RESEARCH ARTICLE

Protective effects of organic extracts of *Alpinia oxyphylla* against hydrogen peroxide-induced cytotoxicity in PC12 cells

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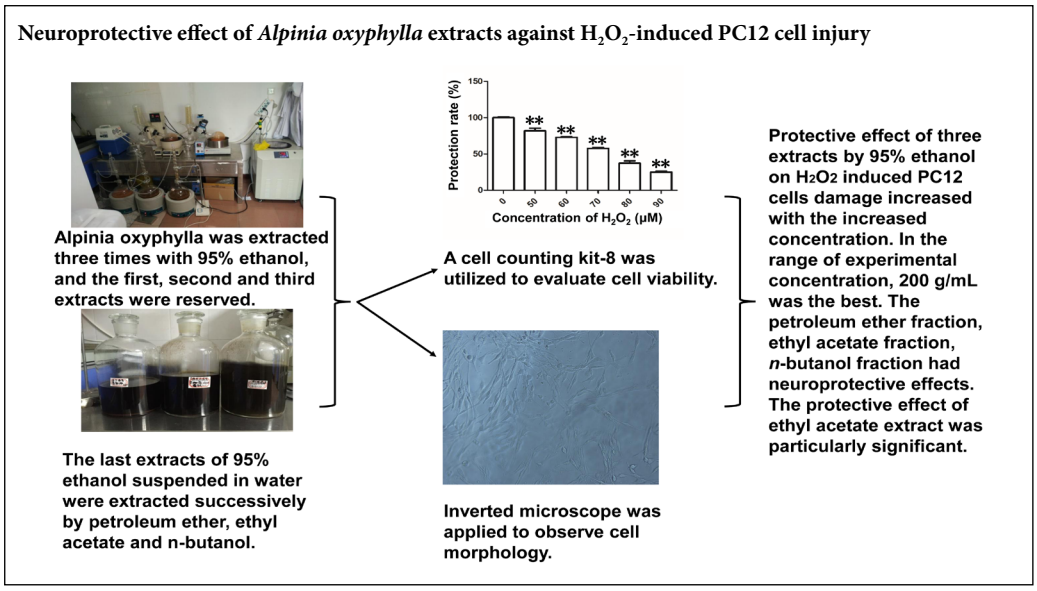
Graphical Abstract



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Abstract

Alpinia oxyphylla, a traditional herb, is widely used for its neuroprotective, antioxidant and memory-improving effects. However, the neuroprotective mechanisms of action of its active ingredients are unclear. In this study, we investigated the neuroprotective effects of various organic extracts of *Alpinia oxyphylla* on PC12 cells exposed to hydrogen peroxide-induced oxidative injury *in vitro*. *Alpinia oxyphylla* was extracted three times with 95% ethanol (representing extracts 1–3). The third 95% ethanol extract was dried and resuspended in water, and then extracted successively with petroleum ether, ethyl acetate and *n*-butanol (representing extracts 4–6). The cell counting kit-8 assay and microscopy were used to evaluate cell viability and observe the morphology of PC12 cells. The protective effect of the three ethanol extracts (at tested concentrations of 50, 100 and 200 μg/mL) against cytotoxicity to PC12 cells increased in a concentration-dependent manner. The ethyl acetate, petroleum ether and *n*-butanol extracts (each tested at 100, 150 and 200 μg/mL) had neuroprotective effects as well. The optimum effective concentration ranged from 50–200 μg/mL, and the protective effect of the ethyl acetate extract was comparatively robust. These results demonstrate that organic extracts of *Alpinia oxyphylla* protect PC12 cells against apoptosis induced by hydrogen peroxide. Our findings should help identify the bioactive neuroprotective components in *Alpinia oxyphylla*.

Key Words: *active ingredients; Alpinia oxyphylla; apoptosis; ethanol crude extract; fraction; hydrogen peroxide; nerve regeneration; neuroprotective agent; neuroprotective effects; PC12 cells; traditional herb*

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Introduction

Alpinia (A.) oxyphylla is commonly used in traditional Chinese medicine to treat dyspepsia, diarrhea (Zhang et al., 2013; Wang et al., 2015), abdominal pain (Song et al., 2014; Zhang et al., 2015a), poor memory (Shi et al., 2015; He et al., 2019), inflammatory conditions (He et al., 2010; Zhang et al., 2018; Qi et al., 2019) and cancer (Lin et al., 2013). Recently, the medicinal properties of *A. oxyphylla* and its pharmaceutical products have received considerable attention (Bian et al., 2013; Zhang et al., 2015b, 2018). The protective effects of *A. oxyphylla* extract in chronic kidney disease have been explored using metabolomics (Li et al., 2016). Various biomarkers, such as agmatine, CAMP and 7-methylguanine, are restored to control levels after treatment with *A. oxyphylla* extract, suggesting that it has protective effects. Wang et al. (2015) showed that the 95% ethanol extract and 90% ethanol-eluted fractions of *A. oxyphylla* have antidiarrheal activity. Some studies focusing on the effectiveness of *A. oxyphylla* extract ignored identifying the bioactive components. *A. oxyphylla* contains numerous potentially bioactive compounds, including flavonoids (Zhang et al., 2015b; Sun et al., 2016), tepenes (Lv et al., 2011; Xie et al., 2014; Hou et al., 2015; Zhao et al., 2015), alkaloids (Zhou et al., 2013) and diphenylheptanes (Bian et al., 2013).

