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The Neuroprotective Effect of Batch-2, an Aqueous Extract From Cat′s Claw (*Uncaria tomentosa*) on 6-OHDA-Induced SH-SY5Y Cell Damage*

SHI Zhen-Hua[1], XI Zhao-Wilson[2], WANG Hao[1,3], GUAN Peng[1], YU Peng[1], CHANG Yan-Zhong[1], DUAN Xiang-Lin[1]**, ZHAO Bao-Lu[4]**

([1] *The Institute of Molecular Neurobiology and Neuropharmacology, Hebei Normal University, Shijiazhuang* 050016, China; [2] *BioMarker Pharmaceuticals, Inc. 5941 Optical Court, San Jose, CA* 95138, USA; [3] *Pony Testing International Group Co.Ltd, Beijing* 100080 ,China; [4] *State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, The Chinese Academy of Sciences, Beijing* 100101, China)

Abstract Previous pharmacological studies found that a water-soluble extract (C-MED-100™) of *Uncaria tomentosa* has antioxidant activity and beneficial effects on DNA repair and immune function. Batch-2 is a novel aqueous extract from cat's claw, however, the free radical scavenging ability and neuroprotective effect of batch-2 have not been reported. Firstly, the neuroprotective effect of batch-2 on the 6-OHDA-induced SH-SY5Y cell damage was detected. Secondly, the component analysis of batch-2 was tested by infrared spectroscopy, HPLC and spectrophotometry.The results showed that batch-2 has scavenging ability to several kinds of free radicals, especial the hydroxyl radical (25 mg/L of batch-2 can scavenge 60% of the hydroxyl radical). The dose dependently attenuated 6-OHDA-induced cell death, lipid peroxidation, mitochondrial potential loss and increase of intracellular reactive oxygen species (ROS) and nitric oxide (NO) in SH-SY5Y cells were also observed. Meanwhile, batch-2 can suppress the 6-OHDA induced increase of expression of iNOS and activity of NF-κB. The results show that the neuroprotective effect of batch-2 on 6-OHDA-induced damage of SH-SY5Y cells by scavenging ROS and NO, inhibiting activation of iNOS and NF-κB. Component analysis shown that the content of polyphenol and quininic acid from Batch-2 was 6.43% and 0.0958% respectively. These results provided evidence to support that the mechanism of batch-2 for antioxidative activity and neuroprotective effect may be similar with (−)-epigallocatechin-3-gallate(EGCG) partly. It is obvious that batch-2 is a potential neuroprotective antioxidant for the precaution candidate of Parkinson's disease.

Key words cat's claw(*Uncaria tomentosa*), antioxidant, Parkinson's disease, ROS, 6-OHDA, ESR
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Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopamingic neurons in pars compacta of the substantia nigra [1]. Although the underlying biochemical and molecular mechanism leading to neuronal degeneration in PD remains unclear, 6-hydrooxydopamine (6-OHDA) is a neurotoxin and can induce cell death of dopamingic neurons. Induced cell death by 6-OHDA in dopamingic neurons has been most frequently used as a PD model for testing new pharmacological therapies [2]. SH-SY5Y cells are dopamingic neurons and have been widely used to establish the Parkinsonism cell model.

It has been established that oxidative stress which can be caused by 6-OHDA, contributes to the cascade leading to dopamine cell degeneration in PD [2-3]. The excessive formation of reactive oxygen species (ROS) and nitrogen species may damage key cellular components such as lipids, proteins and DNA and impair cell viability in PD. There is convincing evidence for the involvement of nitric oxide (NO) that reacts with superoxide to form more reactive peroxynitrite[4-5].

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**Corresponding author.
DUAN Xiang-Lin. Tel: 86-311-86267215, E-mail: xlduan0311@163.com
ZHAO Bao-Lu. Tel: 86-10-64888569, E-mail: zhaobl@sun5.ibp.ac.cn

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NF-κB is an inducible transfactor and iNOS is an inducible NO synthase in the cytoplasm which plays an important role in human immune and inflammatory responses. An increased level of NF-κB and iNOS has been observed in the brain of patients with various neurodegenerative disorders including Parkinson's disease or in models treated with 6-OHDA[2, 6].

Cat's claw, *Uncaria tomentosa*, grows in the Amazon forests of Peru and is widely used in traditional Peruvian medicine for the treatment of several diseases, in particular as a potent anti-inflammatory agent [7]. Additionally, its antioxidant activity has been evaluated by reaction with the free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion ($O^{\cdot}_2$), peroxy ($ROO^{\cdot}$), and hydroxyl ($HO^{\cdot}$) radicals, hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) as well [7-8]. Quinic acid (QA) is a biologically active component of the *Uncaria tomentosa* extract and it can inhibit the expression of NF-κB. It is unknown whether QA has antioxidant activity. Batch-2 is a novel aqueous extract from cat's claw. In the present study, we use QA as an internal control to investigate the antioxidative activity and protective effect of batch-2 on apoptosis by 6-OHDA in SH-SY5Y cells.

