Antioxidative and Neuroprotective Effects of the Cytochalasans From Endophytes

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Abstract

Six 10-indolyl-cytochalasans, chaetoglobosin F (1), chaetoglobosin Fex (2), chaetoglobosin E (3), cytoglobosin A (4), peno- chalasin C (5), and isochaetoglobosin D (6), and 2 10-phenyl-cytochalasans, cytochalasin H (7) and 18-methoxy- cytochalasin J (8) were isolated from 2 plant endophytes, Chaetomium globosum WQ and Phomopsis sp. IFB-E060, respectively. These cytochalasans were investigated with radical-scavenging activity assay and hydrogen peroxide (H₂O₂)/N-methyl-4-phenylpyridinium iodide (MPP⁺)-induced pheochromocytoma cell line 12 (PC12) cell models, respectively. Results showed that 7 compounds had antioxidative effects except 5, with scavenging 2,2-diphenyl-1-picrylhydrazyl radical effect 1 = 6 > 7 > 2 > 4 > 3 > 8 and scavenging 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical effect 1 = 6 > 7 > 2 > 3 > 8 > 4. They could also inhibit H₂O₂/MPP⁺-induced damage in PC12 cells by increasing cell viability and decreasing lactate dehydrogenase release. Compounds 1, 6, and 7 exhibited the strongest antioxidative potencies, which are more potent than vitamin E. Additionally, antioxidative and neuroprotective effects of 1–8 showed some structure–activity relationship. These findings would be useful for looking for cytochalasin-related new lead compounds or drugs to prevent and treat Parkinson’s disease.

Keywords
cytochalasin, antioxidative potency, neuroprotective effect, parkinson’s disease, PC12 cells

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Parkinson’s disease (PD) is a common long-term degenerative disorder of the central nervous system. It might be caused by a progressive selective degeneration and loss/death of dopaminergic neurons in the substantia nigra, as well as the decrease of dopamine content in the striatum.¹ There is still no effective drug to cure it radically.² It has been recognized that the causes of PD are genetic polymorphism and susceptibility combined with environmental factors. Oxidative stress, mitochondrial dysfunction, proteolysis stress, and immune abnormality eventually lead to the apoptosis of dopaminergic neurons, which increases the susceptibility of PD.³ Researches showed that apoptosis was the main form of neuronal loss and oxidative stress was one of the major pathogenesis of PD. Natural products and natural product-derived drugs have many advantages in clinical trials. Therefore, neuroprotective natural product with antioxidative stress and antiapoptosis activity would be a potential leading compound for curing PD.⁴

Endophytic fungi are an important source of natural products that exhibit a wide range of bioactivities. Cytochalasans are a class of fungal secondary metabolites containing a parent nucleus of hydrogenated isoindolone structure fused with a macrocyclic ring (a carbocycle, a lactone, or a cyclic carbonate).⁵ Many cytochalasans have been found in fungi. For example, Buchanan et al have isolated 15 new...
10-phenyl-[11]-cytochalasans, a 10-phenyl-22-oxa-[12]-cytochalasin, and 1 known 10-phenyl-[11]-cytochalasin from one Japanese fungus *Daldinia* sp. Initially, cytochalasans showed the distinctive effects on the cellular structure and movement in vitro. Gradually, cytochalasans were found to have various bioactivities, such as cytotoxic, immunomodulatory, anti-inflammatory, antimicrobial, antiparasitic, antiviral, and nematocidal properties. More and more scientists realize the potential of cytochalasans as anticancer agents; however, the function of cytochalasans on neuroprotection effect is still neglected.

