Neurochemistry International 62 (2013) 940–947

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

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

Neuroprotective effects of aqueous extracts of *Uncaria tomentosa: Insights from* 6-OHDA induced cell damage and transgenic *Caenorhabditis elegans* model

Zhenhua Shi [a,1], Zhongbing Lu [b,1], Yashuo Zhao [a], Yueqi Wang [a], Xi Zhao-Wilson [c], Peng Guan [a], Xianglin Duan [a], Yan-Zhong Chang [a,*], Baolu Zhao [d,e,*]

[a] *The Institute of Molecular Neurobiology and Neuropharmacology, Hebei Normal University, Shijiazhuang 050016, China*
[b] *College of Life Sciences, University of Chinese Academy of Science, Beijing 100049, China*
[c] *BioMarker Pharmaceuticals, Inc., 5941 Optical Court, San Jose, CA 95138, USA*
[d] *Pony Testing International Group Co. Ltd, Beijing 100080, China*
[e] *State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Science, Beijing 100101, China*

ARTICLE INFO

Article history:
Received 5 December 2012
Received in revised form 26 February 2013
Accepted 3 March 2013
Available online 14 March 2013

Keywords:
AC11
Oxidative stress
Parkinson's disease
α-Synuclein aggregation

ABSTRACT

Previous pharmacological studies have indicated that AC11 (a standardized aqueous extract of *Uncaria tomentosa*) has beneficial effects on DNA repair and immune function. However, its benefits go beyond this. The present study utilized electron spin resonance (ESR) and spin trapping technique, as well as the 6-OHDA-induced cell damage and transgenic *Caenorhabditis elegans* models, towards exploring the antioxidant and neuroprotective ability of AC11. Our results showed that AC11 could scavenge several types of free radicals, especially hydroxyl radicals (60% of hydroxyl radicals were scavenged by 30 μg/ml of AC11). In SH-SY5Y cells, we found that AC11 could dose dependently protect 6-OHDA induced cell damage by increase cell viability and mitochondrial membrane potential. AC11 pretreatment also significantly decreased the level of lipid peroxidation, intracellular reactive oxygen species and nitric oxide in 6-OHDA treated cells. In NL5901 *C. elegans*, 10 μg/ml AC11 could reduce the aggregation of α-synuclein by 40%. These findings encourage further investigation on AC11 and its active constituent compounds, as possible therapeutic intervention against Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in pars compacta of the substantia nigra and aggregation of protein α-synuclein (Schapira et al., 1998). Although the underlying biochemical and molecular mechanisms leading to neuronal degeneration in PD remain unclear, the oxidation of dopamine is known to generate so-called reactive oxygen species (ROS), and an unbalanced overproduction of ROS inducing neuronal damage, ultimately leading to neuronal death via apoptosis or necrosis, has been implicated in the pathological process of PD (Fahn and Cohen, 1992; Beal, 1995). As shown in our previous studies (Guo et al., 2005; Guo et al., 2007), nitric oxide (NO) was also shown to be involved in this pathological mechanism by reacting with superoxide to form more reactive peroxynitrite. The excessive formation of ROS and reactive nitrogen species (RNS) may damage key cellular components such as lipids, proteins and DNA and impair cell viability in PD. Therefore, antioxidants may hold the key to preventative measures against neurodegenerative diseases.

Uncaria tomentosa, popularly known as Cat's claw, is widely used in traditional Peruvian medicine for the treatment of several diseases, particularly as a potent anti-inflammatory agent (Mammone et al., 2006). It has been shown that the aqueous extract of *U. tomentosa* can protect against oxidant-induced stress in human erythrocytes (Bors et al., 2011) and attenuate indomethacin-induced chronic intestinal inflammation in rats (Sandoval-Chacon et al., 1998; Sandoval et al., 2002). *U. tomentosa* extract was also found to prevent the activation of the nuclear factor kappa beta (NF-κB), a potential mechanism for the anti-inflammatory activity of AC11 (Allen-Hall et al., 2010). As a commercially available, standardized aqueous extract of *U. tomentosa*, AC11 is relatively alkaloid free (<0.05%), unlike many other commercial preparations from this species. Animal and human studies have demonstrated the beneficial effect of AC11 on enhancing DNA repair and immune function (Sheng et al., 2000, 2001). However, the protective action of AC11 in neurodegenerative disease is still unknown. Here we

* Corresponding authors. Address: Laboratory of Molecular Iron Metabolism, College of Life Science, Hebei Normal University, Shijiazhuang 050016, Hebei Province, China. Tel.: +86 311 86267215; fax: +86 311 87888526 (Y. Chang). Address: State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China. Tel.: +86 10 64888569; fax: +86 10 64871293 (B. Zhao).
E-mail addresses: frankyzchang@yahoo.com.hk (Y.-Z. Chang), zhaobl@sun5.ibp.ac.cn (B. Zhao).
[1] These authors contributed equally to this work.

0197-0186/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuint.2013.03.001

used a classical PD cell model, 6-hydroxydopamine (6-OHDA)-induced apoptosis in SH-SY5Y cells, and a transgenic *Caenorhabditis elegans* model NL5901 which has human α-synuclein–YFP expression in the muscles (Punc-54::α-synuclein::YFP + unc-119) to evaluate the neuroprotective properties of AC11. To our knowledge, the data presented here revealing the new beneficial effects of AC11 has not been reported elsewhere.

2. Materials and methods

2.1. Materials

AC11 (Optigenex, Inc., New York, NY) was dissolved in distilled water before use. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Gibco BRL (Grand Island, NY, USA). Quinic acid (QA), 6-OHDA, 2′,7′-dichlorofluorescein diacetate (DCF-DA), 4,5-diaminofluorescein diacetate (DAF-2DA), 5,5-dimethyl-pyrroline-oxide (DMPO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2, 6, 6-tetramethyl-4-piperidone hydrochloride (TEMP), Hoechst 33258 and 2,2-azino-bisc3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma (St. Louis, MO, USA). 2-butylamino-2-demethoxy-hypocrellin B (2-BA-2-DMHB) was synthesized as previously reported (Yang et al., 2001). Rabbit polyclonal antibodies for NF-κB, iNOS, TNF-α and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals made in China were of analytical grade.