1 Materials and methods

1.1 Materials

Batch-2 was extracted from *Uncaria tomentosa* which is a brown powder and should be dissolved in didistilled water before use. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, Hepes and 3- (4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) were purchased from Gibco BRL (Grand Island, NY, USA). Quinic acid (QA), 5,5-Dimethyl-pyrroline-oxide (DMPO), 1,1,-diphenyl-2-picryl-hydrazyl (DPPH), 6-OHDA, TBA, BHT, 2-BA-2-DMHB, 2, 2, 6, 6-tetramethyl-4-piperidone hydrochloride (TEMP), 2-butylamino-2-demethoxy-hypocrellin B(2-BA-2-DMHB), 2,2-Azino-bisc3-ethylbenzothiazoline-6-sulfonicacid (ABTS) were purchased from Sigma. Rabbit polyclonal antibody for NF-κB, inducible nitric oxide synthase (iNOS) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals made in China were analytical grade.

1.2 Cell culture and cell treatment

SH-SY5Y cells were grown in DMEM supplemented with 10% fetal bovine serum (Gibicol BRL), 100 U/ml penicillin and 100 mg/L streptomycin. Cells were kept at 37℃ in a humidified 5% CO_2/95% air incubator. The cells were pretreated with 50, 100 and 200 mg/L batch-2 for 1 h, and then, 6-OHDA was added to a final concentration of 100 μmol/L. All of the determinations were performed 24 h later.

1.3 Cell viability

Cell viability was measured in 96-well plates by quantitative colorimetric assay with MTT, which is an indicator of the mitochondrial activity of living cells[9]. Briefly, at the indicated time after the treatment, 500 mg/L MTT (final concentration) was added to the medium, which continued to incubate at 37℃ for 3 h. The MTT solution was removed and the colored formazan crystal was dissolved in dimethyl sulfoxide (DMSO). The absorbance at 595 nm of each aliquot was determined using a Bio-Rad 3350 microplate reader. Cell viability was expressed as the ratio of the signal obtained from treated cultures and control cultures.

1.4 The assay of scavenging of ABTS, DPPH, singlet oxygen, hydroxyl radical and superoxide anion free radical *in vitro* by ESR and spectrophotometry

1.4.1 Reaction with stable free radicals ABTS and DDPH. ABTS (2,2-Azino-bisc3-ethylbenzothiazoline-6-sulfonicacid) reducing activity was determined by Parihar *et al*[10]. The final volume (1 ml) contained 0.1 ml of batch-2 at various concentrations, 0.75 ml of phosphate buffer (100 μmol/L, pH 7.4) and 0.15 ml of ABTS radical [prepared by mixing of 100 ml of potassium persulphate (70 μmol/L) and 25 ml of ABTS solution (2 μmol/L) and was used after 6 h of preparation]. The absorbance was recorded at 734 nm immediately after addition of ABTS radical, blank was performed by similar manner without batch-2. A batch-2 rate kinetic study with ABTS was carried by using IC_{50} concentration (17.65 mg/L). For DPPH reducing activity, equal volume of batch-2 in different concentration was incubated with methanolic DPPH (100 μmol/L) at room temperature. Absorbance was recorded at 517 nm after 20 min. The comparison to blank percentage scavenging was calculated in both the models. The batch-2 rate kinetic study with DPPH was carried by using IC_{50} concentration (33.17 mg/L).

1.4.2 Scavenging singlet oxygen, superoxide anion and hydroxyl radical. Scavenging singlet oxygen, hydroxyl radical and superoxide anion was detected by ESR. The production of singlet oxygen, superoxide

anion and hydroxyl radical was by 2-BA-2-DMHB[11], riboflavin and H_2O_2 +Fe $(SO_4)_2$ respectively [12]. For singlet oxygen assay, the final volume(45 μl) contained 30 μl PBS (pH7.4), 5 μl TEMP (30 mmol/L), 5 μl 2-BA-2-DMHB, 5 μl different concentration of batch-2, testing with ESR after 5 min light. For superoxide assay, the final volume (30 μl) contained 10 μl DMPO (150 mmol/L), 5 μl EDTA (10 mmol/L), 5 μl DETAPAC(3 mmol/L), 5 μl ovoflavin(0.5 mmol/L), and 5 μl different concentration of batch-2, testing after 2 min light. For hydroxyl radical assay, the final volume (50 μl) contained 25 μl PBS (pH7.4), 10 μl DMPO, 5 μl $FeSO_4$, 5 μl H_2O_2 and 5 μl different concentrations of batch-2, testing after 2 min. ESR spectra was recorded at room temperature in a quartz tube with an ER-200 spectrometer (Bruker, Karlsruhe, Germany) operating at X-band with 100 kHz modulation, modulation amplitude 1 G, microwave power 20 mW, scan width 200 G, time constant 0.2 s.

1.5 Assay of intracellular ROS

The level of intracellular ROS was quantified by fluorescence with 2′, 7′-dichlorofluorescin diacetate (DCF-DA)[13]. After treatment, the cells were collected and washed three times with PBS. Cells were then incubated with 5 μmol/L DCF-DA for 45 min at 37℃ in the dark. Then the cells were washed three times with PBS and resuspended in BSS buffer containing 130 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 15 mmol/L glucose and 5 mmol/L HEPES, pH 7.4. The relative levels of fluorescence were quantified by a fluorescence spectrophotometer (Hitachi F-4500, Tokoyo, Japan, 485 nm excitation and 535 nm emission). The data were expressed as a percentage of the fluorescence relative to the fluorescent value in control cells.