Our previous studies have reported six 10-indolyl-cytochalasans isolated from *Chaetomium globosum* WQ, an endophytic fungus in *Imperata cylindrical*, and two 10-phenyl-cytochalasans from the endophyte, *Phomopsis* sp. IFB-E060 in *Vatica mangachapoi*, respectively. Among them, cytochalasin H was found to be able to inhibit N-methyl-4-phenylpyridinium iodide (MPP⁺)-induced apoptosis in pheochromocytoma cell line 12 (PC12) cells, which was closely relative to the reduction of the intracellular reactive oxygen species (ROS) level. The protective effect of cytochalasin H to MPP⁺-damaged PC12 cells implied the potential application of cytochalasans in developing new anti-PD drugs. In order to investigate the antioxidative and neuroprotective effects of cytochalasans furtherly, radical-scavenging activity assay, hydrogen peroxide (H₂O₂) and MPP⁺-induced PC12 cell models were applied for these 8 natural cytochalasans in the present study.

Antioxidative capacity of the 8 cytochalasans (ie, 1 chaetoglobosin F; 2 chaetoglobosin F₆; 3 chaetoglobosin E; 4 cytochalasin A; 5 penochalasin C; 6 isochaetoglobosin D; 7 cytochalasin H; 8 18-methoxycytochalasin J) was investigated with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals scavenging activity assay in vitro. The structures of the 8 cytochalasans are shown in Figure 1, and their antioxidative scavenging DPPH radical effect ordered as followed: 1 = 6 = 7 > 3 > 8, while the scavenging ABTS radical effect ordered as followed: 1 = 6 = 7 > 3 > 8 > 4.

H₂O₂ can cause oxidative damage and cell death in different types of cells, including PC12 cells. The 8 cytochalasans had no cytotoxicity to the PC12 cells (unpublished data) but mostly could protect the PC12 cells from the H₂O₂-induced damage. Similar to the radical scavenging assay in vitro, 1, 6, and 7 also represented the best protective effects against the oxidative damage induced by H₂O₂. Their values of maximum effect (Fₘₐₓ) were all more than 90%, while half-maximal effective concentration (EC₅₀) were all less than 0.01 μmol/L. Besides, 2, 3, and 4 had Fₘₐₓ values above 70%. Lactate dehydrogenase (LDH), an enzyme released during damage in nearly all the living cells, was used as a marker to analyze the injury level of cells here. Compared with the model, the 7 compounds could reduce significantly LDH release. Among them, 6 was the best one with LDH value approximate to the standard Vₑ, while LDH values of 4, 3, and 8 were from 67.4 ± 5.0 to 71.1 ± 6.1 U/dL (P < 0.01 vs. the model) (Table 3).

MPP⁺ causes cellular damage mainly by promoting the formation of radical ROS and leading death of the dopaminergic neurons, which ultimately displays as symptoms of PD. Except compounds 5 and 6, 6 compounds could enhance the cell viability of MPP⁺-induced PC12 cells to some degree. Compounds 1, 6, and 7 improved the cell viability in a dose-dependent manner, whose Fₘₐₓ values were all above 99% and

![Figure 1. Structures of compounds 1-8.](image)

7 of the 8 cytochalasans had an obvious effect on the radicals scavenging, which affirmed their antioxidative role (Table 2). The radical scavenging rate was proportional to the concentration of the samples. Compounds 1, 6, and 7 exhibited the strongest antioxidative capacity, which was even better than the positive standard vitamin E (Vₑ). The scavenging DPPH radical effect of the compounds ordered as followed: 1 = 6 = 7 > 2 > 4 > 3 > 8, while the scavenging ABTS radical effect ordered as followed: 1 = 6 = 7 > 3 > 8 > 4.
The protective effects of the compounds against MPP+ induced damage in PC12 cells ordered as followed:

1 = 6 >7 >2 >4 >3. The control had a low LDH activity and ROS level, while the MPP+ induced model increased to 5.35 folds of LDH and 13.1 folds of ROS (P < 0.001). Compared with the model, the 5 compounds could reduce both LDH and ROS release except 4, 5, and 8 (Table 4). Among them, 1 had the strongest protective effects with only 1.9 folds of LDH and 1.1 folds of ROS (P < 0.001 vs. the model). LDH catalyzes the conversion of lactate to pyruvic acid, which is released during tissue damage. Overexpression of ROS during oxidative stress is harmful to both deoxyribonucleic acid and proteins, causing cellular death and disease. The capacity of the cytochalasans to decrease LDH and ROS indicated that they could be used to alleviate harmful effects and protect the cells from oxidative impairment.

