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Biruloquinone, an Acetylcholinesterase Inhibitor Produced by Lichen-Forming Fungus *Cladonia macilenta*

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At present, acetylcholinesterase (AChE) inhibitors are the first group of drugs to treat mild to moderate Alzheimer's disease (AD). Although beneficial in improving cognitive and behavioral symptoms, the effectiveness of AChE inhibitors has been questioned since they do not delay or prevent neurodegeneration in AD patients. Therefore, in the present study, in order to develop new and effective anti-AD agents from lichen products, both the AChE inhibitory and the neuroprotective effects were evaluated. The AChE inhibitory assay was performed based on Ellman's reaction, and the neuroprotective effect was evaluated by using the MTT method on injured PC12 cells. One AChE inhibitor (IC_{50} = 27.1 μg/ml) was isolated by means of bioactivity-guided isolation from the extract of lichen-forming fungus *Cladonia macilenta*, which showed the most potent AChE inhibitory activity in previous screening experiment. It was then identified as biruloquinone by MS, and ^{1}H- and ^{13}C-NMR analyses. The inhibitory kinetic assay suggested that biruloquinone is a mixed-II inhibitor on AChE. Meanwhile, biruloquinone improved the viability of the H_2O_2- and β-amyloid-injured PC12 cells at 1 to 25 μg/ml. The protective effects are proposed to be related to the potent antioxidant activities of biruloquinone. These results imply that biruloquinone has the potential to be developed as a multifunctional anti-AD agent.

Key words: Acetylcholinesterase inhibitor, biruloquinone, *Cladonia macilenta*, lichen-forming fungus

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Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory loss and cognitive impairment. It is the most common type of dementia in the aging population, affecting approximately 3% of the population between the ages of 65 and 74, and nearly 50% of those 85 years and older. AD has become one of the most costly diseases, which brings heavy social and financial burden to both society and families [20]. At present, acetylcholinesterase (AChE) inhibitors are the first group of drugs approved by the Food and Drugs Administration (FDA) to treat mild to moderate Alzheimer's disease. AChE hydrolyzes acetylcholine at cholinergic synapses to terminate nerve impulse transmission. Inhibition of AChE increases the availability of acetylcholine to sustain nerve cell communications, and therefore chemical inhibitors as possible leads for nervous system disorders are being searched for [7]. Although beneficial in improving cognitive and behavioral symptoms, the effectiveness of AChE inhibitors has been questioned since they do not delay or prevent neurodegeneration [2, 16, 19]. Increasing evidence supports the role for oxidative damage in the pathogenesis of AD, and neuronal degeneration of AD patients is associated with oxidative damage to all biomacromolecule types [8, 14]. These implicated that the therapeutic strategies aimed at removal of free radicals or prevention of their formation might be a reasonable choice for the management of the disease, so antioxidants have been considered as a potential treatment of AD [15].

Lichens synthesize a great variety of secondary metabolites that are often structurally unique, with only a small number of them being found in other fungi and higher plants [17]. Recently, much attention has been paid to the biological roles of lichen secondary substances; many lichen substances have been found to have numerous bioactivities,

such as antitumour, antibacterial, antifungal, antiviral, antiinflammatory, and also antioxidant activities [12]. However, the therapeutical utilization of lichen substances against AD had never been evaluated.

For the purpose of developing new anti-AD agents from lichen secondary metabolites, we conducted a screening for 109 extracts of lichen-forming fungi (LFF), and found that the extract of *Cladonia macilenta* exhibited the most potent AChE inhibitory activity (60.5%). Since lots of lichen secondary metabolites are phenolic compounds and possess potent antioxidant activities, we hypothesized that the AChE inhibitor in the extract of *C. macilenta* could be both AChE-inhibitory to alleviate symptoms of AD, and an antioxidant to protect the neurons from reactive oxygen species (ROS) elicited neurodegeneration.

Therefore, the purposes of this study were (i) to isolate and identify the AChE inhibitor(s) from the extract of *C. macilenta* through bioactivity-guided isolation, (ii) to investigate the action mechanisms of the AChE inhibitor(s), and (iii) to evaluate the neuroprotective effect of the AChE inhibitor(s) on injured PC12 cells.