2.2. Assay for free radical scavenging activity in vitro

ABTS and DPPH reducing activity were determined by measuring the changes in absorbance changes at 734 nm and 517 nm respectively as described previously (Parihar et al., 2007). The AC11 kinetic analysis with ABTS and DPPH was carried out using the IC_{50} concentration.

Electron spin resonance (ESR) and spin trapping technique were used to determine the scavenging effect of AC-11 on singlet oxygen, hydroxyl radicals and superoxide anion. Singlet oxygen, superoxide anion and hydroxyl radicals were produced by 2-BA-2-DMHB (Lu et al., 2006a, 2006b), riboflavin and $H_2O_2 + Fe(SO_4)_2$, respectively (Noda et al., 1997). For the singlet oxygen assay, the final reaction volume (45 μl) contained 30 μl PBS (pH 7.4), 5 μl TEMP (30 mM), 5 μl 2-BA-2-DMHB and 5 μl of AC11 at different concentrations, and the ESR spectrum was recorded after 5 min of illumination. For the superoxide assay, the final reaction volume (30 μl) contained 10 μl DMPO (150 mM), 5 μl EDTA (10 mM), 5 μl diethylenetriaminepentaacetic acid (DETAPAC) (3 mM), 5 μl ovoflavin (0.5 mM) and 5 μl AC11 at different concentrations, and the ESR spectrum was recorded after 2 min of illumination. For the hydroxyl radical assay, the final reaction volume (50 μl) contained 25 μl PBS (pH 7.4), 10 μl DMPO, 5 μl $Fe(SO_4)_2$, 5 μl H_2O_2 and 5 μl AC11 at different concentrations, and the ESR spectrum was recorded after 2 min of illumination. 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.

2.3. Cell culture and treatment

SH-SY5Y cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a humidified 5% CO_2/95% air incubator. The cells were pre-treated with 50,100 or 200 μg/ml AC11 for 1 h, and then 6-OHDA was added to a final concentration of 100 μM. All of the determinations were performed 24 h later. Selection of AC11 concentrations was based on a previous report (Allen-Hall et al., 2010). Cell viability, nuclear morphology, lipid peroxidation, mitochondrial membrane potential and intracellular ROS/NO were measured as previous reported (Guo et al., 2005). TUNEL staining was performed with a kit from Roche Group.

2.4. Western blotting

The cells were grown in 75 mm^2 sterile culture flasks and treated with different concentrations of AC11 and 100 μM 6-OHDA for 24 h. After incubation, the medium was removed, and the cells were washed with PBS and lysed with 200 μl RIPA lysis buffer containing 100 μg/ml PMSF, 1 μg/ml aprotinin. The lysate was collected, kept on ice for 15 min and centrifuged at 12,000×*g* for 10 min at 4 °C. The pellet (containing nuclei) was used to detect NF-κB.

For the detection of iNOS, the cells were lysed on ice for 30 min with lysis buffer (50 mM Tris–Cl, 150 mM NaCl, 100 μg/ml PMSF, 1 μg/ml aprotinin and 1% Triton X-100). The lysates were then centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the protein concentration was determined using 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 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20, 5% BSA) overnight at 4 °C. Thereafter, the membrane was incubated for 2 h with antibodies against iNOS and NF-kB at 1:200 dilution and an anti-β-actin antibody in 1:400 dilution. The samples were then incubated with a peroxidase-conjugated secondary antibody for 1 h with constant agitation. After incubation with the secondary antibody, the samples were washed, reacted with the Supersignal chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed to Kodak-XAR film. The film was digitized and analyzed using NIH imaging software.

2.5. C. elegans culture and AC-11 treatment

NL5901 strain was obtained from the Caenorhabditis Genetics Center (University of Minnesota). Worms were raised on the OP50 seeded standard Nematode Growth Medium (NGM) and grown at 22 °C. AC11 was diluted in OP50 before seeding onto NGM plates. The plates were incubated overnight for optimum growth of bacteria OP50 following which, age synchronized worms were grown on the plates, for further studies.

2.6. Assay for analysis of α-synuclein protein aggregation

Aggregation of α-synuclein protein was observed in control and AC-11 treated (10 μg/ml) NL5901 strain of *C. elegans* as previous report (Jadiya et al., 2011; Van Ham et al., 2008). After 48 h of treatment, worms were washed thrice with M-9 buffer to remove adhering bacteria and transferred to agar padded slides (2% agarose) and sealed with a cover slip. Worms were immobilized with 100 mM sodium azide. Imaging of live (immobilised) worms (15–20 worms in each group) using confocal microscopy (Olympus FV500, Tokyo, Japan) was carried out to monitor the α-synuclein–YFP protein with excitation/emission filter (500/545 nm). The aggregation was quantified by measuring fluorescence intensity in each worm with image J software (Image National Institutes of Health, Bethesda, MD).

2.7. Statistical analysis

All values are expressed as mean ± standard error. Statistical significance was defined as $p < 0.05$. One-way analysis of variance (ANOVA) was used to test each variable for differences among the treatment groups with StatView (SAS Institute Inc.). If ANOVA demonstrated a significant effect, pair wise post hoc comparisons were made with Fisher's least significant difference test.

3. Results

3.1. Free radical scavenging capacity of AC11 in solution

As shown in Fig. 1A and B, AC11 scavenged ABTS and DPPH radicals in a concentration-dependent manner, and the maximum scavenging rates of 74.8% and 86.1% were observed at 30 μg/ml and 64 μg/ml, respectively. IC50 values for ABTS and DPPH radicals were determined to be 20.1 μg/ml and 32.2 μg/ml, respectively. With ESR and spin trapping technique, we have measured the scavenging capacity of singlet oxygen, hydroxyl radicals and superoxide anions. As shown in Fig. 1C and Table 1, AC11 could scavenge all those free radicals and the capacity of AC11 at 25 μg/ml was 55.71% on hydroxyl radicals, 21.06% on superoxide anion, 14.21% on singlet oxygen and 28.89% on lipid peroxidation. Although QA has been identified as one of the active ingredient of AC11, it showed no scavenging effect on free radicals at 25 μg/ml, and the scavenging rate on hydroxyl radicals was only about 15% at 5 mg/ml.