The previous review outlined some structure–activity relationship of cytochalasans: palladium-catalyzed hydrogenation reduced cytochalasin D skin-irritating effects while retained cytotoxicity; the hydroxy function at C-7 and the C-3 benzyl group was important to biological activity; derivatives with bromo, iodo, or azido functions significantly reduced the effectiveness. In this study, the 8 cytochalasans all had a similar parent nucleus of hydrogenated isoindolone structure with an indolyl or phenyl ring substituted at C-10. Various substituents in the parent nucleus and macrocycle should cause a difference

### Table 1. Results of In Silico pkCSM Prediction of BBB Property.

| Compound | SMILES                                                                 | BBB permeability (logBBB) |
|----------|------------------------------------------------------------------------|---------------------------|
| 1        | O = C1N[C@@H](CC2 = CNC3=C2C = CC = C3)[C@@H](CC6 = O)C1=C6=O | −0.647                    |
| 2        | O = C1N[C@@H](CC2 = CNC3=C2C = CC = C3)[C@H](CC4=O4O)C1=C6=O | −0.608                    |
| 3        | O = C1N[C@@H](CC2 = CNC3=C2C = CC = C3)[C@H](CC=C)C1=C6=O | −0.628                    |
| 4        | O = C1N[C@@H](CC2 = CNC3=C2C = CC = C3)[C@H](CC=C)C1=C6=O | −0.62                     |
| 5        | O = C1N[C@@H](CC2 = CNC3=C2C = CC = C3)[C@H](CC=C)C1=C6=O | −0.429                    |
| 6        | O = C1N[C@@H](CC2 = CNC3=C2C = CC = C3)[C@H](CC=C)C1=C6=O | −0.63                     |
| 7        | O = C1N[C@@H](CC2 = CNC3=C2C = CC = C3)[C@H](CC=C)C1=C6=O | −0.626                    |
| 8        | O = C1N[C@@H](CC2 = CNC3=C2C = CC = C3)[C@H](CC=C)C1=C6=O | −0.456                    |

BBB, blood–brain barrier.

1, chaetoglobosin F; 2, chaetoglobosin Fex; 3, chaetoglobosin E; 4, cytoglobosin Λ; 5, penochalasin C; 6, isochaetoglobosin D; 7, cytochalasin H; 8, 18-methoxycytochalasin J.

### Table 2. EC50 and Emax of the 8 Cytochalasans for Scavenging DPPH/ABTS Radical, and Their TEAC (X ± SD, N = 3).

| Compound | EC50 (mmol/L) | Emax (%) | EC50 (mmol/L) | Emax (%) | TEAC (mol trolox/mol) |
|----------|---------------|----------|---------------|----------|-----------------------|
| 1        | 0.002 ± 0.001 | 92.8 ± 3.0 | 0.002 ± 0.004 | 62.4 ± 7.5 | 359                   |
| 2        | 0.194 ± 0.094 | 58.2 ± 2.6 | 0.119 ± 0.092 | 34.0 ± 6.0 | 6.034                 |
| 3        | 1.068 ± 0.350 | 42.1 ± 3.0 | 0.419 ± 0.048 | 30.5 ± 4.5 | 1.714                 |
| 4        | 0.879 ± 0.348 | 47.2 ± 2.8 | 472.870 ± 0.543 | 22.4 ± 3.2 | 0.002                 |
| 5        | 0.002 ± 0.001 | 95.2 ± 1.9 | 0.002 ± 0.001 | 63.4 ± 1.3 | 359                   |
| 6        | 0.002 ± 0.001 | 93.0 ± 0.7 | 0.001 ± 0.001 | 60.1 ± 3.0 | 718                   |
| 7        | 481.121 ± 13.155 | 35.2 ± 2.4 | 1.052 ± 0.357 | 29.4 ± 1.5 | 0.683                 |
| Vitamin E | 0.079 ± 0.001 | 93.8 ± 2.6 | 0.718 ± 0.008 | 70.71 ± 15.1 | 1.0                   |

