ORIGINAL RESEARCH

Ethanolic Extract of Fructus *Alpinia oxyphylla* Protects Against 6-Hydroxydopamine-Induced Damage of PC12 Cells In Vitro and Dopaminergic Neurons in Zebrafish

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Abstract In an attempt to understand the neuroprotective effect of Fructus *Alpinia oxyphylla* (AOE) and to elucidate its underlying mechanism of action, the ethanolic extract of AOE was investigated using zebrafish and PC12 cell models. AOE prevented and restored 6-hydroxydopamine (6-OHDA)-induced dopaminergic (DA) neuron degeneration and attenuated a deficit of locomotor activity in a zebrafish (*Danio rerio*) model of Parkinson's disease (PD). Treatment with AOE increased the viability of 6-OHDA-treated PC12 cells in vitro in a dose-dependent manner by attenuating cellular apoptosis. However, protocatechuic acid (PCA) and chrysin, two known polyphenol components of AOE, could not reproduce the neuroprotective activity of AOE in the PD zebrafish or PC12 cell models. A mechanistic study found that the protective effect of AOE against 6-OHDA-induced neuronal injury involved anti-inflammatory action (down-regulation of gene expression of IL-1β and TNF-α) and anti-oxidative action (inhibition of NO production and iNOS expression in PC12 cells). Moreover, the PI3K-AKT pathway might be part of the mechanism of neuroprotection of AOE. The results of this research are expected to provide a scientific rationale for the use of AOE in the treatment of PD. However, it is important that the active components that contribute to the neuroprotective action of AOE are identified and characterized.

Keywords Fructus *Alpinia oxyphylla* · Neuroprotection · Parkinson's disease · Zebrafish · PC12 cell

List of Abbreviations

AOE	Ethanolic extract of Fructus *Alpinia oxyphylla*
PCA	Protocacheuic acid
F-12K	Kaighn's modification of Ham's F12 medium
MTT	3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
PBS	Phosphate-buffered saline
6-OHDA	6-Hydroxydopamine
IL-1b	Interleukin 1 beta
TNF-a	Tumor necrosis factor alpha
L-NAME	Nω-nitro-L-arginine methyl ester hydrochloride
SNP	Sodium nitroprusside dehydrate
CNS	Central nervous system
PD	Parkinson's disease
DA	Dopaminergic
dpf	Days post fertilization
nom	Nomifensine

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Introduction

Parkinson's disease (PD) in humans results primarily from the death of dopaminergic (DA) neurons in the substantia nigra. Recent research in the pharmacotherapy of PD has identified numerous agents for the symptomatic control of motor impairments, but none is able to prevent, slow, or halt the progression of the disease (Dunnett and Bjorklund 1999). The main obstacle to developing neuroprotective therapies is our limited understanding of the key molecular events that provoke neurodegeneration. Earlier studies highlighted the pathological involvement of oxidative stress, neuroinflammation, excitotoxicity, and apoptosis in neurodegenerative diseases (Barnham et al. 2004; Lin and Beal 2006). Because PD, as well as other neurodegenerative disorders, usually has multi-factorial etiopathogenesis, multiple drug therapy is required to address the varied pathological aspects (Li et al. 2009; Weinreb et al. 2010). Multiple drug strategy has been the essence of the rationales used for formulating traditional Chinese medicines (TCMs) for thousands of years. TCMs contain a mixture of chemical components from a single herb or a combination of several herbs and thus versatile functions, and possess great potential in the multi-target approach for the improved treatment of complicated diseases, such as PD.

Fructus *Alpinia oxyphylla* is called Yi Zhi in Chinese, which translates into English as beneficial to intelligence, is commonly used in TCMs and as plant food. In traditional systems of folk medicine, it has been used for treating diarrhea with splenic cold, polyuria, gastralgia, spontaneous salivation, kidney asthenia with enuresis, spermatorrhea, and turbid urine (But 1997). Recently, there has been increasing evidence of the beneficial effects of Fructus *Alpinia oxyphylla* (AOE) on various neurodegenerative diseases. Treatment with the aqueous extract of AOE attenuated the death of cortical astrocytes induced by amyloid-β (Aβ) in vitro, prevented ischemia-induced learning disability and rescued hippocampal CA1 neurons from lethal ischemic damage in mice (Koo et al. 2004). Treatment with the ethanolic extract of AOE in the presence of glutamate significantly enhanced viability and reduced apoptosis in cortical neuron culture (Yu et al. 2003). Moreover, protocatechuic acid (PCA), a phenolic acid naturally present in AOE, was shown to protect PC12 cells against hydrogen peroxide and MPP$^+$-induced apoptosis, as well as rotenone-induced mitochondrial dysfunction (Guan et al. 2006a, b; Liu et al. 2008) and effectively promoted cell proliferation and reduced basal apoptosis in cultured neuronal stem cells (NSCs) (Guan et al. 2009). Chrysin, a flavonoid naturally occurring in many food plants, including AOE, protected mesencephalic cultures from injury caused by MPP$^+$ (Mercer et al. 2005) and was found to be a potent monoamine oxidase inhibitor (MAOI)

(Sloley et al. 2000). These results show that AOE is a promising candidate for developing effective agents for the treatment of neurodegenerative clinical problems.

To our knowledge, all the studies of AOE in the literature used a neuronal cell damage model in vitro, and there is no report of a PD animal model in vivo. PD is a multifactorial neurodegenerative disorder; however, the protective effect of AOE against other etiopathological factors, such as the neurotoxin 6-hydroxydopamine (6-OHDA), is unknown and needs to be evaluated. The mechanisms underlying the neuroprotective effect of AOE on PD and the factors that contribute to this effect remain to be fully explored. The present study investigated the neuroprotective effect and the underlying mechanism of AOE and two of its naturally occurring polyphenol components, PCA and chrysin, in a 6-OHDA-treated zebrafish model and in PC12 cell culture.

Materials and Methods

Plant Material, Preparation, and Qualitative Analysis of the AOE

The AOE raw material was purchased from the Zhi-xin Herbal Company (Hong Kong) and authenticated by Dr Yu-ying Zong, an experienced pharmacognosist. A voucher herbarium specimen of the material used in this study is deposited with the Herbarium of the Institute of Chinese Medical Sciences (No. 0011), University of Macau, Macau SAR, China. For the preparation of AOE, the dried herbal material was ground to a powder in an electrical blender, 80% (v/v) aqueous ethanol was added, and the mixture was sonicated in an ultrasonic bath at 50°C for 30 min. The mixture was filtered using Whatman filter paper, and the residue was extracted twice more with 80% aqueous ethanol. The three filtrates were combined, concentrated in a rotary evaporator, dried in a moving air current and then stored at −20°C.

For quality control of AOE, chromatographic separation was done with an Agilent 1100 series HPLC system (Agilent Technologies, CA, USA) coupled to an ABI2000-QTRAP quadrupole-linear ion trap mass spectrometer (MS; Applied Biosystems/MDS Sciex, Concord, ON, Canada) with a TurboIon Spray ion source in both positive and negative mode, and a vacuum degasser using a GL Sciences Inertsil ODS-4 column (length, 250 mm; inner diameter, 4.6 mm; particle size, 5 μm) at ambient temperature. The mobile phase was 0.1% trifluoroacetic acid (TFA) (A) and acetonitrile (B) using a gradient elution of 15–70% B in 0–100 min, 70–100% B in 100–110 min. The flow rate was 0.8 ml/min, and the output from the column was monitored by measuring UV absorbance at 254 nm.

The flow was then directed to the electrospray ionization source of an API2000-QTRAP quadrupole-linear ion trap mass spectrometer. Mass spectra were collected in alternating polarity mode over a scan range of 100–1200 m/z. The system control software was an integration of Agilent Chemstation A.09.03 and Brucker MSD Trap Control 5.1. Evaluation of the UV and MS data was done with Agilent Chemstation A.09.03 and DataAnalysis 2.2, respectively. PCA and chrysin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, P.R. China), which both occur naturally in AOE, were selected as the chemical markers.