1.6 Measurement of intracellular NO level

The intracellular NO level was measured by using 4,5-diaminofluorescein diacetate (DAF-2DA) as a probe[14]. Cells were incubated with 10 μmol/L DAF-2DA final concentration at 37℃ with constant shaking in the dark for 30 min. Then the cells were washed, re-suspended in 0.01 mol/L PBS, and transferred to a quartz cuvette of Hitachi spectrofluorometer (Model 4500). Fluorescence was measured at 37℃ with excitation wavelength at 492 nm and emission wavelength at 519 nm.

1.7 Thiobarbituric acid reactive substances (TBARS)

The level of lipid peroxidation was measured by determining TBARS[15]. The cells after treatment were collected and lysed on ice for 20 min by lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1% Triton X-100). Then, the samples were mixed with 1 ml of 20% (*w*/*v*) trichloroacetic acid, 0.8 ml dd-water and 1 ml of 0.67% (*w*/*v*) 2-thiobarbituric acid. After concussing, the samples were incubated for 60 min in boiling water, and TBARS were extracted in 3 ml of *n*-butanol. Following centrifugation at 4 400 *g* for 10 min, the absorption of the butanol layer was measured at 532 nm. 1, 1, 3, 3-tetraethoxypropane (TMP) served as MDA standards. The TBARS levels were expressed as the ratio of the value obtained from treated cultures and control cultures.

1.8 Measurement of mitochondrial membrane potential

The change in mitochondria membrane potential was assayed by measuring the retention of rhodamine 123 (Rh123)[16]. Briefly, after treatment, the medium was removed and replaced with fresh nonserum medium containing 10 μmol/L Rh123 for 15 min at 37℃ in dark. The cells were then washed three times with PBS. Rh123-specific fluorescence intensity was monitored at an excitation wavelength of 490 nm and an emission wavelength of 515 nm using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The data were expressed as a percentage of the fluorescence relative to the fluorescent value in control cells.

1.9 Western blotting

The cells were grown in 75 mm^2 sterile culture flasks and treated with different concentrations of batch-2 and 100 μmol/L 6-OHDA for 24 h. After incubation, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS) and lysed with 200 μl RIPA lysis buffer containing 100 mg/L PMSF, 1 mg/L aprotinin. The lysate was collected, kept on ice for 15 min, and centrifuged at 12 000 *g* at 4℃ for 10 min. The pellet (indicated nucleus) was used to detect NF-κB.

For the detection of iNOS, the cells were lysed on ice for 30 min by lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 100 mg/L PMSF, 1 mg/L aprotinin and 1% Triton X-100). The lysates were then centrifuged at 12 000 *g* for 20 min at 4℃. The supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein content was estimated by a BCA kit (Pierce Inc., USA). Proteins were separated on gels and transferred to a

nitrocellulose membrane. The membrane was incubated in TBST-M (20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 0.1% Tween-20, 5% BSA) overnight at 4℃. After that, the membrane was incubated for 2 h with antibodies against iNOS and NF-κB in 1∶200 dilution and anti-β-actin antibody in 1∶400 dilution. The samples were then incubated with peroxidase-conjugated secondary antibody for 1 h with constant agitation. After incubation with the secondary antibody, the samples were washed and reacted with the supersignal chemiluminescent substrate (Pierce Biotechnology, IL, USA), and exposed to Kodak-XAR film. The film was digitized and analyzed by NIH imaging software.

1.10 Component analysis of batch-2

1.10.1 Infrared spectrum assay of batch-2(KBr pellet method). The major component was measured by infrared spectroscopy [17]. Briefly, The homogeneous mixture including 2 mg batch-2 and 300 mg dry KBr powder was shifted to tooting and the pump pressure was set to $5\times10^7\sim10\times10^7$ Pa for 2 min under evacuation. The transparent thin slice was put to sheeting box to make the infrared spectrum scan as control of KBr blank pellet. The ingredient identification of spectrogram peak position, shape and relative intensity was done by looking at the spectral line index.

1.10.2 Assay of total polyphenol content (Folin-Ciocalteu colorimetric method). The total polyphenol content was measured by Folin-Ciocalteu colorimetric method[18]. Briefly, 1 ml extract solution (1 g/L) were mixed with 1 ml Folin-Ciocalteu reagent, 2 ml 15% Na_2CO_3 and 6 ml distilled water. After concussing, the mixtures were incubated for 60 min at 30℃ in dark. The absorption was measured at 760 nm using SHIMADZU UV-1601 ultraviolet spectrophotometer. The gallic acid were used as standard and the values were expressed as total polyphenol content(μg/mg).

1.10.3 Assay of quininic acid. Quininic acid was detected by HPLC[19]. Briefly. The standard curve was set up with quininic acid (purity 98%), 1 g/L batch-2 resolved in mobile phase was treated with 0.45 μm filter membrane and then the filtrate was measured by Aglient 1200 chromatographic system. The chromatographic condition was as follow: Accurasil C18 chromatographic column (250 mm × 4.6 mm × 5 μm), sample size 20 μl, flow rate 0.5 ml/min, detection wave length 210 nm, column temperature 30℃, mobile phase 0.01 mol/L K_2HPO_4 phosphate buffer and 3% methanol.