EC50, half maximal effective concentration; Emax, maximum effect; TEAC, trolox equivalent antioxidative capacity; SD, standard deviation; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.
in the function of cytochalasans markedly. First, compounds containing epoxy group sometimes had strong bioactivity, and the 6,7- epoxy group was also critical for cytochalasans. Chaetoglobosin F (1) with the 6,7- epoxy group had stronger activities than its epoxy ring- opening analogs, chaetoglobosin Fex (2) and chaetoglobosin E (3). The previous study also reported that the epoxy group significantly increased the in vitro cytotoxicity of the trichothecene macrolides.22 Second, the difference between hydroxyl and carbonyl determined their activity. In general, the antioxidative potency of compounds is associated with the number of free hydroxyls.23 However, here the position of the hydroxyl group in these cytochalasans seemed more important. Cytochalasin H (7) had a C-18 hydroxyl and a C-21 acetoxy in its macrocycle, while 18- methoxycytochalasin J (8) had a C-18 methoxyl and a C-21 hydroxyl. Antioxidative capacity of 7 was much better than that of 8 with above 1000 times of efficiency on scavenging radicals. In addition, oxidation of 20-hydroxyl to carbonyl enhanced the activity. Isochaetoglobosin D (6) had a better antioxidative capacity and neuroprotective effect than chaetoglobosin Fex (2) mainly due to the C-20 carbonyl group. Interestingly, penochalasin C (5) with a pyrrole ring in the macrocycle not only had no antioxidative potency but also aggravated the cell damage.

In conclusion, 7 among the 8 cytochalasin compounds, chaetoglobosin F (1), chaetoglobosin Fex (2), chaetoglobosin E (3), cytoglobosin A (4), penochalasin C (5), isochaetoglobosin D (6), cytochalasin H (7), and 18-methoxycytochalasin J (8), isolated from endophytes C. globosum WQ and Phomopsis sp. IFB-E060 exhibited in vitro antioxidative potency to a different extent. They could scavenge DPPH and ABTS radicals, as well as inhibit H2O2- induced damage in PC12 cells effect. The cell viability of PC12 cells was improved, while the release of LDH was reduced. Six cytochalasin compounds could resist the damage from MPP+ basing on the results of cell viability, cell morphology, LDH release, and ROS level. Among these compounds, chaetoglobosin F (1), isochaetoglobosin D (6), and cytochalasin H (7) had the strongest protective effect on PC12

| Table 3. Effects of the 8 Cytochalasans (0.005 μmol/L) for Inhibiting Hydrogen Peroxide-Induced PC12 Cell Damage (x ± SD, N = 3). |
|---------------------------------|-------------|----------------|----------------|
| Compound | EC50 (μmol/L) | Emax (%) | LDH value (U/dl) |
|---------|--------------|----------|-----------------|
| 1       | 0.003 ± 0.0003 | 96.6 ± 1.7 | 34.7 ± 7.5*** |
| 2       | 0.030 ± 0.002 | 76.7 ± 2.2 | 36.4 ± 1.9*** |
| 3       | 0.240 ± 0.236 | 73.4 ± 0.8 | 68.2 ± 9.0*** |
| 4       | 0.023 ± 0.004 | 74.6 ± 2.2 | 67.4 ± 5.0*** |
| 5       | -             | 40.3 ± 2.9 | 107.9 ± 7.7    |
| 6       | 0.009 ± 0.001 | 96.9 ± 1.4 | 22.7 ± 2.6***  |
| 7       | 0.004 ± 0.002 | 93.5 ± 1.5 | 31.4 ± 6.8***  |
| 8       | -             | 55.5 ± 1.8 | 71.1 ± 6.1***  |
| Control | -             | -         | 16.9 ± 1.4     |
| Model   | -             | -         | 109.1 ± 2.5### |
| Vitamin E | -             | -         | 19.8 ± 2.5###  |