Fish Maintenance

The AB strain of wild-type zebrafish was used for this study. Embryos were collected after natural spawning, staged according to standard criteria, and raised synchronously at 28.5°C in embryo medium (13.7 mM NaCl, 540 μM KCl, pH 7.4, 25 μM Na_2HPO_4, 44 μM KH_2PO_4, 300 μM $CaCl_2$, 100 μM $MgSO_4$, 420 μM $NaHCO_3$, pH 7.4). No additional maintenance was required because the embryos receive nourishment from the attached yolk ball. Ethical approval for the animal experiments was granted by the Animal Research Ethics Committee, University of Macau, Macau SAR, China.

Anti-tyrosine Hydroxylase (TH) Whole Mount Immunostaining

Zebrafish larvae were fixed in 4% (v/v) paraformaldehyde in PBS for 5 h, rinsed, and stored at −20°C in EtOH. Whole-mount immunostaining was done by standard methods (Zhang et al. 2010). Briefly, fixed samples were blocked by 2% (v/v) lamb serum and 0.1% (w/v) BSA in PBST for 1 h at room temperature. A mouse monoclonal anti-tyrosine hydroxylase antibody (diluted 1:200 (v/v) in blocking buffer, MAB318, Millipore) was used as the primary antibody and incubated with the sample overnight at 4°C. The next day, samples were washed six times with PBST (30 min each wash), followed by incubation with secondary antibody according to the instructions that accompany the Vectastain ABC kit (Vector Laboratories, Inc.). After color development, the zebrafish were flat-mounted with 3.5% methylcellulose and photographed.

Locomotor Behavioral Test of Zebrafish

Zebrafish larvae at 3 dpf were treated with 6-OHDA in the absence or in the presence of AOE for 4 days. At 7 dpf, fish were transferred into 96-well plates (1 fish/well). Swimming behavior was monitored by an automated video tracking system (Zebrabox, Viewpoint, LifeSciences). The 96-well plate and camera were housed inside a Zebrabox, and the swimming pattern of each fish was recorded for 10 min. The total distance moved was defined as the distance (in cm) that the fish moved during one session (10 min).

Total RNA Extraction and Reverse Transcription and Quantitative Real-time PCR

Zebrafish embryos at 24 hpf were treated for 48 h with 6 μM or 12 μg/ml AOE in the presence or in the absence of 6-OHDA. Total RNA was extracted from 40 zebrafish embryos of each treatment group using the RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions. RNA was reverse transcribed to single-stranded cDNA using the SuperScript^{TM} III first-strand synthesis system for RT-PCR (Invitrogen^{TM}, USA), followed by real-time PCR using the TaqMan® Universal PCR Master Mix and TaqMan® gene expression assay primer for zebrafish interleukin 1 beta (IL-1β, assay ID Dr03114368_m1) and tumor necrosis factor alpha (TNF-α, assay ID Dr03126849_g1) in the ABI 7500 real time PCR system (all materials were from Applied Biosystems). The expression level of each gene was expressed as relative fold change ($\log_2$ ratio) calculated using the comparative C_t method as described (Livak and Schmittgen 2001), and β-actin was used as the internal reference.

Cell Culture

Stock cultures of rat pheochromocytoma cells (PC12) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in a humidified 5% (v/v) CO_2 atmosphere at 37°C in F-12K medium supplemented with 15% (v/v) heat-inactivated horse serum, 2.5% (v/v) FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cell culture materials were purchased from Gibco Invitrogen (Carlsbad, CA, USA). Wortmannin (cat. no. 681675), AKT inhibitor IV (cat. no. 124011), and ERK inhibitor (cat. no. 328006) were purchased from Calibochem (La Jolla, CA, USA).

MTT Assay

3-(4,5-Dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) is a tetrazolium salt that can be reduced to purple formazan by living cells. Cells were incubated at 37°C for 4 h in MTT solution (prepared in fresh 0.5% (v/v) heat-inactivated horse serum; final concentration 0.5 mg/ml). The medium was then discarded, and 150 μl of DMSO was added to each well to dissolve the violet formazan crystals in intact cells. The absorbance at 490 nm was measured by a multi-label counter (Wallac VICTOR3TMV, Perkin Elmer,

Netherlands). Cell viability was expressed as a percentage of the control (untreated cells). All assays were done three times in eight replicates.

LDH Assay

Cell viability was determined by measuring the activity of lactate dehydrogenase (LDH) released into the incubation medium when cellular membranes are damaged. Cells were plated in 96-well plates, and the amount of LDH released was measured. Determination of total and released LDH activity was according to the instructions that accompany the Cytotoxicity Detection Kit (Roche).

Hoechst 33342 Staining

PC12 cells (8×10^4/well) grew in 12-well plate. After drug treatment, cells were washed with ice-cold phosphate-buffered saline (PBS) and fixed with 1% (v/v in PBS) formaldehyde, then stained with 10 μg/ml Hoechst 33342 (in PBS containing 5 μg/ml RNase) for 15 min at room temperature. The nuclei were visualized with a fluorescence microscope (Carl Zeiss, Axiovert 200, USA) equipped with a camera (Carl Zeiss, AxioCam HRc, USA).

Intracellular Nitric Oxide (NO) Staining

Intracellular NO was evaluated with the fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate). The cells were seeded in black, 96-well, clear-bottomed plates. After treatment, cells were washed with PBS, and 2.5 μM DAF-FM diacetate (diluted in PBS) was added for staining in the dark at room temperature for 30 min. The cells were then washed twice in PBS, and the fluorescence was evaluated in a microplate reader at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The increase in fluorescence for each treatment was calculated as the relative fluorescence of each treatment compared to that of the untreated control cells.

Western Blot Analysis

After treatment, PC12 cells were washed three times with PBS and then with RIPA lysis buffer containing 1 mM PMSF and 1% Protease Inhibitor Cocktail and incubated for 30 min on ice. Cell lysates were centrifuged at 12,500g for 20 min at 4°C, the supernatant was separated, and the amount of protein was determined using the BCA protein assay kit. Protein samples (30 μg) were separated by SDS-PAGE (12% (w/v) polyacrylamide gel) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Subsequently, the membrane was blocked with 5% (v/v) non-fat milk in PBST (PBS containing 0.1% (v/v) Tween 20) at room temperature for 1 h. The blots were incubated overnight at 4°C with primary antibodies (1:1000, Cell Signaling Technology, Beverley, MA, USA), and β-actin was used as the internal reference. After three washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, Cell Signaling Technology) in PBST with 5% non-fat milk at room temperature for 1 h. After repeated washing with PBST, proteins were visualized with an ECL advanced western blotting detection kit (Amersham, UK) according to the manufacturer's protocol. Photographs of protein bands were taken with a Molecular Imager ChemiDoc XRS (Bio-Rad, Hercules, CA). Quantitative assessment of protein bands was done with Gel DocTM XRS equipped with QuantityOne software detection kit (Amersham, UK) according to the manufacturer's protocol. Photographs of protein bands were taken with a Molecular Imager Chemi-Doc XRS (Biorad, USA). Quantitative assessment of protein blots was performed using Gel Doc^{TM} XR (Biorad, Hercules, CA) equipped with QuantityOne software.

Statistical Analysis

Measurements were done three times independently for multiple biological samples. The data were analyzed using GraphPad Prism V4.0 (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) and Dunnett's test were used to evaluate the statistical differences. The value of statistical significance was set at $P < 0.05$.

Result

Chemical Analysis of AOE

The yield of dried AOE was 914.5 g from 10 kg of fresh AOE. Figure 1 shows the HPLC chromatograms and MS analysis of AOE and the reference chemical standards PCA and chrysin. The two standards were identified in AOE by direct comparison of retention time, UV absorption profile, and MS data with the authentic reference standards. The PCA and chrysin contents of AOE were 3.75 and 4.35%, respectively. The established HPLC fingerprinting of AOE could be used as a reference for the purpose of quality assurance for future experiments.