Peripheral nerve injuries, such as those caused by accidental trauma, birth injury, ischemia or iatrogenic injury, often result in temporary or life-long neurological dysfunctions, which can be devastating and severely impact the patient's quality of life (Cao et al., 2019; Han et al., 2019; Zhang et al., 2019). It is necessary to promote neural cell proliferation to restore the injured nerves in adults. A few studies have shown that protocatechuic acid modulates the MAPK (ERK1/2, JNK and p38)/PA (uPA, tPA)/MMP (MMP2, MMP9) regeneration and migration signaling pathways in Schwann cells (Ju et al., 2015a). Furthermore, protocatechuic acid promotes cell proliferation and survival via the insulin-like growth factor-I signaling pathway (Ju et al., 2015b). Li et al. (2016) reported that a novel lead compound, oxyphylla A, is a neuroprotective agent for Parkinson's disease.

In the present study, we investigated the neuroprotective effects of various organic extracts of *A. oxyphylla* on hydrogen peroxide (H_2O_2)-induced apoptosis in cultured PC12 cells. Our aim is to lay the foundation for the purification and identification of the bioactive components in *A. oxyphylla* for use in future clinical application.

Materials and Methods

Materials

Ethanol, petroleum ether, ethyl acetate and *n*-butanol were of analytical grade (Guangdong Guanghua Sci-Tech Co., Ltd., Guangzhou, China). Undifferentiated rat PC12 cells were from Procell Life Science & Technology Co., Ltd., Wuhan, China.

Sample extraction and fractionation

The air-dried fruits of *A. oxyphylla* (10.0 kg) were extracted three times for 1.5 hours each by refluxing in 95% ethanol (1:10, w/v) to obtain 95% ethanol extracts 1–3 (95% EE-1–3). A portion of 95% EE-3 was concentrated by vacuum evaporation and dried by water bath evaporation, and then resuspended and dissolved in ultrapure water by ultrasonication for 30 minutes, resulting in a brown-yellow suspension. The suspension was extracted with petroleum ether several times until the upper layer of the extract was colorless to obtain the petroleum ether fraction (PF). The residual extract was extracted with ethyl acetate until the upper layer of the extract was colorless to obtain the ethyl acetate fraction (EF). The final raffinate was extracted with *n*-butanol until the upper layer of the extract was colorless to obtain the *n*-butanol fraction (BF). All six extracts were concentrated by rotary evaporation and dried in a vacuum at 45°C.

Cell culture

PC12 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco) in a water-saturated atmosphere of 5% CO_2 in 96-well plates at 37°C for 24 hours. Five wells each were treated with vehicle alone or different concentrations of the six *A. oxyphylla* extracts. The cultures were incubated for 24 hours. After culture for 48 hours, cell viability was analyzed with the cell counting kit-8 assay (Biosharp, Hefei, China), and morphology was observed on an inverted microscope (MZ16FA; Leica, Wetzlar, Hesse-Darmstadt, Germany).

Analysis of cell viability

The cell counting kit-8 assay was used to evaluate cell viability. PC12 cells were seeded into 96-well plates at a density of 4×10^4 cells/well (100 μL/well) for 24 hours, and then pretreated with vehicle alone or different concentrations of the various *A. oxyphylla* extracts for 24 hours. The supernatants were discarded, the wells were washed twice with phosphate-buffered saline, and 1 mL serum-free 1640 medium and 100 μL cell counting kit-8 solution were added, followed by incubation for 3 hours at 37°C. The optical density was determined at 450 nm. Five parallel experiments were done. The cell viability of the tested compounds was calculated using the following equation:

Cell viability (%) = (cell viability of drug group – cell viability of model group)/(cell viability of control group – cell viability of model group) × 100.

Cell viability was assessed with the cell counting kit-8 assay to identify the optimal H_2O_2 concentration (70 μM in this study). The cells were exposed to 70 μM H_2O_2 for 2 hours before supernatant removal to observe the protective effects of the different concentrations of the various *A. oxyphylla* extracts.

Statistical analysis

Each experiment was performed at least three times, and the results were expressed as the mean ± SD. The values followed a Gaussian distribution. Differences between means were

compared by one-way analysis of variance followed by Dunnett's *post hoc* test using SPSS 22.0 software (IBM, Armonk, NY, USA).

Results

Effects of the different extracts of *A. oxyphylla* on cell viability

PC12 cells were treated with different concentrations of the various *A. oxyphylla* extracts for 24 hours. Each extract was tested using a different concentration range, based on pilot studies. The EF, 95% EE-1 and EE-3 extracts were tested at 0–400 μg/mL; 95% EE-2 was tested at 0–300 μg/mL; PF was tested at 0–100 μg/mL; BF was tested at 0–200 μg/mL.