Materials and Methods

Chemicals

Acetylcholinesterase (AChE) (E.C. 3.1.1.7, type VI-S from electric eel), acetylthiocholine iodide (ATCI), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 1,5-bis(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide, dimethylsulfoxide (DMSO), acetone, ethyl acetate, toluene, formic acid, chloroform, methanol, H_2O_2, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 (with L-glutamine), fetal bovine serum (FBS), horse serum, and antibiotic/antimycotic for cell culture were purchased from PAA: The Cell Culture Company (PAA-Strasse 1.4061 Pasching, Austria). Difco Potato Dextrose Broth was purchased from Becton, Dickinson and Company, USA. β-Amyloid (25–35) peptide was purchased from GenScript Company, USA.

Cells and Cell Culture

PC12 cells were purchased from the Korean Cell Line Bank (KCLB). They were cultured in growth medium RPMI-1640 with L-glutamine, 5% fetal bovine serum (FBS), 10% horse serum, and antibiotics. The cells were maintained in collagen-coated plates in 5% CO_2/95% air at 37°C. The culture medium was changed twice every week and the cells were split 1:4 every week.

Activity Guided Isolation and Identification of the AChE Inhibitor

Cladonia macilenta Hoffm. was collected in Yunnan Province, China in 2005. The voucher specimen (Specimen No. CH050136) was identified by Lisong Wang, curator of the lichen herbarium of Kunming Botanical Institute (L-KUN), and deposited at the herbarium. The lichen-forming fungus (LFF) of *C. macilenta* was isolated using ascospores by the discharged spore method [21] and deposited at the Korean Lichen and Allied Bioresource Center (KOLABIC), Sunchon National University, Korea. The LFF was subcultured in a bioreactor (20 L) with 15 L of potato dextrose broth medium and a continuous filtrated-air supply (10 L/min) in the dark at 15°C. After a 2-month incubation, the fermentation broth (12 L) was extracted with an equal volume of ethyl acetate for 24 h. The organic layer was filtrated through Whatman filter paper No. 1 to remove mycelia. This process was repeated twice, and the extracts were combined and concentrated in vacuum at 40°C. The concentrated extract (20 ml) was then loaded on a silica gel column (230–400 mesh, 3.580 cm), and eluted with toluene–ethyl acetate–formic acid [90:20:2 (v/v), 5 L]. This gave 12 fractions (Fr0–Fr11). Each fraction was concentrated in vacuum at 30°C. The AChE inhibitory assay was conducted for the 12 fractions based on Ellman's reaction [4], where Fr9 showed the strongest AChE inhibitory activity. The residue (1.2 g) was then further purified by recrystallization with chloroform–methanol [1:1 (v/v)] to give 410 mg of dark purple crystals. The purple crystals were then identified by MS and ^{1}H- and ^{13}C-NMR analyses in the Korea Research Institute of Chemical Technology (KRICT), Daejeon, Korea.

IC_{50} and Inhibition Kinetics Assay

IC_{50} (concentration of drug causing 50% enzyme activity inhibition) values were calculated by SPSS 15.0 software based on the concentration-inhibition curves. The inhibition curves were performed in triplicate by incubating five gradient concentrations of biruloquinone (6.25–100 μg/ml, final concentration) with the reaction mixture, as mentioned above. One triplicate sample without inhibitor was always present to yield 100% of AChE activity.

Kinetic studies were performed using AChE from electric eel. Enzyme activities were determined at 25°C using four concentrations of substrate (250, 500, 1,000, and 2,000 μM) in the presence or absence of two concentrations of biruloquinone (61 μM, 122 μM) against AChE. The inhibition type of biruloquinone on the enzyme was assayed by a Lineweaver–Burk plot. Plots of the slopes or intercepts versus the inhibitor concentrations gave estimates of K_I (the dissociation constant for inhibitor binding to enzyme) and K_{IS} (the dissociation constant for inhibitor binding to enzyme–substrate complex).

Cell Viability Assays

The cell viability was determined by conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [15]. PC12 cells (10^4/well) were plated into 96-well collagen-coated plates 24 h before the experiments. The cells were pre-incubated with different concentrations (25, 12.5, 6.75, and 1 μg/ml) of biruloquinone or with DMSO (0.5%, as negative control) for 30 min. Cells were then treated with 200 μM H_2O_2 for 2 h or with 50 μM $A\beta_{25-35}$ for 6 h at 37°C. MTT solution in phosphate buffered saline (pH 7.4) was added to the medium with the final concentration of 0.5 mg/ml. After 4 h incubation with MTT, cells were lysed in DMSO and the amount of MTT formazan was quantified by determining the absorbance at 570 nm using a microplate reader (VERSAmax, Molecular Devices, CA, USA). Cell viability was expressed as a percent of the control culture value.