AOE, but not PCA or Chrysin, Prevented 6-OHDA-Induced DA Neuron Loss in Zebrafish

All the tyrosine hydroxylase-positive neurons in zebrafish diencephalons have been shown to be DA neurons (Rink
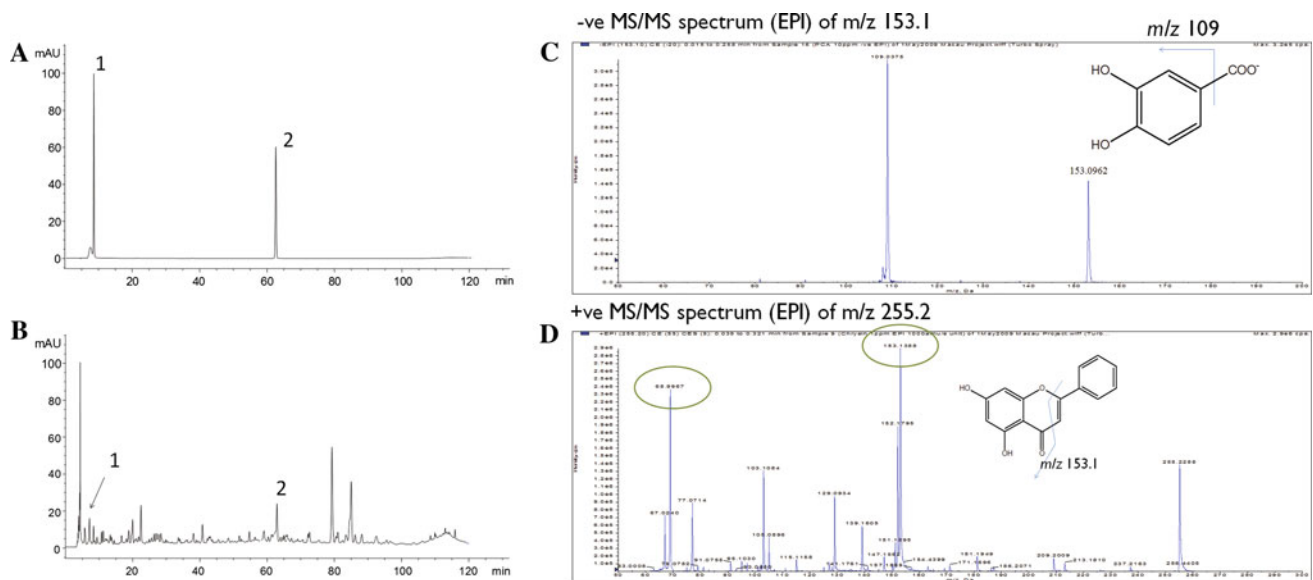
Fig. 1 Qualitative and quantitative analysis of AOE. Typical HPLC chromatograms of two chemical standards (**a**) and AOE (**b**). *Peak 1* PCA, *peak 2* chrysin. MS/MS spectra of **c** PCA (M$^-$, m/z 153) and **d** chrysin (M$^+$, m/z 255)

and Wullimann 2001). 6-OHDA is a hydroxylated analog of the natural neurotransmitter dopamine (Blum et al. 2001). Its induced toxicity is relatively selective for catecholaminergic neurons, resulting from a preferential uptake of 6-OHDA by dopamine and noradrenergic transporter molecules (Luthman et al. 1989). Exposure of 1 dpf zebrafish embryos to 250 μM 6-OHDA for 48 h resulted in a dramatic reduction of DA neurons in the ventral diencephalic clusters (Fig. 2b). Co-treatment with AOE attenuated 6-OHDA-induced DA neuron injury in a dose-dependent manner (Fig. 2d–f). Nomifensine (a dopamine transporter inhibitor), which can inhibit the transportation of 6-OHDA into DA neurons and abolish the neurotoxic effect of 6-OHDA (Lees et al. 1984), was used as a positive control. Nomifensine was found to prevent the loss of DA neurons in zebrafish caused by 6-OHDA (Fig. 2c).

PCA and chrysin, two naturally occurring polyphenols in AOE, have been demonstrated to be neuroprotective against oxidative stress, MPP$^+$, and amyloid-β (Aβ) (Barthwal et al. 2001; Mercer et al. 2005; Guan et al. 2006a, b; Liu et al. 2008), did not show a significant protective effect against 6-OHDA-induced DA neuron loss in zebrafish (Fig. 3). Toxicity of PCA to DA neurons was observed at the highest dose tested (100 μM PCA).

AOE Restored 6-OHDA-Induced DA Neuron Loss in Zebrafish

To further investigate if AOE exerted rescue effect in addition to above mentioned preventive effect against 6-OHDA induced DA loss, 1 dpf zebrafish embryos were treated with 6-OHDA for 48 h first and then replaced with

AOE for another 2 days. As shown in Fig. 4, compared to control group, 6-OHDA treatment resulted in about 30% DA neuron loss when the larvae were examined at 5 dpf. AOE dose-dependently restored DA neuron from injury caused by pre 6-OHDA treatment, and importantly, the DA neuron was almost completely recovered by high doses of AOE (6 and 12 μg/ml).

AOE Rescued the Deficit of Locomotor Activity in 6-OHDA-Treated Zebrafish

In mammals, injury of DA neurons affects mobility. In this study, treatment with 6-OHDA markedly reduced the swimming activity of zebrafish larvae. Typical swimming paths of untreated control and 6-OHDA-treated zebrafish larvae are shown in Fig. 5. The total swimming distance in 10 min was decreased significantly from 132.35±11.81 cm in the control group to 80.90±18.37 cm in the 6-OHDA treatment group ($P < 0.01$) (Fig. 5). AOE was as effective as the positive control drug nomifensine rescued the deficit of locomotor activity of 6-OHDA-treated zebrafish to normal. AOE alone did not significantly affect the locomotor behavior of normal zebrafish (Fig. 5).

AOE Reversed 6-OHDA-Induced Expression of Pro-inflammatory Genes in Zebrafish

Neuro-inflammation plays a key role in 6-OHDA-induced DA neuron damage in vivo (Cicchetti et al. 2002). Treatment with 6-OHDA increased the expression of IL-1β and TNF-α to several-fold higher than that of untreated control fish (Fig. 6). Co-treatment with AOE markedly decreased
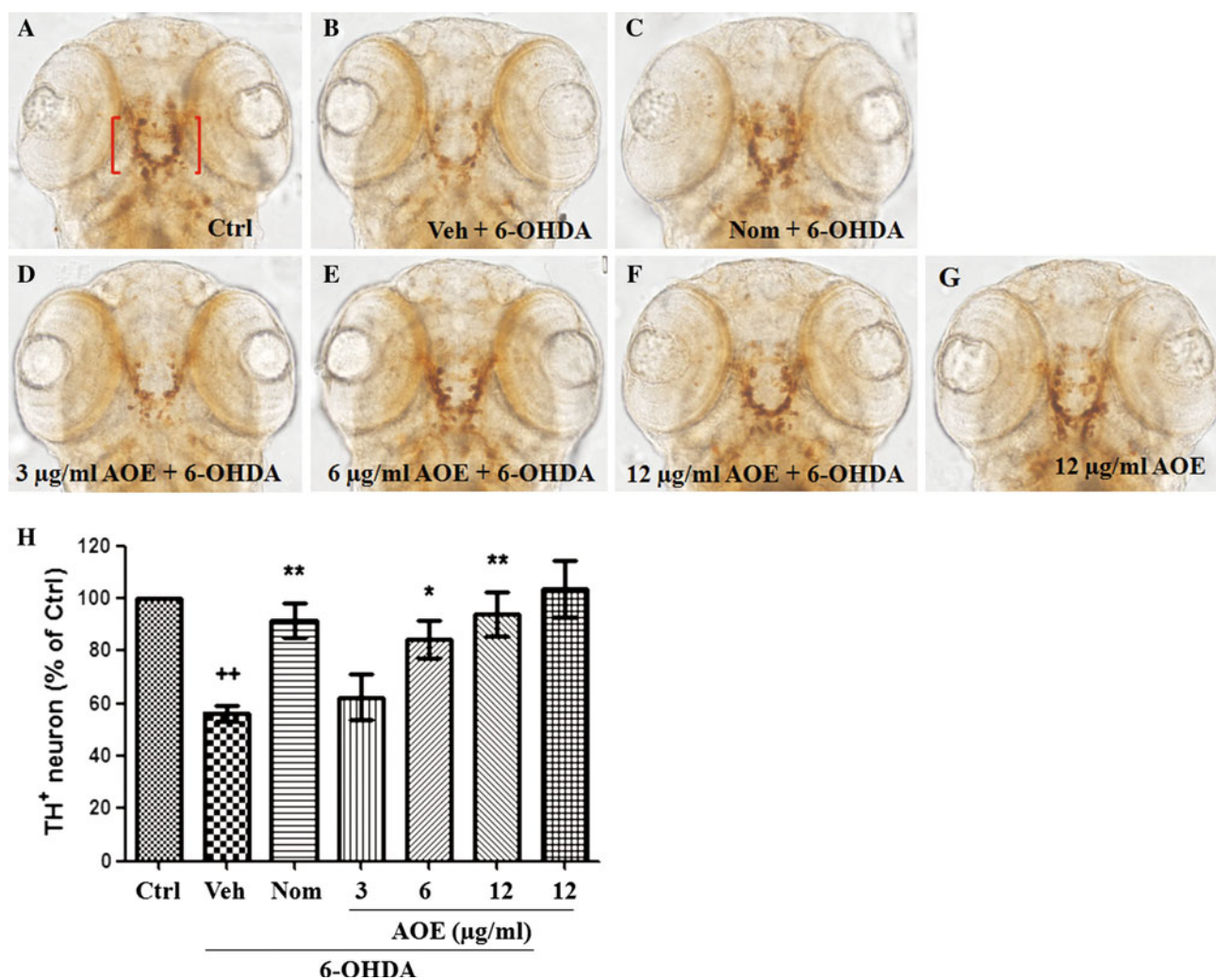
Fig. 2 AOE protected against 6-OHDA-induced DA neuron loss in zebrafish. Zebrafish at 1 dpf were exposed to different concentrations of AOE with or without 6-OHDA for 48 h. Then fish were fixed for whole mount immunostaining. **a–g** Representative morphology of DA neurons in zebrafish brain indicated by immunostaining with antibody against tyrosine hydroxylase (TH). TH⁺ neurons in the diencephalic region are within the brackets. Ventral view, head on top. **h** Statistical analysis of TH⁺ neurons in each group, 20 fish/group were used and experiments were repeated three times. Data are expressed as a percentage of the control group. $^{++}P < 0.001$ versus control (Ctrl) group (without 6-OHDA treatment); $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle (Veh) + 6-OHDA-treated group