3.2. AC11 attenuated 6-OHDA induced cell viability loss and apoptosis

Cell viability was expressed as an MTT conversion rate. No significant difference in cell viability was seen for 24 h among the different concentrations of AC11 and QA treatment (Fig. 2A and C). However, cell viability was decreased by about 55% in the presence of 100 μM 6-OHDA alone for 24 h. When the SH-SY5Y cells were pre-treated with different concentrations of AC11 and QA for 1 h, followed by 24 h of incubation with 100 μM 6-OHDA, the cell viability increased with increasing concentrations of AC11 compared with cells treated with 6-OHDA alone (Fig. 2B). No significant change of cell viability was observed with increasing concentrations of QA compared with cells treated with 6-OHDA alone (Fig. 2D). These results indicate that AC11 could dose-dependently enhance the viability of SH-SY5Y cells in the presence of 6-OHDA. While QA showed no protective effect on SH-SY5Y cells against
6-OHDA-induced cell damage. Another representative antioxidant, α-tocopherol (Vitamin E, Ve), at the same concentration (200 μg/ml) showed much weaker protective effect on cell viability as compared with AC11 (Fig 2).

To further study the neuroprotective effects of AC11, its influence on cell apoptosis was determined by detecting morphological changes induced by 6-OHDA treatment. As shown in Fig. 3, the majority of cells in the control group showed a normal nuclear staining pattern (Fig. 3A), while exposure to 100 μM 6-OHDA for 24 h led to typical apoptotic morphology (condensed chromatin and bright staining) in SH-SY5Y cells (Fig. 3B). Such nuclear morphology changes were attenuated after AC11 treatment (Fig. 3C–E).

The cell apoptosis rate was also confirmed by TUNEL staining. As shown in Fig 3F–J, 6-OHDA treatment dramatically increased the number of TUNEL-positive cells (compare G to F). When cells were pretreated with different dose of AC11, the 6-OHDA-induced apoptosis was greatly inhibited (compare I/J to G).

3.3. AC11 suppressed 6-OHDA induced intracellular ROS

As shown in Fig. 4A, intracellular ROS levels were examined using DCF-DA. SH-SY5Y cells treated with 100 μM 6-OHDA for

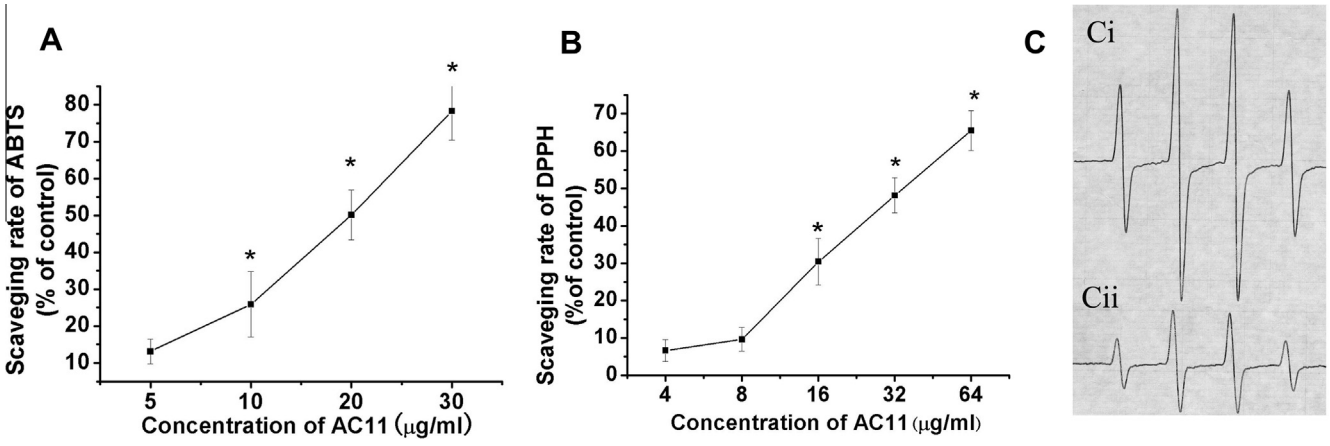

Fig. 1. Effects of AC11 on scavenging ABTS (A), DPPH (B) and hydroxyl radicals (Ci, control; Cii, AC11 treated) in vitro by absorption spectrometry and ESR. For A and B, Data were expressed as a percentage of the untreated control, $n = 3$, $^{*}p < 0.01$ compared with control.

Table 1
Comparing the effect of AC11 with QA and positive control Epigallocatechin gallate (EGCG) on free radical scavenging and lipid peroxidation inhibiting in solution.

	Scavenging capacity (% of control)			
	Hydroxyl radical	Superoxide anion	Singlet oxygen	Lipid peroxidation
AC-11 (25 μg/ml)	55.71 ± 6.2*	21.06 ± 2.14*	14.21 ± 1.28*	28.89 ± 4.2*
QA (5 mg/ml)	15.47 ± 1.2*	–	–	–
EGCG (25 μg/ml)	76.65 ± 4.2*	67.983.7*	79.37 ± 4.8*	46.04 ± 3.5*

* $p < 0.01$ compare with blank control.