1.11 Statistical analysis

The values in the figures are $\bar{x}\pm s$. Data analysis was performed by a one-way analysis of variance (ANOVA) and $P<0.05$ was considered significant.

2 Results

2.1 Batch-2 ameliorated the cell viability decrease induced by 6-OHDA

The cell viability was expressed as an MTT conversion rate. No significant difference in cell viability among the different concentrations of batch-2 and quinic acid treatment respectively for 24 h was seen (Figure 1a and c). However, cell viability was decreased by about 55% in the presence of 100 μmol/L 6-OHDA alone for 24 h. When the SH-SY5Y cells were pretreated with different concentrations of batch-2 and quinic acid for 1 h, and followed by 24 h incubation with 100 μmol/L 6-OHDA, the cell viability was increased with the increasing of concentration of batch-2 compared with cells treated with 6-OHDA alone (Figure 1b). But no significant change of the cell viability was observed with the increasing of concentration of quinic acid compared with cells treated with 6-OHDA alone (Figure 1d). These results depicted that batch-2 can enhance the cell viability in the SH-SY5Y cell induced by 6-OHDA in a dose-dependent manner. Quinic acid is one of the main components of Cat's claw but it has no protective effect on SH-SY5Y cells against 6-OHDA induced-cell damage.

2.2 Batch-2 repress the increase of intracellular ROS induced by 6-OHDA

As shown in Figure 2a, intracellular ROS levels were examined by using DCF-DA. SH-SY5Y cells treated with 100 μmol/L 6-OHDA for 24 h exhibited a significant increase in the DCF signal relative to the control($P<0.01$). However, this effect was significantly attenuated by different concentrations of batch-2. The result indicated that batch-2 can inhibit the increasing of intracellular ROS induced by 6-OHDA in the SH-SY5Y in a dose-dependent fashion.

2.3 Batch-2 attenuate 6-OHDA-induced mitochondrial membrane potential loss

The levels of mitochondrial membrane potential (MMP) imply the viability of cells. Exposure of SH-SY5Y cells to 6-OHDA for 24 h decreased the fluorescent intensity of Rhodamine 123 staining, representing a fall in the mitochondrial membrane

potential. Batch-2 inhibited the fall of mitochondrial membrane potential caused by 6-OHDA at 50, 100 and 200 mg/L in a concentration-dependent manner (Figure 2c).

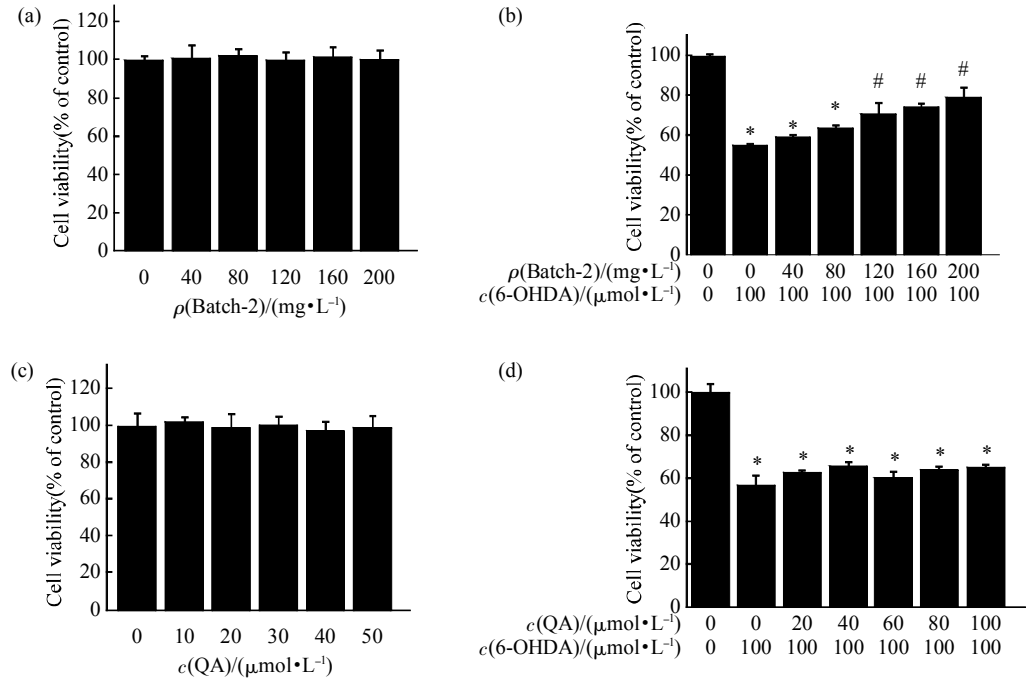

Fig. 1　Effect of batch-2, QA and 6-OHDA on SH-SY5Y cell viability

Cells were incubated in drug-free medium or medium containing different concentrations of batch-2 (a) and QA (c) for 24 h or cells were preincubated with various concentrations of batch-2 (b) and QA (d) for 1 h, 6-OHDA (100 μmol/L) was added for an additional 24 h. Data were expressed as a percentage of the untreated control ± s, n = 3. *P < 0.01 significantly different from control cells; $^{\#}P$ < 0.05 compared with only 6-OHDA group cells by ANOVA .