the expression of those pro-inflammatory genes compared to 6-OHDA treatment alone; revealing that the anti-inflammatory activity of AOE is involved in its neuroprotective effect in the zebrafish model of 6-OHDA-induced neuronal damage.

AOE, but not PCA or Chrysin, Protected PC12 Cells from 6-OHDA-Induced Damage

To further study the neuroprotective effects of AOE in vitro, PC12 cells were treated for 12 h with AOE, which displayed no cytotoxicity to PC12 cells up to a maximum concentration of 50 μg/ml (Fig. 7a). Treatment with AOE increased the viability of 6-OHDA-treated PC12 cells in a dose-dependent manner (Fig. 7b) and significantly reduced LDH leakage,

a biochemical marker of cell membrane integrity, caused by treatment with 6-OHDA (Fig. 7c). The protective effect of AOE was greater than that of L-NAME, an NO inhibitor (LN, Fig. 7b, c). Moreover, similar to the results obtained in vivo, PCA and chrysin showed no protective effect against 6-OHDA-induced PC12 cell damage (Fig. 7d).

AOE Attenuated 6-OHDA-Induced Apoptosis in PC12 Cells

Apoptosis is morphologically characterized by cell shrinkage, chromatin condensation, and nuclear fragmentation (Bonfoco et al. 1995). Untreated control cells were visualized as circles or ellipses, and no condensation of the nucleus was observed (Fig. 8a). In contrast, bright
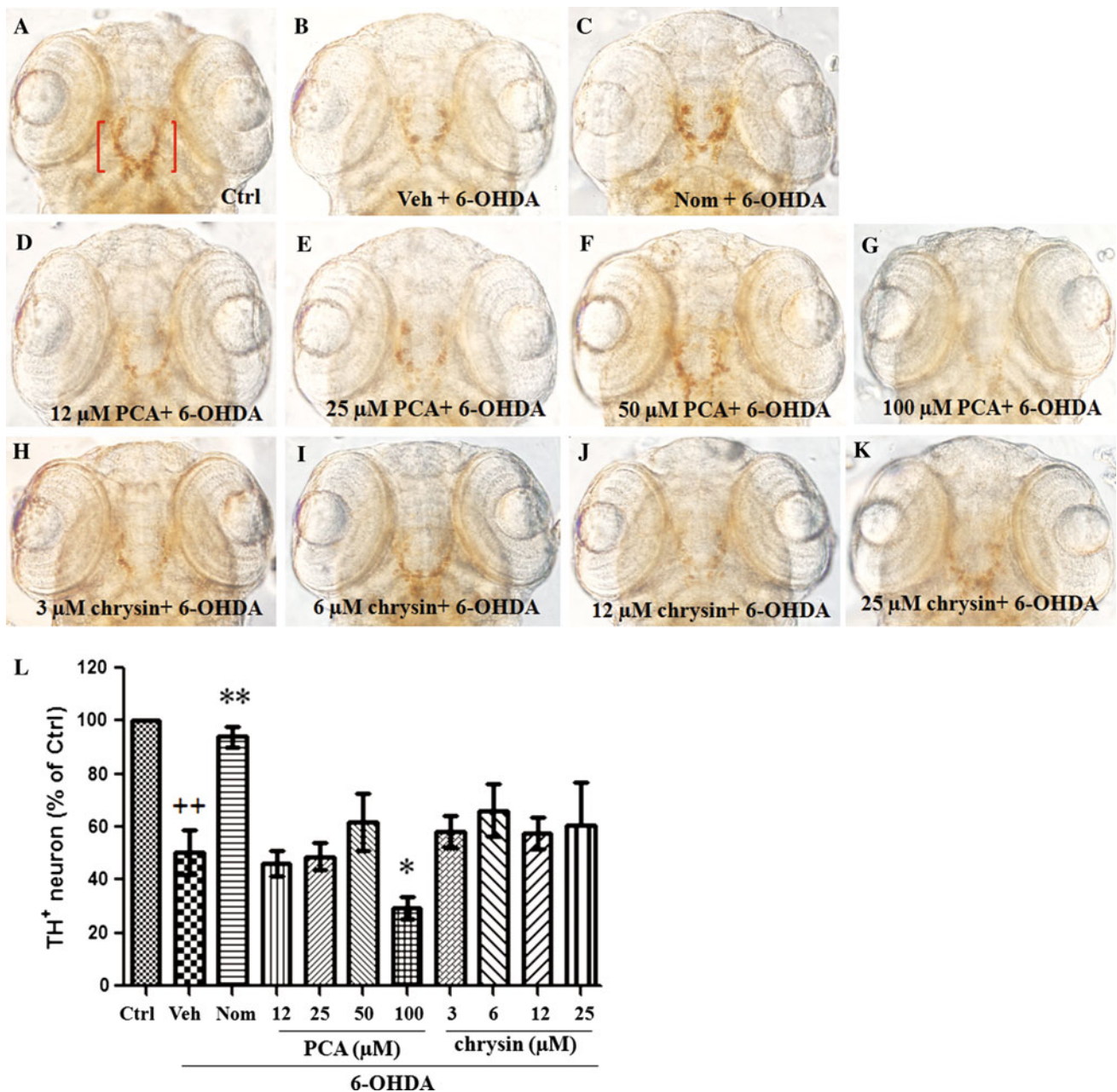
Fig. 3 PCA and chrysin did not protect against 6-OHDA-induced DA neuron loss in zebrafish. Zebrafish embryos at 1 dpf were exposed to different concentrations of PCA or chrysin with or without 6-OHDA for 48 h. Then, 3 dpf larvae were fixed for whole mount immunostaining. **a–k** Representative morphology of DA neurons in zebrafish brain indicated by immunostaining with antibody against TH. **l** Statistical analysis of TH^+ neuron in each group, 20 fish/group were used and experiments were repeated three times. Data are expressed as a percentage of the control group. $^{++}P < 0.001$ versus control (Ctrl) group (without 6-OHDA treatment), $^*P < 0.05$ and $^{**}P < 0.01$ versus Veh + 6-OHDA-treated group

condensed dots known as apoptotic bodies (indicated by arrows in Fig. 8b) were observed after exposure to 1 mM 6-OHDA for 6 h. Apoptotic bodies are generated when chromatin fragments are packaged in apoptotic cells and are commonly accepted as a marker of apoptosis. These changes in nuclear characteristics of apoptosis were attenuated when the cells were treated with different concentrations of AOE (Fig. 8d–f) and with L-NAME (Fig. 8c).