Cellular Oxidative Stress Assay

The intracellular oxidative stress was quantified based on the 2',7'-dichlorofluorescein diacetate (DCFH-DA) method described by Keston and Brandt [5]. Viable cells (10^4/well) were plated into 96-well collagen-coated plates 24 h before the experiments. On the

experiment day, the cells were cultured with 100 μM DCFH-DA in the medium in collagen-coated plates in 5% CO_2/95% air at 37°C for 30 min. After DCFH-DA was removed, the cells were washed and incubated with RPMI medium. PC12 cells were then pretreated with various concentrations of biruloquinone (25, 12.5, 6.75, and 1 μg/ml) or with DMSO (0.5%, as negative control) for 30 min. The cells were then treated with or without 200 μM H_2O_2 for 30 min and the fluorescence was finally quantified using a fluorescent microplate reader (SpectraMax, Molecular Devices, CA, USA) equipped with 485 nm excitation and 530 nm emission filters. The results were expressed in percent relative to the oxidative stress of the control cells set to 100%.

In Vitro Toxicity Assay on PC12 Cells

PC12 cells were split and counted by trypan blue exclusion. Viable cells (10^4/well) were plated into 96-well collagen-coated plates 24 h before the experiments. On the experiment day, the cells were cultured with or without 10 μl of DMSO (final concentration: 0.5%) or gradient concentration of biruloquinone (6.25–100 μg/ml) for 3 h. The cell viability was then determined by the MTT method as mentioned above.

Statistical Analysis

All data are expressed as means ± SD. One-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test was performed to compare groups. Differences were considered statistically significant when $p < 0.05$.

Table 1. ^{1}H and ^{13}C NMR data for biruloquinone isolated from *Cladonia macilenta* in trifluoroacetic acid-d.

No.	^{13}C[a]	^{1}H(multi.)[b]
1	155.08	
1-OH		11.49(s)[c]
2	157.32	
3	124.06	7.12(s)
4	140.11	
4a	155.08	
4b	114.20	
5	117/05	
6	157.32	
7	110.44	7.00(s)
8	172.33	
8-OH		11.52(s)[c]
8a	107.99	
9	183.63*	
10	183.95*	
10a	111.26	
11	58.10	4.03(s)
12	161.73	
13	25.43	2.80(s)

[a]125 MHz; [b]500 MHz
[c]These values may be interchanged.

RESULTS AND DISCUSSION

One active compound was isolated from secondary metabolites of *C. macilenta* LFF as dark crystals, and the MS (Fig. 2) and ^{1}H and ^{13}C NMR data (Table 1) of the isolated compound were identical to those of biruloquinone ($C_{17}H_{10}O_7$, Fig. 1) in the literature [1]. According to available literatures, biruloquinone was previously isolated from lichen thalli of *Parmelia birulae* (Parmeliaceae) [6]. Biruloquinone belongs to the *ortho*-phenanthraquinone compounds, which are extremely rare compounds among natural quinones [6]. In the present study, this compound was first isolated from fermentation broth of LFF belonging to the Cladoniacea family, and this is the first report describing its bioactivity.

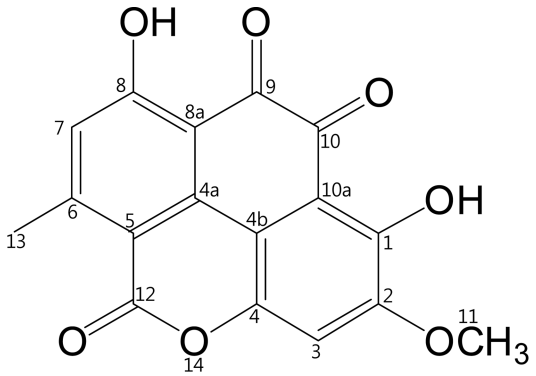

Fig. 1. Chemical structure of biruloquinone from *Cladonia macilenta.*

The inhibition of biruloquinone on AChE was in a dose-dependent manner. The inhibition percentage increased rapidly with the increase of biruloquinone concentration from 0 to 100 μg/ml. The concentration that was required for the 50% enzyme inhibition (IC_{50}) was 27.1 μg/ml (83.1 μM). Comparing with other AChE inhibitors, biruloquinone exerted lower inhibition than tacrine, which has been used in the treatment of neuronal disorder, and much lower potency than donepezil, an anti-Alzheimer's drug [11], but its activity is comparable with many other AChE inhibitors isolated from natural extracts [10].