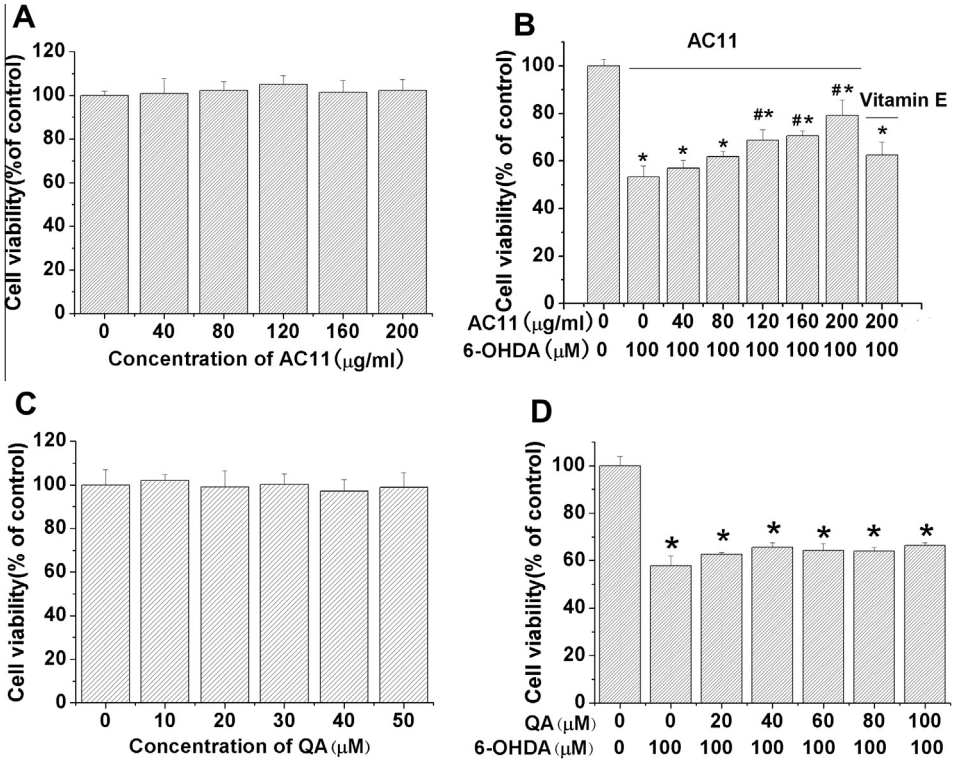

Fig. 2. Effects of AC11, QA and 6-OHDA on SH-SY5Y cell viability. Cells were incubated in drug-free medium or medium containing different concentrations of AC11 (A) and QA (C) for 24 h or pre-incubated with various concentrations of AC11 or 200 μg/ml Vitamin E as positive control (B) and QA (D) for 1 h, and then 6-OHDA (100 μM) was added for an additional 24 h. Data are expressed as a percentage of the untreated control ± SE, $n = 3$. $*p < 0.01$ compared with control cells; $^{\#}p < 0.05$ compared with cells treated with 6-OHDA alone.

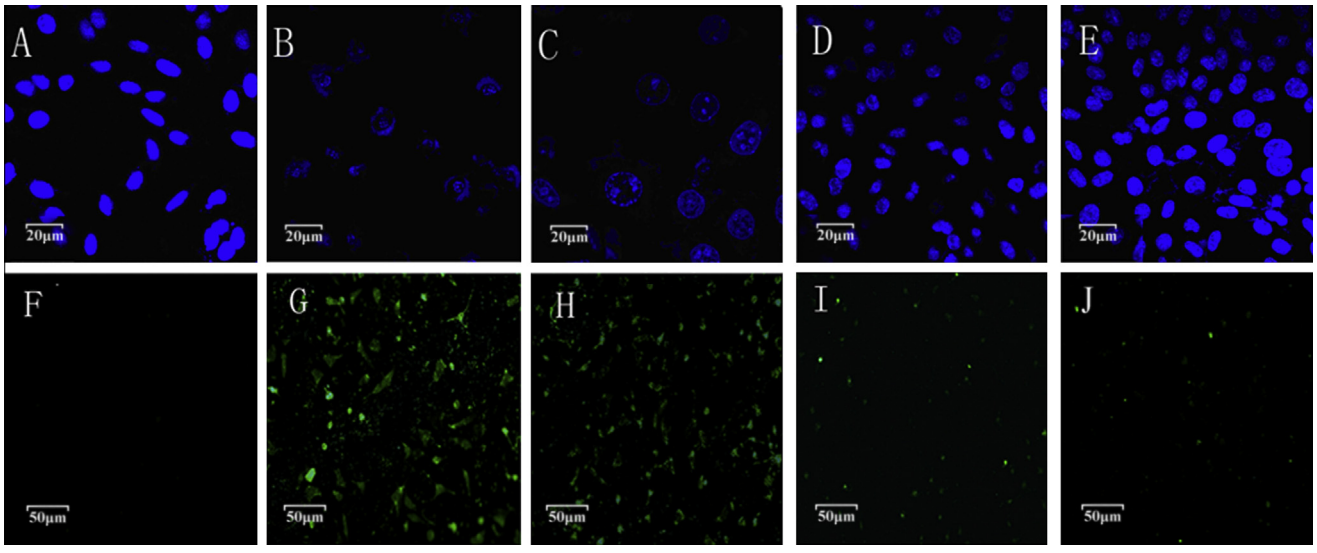

Fig. 3. AC11 decreased cell apoptosis induced by 6-OHDA. Cells were cultured with 100 μM 6-OHDA in the presence or absence of different concentrations of AC11 for 24 h. Nuclear morphological changes were detected as described in Section 2. (A) Control, (B) 6-OHDA, (C) 6-OHDA plus 50 μg/ml AC11, (D) 6-OHDA plus 100 μg/ml AC11, (E) 6-OHDA plus 200 μg/ml AC11. Cell apoptosis were also detected by TUNEL staining: (F) Control, (G) 6-OHDA, (H) 6-OHDA plus 50 μg/ml AC11, (I) 6-OHDA plus 100 μg/ml AC11, (J) 6-OHDA plus 200 μg/ml AC11.

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 AC11. The results indicate that AC11 could dose-dependently inhibit the increase of intracellular ROS induced by 6-OHDA in SH-SY5Y cells.