AOE Inhibited NO Production and Down-Regulated Inducible Nitric Oxidase Synthetase (iNOS) Expression in 6-OHDA-Treated PC12 Cells

Treatment with 6-OHDA resulted in ~2.5-fold increase of NO production compared to the control group (Fig. 9a). The increased level of NO was reduced in a concentration-dependent manner by treatment with AOE for 12 h
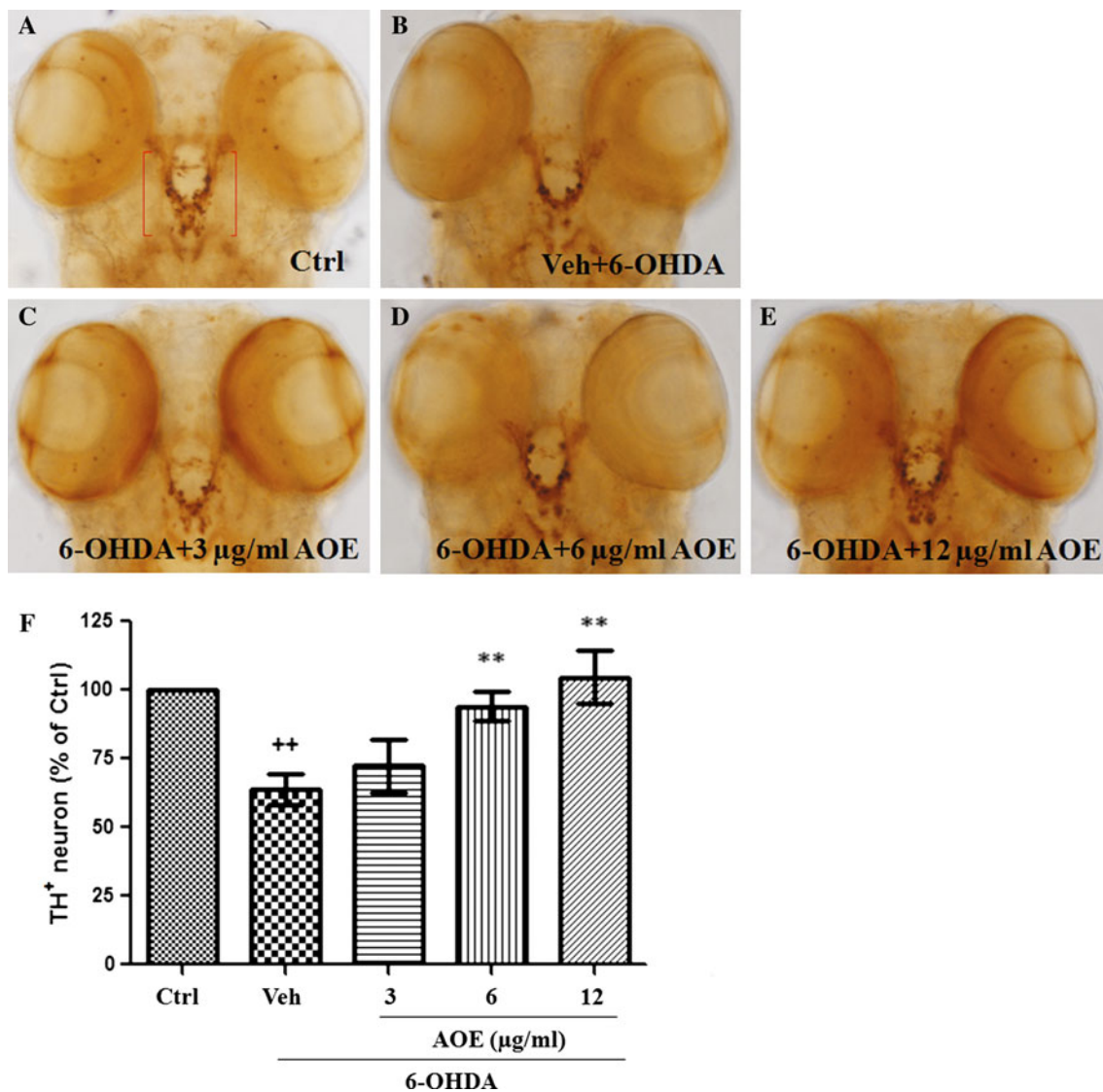
Fig. 4 AOE restored DA neuron loss induced by pre-6-OHDA treatment in zebrafish. Zebrafish embryos at 1 dpf were treated with 250 μM 6-OHDA for 48 h; then, 6-OHDA was washed out and replaced with AOE for another 48 h. Then, 5 dpf larvae were fixed for whole mount immunostaining. **a–e** Representative morphology of DA neurons in zebrafish brain indicated by immunostaining with antibody against TH. **f** Statistical analysis of TH$^+$ neuron in each group, 20 fish/group were used and experiments were repeated three times. Data are expressed as a percentage of the control group. $^{++}P < 0.01$ versus control (Ctrl) group (without 6-OHDA treatment), $^{**}P < 0.01$ versus Veh + 6-OHDA-treated group

(Fig. 9a). Treatment with L-NAME also significantly reduced NO production compared to that of the 6-OHDA group. The ability of AOE to reduce NO production at higher concentrations (25 and 50 μg/ml AOE) was better than that of L-NAME (Fig. 9a). We then examined whether AOE affected expression of the iNOS protein; exposure to 6-OHDA induced a threefold increase of the level of iNOS protein expression, and this up-regulation was diminished by pretreatment with AOE in a concentration-dependent manner (Fig. 9b, c).

The Neuroprotective Effect of AOE Involved Modulation of the PI3K-AKT Signaling Pathway

