crystal structure of NMDA (PDB:3qel) was selected for this docking simulation. Accelrys Discovery Studio Visualizer 4.5 was used for graphic display.

3.12. Statistical analysis

Data were presented as means ± standard deviation (S.D.) from three independent experiments. Group differences were analyzed with one-way analysis of variance (ANOVA) followed by the post hoc LSD test. A value of $P < .05$ was considered to be significant.

4. Discussion

Recent years, dark tea has attracted great attention because of being abundant in secondary metabolites including flavonoids and catechins, which have been considered to be the major active component and beneficial for people's healthy. In addition, some structures with biological activities often modified via a fermentation process, along with the alteration of activities [3]. It has been reported that during the microbial post-fermentation in Fuzhuan brick tea, catechin derivatives were degraded to several kind of B-ring fusion derivatives [5–7], while in Pu-erh tea, some A-ring derivatives were isolated [8,9,23]. In our study, we isolated and identified two novel compounds with A-ring methoxycarbonyl group substitute derived from catechin from Anhua Dark tea suggesting that similar process might also occur in post-fermentation of Anhua dark tea and Pu-erh tea. Numerous pharmacological activities such as anticancer, antioxidant and cytoprotective effects of catechin derivatives have been reported during recent decades [3]. We hypothesized that the catechin derivatives we isolated from the dark tea might also have cytoprotective effects. Thus, we evaluated the neuroprotective effects of these two catechin derivatives and other isolated compounds against NMDA induced cell injury in SH-SY5Y cells. Among the isolated catechin derivatives compound **1** showed good cytoprotective effects at 10 μM against the NMDA-induced cell injury.

NMDARs are critical ion channels of calcium and glutamate-gate. It involves in neurologic disorders, synaptic plasticity and excitotoxic cell death and underpin learning and memory [11,24]. NMDARs dysfunction often lead to neurodegenerative diseases including Alzheimer's disease, depression, stroke, epilepsy and schizophrenia [25,26]. Therefore, NMDARs have been considered as a promising target for therapy of neurodegenerative diseases. Ifenprodil is a structurally unique modulator of the NMDA receptor which exhibits subunit-specific affinity for NR2B [27]. Ifenprodil showed great protective effects in cerebral ischemic model [28,29]. However, it is still necessary to develop new effective NMDA inhibitors. In our study, both two new catechin derivatives compound **1** and **2** effectively protect the SH-SY5Y cells against neurotoxicity induced by NMDA. Compared to the catechins without A-ring methoxycarbonyl group substitute, compound **1** and **2** showed equivalent or better neuroprotective activities against NMDA induced cell excitatory injury, indicating this structure modify improves or doesn't affect their neuroprotective activities. In addition, compounds **1** and **2** showed less toxicity as major components of various teas available [30]. Phosphoinositide 3-kinase-Akt signaling pathway was reported to involve of NMDA-induced cell injury [31]. Compound **1** improved the cell viability via increasing the p-Akt in NMDA-treated SH-SY5Y cells. In addition, Compound **1** also restored the p-ERK decreased by NMDA treatment. Taken together, Compound **1** would be a potent dietary therapy reagent for prevention of excitable brain injury.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

Acknowledgements

This work was supported by the Natural Sciences Foundation of Liaoning Province (grant no. 201602712) and the National Natural Science Foundation of China (grant no. 81860609). The authors are grateful to Hunan Xiao Hei Shen Development Co., Ltd. for providing the crude material of Anhua dark tea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2019.104240.

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