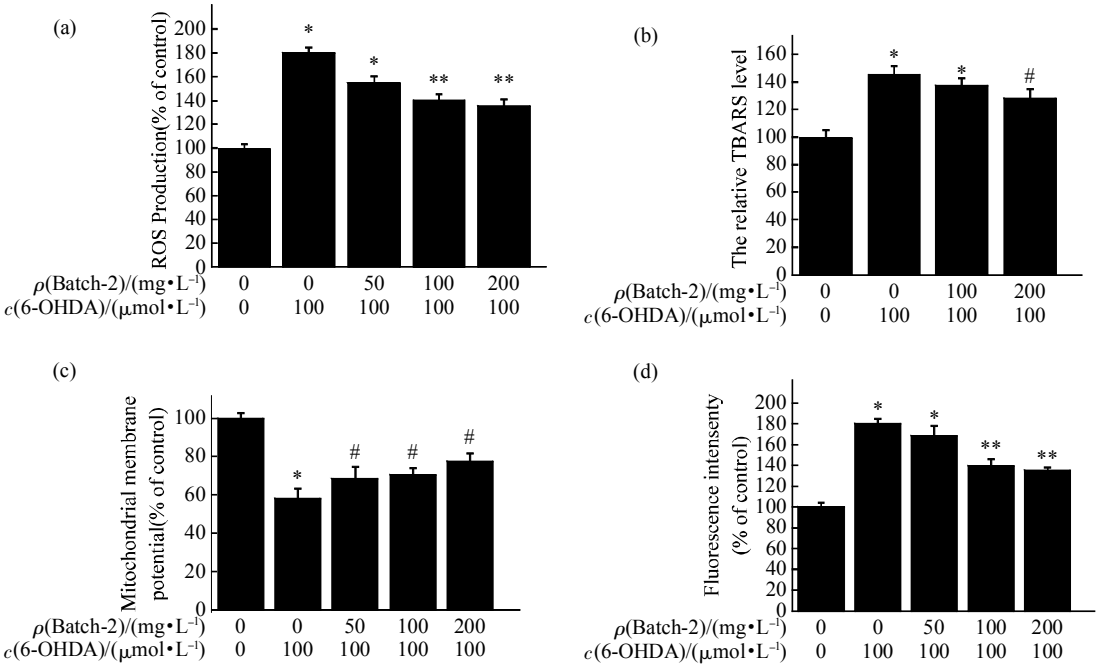

Fig. 2　Batch-2 attenuated 6-OHDA-induced accumulation of ROS(a), decrease of mitochondrial membrane potential(b), increase of TBARS(c) and intracellular NO(d)

Cells were cultured with 100 μmol/L 6-OHDA in the presence or absence of batch-2 for 24 h. The cell viability was measured as described in the **Materials and methods**. Data are expressed as a percentage of the untreated control ± s, n = 3. *P < 0.01 compared with control cells, **P < 0.01 compared with 6-OHDA-treated cells alone. $^{\#}P$ < 0.05 compared with 6-OHDA-treated cells alone.

2.4 Batch-2 decreased the TBARS level

The TBARS concentration, an end product of lipid peroxidation, in the SH-SY5Y cells is shown in Figure 2b and Table 1. The lipid peroxide level increased about 45% with the treatment of 6-OHDA compared with the control group. Administration of 100 mg/L and 200 mg/L significantly decreased the TBARS level about 8% and 10% respectively. When the concentration of batch-2 was 25 mg/L, the scavenging rate of lipid peroxide was 32% in solution.

Table 1 Comparing the effect of batch-2 with EGCG and QA on scaveging hydroxyl radical, superoxide anion, singlet oxygen and inhibing lipid peroxidation in solution

	Scavenging capacity(% of control)			
	Hydroxyl radical	Superoxide anion	Singlet oxygen	Lipid peroxidation
EGCG(25 mg/L)	76.65 ± 4.2	67.98 ± 3.7	79.37 ± 4.8	46.04 ± 3.5
Batch-2(25 mg/L)	59.89 ± 5.1	20.96 ± 1.0	10.91 ± 0.8	32.63 ± 2.1
QA(5 g/L)	15.47 ± 1.2	–	–	–

2.5 Effects of batch-2 on intracellular NO level and expression of iNOS and NF-κB

As shown in Figure 2d, intracelluar NO levels increased significantly after treatment with 6-OHDA alone compared with the control group, and this effect was reduced in a concentration-dependent manner by pretreatment with batch-2 in 50, 100 and 200 mg/L. 6-OHDA exposure induced up-regulation of iNOS and NF-κB as revealed by Western blot analysis (Figure 3a, b and c). Such up-regulation was dose-dependently inhibited by co-application of batch-2.