LDH, lactate dehydrogenase; SD, standard deviation; EC50, half-maximal effective concentration; Emax, maximum effect. 
ΔΔΔ indicate P < 0.001 compared to the control group; ** and *** indicate P < 0.01 and P < 0.001, respectively compared to the H2O2 model group.

| Table 4. Effects of the 8 Cytochalasans (0.025 μmol/L) for Inhibiting MPP+-Induced PC12 Cell Damage (x ± SD, N = 3). |
|---------------------------------|-------------|----------------|----------------|
| Compound | EC50 (μmol/L) | Emax (%) | LDH value (U/dL) | ROS (fold change) |
|---------|--------------|----------|-----------------|------------------|
| 1       | 0.009 ± 0.007 | 99.8 ± 7.2 | 48.2 ± 2.6*** | 1     |
| 2       | 0.140 ± 0.182 | 80.7 ± 6.7 | 88.9 ± 2.0*** | 13.1 ± 0.11ΔΔΔ |
| 3       | 6.100 ± 0.007 | 71.8 ± 5.4 | 85.5 ± 2.0*** | 1.1 ± 0.11***   |
| 4       | 0.355 ± 0.242 | 79.8 ± 3.5 | 128.9 ± 4.7    | 12.3 ± 0.29**   |
| 5       | -             | 55.4 ± 5.0 | 143.2 ± 4.7    | 11.2 ± 0.22**   |
| 6       | 0.025 ± 0.012 | 99.4 ± 0.3 | 52.9 ± 7.2***  | 12.6 ± 0.33     |
| 7       | 0.040 ± 0.011 | 100.2 ± 0.5 | 53.4 ± 1.3*** | 13.4 ± 0.16     |
| 8       | -             | 51.6 ± 2.9 | 130.2 ± 7.9    | 2.0 ± 0.01***   |
| Control | -             | -         | -               | 5.4 ± 0.42***    |
| Model   | -             | -         | 134.9 ± 8.7### | 12.7 ± 0.27     |
| Vitamin E | -             | -         | ND              | ND               |

ND, not determined; EC50, half-maximal effective concentration; Emax, maximum effect; LDH, lactate dehydrogenase; ROS, reactive oxygen species; MPP+, N-methyl-4-phenylpyridinium iodide. 
ΔΔΔ indicate P < 0.001 compared to the control group; ** and *** indicate P < 0.01 and P < 0.001, respectively compared to the MPP+ model group.
cells; however, penochalasin C (5) enhanced the cell damage on the contrary. In addition, antioxidative and neuroprotective effects of the cytochalasins showed some structure–activity relationship. It was the first report that the cytochalasins chaetoglobosin F (1) and isochaetoglobosin D (6) possessed great antioxidative and neuroprotective effects, whose mechanisms were worthy of further study. This implied the possible application of cytochalasans in developing new anti-PD drugs.

**Experimental**

**Materials and Chemicals**

The rat PC12 was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Dulbecco’s modified Eagle medium (DMEM) was purchased from Gibco/Invitrogen (Gaithersburg, MD, USA). The standard VE with purity >98.8% was obtained from the Chinese National Institute For Food and Drug Control (Beijing, China). MPP+ and DPPH were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were bought from AMROSCO LLC (Solon, OH, USA). Fetal bovine serum (FBS) was obtained from Zhejiang Tianhan Biotechnology Co., Ltd (Hangzhou, Zhejiang, China). Penicillin, streptomycin, and ABTS radical scavenging activity assay kit were bought from Beyotime Institute of Biotechnology (Shanghai, China). LDH and ROS assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). All the chemicals and reagents were of the analytical grade.