The inhibition behavior of biruloquinone on AChE is shown in Fig. 3. The double-reciprocal plots yield a family of lines with different slopes and different intercepts, and they intersect one another in the third quadrant. The inhibition behavior of biruloquinone gave increasing slopes and growing y-axis intercepts with higher inhibitor concentration, indicating that biruloquinone is a mixed-II inhibitor (Fig. 3A). The equilibrium constants for the inhibitor binding with enzyme, K_I, and with enzyme–substrate complex, K_{IS}, are obtained from the second plots of the K_m/V_m (Fig. 3B) and $1/V_m$ (Fig. 3C) versus concentration of biruloquinone, respectively, which are linear. The K_I

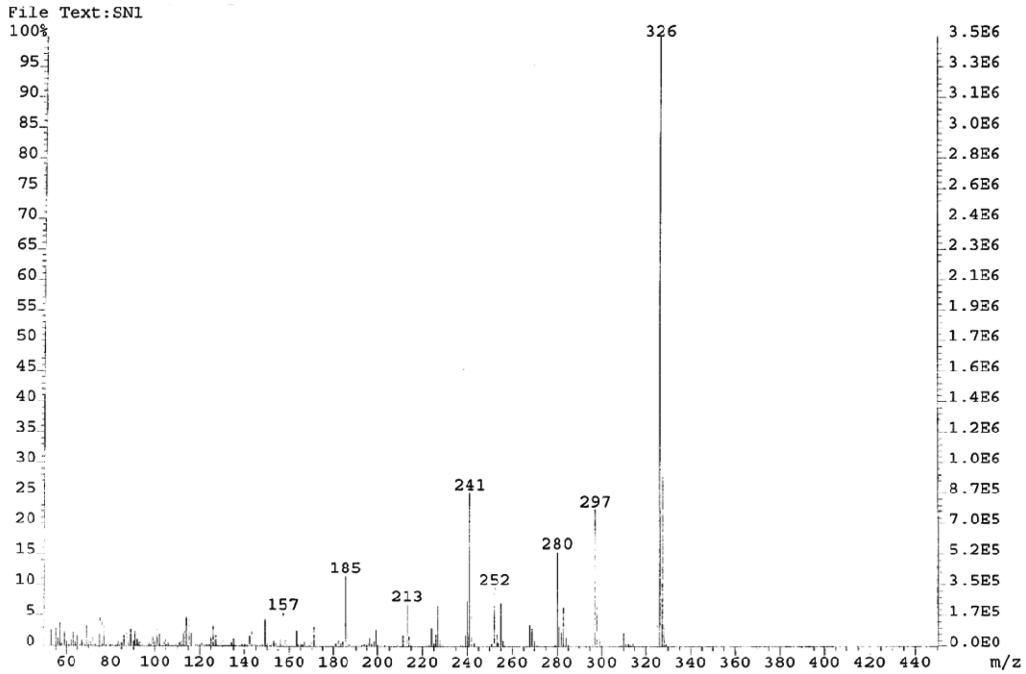

Fig. 2. The electron impact mass spectrum of biruloquinone.
Electron impact MS, *m/z* (%): 328 (6), 327 (29), 326 (100), 297 (23), 280(15), 252 (10), 241 (25).

(204 μM) was larger than that of K_{IS} (12.5 μM), indicating that the affinity of inhibitor for free enzyme is weaker than that for the enzyme–substrate complex.

The protective effects of biruloquinone on H_2O_2-induced cell injury are summarized in Fig. 4. As determined by MTT assay, cell viability was markedly decreased to about 50% after a 2 h exposure to 200 μM H_2O_2. However, when cells were pretreated with biruloquinone for 30 min, cell toxicity was significantly attenuated in a dose-dependent manner, indicating a potent neuroprotective activity of biruloquinone. Likewise, biruloquinone also showed protective effects on $A\beta_{25-35}$-induced cell injuries. As shown in Fig. 5, the viability of PC12 cells was significantly reduced to 60.3% of untreated cells after exposure to $A\beta_{25-35}$, the most