A time course study of 6-OHDA-treated PC12 cells showed that the level of expression of the phosphorylated form of AKT was decreased after treatment with 6-OHDA for 4 h, and this was maintained up to 8 h (data not shown). We found that treatment with 6-OHDA for >2 h reduced the phosphorylation of ERK (data not shown). We investigated which pathway was involved in
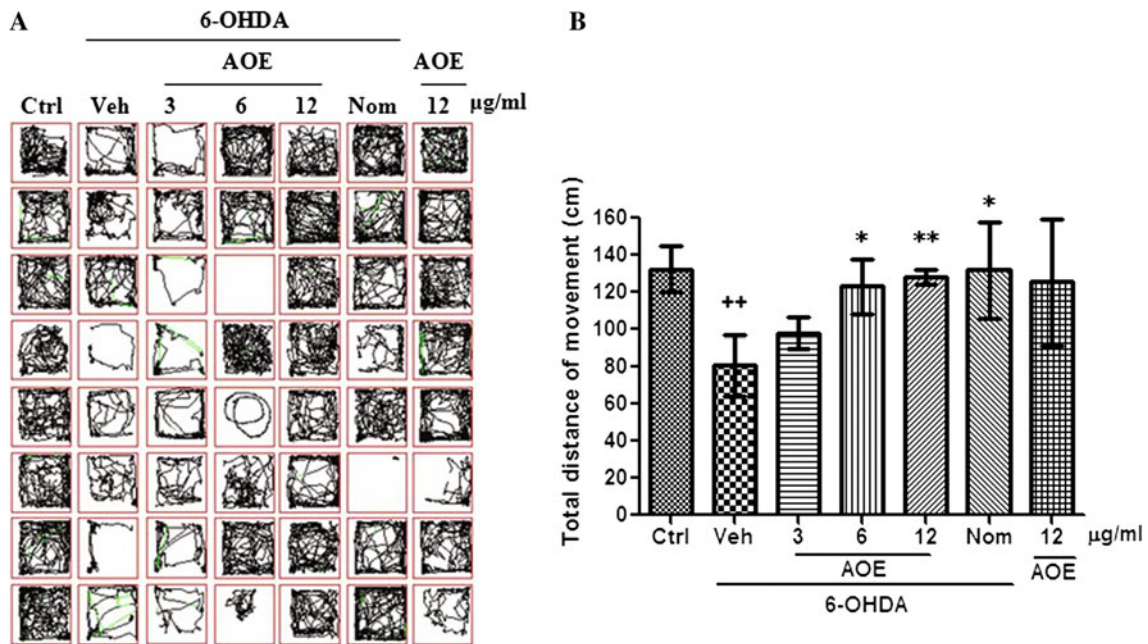
Fig. 5 AOE had a stimulatory effect on the locomotor behavior in 6-OHDA-treated zebrafish. Zebrafish larvae at 3 dpf were exposed to different concentrations of AOE with or without 6-OHDA for 4 days. After treatment, the locomotor activity of each treatment group was monitored. **a** Typical swimming patterns of control and different treatment group. **b** Quantitative analysis of total distance moved of each treatment group, eight fish/group were used and experiments were repeated three times. $^{++}P < 0.01$ versus control (Ctrl) group; $*P < 0.05$ and $**P < 0.01$ versus vehicle (Veh) + 6-OHDA-treated group
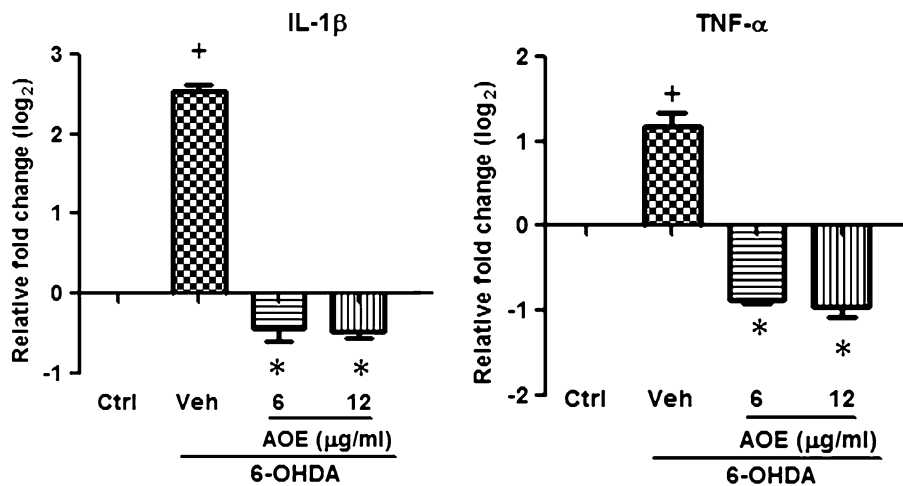
Fig. 6 AOE down-regulated mRNA expression of the 6-OHDA-activated pro-inflammation gene in zebrafish. Zebrafish embryos at 1 dpf were exposed to 6-OHDA with or without AOE for 48 h. After treatment, mRNA was extracted from each group, and quantitative real-time PCR analysis of pro-inflammatory genes IL-1β and TNF-α expression was done. The results are illustrated as the change in fold (log$_2$ ratio) calculated by the relative C_t method using β-actin as the internal reference. 30 fish/groups were used and experiments were repeated three times. $^{+}P < 0.05$ versus the control (Ctrl) group; $*P < 0.05$ versus vehicle (Veh) + 6-OHDA-treated group

the neuroprotective effect of AOE and found that pre-treatment with AOE could reverse the changes of the protein phosphorylation state induced by 6-OHDA; that is, up-regulating AKT and ERK phosphorylation (Fig. 10a, b). We asked whether the modulation of Akt and ERK signaling is essential for the neuroprotective effect of AOE, selective inhibitors of PI3K, Akt, and ERK were tested for their effect on the protective action

of AOE. We found that both Wortmannin (a PI3K inhibitor, cat. no. 681675, Calbiochem) and AKT inhibitor IV (Cat. No. 124011, Calbiochem) profoundly inhibited the neuroprotective action of AOE on 6-OHDA-induced PC12 cell damage (Fig. 10c), whereas ERK inhibition (ERK inhibitor, cat. no. 328006, Calbiochem) did not significantly affect the neuroprotective action of AOE (Fig. 10c).

Fig. 7 Effect of AOE, PCA, and chrysin on 6-OHDA-induced PC12 cell damage. PC12 cells were pretreated with AOE, PCA, or chrysin for 12 h and then exposed to 1 mM 6-OHDA for a further 12 h to induce cell damage. In 6-OHDA-induced cell damage, 250 μM L-NAME (LN) pretreatment for 12 h was used as the positive control.
a Cytotoxic assessment of AOE for 12 h exposure to PC12 cell by MTT assay.
b–d Neuroprotective effect of AOE, PCA, and chrysin on 6-OHDA-induced PC12 cell damage. Cell viability was measured by MTT assay (**b**, **d**) and by LDH release assay (**c**). $^{+++}P < 0.0001$ versus control (Ctrl) group (without 6-OHDA treatment); $*P < 0.05$, $**P < 0.01$ and $***P < 0.0001$ versus vehicle (Veh) + 6-OHDA treatment group, respectively
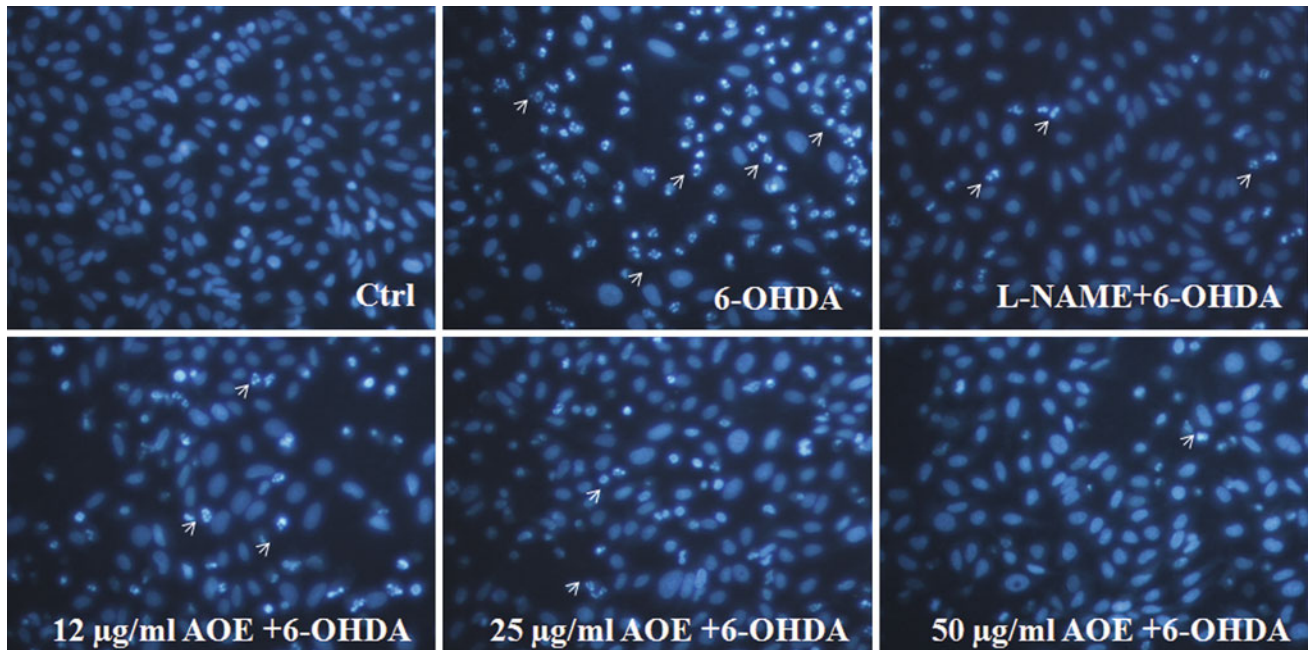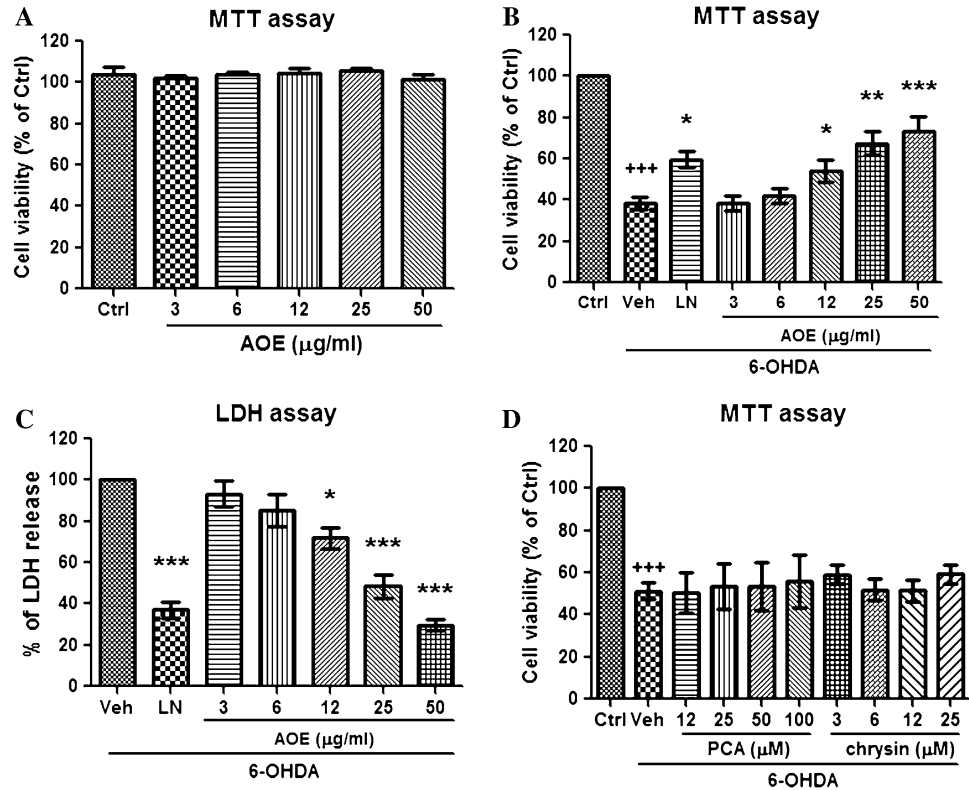