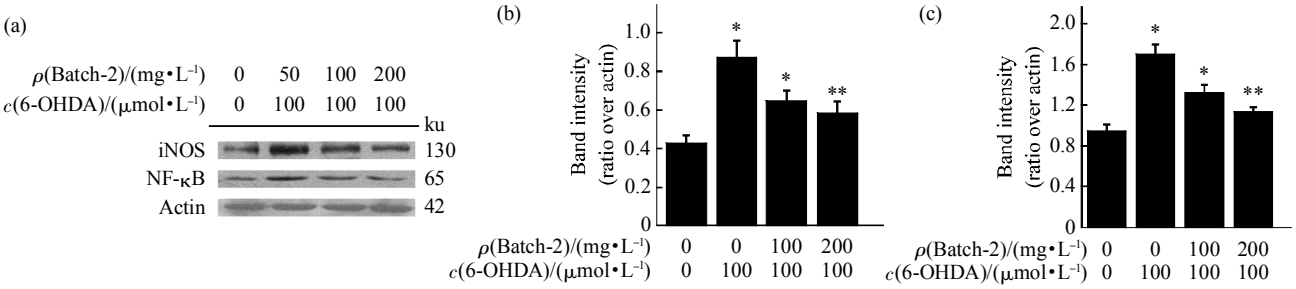

Fig. 3 Effect of batch-2 and 6-OHDA on the expression of NF-κB and iNOS

SY5Y cells were exposed to 6-OHDA (100 μmol/L) with various concentrations of batch-2 for 24 h. The NF-κB and iNOS were detected in the cell lysates by Western blot (a), and the protein levels for NF-κB (b) and iNOS (c) were also analyzed. The data were calculated by the ratio of the band intensity of NF-κB and iNOS over that of actin, expressed as ratio ± s, n =3. *P < 0.01 compared with control cells, **P < 0.05 compared with 6-OHDA-treated cells.

2.6 Free radicals scavenging ability of batch-2 in solution

Using the ESR and spectrophotometry techniques, we observed the scavenging of free radicals of ABTS, DPPH, singlet oxygen, hydroxyl radical and superoxide anions as shown in Figure 4 and Table 1. Batch-2 scavenged DPPH and ABTS radicals in a concentration-dependent manner and maximum scavenging 75.9% and 85% was observed at 64 mg/L and 30 mg/L, respectively. IC_{50} for DPPH and ABTS radicals were observed 33.17 mg/L and 17.65 mg/L, respectively. Batch-2 scavenged hydroxyl radicals were much stronger than scavenging singlet oxygen and superoxide anion radicals at 5 mg/L. There was some difference from EGCG.

2.7 The major components of batch-2 were polyphenol and quininic acid

As shown in Figure 5a, the infrared spectrum has obvious O—H group characteristic absorption peak in 3 650 to 3 200 cm^{-1}, characteristic absorption peak of

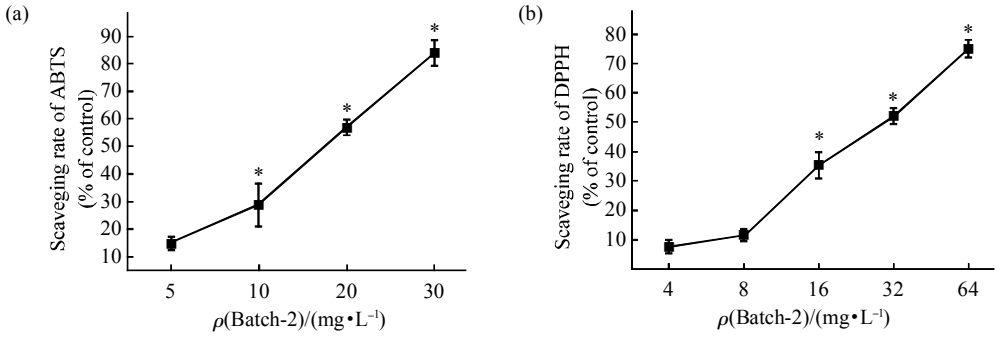

Fig. 4　Eeffect of batch-2 on scavenging ABTS (a) and DPPH (b) *in vitro* by absorption spectrometry

Data are expressed as a percentage of the untreated control ± s, n = 3, *P < 0.01 compared with control.