3.4. AC11 attenuated 6-OHDA induced mitochondrial membrane potential loss

The levels of mitochondrial membrane potential correspond to the viability of cells. Exposure of SH-SY5Y cells to 6-OHDA for 24 h

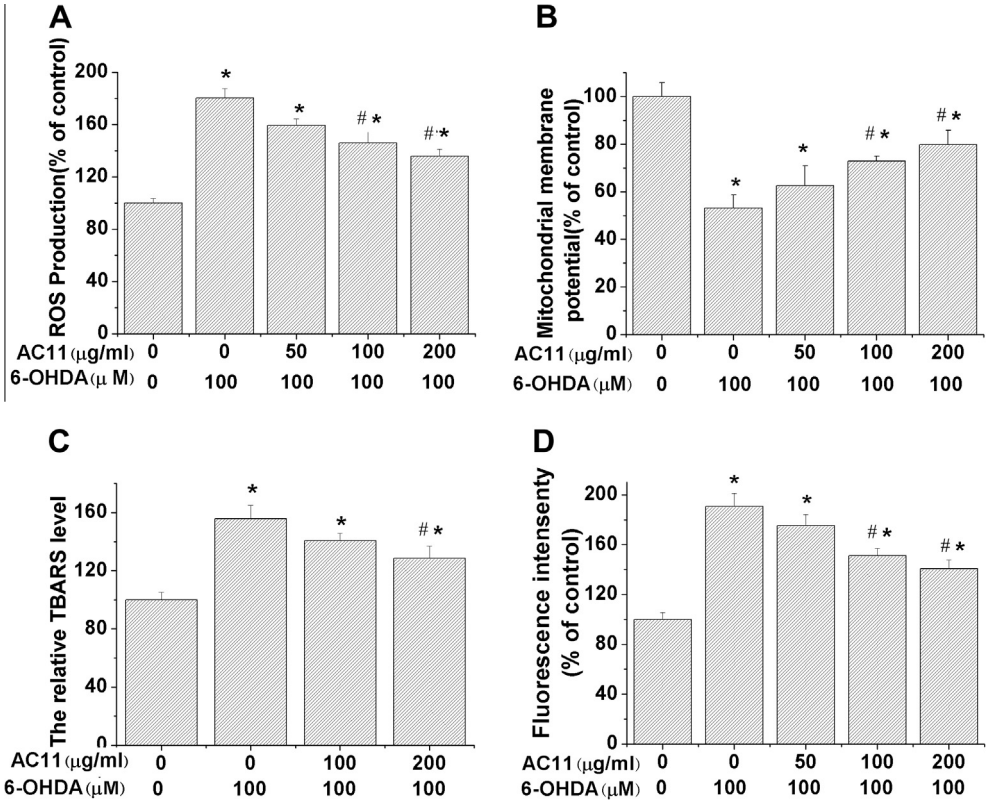

Fig. 4. AC11 attenuated 6-OHDA induced accumulation of ROS (A), decrease of mitochondrial membrane potential (B), increase of TBARS (C) and intracellular NO (D). Cells were cultured with 100 μM 6-OHDA in the presence or absence of AC11 for 24 h. Cell viability was measured as described in Materials and Methods. Data are expressed as a percentage of the untreated control ± SE, n = 3. $^{*}p < 0.01$ compared with control cells, $^{\#}p < 0.05$ compared with cells treated with 6-OHDA alone.

decreased the fluorescent intensity of Rhodamine 123 staining, representing a fall in the mitochondrial membrane potential. AC11 attenuated the decrease of mitochondrial membrane potential caused by 6-OHDA at 50,100 and 200 μg in a concentration-dependent manner (Fig. 4B).

3.5. AC11 attenuated lipid peroxidation in 6-OHDA treated cells

TBARS, an end product of lipid peroxidation, were measured in SH-SY5Y cells under various conditions as indicated in Fig. 4C. The results demonstrate that the lipid peroxide level increased by about 40% with 6-OHDA treatment compared with the control group. Co-administration of 100 μg/ml and 200 μg/ml AC11 with 6-OHDA decreased the level of TBARS by about 8% and 20%, respectively, which were statistically significant.

3.6. Effects of AC11 on intracellular NO level and expression of iNOS, NF-κB and TNF-α

As shown in Fig. 4D, intracellular 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 pre-treatment with AC11 at 50, 100 and 200 μg/ml. 6-OHDA exposure up-regulated iNOS, NF-κB and TNF-α as revealed by Western blot analysis (Fig. 5A–D), and these increases were dose-dependently attenuated by co-treatment of AC11.

3.7. AC11 reduced α-synuclein protein aggregation

Worms of untreated and AC11 treated groups were observed under confocal microscope for assaying their α-synuclein aggregation pattern. Phenotypically, the worms appeared normal and optimally fed. A marked and significant reduction in the aggregation of α-synuclein in case of NL5901 worms treated with AC11 was observed as compared to that of control group (Fig. 6A). The images for fluorescence intensity of α-synuclein aggregation were quantified using Image J software. Treatment of worms with AC11 showed significantly reduced fluorescence intensity of aggregation as compared to that of untreated worms. The mean fluorescence (GFP) intensity was 5.808 ± 0.728 arbitrary units in control worms and 3.582 ± 0.515 arbitrary units in AC11 treated subjects (Fig. 6), indicating 40% reduction ($p < 0.05$) in α-synuclein protein aggregation after AC11 treated. Treatment of same concentration of α-tocopherol caused 25% reduction ($p < 0.05$ compare with control group).

4. Discussion

To the best of our knowledge, this is the first report assessing the antioxidant and neuroprotective effect of AC11. The major new finding is that AC11 significantly attenuated 6-OHDA induced cell death, oxidative stress in SH-SY5Y cells. AC11 also significantly reduced the α-synuclein aggregation in NL5901 *C. elegans*. The antioxidant activity, especially the hydroxyl radicals scavenging capacity, may be responsible for the neuroprotective effects of AC11.