**Sample Preparation**

Eight cytochalasan compounds (ie, 1, chaetoglobosin F; 2, chaetoglobosin F6; 3, chaetoglobosin E; 4, cytochalosin A; 5, penochalasin C; 6, isochaetoglobosin D; 7, cytochalasin H; 8, 18-methoxycytochalosan; J; Figure 1) were obtained from our lab in Medical College of Yangzhou University, China. Compounds 1–6 were isolated from endophyte *C. globosum WQ*,12 and compounds 7 and 8 were isolated from endophyte *Phomopsis* sp. IFB-E060.13 Purity of each compound was checked by high-performance liquid chromatography and thin-layer chromatography, and structures of these compounds were determined by high-resolution electrospay ionization mass spectrometry and nuclear magnetic resonance.12,13 The 8 compounds were dissolved, respectively, in DMSO (final concentration <0.1%) to provide the stock solution. The stock concentration of compound 7 was 1.08 mmol/L, while all the others were 1.89 mmol/L. The stock solutions were stored at −4°C in a refrigerator. The stock solutions of 1–8 were diluted by distilled water to a series test solution at different concentrations, respectively, for antioxidative capacity assays in vitro; while 1–8 were diluted by DMEM for neuroprotection effect determination in cell models.

**Assay of DPPH Radical Scavenging Activity**

The DPPH radical scavenging activity of 8 cytochalasan compounds was examined via spectrophotometric analysis.24 Different concentrations (10−6, 10−5, 10−4, 10−3, and 10−2 mmol/L) of samples were prepared and standard VE was assayed as the positive control. The solution 0.01% DMSO without the tested sample was used as the blank control. The absorbance was then recorded at 517 nm, and the scavenging activity was expressed as the percentage of scavenged DPPH radical in the assay system. The EC50 (expressed as mmol/L) value, denoting the concentration of the sample required to scavenge 50% DPPH free radicals, was calculated by graphical regression analysis, and E_max (expressed as %) was calculated by Scott Law.25

**Assay of ABTS Radical Cation Scavenging Activity**

The ABTS radical cation (ABTS+) scavenging activity of the sample was performed using the method of Cai et al.26 Different levels of cytochalasan compound samples (10−6, 10−5, 10−4, 10−3, and 10−2 mmol/L) and trolox, an analog of VE, were prepared and standard sample was performed using the method of Cai et al.26 Different levels of cytochalasan compound samples (10−6, 10−5, 10−4, 10−3, and 10−2 mmol/L) and trolox, an analog of VE, standard solution (6, 3, 2, 1, 0.2, and 0.1 mmol/L) were prepared and assayed under the same conditions. The absorbance of 734 nm was recorded to get the percentage of scavenged ABTS radical curve. Results were expressed in terms of trolox equivalent antioxidative capacity (TEAC), that is, mol trolox/mol. Similar to DPPH assay, EC50 and E_max of ABTS assay were also calculated.

**Cell Culture and Treatment**

PC12 cells with cell composition and function close to the mesencephalic dopamine neuron have become a common cell model of PD in vitro.27 PC12 cells were cultured in DMEM containing 10% FBS, 1% streptomycin and penicillin at 37°C in a 5% carbon dioxide humidified NAPCO 5400 incubator (JOUAN, France). The cells in the period of logarithmic phase were used for all experiments. H2O2 and/or MPP+ induced PC12 cell models were established based on our previous work (unpublished data) and other reports.28

For H2O2-induced model, the cells in 96-well or 24-well plates (1 × 10^5 cells/mL) were pretreated with the different concentration of test compounds (0.001, 0.0025, 0.005, 0.01, 0.05, 0.1, and 0.2 μmol/L) for 0.5 hours, respectively. Then the sample cells were stimulated with H2O2 solution (final concentration 200 μmol/L) for another 4 hours. DMEM without cells was used as the blank. The solution 0.01% DMSO in DMEM with H2O2 stimulation was used as the H2O2−induced model, while without H2O2 was the control. The positive control chose 1057.6 μmol/L VE.