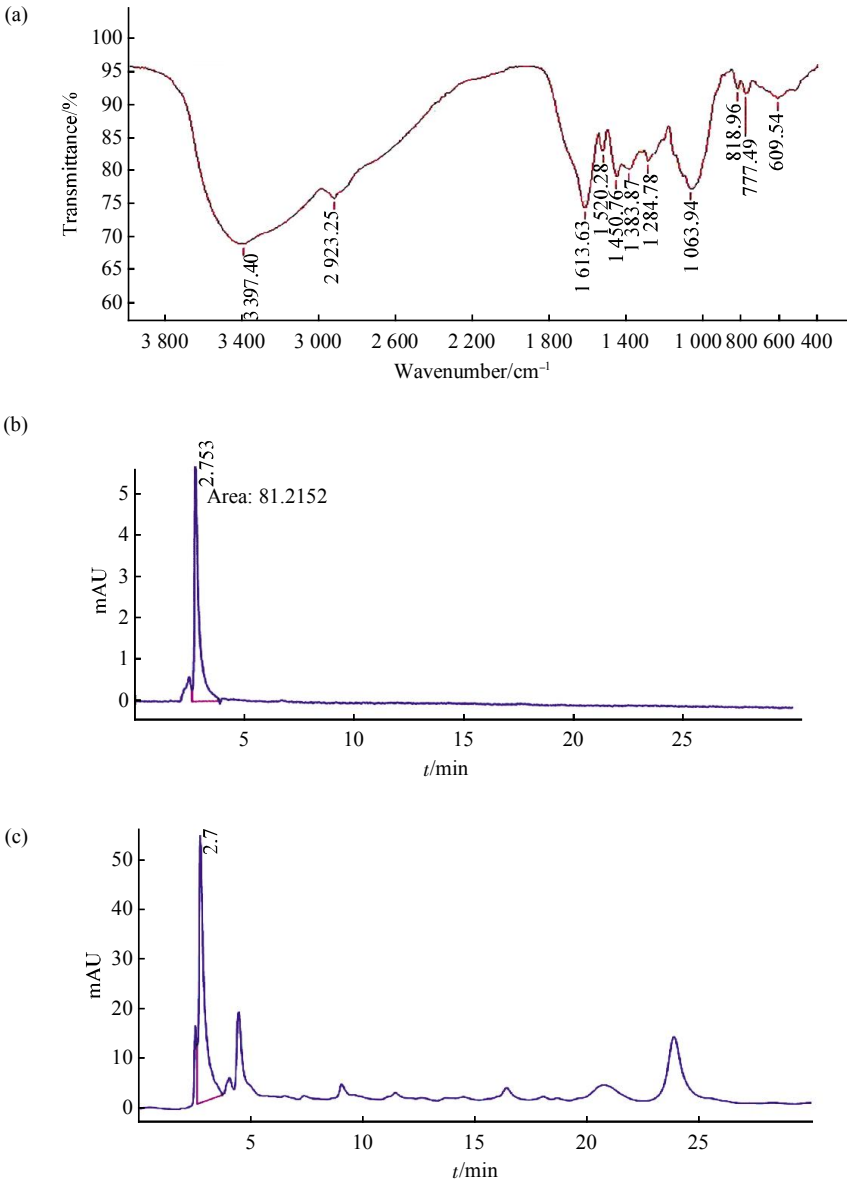

Fig. 5　Component analysis of batch-2

Infrared spectrum of batch-2(a), HPLC chromatogram of QA standard sample (b) and batch-2(c).

unsaturation C—H groups from benzene ring in 3 100～2 800 cm^{-1} and absorption peak of C═O stretching vibration in 1 900 to 1600 cm^{-1}, which indicated that batch-2 probably contained polyphenols or organic acids. Further analysis with HPLC (as shown in Figure 5 b and c) and spectrophotometric technique, we determined that batch-2 has 6.43% total polyphenol and 0.095 8% quininic acid.

3 Discussion

Although Pharmacological therapy of Parkinson's disease has made many advances over the past years, there is still no drug to cure PD. Recently, attention has been focused on the neuroprotective strategy for this neurological disorder[20–23]. It is known that some antioxidants such as EGCG[13, 20] may play an important role in protecting the neural cells.

Evidence demonstrates that the free radicals of 6-OHDA-derived can damage the cellular enzymes and induce the oxidation of the membrane component, which may be responsible for the disruption of Ca^{2+} homeostasis[24]. ROS induced by 6-OHDA react with biological target molecules, induce lipid peroxidation, and damage the mitochondrial membrane, resulting eventually in the collapse of the mitochondrial membrane potential.

The present study was to investigate the antioxidative activity and protective action of batch-2 against 6-OHDA-induced cytotoxicity in SH-SY5Y neuronal cells and its active mechanism. After treatment of 100 μmol/L 6-OHDA for 24 h, the SH-SY5Y cell viability was reduced by about 50% as compared with the control group. The addition of batch-2 to the culture mixture significantly increased the variability of SH-SY5Y cells. Previous studies found that QA was one of the major components of extracts of Cat's claw and has health beneficial biological activities[25]. Here we tested the component of batch-2 and used QA as an internal standard of extracts to investigate the antioxidative mechanism. Our study showed that QA has little antioxidative activity and effect on protecting SH-SY5Y cells apoptosis induced by 6-OHDA.

To elucidate the antioxidative mechanism of batch-2 against 6-OHDA induced cytotoxicity in SH-SY5Y cells, the level of the intercellular ROS, NO and lipid peroxidation were studied. It was found that SH-SY5Y cells underwent apoptosis after 24 h of exposure to 6-OHDA. Batch-2 inhibited the production of ROS, NO and TBARS in a dose-dependent manner. Several lines of evidence suggest that ROS-mediated 6-OHDA toxicity is due to the formation of highly reactive quinone compounds and superoxide radicals *via* a non-enzymatic self-autioxidation process[26]. It was reported that ROS and NO possess played very important roles in the 6-OHDA- induced apoptosis[13, 20]. The production of $ONOO^-$ *in vivo* is highly dependent on the metabolic pathways of NO and superoxide. Under pathological conditions, the production of ROS and NO increases and they can thus form $ONOO^-$[2, 27]. Recent studies have shown that $ONOO^-$ in particular is involved in apoptotic cell death in arteriolar smooth muscle cells, leukemia cells, and neurons[28–29]. The present study implied that batch-2 might control the ROS-NO pathway against 6-OHDA which is similar to previous studies[13, 20].