Many advances in pharmacological therapies for PD have been made over the past few years; however, there is still no drug currently available to cure or significantly prevent the advancement of this disease. Although the detailed mechanism causing degeneration in dopaminergic (DA) neurons in PD is not well understood, oxidative stress has been regarded as one of the intermediary risk factors that can initiate and/or promote degeneration of DA neu-

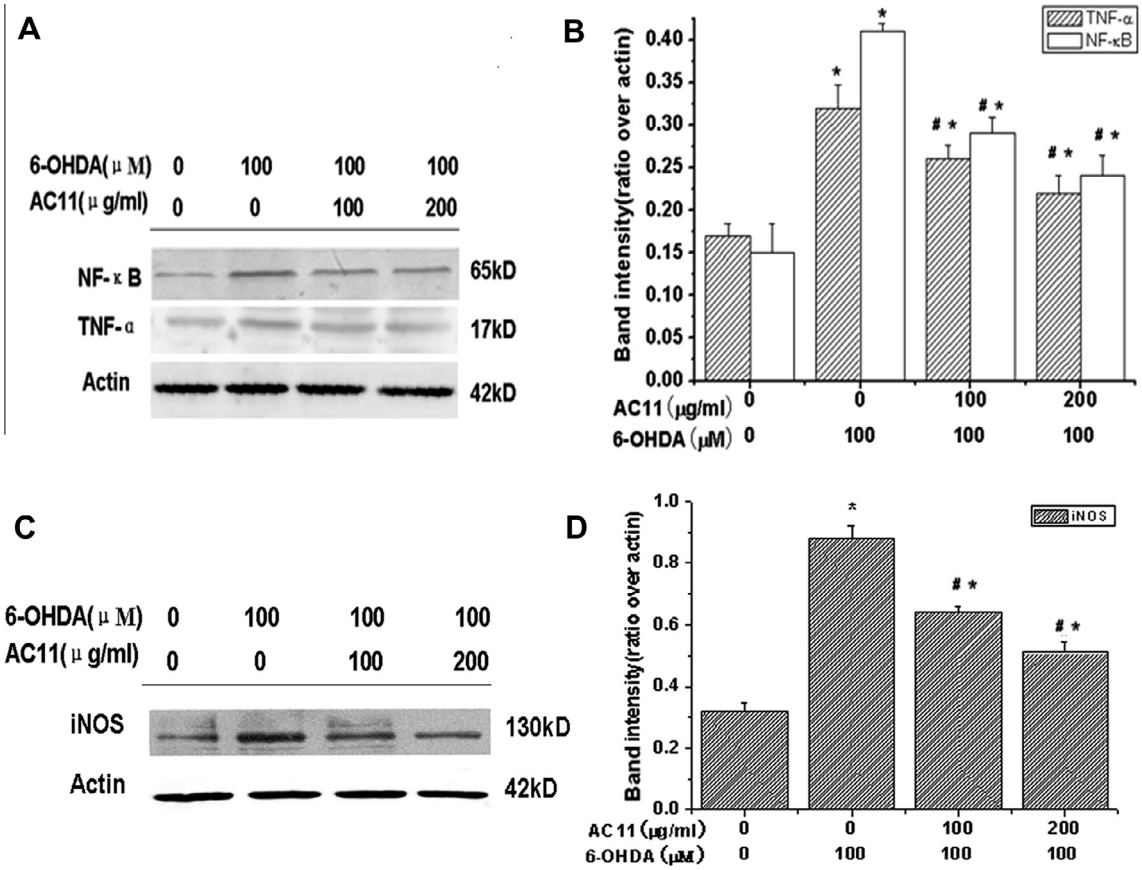

Fig. 5. Effects of AC11 and 6-OHDA on the expression of NF-κB, TNF-α and iNOS. Cells were exposed to 6-OHDA (100 μM) with various concentrations of AC11 for 24 h. NF-kB, TNF-α and iNOS were detected in the cell lysates by Western blot (A, C), and quantified (B, D). The data were calculated as the ratio of the band intensity of NF-kB, TNF-α or iNOS over that of actin and expressed as the mean ± SE, $n = 3$. $^{*}p < 0.05$ compared with control cells, $^{\#}p < 0.05$ compared with cells treated with 6-OHDA alone.

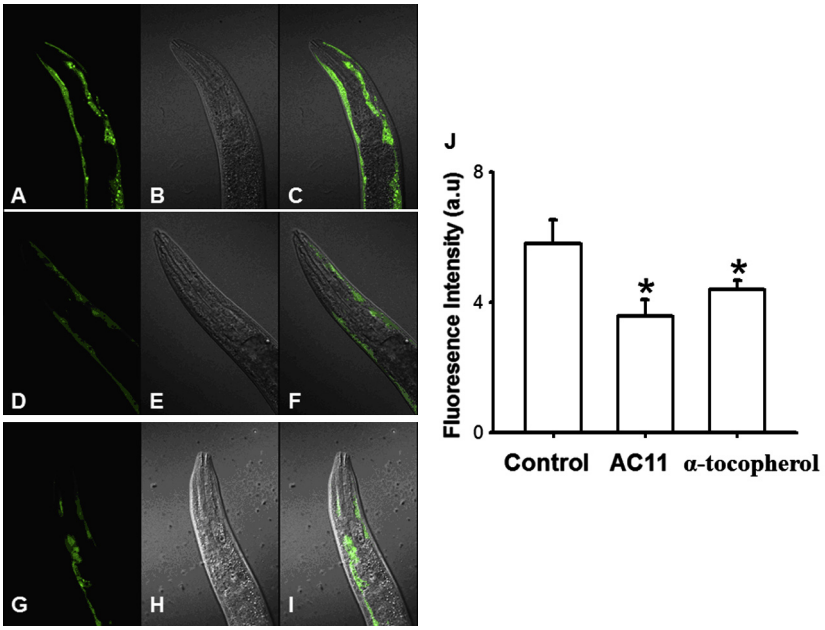

Fig. 6. α-Ynuclein aggregation in NL5901 strain of *C. elegans* fed on OP50 (A–C), OP50 plus 10 μg/ml AC11 (D–F) and OP50 plus 10 μg/ml α-tocopherol (G–I). Images A, D and G are fluorescent images; B, E and H are images grabbed using Differential Interference Contrast (DIC) optics; C, F and I are overlap images. J is graphical representation for fluorescence intensity of the nematodes as quantified using Image J software from 20 worms each group. $^{*}p < 0.05$ compare with control group.

rons (Fahn and Cohen, 1992; Beal, 1995). Therefore, supplementation with antioxidants may prevent or reduce the rate of progression of this disease (Prasad et al., 1999; Zhao, 2009). For example, our previous studies have demonstrated the protective effect of green tea polyphenols on neurons against apoptosis of cellular and animal PD models (Guo et al., 2005, 2007). Currently,

U. tomentosa is one of the most popular herbal remedies in the United States due to its beneficial effects on DNA repair and immune function (Sheng et al., 2000, 2001). There is growing evidence to support its use in treating cancer, inflammation, viral infection and vascular conditions, as well as for its use as an immunostimulant, antioxidant and antibacterial agent (Heitzman et al., 2005). Here we found that AC11 significantly attenuated the cell death induced by 6-OHDA (Figs. 2 and 3), suggesting AC11 may act as a potential neuroprotective agent in PD. To clarify the protective mechanism of AC11 against 6-OHDA-induced cytotoxicity, the levels of intercellular ROS and lipid peroxidation were studied. In agreement with a number of previous reports (Lu et al., 2006a, 2006b), we have shown that 6-OHDA does indeed induce oxidative stress, as demonstrated by the increase of intracellular ROS levels and TBARS content (Fig. 4A and C), whereas AC11 pretreatment significantly attenuated these increases in a dose-dependent manner, suggesting that the protective effect of AC11 is at least partially through its antioxidant property.