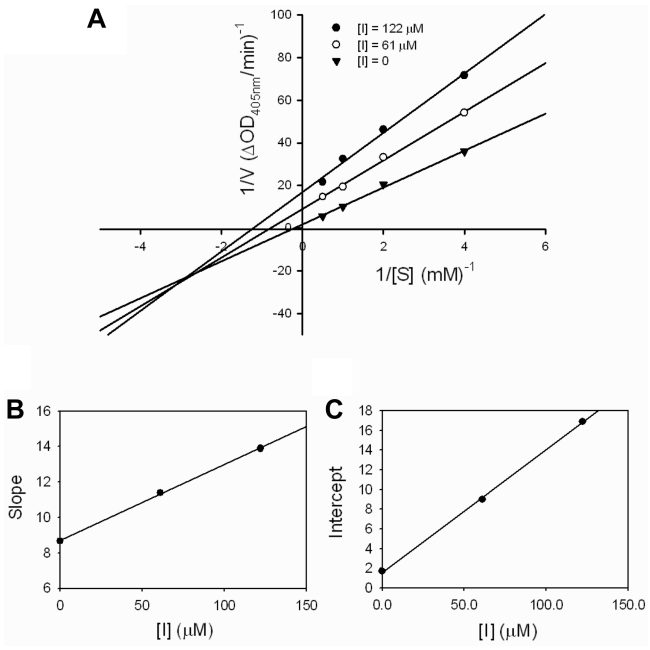

Fig. 3. The Lineweaver–Burk plot (**A**) and the plots of the K_m/V_m (**B**) and $1/V_m$ (**C**) versus concentration of biruloquinone on AChE.

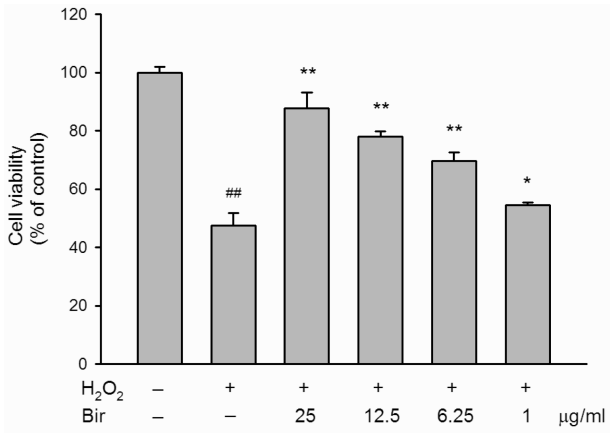

Fig. 4. The protective effects of biruloquinone on H_2O_2-induced cell injuries.
All data are presented as mean ± SD of three independent experiments. A difference was considered statistically significant when $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. H_2O_2-treated control groups or $^{\#\#}p < 0.01$ vs. DMSO-treated control groups.

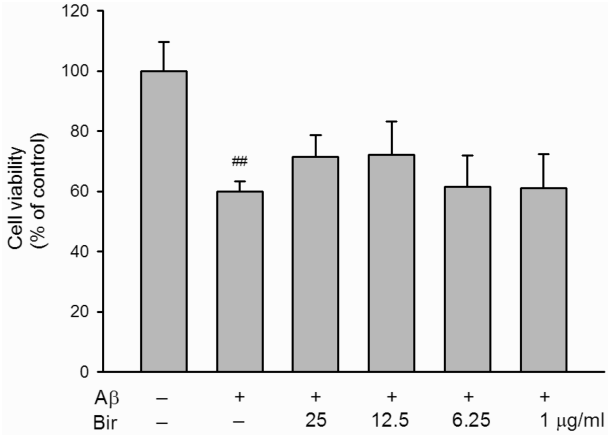

Fig. 5. The protective effects of biruloquinone on $A\beta_{25-35}$-induced cell injuries.
All data are presented as mean ± SD of three independent experiments. A difference was considered statistically significant when $^{\#\#}p < 0.01$ vs. DMSO-treated control groups.

toxic peptide fragment derived from amyloid precursor for 6 h, which indicates a serious cell toxicity of Aβ. However, when cells were pretreated with biruloquinone (25 μg/ml) for 30 min, the cell viability was increased to 71.5%, suggested a protective effect of biruloquinone, whereas it was not significantly different from that of untreated cells.

PC12 cells exposed to H_2O_2 for 30 min displayed a significant increase (about 1.5-fold) in the intracellular level of ROS relative to the levels seen in control cells. Cells pretreated with gradient concentrations of biruloquinone (1 μg to 25 μg) before H_2O_2 exposure markedly reduced the ROS levels. However, 6.25 μg and 1 μg of biruloquinone had no significant effect on the ROS levels of the H_2O_2-treated PC12 cells (Fig. 6).