The disruption of the mitochondrial membrane potential ($\Delta\Psi_m$) is one of the critical events in cell apoptosis. It was reported that 6-OHDA at high concentrations directly acts as a mitochondrial inhibitor[30]. Our results showed that batch-2 suppressed the decreasing of mitochondrial membrane potential induced by 6-OHDA. It was reported that there were elevated levels of iNOS and NO in PD brain[20]. The elevated iNOS and NO, together with iNOS and nNOS, is known to be involved in the pathogenesis of PD. Our results showed that batch-2 suppressed the expression of iNOS, and so inhibited the production of NO to decrease the level of $ONOO^-$. Elevated levels of iNOS have been reported in PD brain[31], which indicated one of the protective mechanisms of batch-2 in SH-SY5Y cells against apoptosis induced by 6-OHDA through modulating the intracellular NO level.

NF-κB is the main transcription factor in inflammation and tissue injury including the PD process[32–33]. It was reported that Cat's claw inhibited the expression of TNFα by decreasing of NF-κB[34]. The present data showed that 100 μmol/L 6-OHDA induced activation of NF-κB in SH-SY5Y cell nucleus (as indicated in Figure 3), and this activation was significantly inhibited by batch-2. It appears that batch-2 might play a role in NF-κB-TNFα regulation pathway, which may partially explain the neuroprotective activity and other health benefit of the tested compound.

EGCG possesses efficacious antioxidative activities[13, 17]. In order to investigate the antioxidant

activities of batch-2 *in vitro* comparing to EGCG, we tested the power of them to scavenge free radicals of DPPH, ABTS, hydroxyl radical, superoxide anion, singlet oxygen and lipid peroxidation by ESR or spectrometry. The IC_{50} value for DPPH and ABTS are 17.65 mg/L and 33.17 mg/L respectively. Our data showed that batch-2 might play a role in protecting SH-SY5Y cell from 6-OHDA-induced apoptosis by scavenging hydroxyl radical and lipid peroxidation. Otherwise, EGCG is a major component of tea polyphenols and its antioxidative mechanism was dependent upon its phenolic hydroxyl group. Interestingly,there exist plenty of polyphenol in Batch-2. Our results provided evidence to support that the mechanism of batch-2 for antioxidative activity and neuroprotective effect may be similar with EGCG partly.

In summary, batch-2 has neuroprotective properties against 6-OHDA-induced damage in SH-SY5Y cells by scavenging ROS and NO and then inhibiting activation of iNOS and NF-κB. It is obvious that batch-2 is a potential neuroprotective antioxidant for the mitigation of Parkinson's disease and for research and development as a therapeutic candidate for neurodegenerative disorders.

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钩藤水提物 Batch-2 对 6-羟多巴胺诱导的 SH-SY5Y 细胞损伤的神经保护作用 *

石振华[1)] XI Zhao-Wilson[2)] 王 浩[1,3)] 关 鹏[1)]
于 鹏[1)] 常彦忠[1)] 段相林[1)**] 赵保路[4)**]

([1)] 河北师范大学生命科学学院，石家庄 050016；[2)] *BioMarker Pharmaceuticals, Inc*. 5941 *Optical Court, San Jose, CA* 95138, USA；
[3)] 北京普尼公司，北京 100080；[4)] 中国科学院生物物理研究所，北京 100101)

摘要 以前的药理学研究表明，钩藤水溶性提取物 C-MED-100™ 不仅具有抗氧化活性，而且还具有很好的 DNA 修复和免疫功能．Batch-2 是一种新的水溶性钩藤提取物，而它的自由基清除能力及神经保护作用还未见报道．首先检测了 batch-2对六羟多巴胺诱导的 SH-SY5Y 细胞损伤的神经保护作用机制，然后利用红外光谱、HPLC 和分光光度技术对 batch-2 的组成成分进行了分析．结果表明，batch-2 具有清除各种自由基的能力，尤其是对羟自由基的清除(25 mg/L 的 batch-2 对羟自由基的清除率为 60%)，batch-2 可剂量依赖性地抑制 6-羟多巴胺诱导的细胞凋亡、脂质过氧化水平、线粒体膜电位的降低和细胞内活性氧和一氧化氮的增加．同时，batch-2 抑制了由 6-羟多巴胺诱导的 SH-SY5Y 细胞内 iNOS 和 NF-κB 蛋白的上调．结果表明，batch-2 对六羟多巴胺诱导的 SH-SY5Y 细胞损伤的神经保护作用是通过清除活性氧和一氧化氮、抑制 iNOS 和 NF-κB 表达实现的．成分分析表明，batch-2 中的多酚和奎宁酸含量分别为 6.43%和 0.095 8%．上述结果显示，batch-2 的抗氧化机制部分类似于 EGCG．对于帕金森病的预防，batch-2 是一个潜在具有很好的神经保护作用的天然抗氧化剂．

关键词 绒毛钩藤(*Uncaria tomentosa*)，抗氧化剂，帕金森氏病，活性氧，6-羟多巴胺，电子顺磁共振
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** 通讯联系人.
段相林. Tel: 0311-86267215, E-mail: xlduan0311@163.com
赵保路. Tel: 010-64888569, E-mail: zhaobl@sun5.ibp.ac.cn
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