The neuroprotection by the selective iNOS inhibitor GW274150 in a model of PD demonstrated the important role of activation of iNOS in the pathogenesis of PD (Broom et al., 2011). The present study, as well as our previous study (Guo et al., 2005), showed that 6-OHDA treatment caused increases of iNOS expression and intracellular NO levels. AC11 pretreatment significantly decreased iNOS induction, thereby decreasing the production of NO (Figs. 4D–5C). As the production of $ONOO^-$ *in vivo* is highly dependent on the metabolic pathways of NO and superoxide, our results indicated that AC11 may exert its protection against 6-OHDA cytotoxicity via modulation of intracellular ROS and NO levels, which is consistent with our previous studies (Guo et al., 2005, 2007).

It has been well described that mitochondrial alterations are associated with the cytotoxic effect of 6-OHDA, especially the loss of mitochondrial membrane potential (Tirmenstein et al., 2005). In the present study, 100 μM 6-OHDA treatments caused a significant decrease in the fluorescent intensity of Rhodamine 123. However, AC11 treatment significantly attenuated the mitochondrial membrane potential loss in a dose-dependent manner.

NF-κB is a family of inducible transcription factors that are activated in response to inflammatory stimulation. There is evidence showing that there is a marked increase in NF-κB activation within the midbrain of animals undergoing neurodegeneration as a result of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration, as well as in the substantia nigra pars compacta of PD patients (Hunot et al., 1997; Ghosh et al., 2007), suggesting that targeting the classical pathway of NF-κB may serve as a useful therapeutic approach to the treatment of PD (Flood et al., 2011). Our results showed that 6-OHDA increased the expression of NF-κB and TNF-α in SH-SY5Y cells, and those inductions were significantly attenuated by AC11 treatment (Fig. 4). The modulatory effect of AC11 on NF-κB and TNF-α regulatory pathway is in agreement with previous reports demonstrating that *U. tomentosa* acts as a potent TNF-α inhibitor through NF-κB.

PD is also characterized by the aggregation of several proteins involved in vesicle recycling and protein degradation such as α-synuclein, parkin, and synphilin-1. It has been suggested that aggregation of these proteins lead to oxidative stress, suggesting that compounds that can inhibit protein aggregation can be useful in the treatment of PD. In NL5901 *C. elegans*, 10 μg/ml AC11 could reduce the aggregation of α-synuclein by 40%, indicating the neuroprotective effect of AC11 is also associated with reducing protein aggregation. It has been suggested that therapeutic drugs for PD require aromatic elements for binding to the α-synuclein monomer/oligomer and vicinal hydroxyl groups on a single phenyl ring (Caruana et al., 2011). It has also been indicating that decrease oxidative stress by antioxidant could reduce α-synuclein aggregation (Shastry, 2003). Using infrared spectrum analysis (figures not shown), we found that polyphenols was one of major components in AC11. Meanwhile, we also found AC11 could scavenge several kinds of free radical (Fig 1 and Table 1) and decrease 6-OHDA induced oxidative stress in SH-SY5Y cells (Fig 4). So there may be two possibilities which explain the anti α-synuclein aggregation effect of AC11: one is through direct interaction between the polyphenols ingredient in AC11 with α-synuclein; the other is through decreasing oxidative stress by free radical scavenging.

5. Conclusion

Overall, this study demonstrates that AC11 is a potential neuroprotective antioxidant for the mitigation of PD and a promising therapeutic candidate for neurodegenerative disorders.

Acknowledgements

This work was funded by National Natural Science Foundation of China (31271146) and the Applied Research Project of Hebei Province (10966120D). Zhongbing Lu gratefully acknowledges the support of State Key Laboratory of Biomacromolecules (2012kf03) and Hundred Talents Program of the Chinese Academy of Sciences.

References

Allen-Hall, L., Arnason, J.T., Cano, P., Lafrenie, R.M., 2010. *Uncaria tomentosa* acts as a potent TNF-alpha inhibitor through NF-kappaB. J. Ethnopharmacol. 127, 685–693.

Beal, M.F., 1995. Aging, energy, and oxidative stress in neurodegenerative diseases. Ann. Neurol. 38, 357–366.

Bors, M., Bukowska, B., Pilarski, R., Gulewicz, K., Oszmianski, J., Michalowicz, J., Koter-Michalak, M., 2011. Protective activity of the *Uncaria tomentosa* extracts on human erythrocytes in oxidative stress induced by 2,4-dichlorophenol (2,4-DCP) and catechol. Food Chem. Toxicol. 49, 2202–2211.

Broom, L., Marinova-Mutafchieva, L., Sadeghian, M., Davis, J.B., Medhurst, A.D., Dexter, D.T., 2011. Neuroprotection by the selective iNOS inhibitor GW274150 in a model of Parkinson disease. Free Radic. Biol. Med. 50, 633–640.

Caruana, M., Hogen, T., Levin, J., Hillmer, A., Giese, A., Vassallo, N., 2011. Inhibition and disaggregation of alpha-synuclein oligomers by natural polyphenolic compounds. FEBS Lett. 585, 1113–1120.

Fahn, S., Cohen, G., 1992. The oxidant stress hypothesis in Parkinson's disease: evidence supporting it. Ann. Neurol. 32, 804–812.