Fig. 8 AOE attenuated 6-OHDA-induced PC12 cell apoptosis. Cells were plated into 12-well plates at a density of 10^5/well and pretreated with AOE (12, 25, and 50 μg/ml) for 12 h, or with L-NAME (250 μM) as the positive control, then treated with 1 mM 6-OHDA for 6 h. Apoptotic cells were detected by staining with Hoechst 33342. *Arrows* indicate apoptotic cells

Discussion

In this study, AOE ethanolic extract was shown to possess neuroprotective and neurorestorative effects in a zebrafish neuronal damage model. AOE prevented and restored 6-OHDA-induced DA neuron loss, rescued the deficit of locomotor behavior as well as suppressed the activation of pro-inflammatory gene expression in zebrafish PD model.
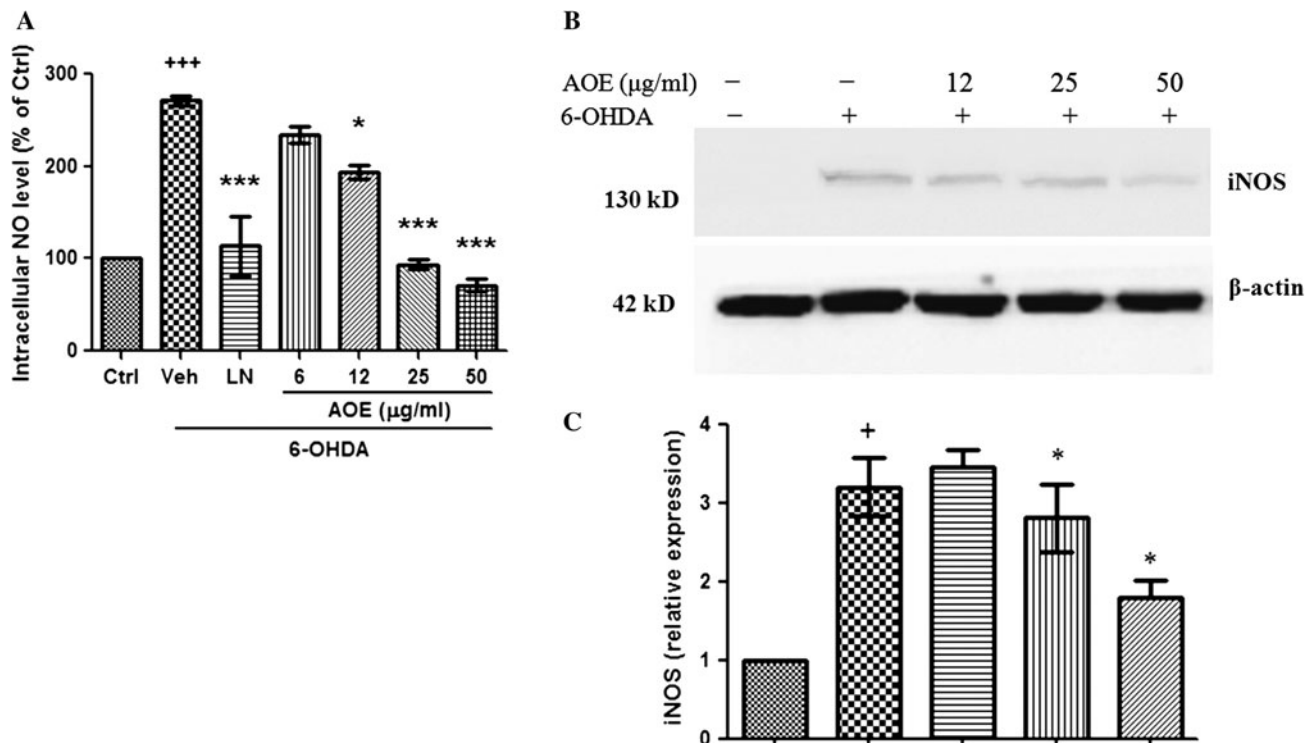
Fig. 9 AOE inhibited 6-OHDA-induced NO production and protein expression of iNOS in PC12 cells. PC12 cells were treated for 12 h with different concentrations of AOE or with 250 μM L-NAME (LN) as the positive control and then exposed to 1 mM 6-OHDA for a further 1 h. After treatment, intracellular NO was measured by DAF-FM diacetate staining and protein expression of iNOS was detected by western blotting. **a** Intracellular NO was measured by the fluorescent indicator DAF-FM diacetate. The NO fluorescence intensity was quantified by a multi-label counter. **b** A representative blot of iNOS expression in different treatment groups. **c** Densitometric analysis of protein blots. The results are expressed as the mean ± SEM for three independent experiments. $^+P < 0.05$ and $^{+++}P < 0.0001$ versus control (Ctrl) group (without 6-OHDA treatment); $^*P < 0.05$ and $^{***}P < 0.001$ versus 6-OHDA-treated group

We demonstrated the ability of AOE to increase the viability of 6-OHDA-treated PC12 cell significantly by preventing cell apoptosis. A mechanistic study showed that anti-inflammation, inhibition of iNOS-NO, and modulation of the PI3K-AKT signaling pathway were involved in the protective effect of AOE on either DA neuron loss of zebrafish and/or PC12 cell damage.

The zebrafish has become a powerful model for drug discovery (Crawford et al. 2008; Alex et al. 2010; Tang et al. 2010). A recent study showed that the anatomy of the DA system in the zebrafish brain is similar to that of mammals (Rink and Wullimann 2001). In the present study, exposure of zebrafish to 6-OHDA resulted in a significant loss of DA neurons and a reduction of locomotor behavior, which is consistent with earlier work (Anichtchik et al. 2004). Co-treatment and post-treatment with AOE prevented and restored the DA neuron loss and rescued the deficit of swimming behavior caused by 6-OHDA in zebrafish larvae. Nominfensine, a DAT inhibitor used as positive control, co-treatment was also effective through inhibition of 6-OHDA uptake by DA neuron to avoid neurotoxicity (Katz et al. 2010).