Similarly, for the MPP+-induced model, the cells were pretreated for 24 hours with a series concentration of cytochalasan compounds (0.005, 0.01, 0.025, 0.05, 0.1, 0.15, and 0.2 μmol/L) as the test groups. The MPP+-damaged experiment was accomplished by stimulating the PC12 cells with 500
μmol/L MPP⁺ for 48 hours. The cell morphology was observed using an XDS-1B Inverted Biomicroscope (Chongqing Optical Instrument Co., Ltd., Chongqing, China).

The PC12 cells in H₂O₂ and/or MPP⁺-induced models were collected and further analyzed the viability by MTT method, while the supernatants were collected for enzyme-linked immunosorbent assay (ELISA) analysis of LDH activity. The MPP⁺-induced PC12 cells were also determined the ROS levels.

**Cell Viability Detection**

Toxicity of the tested compounds to PC12 cells, as well as the influence on H₂O₂ and/or MPP⁺-induced PC12 cells, was analyzed by the determination of the cell viability using MTT method. PC12 cells with different treatments were inoculated in 96-well culture plates (1 × 10⁵ cells/mL) for 48 hours. Each well was added with 10 μL MTT (5 mg/mL) and then continued to culture 4 hours. After removal of the MTT solution, 150 μL DMSO was added to solubilize the purple formazan crystals produced. The absorbance of each well was measured at 490 nm on a Bio-Tek ELx800 Multi-Mode Microplate Reader (BioTek Instruments, Inc. Winooski, VT, USA). The reader was controlled via Hyper Terminal Applet ELISA software. The cell viability was calculated with the following formula and expressed as a percentage. EC₅₀ and Eₖmax of the inhibition on cell damage were also calculated.

\[
\text{Cell viability} (\%) = \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{control}} - OD_{\text{blank}}} \times 100\%
\]

**LDH Activity Assay**

The tested cytochalasan compounds at the optimal concentration were chosen with LDH assay, 0.005 μmol/L for H₂O₂-induced PC12 cell model and 0.025 μmol/L for MPP⁺-induced PC12 cell model. LDH activity in the supernatant was assayed using the ELISA kit. The assay procedure was employed according to the kit protocol booklet instructions. The absorbance was measured at 450 nm on a Bio-Tek ELx800 Multi-Mode Microplate Reader (BioTek Instruments, Inc. Winooski, VT, USA). The reader was controlled via Hyper Terminal Applet ELISA software.

\[
\text{LDH activity (U/dl)} = \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{standard}} - OD_{\text{blank}}} \times C_{\text{standard}} \times 1000
\]

**ROS Assay**

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) detection kit was used to assess the ROS level in MPP⁺-induced PC-12 cells. Briefly, the cells were seeded in 96-well plates (2 × 10^⁵ cells/mL), treated with different samples to incubate for 24 hours and cultured with 500 mmol/L MPP⁺ for 48 hours. After washing cells with phosphate buffer saline (PBS), 2 mL PBS and 2 μL DCFH-DA (final concentration 10 μmol/L) were added to each well and reacted for 30 minutes at 37°C. The cells were collected after dissociated, and fluorescence was recorded by a Becton Dickinson (BD) FACs Calibur Flow Cytometry (BD Biosciences, San Jose, CA, USA) with 488 P excitation and 525 P emission filters. The total fluorescence intensity of cells in each well was noted, and ROS generation was measured as fold of the control.

**Statistical Analysis**

All data presented are mean value ± standard deviation of 3 independent experiments. EC₅₀ and Eₖmax were calculated by SPSS 17.0 and Scott Law, respectively. Figures were obtained using Microsoft Office Excel 2016. SPSS 17.0 was also applied in a one-way analysis of variance. Dunnett test was used to compare with the control group, and the Student–Newman–Keuls test was used to compare among the groups. Differences were considered significant with a P-value <0.05.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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