Flood, P.M., Qian, L., Peterson, L.J., Zhang, F., Shi, J.S., Gao, H.M., Hong, J.S., 2011. Transcriptional Factor NF-kappaB as a Target for Therapy in Parkinson's Disease. Parkinsons Dis. 2011, 216298.

Ghosh, A., Roy, A., Liu, X., Kordower, J.H., Mufson, E.J., Hartley, D.M., Ghosh, S., Mosley, R.L., Gendelman, H.E., Pahan, K., 2007. Selective inhibition of NF-kappaB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease. Proc. Natl. Acad. Sci. USA 104, 18754–18759.

Guo, S., Bezard, E., Zhao, B., 2005. Protective effect of green tea polyphenols on the SH-SY5Y cells against 6-OHDA induced apoptosis through ROS-NO pathway. Free Radic. Biol. Med. 39, 682–695.

Guo, S., Yan, J., Yang, T., Yang, X., Bezard, E., Zhao, B., 2007. Protective effects of green tea polyphenols in the 6-OHDA rat model of Parkinson's disease through inhibition of ROS-NO pathway. Biol. Psychiatry 62, 1353–1362.

Heitzman, M.E., Neto, C.C., Winiarz, E., Vaisberg, A.J., Hammond, G.B., 2005. Ethnobotany, phytochemistry and pharmacology of Uncaria (Rubiaceae). Phytochemistry 66, 5–29.

Hunot, S., Brugg, B., Ricard, D., Michel, P.P., Muriel, M.P., Ruberg, M., Faucheux, B.A., Agid, Y., Hirsch, E.C., 1997. Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease. Proc. Natl. Acad. Sci. USA 94, 7531–7536.

Jadiya, P., Khan, A., Sammi, S.R., Kaur, S., Mir, S.S., Nazir, A., 2011. Anti-Parkinsonian effects of *Bacopa monnieri*: insights from transgenic and pharmacological *Caenorhabditis elegans* models of Parkinson's disease. Biochem. Biophys. Res. Commun. 413, 605–610.

Lu, Z., Nie, G., Belton, P.S., Tang, H., Zhao, B., 2006a. Structure–activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. Neurochem. Int. 48, 263–274.

Lu, Z., Tao, Y., Zhou, Z., Zhang, J., Li, C., Ou, L., Zhao, B., 2006b. Mitochondrial reactive oxygen species and nitric oxide-mediated cancer cell apoptosis in 2-butylamino-2-demethoxyhypocrellin B photodynamic treatment. Free Radic. Biol. Med. 41, 1590–1605.

Mammone, T., Akesson, C., Gan, D., Giampapa, V., Pero, R.W., 2006. A water soluble extract from *Uncaria tomentosa* (Cat's Claw) is a potent enhancer of DNA repair in primary organ cultures of human skin. Phytother. Res. 20, 178–183.

Noda, Y., Anzai, K., Mori, A., Kohno, M., Shinmei, M., Packer, L., 1997. Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. Biochem. Mol. Biol. Int. 42, 35–44.

Parihar, V.K., Dhawan, J., Kumar, S., Manjula, S.N., Subramanian, G., Unnikrishnan, M.K., Rao, C.M., 2007. Free radical scavenging and radioprotective activity of dehydrozingerone against whole body gamma irradiation in Swiss albino mice. Chem. Biol. Interact. 170, 49–58.

Prasad, K.N., Cole, W.C., Kumar, B., 1999. Multiple antioxidants in the prevention and treatment of Parkinson's disease. J. Am. Coll. Nutr. 18, 413–423.

Sandoval, M., Okuhama, N.N., Zhang, X.J., Condezo, L.A., Lao, J., Angeles', F.M., Musah, R.A., Bobrowski, P., Miller, M.J., 2002. Anti-inflammatory and antioxidant activities of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*) are independent of their alkaloid content. Phytomedicine 9, 325–337.

Sandoval-Chacon, M., Thompson, J.H., Zhang, X.J., Liu, X., Mannick, E.E., Sadowska-Krowicka, H., Charbonnet, R.M., Clark, D.A., Miller, M.J., 1998. Antiinflammatory actions of cat's claw: the role of NF-kappaB. Aliment Pharmacol. Ther. 12, 1279–1289.

Schapira, A.H., Gu, M., Taanman, J.W., Tabrizi, S.J., Seaton, T., Cleeter, M., Cooper, J.M., 1998. Mitochondria in the etiology and pathogenesis of Parkinson's disease. Ann. Neurol. 44, S89–S98.

Shastry, B.S., 2003. Neurodegenerative disorders of protein aggregation. Neurochem. Int. 43, 1–7.

Sheng, Y., Bryngelsson, C., Pero, R.W., 2000. Enhanced DNA repair, immune function and reduced toxicity of C-MED-100, a novel aqueous extract from *Uncaria tomentosa*. J. Ethnopharmacol. 69, 115–126.

Sheng, Y., Li, L., Holmgren, K., Pero, R.W., 2001. DNA repair enhancement of aqueous extracts of *Uncaria tomentosa* in a human volunteer study. Phytomedicine 8, 275–282.

Tirmenstein, M.A., Hu, C.X., Scicchitano, M.S., Narayanan, P.K., McFarland, D.C., Thomas, H.C., Schwartz, L.W., 2005. Effects of 6-hydroxydopamine on mitochondrial function and glutathione status in SH-SY5Y human neuroblastoma cells. Toxicol. In Vitro 19, 471–479.

Van Ham, T.J., Thijssen, K.L., Breitling, R., Hofstra, R.M.W., Plasterk, R.H.A., et al., 2008. *C. elegans* model identifies genetic modifiers of α-synuclein inclusion formation during aging. PLoS Genet. 4 (3), e1000027. http://dx.doi.org/10.1371/journal.pgen.1000027.

Yang, H., Wu, T., Zhang, M., Zhang, Z., 2001. A novel photosensitizer, 2-butylamino-2-demethoxy-hypocrellin B (2-BA-2-DMHB) – its photodynamic effects on HeLa cells: efficacy and apoptosis. Biochim. Biophys. Acta 1540, 22–31.

Zhao, B., 2009. Natural antioxidants protect neurons in Alzheimer's disease and Parkinson's disease. Neurochem. Res. 34, 630–638.