In zebrafish, 6-OHDA up-regulated expression of the mRNA of pro-inflammatory genes, such as IL-1β and TNF-α, which play important roles in the etiology of PD. The inhibition of TNF-α has been reported to provide neuroprotection in rats (Hunter et al. 2007). In the present study, co-treatment with AOE significantly suppressed pro-inflammatory gene stimulation by 6-OHDA. Thus, the neuroprotective effect of AOE in the 6-OHDA-induced zebrafish model could be explained, at least in part, by its anti-inflammatory action. Exposure to 6-OHDA results in the formation of reactive nitrogen species (RNS) and reactive oxygen species (ROS), which can contribute to neuronal cell injury and death (Zhang et al. 2010). It has been shown that the oxidative damage induced by 6-OHDA can be prevented by antioxidants (Guo et al. 2005), as well as by L-NAME, a non-specific NOS inhibitor that has been proved to protect PC12 cells against 6-OHDA-induced cytotoxicity by inhibiting NO production, and to exert significant neuroprotection in an in vivo PD model (Barthwal et al. 2001; Gao et al. 2003; Wang et al. 2011) This study showed that AOE has a protective effect in PC12 cell injury induced by 6-OHDA. Moreover, AOE

A

B

C
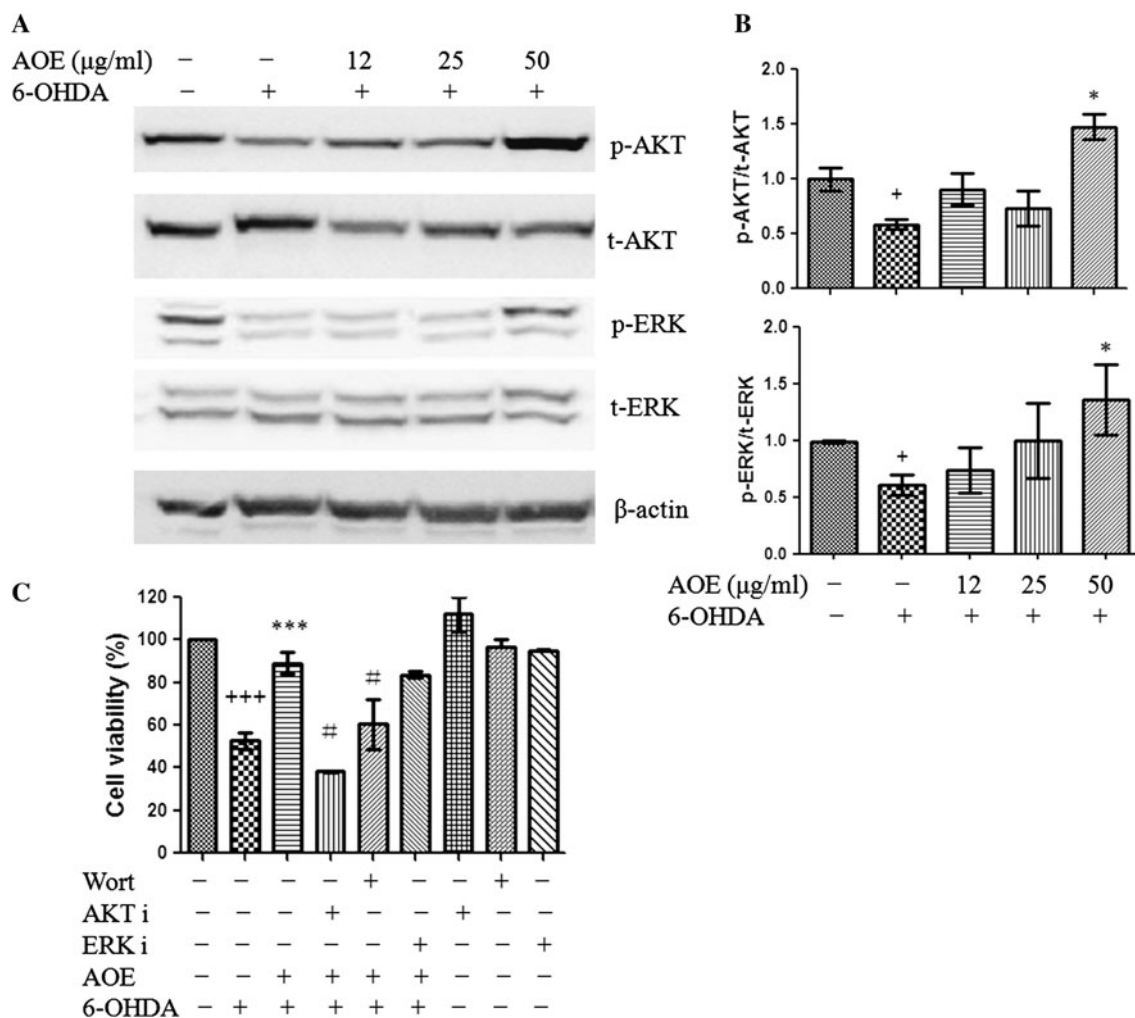
Fig. 10 Roles of ERK and PI3K-AKT signalling pathways in the neuroprotective action of AOE on 6-OHDA-induced PC12 cell damage. **a** PC12 cells were treated with 1 mM 6-OHDA for 4 h or with different concentrations of AOE for 12 h then with 1 mM 6-OHDA for 4 h. Expression of the phosphorylated form of AKT and ERK and the corresponding total proteins were determined by western blot. A representative blot is shown. **b** Densitometric analysis of protein blots. The results are expressed as the mean ± SEM for three independent experiments. $^{+}P < 0.05$ versus control (Ctrl) group (without 6-OHDA treatment); $^{*}P < 0.05$ versus 6-OHDA-treated group. **c** PC12 cells were treated with 0.5 μM Wortmannin (Wort) or 0.1 μM AKT inhibitor IV (AKT i) or 1 μM ERK inhibitor (ERK i) for 2 h, then incubated with or without 50 μg/ml AOE for 12 h before adding 1 mM 6-OHDA for a further 12 h. Cell viability was measured by MTT assay. $^{+++}P < 0.001$ versus control (Ctrl) group (without 6-OHDA treatment); $^{***}P < 0.01$ versus 6-OHDA-treated group; $^{#}P < 0.05$ versus AOE + 6-OHDA-treated group

reduced NO production and down-regulated the expression of iNOS induced by 6-OHDA. The potency of higher concentrations of AOE is stronger than that of L-NAME. These results suggest that AOE exerts a protective effect in the PC12 cell through anti-oxidative action and suppression of the iNOS–NO pathway.

The PI3K-Akt and MAPK pathways play important roles in neuronal survival in both physiological and pathological states (Brunet et al. 2001; Klein and Ackerman 2003; Chong et al. 2005). In this study, we found a decrease of phosphorylation of AKT and ERK after treatment with 6-OHDA, which is consistent with earlier work (Rodriguez-Blanco et al. 2008). Pre-treatment with AOE reversed the changes of the MAPK protein

phosphorylation state induced by 6-OHDA. Moreover, we found that both Wortmannin (a PI3K inhibitor, inhibition of Akt phosphorylation) (Rodriguez-Blanco et al. 2008) and AKT inhibitor significantly inhibited the neuroprotective action of AOE. However, ERK inhibitor only moderately attenuated the neuroprotective action of AOE. Thus, whether up-regulated phosphorylation of ERK is involved in the neuroprotection of AOE requires further investigation. Wortmannin and AKT inhibitor blocked the effect of AOE almost completely, implying that activation of these kinases is a key step in its mechanism of action.

To identify the neuroprotective constituents in AOE, PCA and chrysin were tested in 6-OHDA-induced PD

zebrafish and PC cell models, because these two compounds have been reported to exhibit neuroprotective effects against other etipopathological factors, such as oxidative stress, MPTP, and amyloid-β (Sloley et al. 2000; Guan et al. 2006a, b; Shi et al. 2006; Liu et al. 2008). Moreover, our chemical analysis found that the abundance of PCA and chrysin in AOE is 3.75 and 4.35‰, respectively. In this study, PCA alone (up to 100 μM) and chrysin alone (3–25 μM) did not exhibit a marked protective effect in the zebrafish or PC12 cell model. These results suggest that other components of AOE (as the uncharacterized peaks shown in Fig. 1b) might contribute to its neuroprotective effect against 6-OHDA toxicity. In future study, we will try to isolate and identify the active compounds from the ethanolic extract of Fructus *Alpinia oxyphylla*. Furthermore, the interaction of different AOE components should be investigated for their additive and/or synergistic contribution to neuroprotection.

In conclusion, the present study shows that AOE exerts a protective effect against neuronal insult in zebrafish and PC12 cell damage and multiple mechanisms of action might be involved in this neuroprotective activity. These observations imply that AOE can be of value in the treatment of PD. However, the active components of AOE need to be identified and characterized and the neuroprotective effect of AOE in rodent PD models should be the subject of future studies.

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