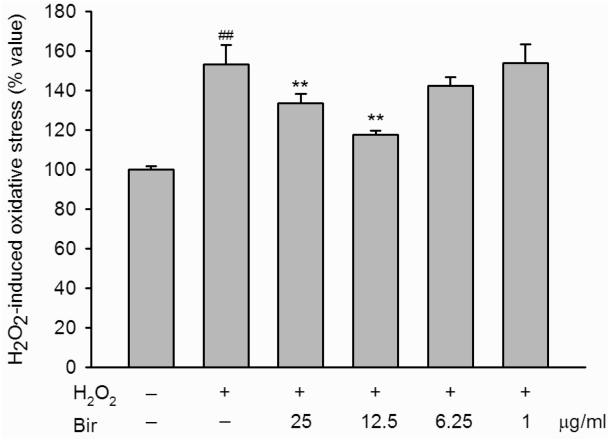

Fig. 6. The effect of biruloquinone on the intracellular oxidative stress of PC12 cells.
All data are presented as mean ± SD of three independent experiments. A difference was considered statistically significant when $^{**}p < 0.01$ vs. H_2O_2-treated control groups or $^{\#\#}p < 0.01$ vs. DMSO-treated control groups.

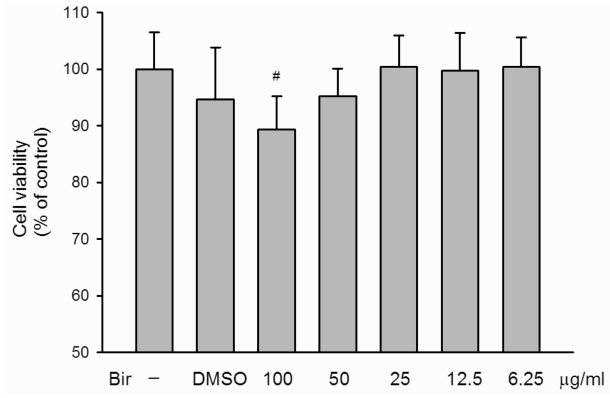

Fig. 7. The *in vitro* toxicity of biruloquinone on nerve cells.
All data are presented as mean ± SD of three independent experiments. A difference was considered statistically significant when $^{\#}p < 0.05$ vs. control groups.

The *in vitro* toxicity of biruloquinone on nerve cells is summarized in Fig. 7. The results showed that the cell viability of PC12 cells treated with biruloquinone (lower than 50 μg/ml) and 0.5% of DMSO was not significantly different with that of untreated cells, indicating the low *in vitro* toxicity of biruloquinone on PC12 cells at the active concentration derived from the AChE inhibitory activity assay and neuroprotective effects assay.

Recently, the accumulation of β-amyloid (Aβ) in the brain has been thought to play a key role in the pathogenesis of AD [13]. Aβ forms soluble oligomers and insoluble aggregates that deposit as senile plaques, and triggers neurodegeneration in AD. Although the mechanism of Aβ-induced neurotoxicity remains obscure, several lines of evidence suggest that enhanced oxidative stress provoked by Aβ is associated with the pathogenesis of AD [2, 3]. Furthermore, it is well established that excessive reactive oxygen species production could also lead to neuronal apoptosis in neurodegenerative disorders [9, 18]. Therefore, antioxidants may be considered as one of the therapeutic strategies to treat Aβ-induced neurotoxicity and to improve neurological outcome in AD. The results of the present study clearly demonstrated that biruloquinone mitigates the intracellular oxidative stress in PC12 cells. Therefore, one possible mechanism of the neuroprotective effects of biruloquinone might be related to its potent antioxidant activities.

These results implied that biruloquinone may not only ameliorate the symptom of AD patients by enhancing their cognition, but may also slow or arrest the symptoms by protecting the injured neurons. Therefore, biruloquinone has great potential to be developed as a multifunctional anti-AD agent.

However, to use biruloquinone as new anti-AD agent as a food supplement or in the pharmaceutical industry, a series of *in vivo* studies should be carried out in future,

because the ability of this compound to cross the blood–brain barrier is still not clear. Moreover, *in vivo* toxicity tests are also necessary to ensure its safety.

Acknowledgments

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