As shown in **Figure 1A**, **B** and **E**, at concentrations of 100–200 μg/mL, 95% EE-1, 95% EE-2 and BF increased cell viability, without affecting cellular morphology. The 200 μg/mL concentration of these extracts increased cell viability to 136.4%, 164.3% and 159.9% of that in the control group, respectively. **Figure 1C** shows that 95% EE-3 slightly increased cell viability at concentrations of 50–200 μg/mL, with the 100 μg/mL concentration increasing cell viability to 118.0% of that in the control group. At concentrations of 25–75 μg/mL, PF markedly increased cell viability, with the 50 μg/mL concentration increasing viability to 163.8% of that in the control (**Figure 1D**). As shown in **Figure 1F**, at concentrations of 25–100 μg/mL, EF increased cell viability, with the 100 μg/mL concentration increasing it to 141.1% of that in the control.

Effects of different extracts of *A. oxyphylla* on the viability of H_2O_2-exposed PC12 cells

The pilot study revealed that H_2O_2 at 50–90 μM induced cell death in a dose-dependent manner (**Figure 2**). To evaluate the cytoprotective effects of the *A. oxyphylla* extracts, PC12 cells were pretreated with the extracts and a moderate concentration (70 μM) of H_2O_2. Cell viability was assessed with the cell counting kit-8 assay. As shown in **Figure 3A–C**, incubation with 70 μM H_2O_2 for 2 hours resulted in a cell viability rate of 36.2% compared with the control. However, viability increased to 48.4%, 66.8% and 73.9% in cells pretreated with 95% EE-1 (100, 150 and 200 μg/mL, respectively) for 24 hours. Cell viability rate increased to 74.4%, 82.0% and 83.4% in cells pretreated with 95% EE-2 (100, 150 and 200 μg/mL, respectively) for 24 hours. Cell viability increased to 56.1%, 61.3% and 65.6% in cells pretreated with 95% EE-3 (50, 100 and 200 μg/mL, respectively) for 24 hours. As shown in **Figure 3D** and **E**, incubation with 70 μM H_2O_2 for 2 hours resulted in a cell viability rate of 41.6% compared with the control. Viability increased to 62.4% in cells pretreated with 25 μg/mL PF for 24 hours, but was only 24.9% in cells pretreated with 75 μg/mL PF (**Figure 3D**). Cell viability rate increased dramatically to 85.4%, 108.5% and 99.2% in cells pretreated with EF (25, 50 and 100 μg/mL, respectively) for 24 hours (**Figure 3E**). Moreover, the cell viability rate increased slightly to 52.1%, 58.5% and 76.3% in cells pretreated with BF (100, 150 and 200 μg/mL, respectively) for 24 hours (**Figure 3F**). Together, these results suggest that extracts of *A. oxyphylla* are neuroprotective against H_2O_2-induced oxidative stress. The neuroprotective effect of the ethyl acetate extract was the most robust.

Under the optical microscope, PC12 cells were small and translucent immediately after passage in suspension. The cells were plump and formed a network after 48 hours (**Figure 4A**). After exposure to H_2O_2 for 2 hours, the cells in the model group were severely damaged (**Figure 4B**). The cells in the various extract treatment groups exhibited varying degrees of morphological changes (**Figures 4C1**, **2**, **3**–**H1**, **2**, **3**). The six extracts of *A. oxyphylla* effectively inhibited H_2O_2-induced cytotoxicity at the different concentrations. The cytoprotective effects of 95% EE-1, 95% EE-2 and 95% EE-3 increased with increasing concentration from 100–200 μg/mL. As shown in **Figure 4C3**, **D3** and **E3**, morphology was good and axons grew well. The EF, PF and BF extracts had optimal effects on morphology at 25, 50 and 200 μg/mL, respectively. Thus, the EF extract had the best effect.

Discussion

H_2O_2 induces apoptosis in many different cell types, including PC12 cells, by initiating mitochondrial dysfunction (Jang et al, 2001; Huang et al, 2015; Chen et al., 2019). H_2O_2, an inducer of neuronal injury, is extensively used to explore the neuroprotective potential of new pharmacotherapies (Porres-Martínez et al., 2016; Liu et al., 2018; Chu et al., 2019). Exploration of natural compounds that support neurite outgrowth against the toxicity of H_2O_2 is critical for treating neurodegenerative diseases.

The H_2O_2 concentration that induces 50% PC12 cell lethality is around 70 μM, when incubated for 2 hours. Other researchers have found it to be around 150 μM for 24 hours of exposure and 750 μM for 6 hours of exposure (Tusi et al., 2014; Cheong et al., 2016). The reasons for the discrepancies may include differences in experimental conditions, including reagent quality.

In recent years, an increasing number of studies have focused on natural substances isolated from Chinese herbal medicines, particularly as synthetic chemicals can have serious adverse effects (Wang et al., 2014; Hu and Sun, 2017; Liu et al., 2017; Ai et ai., 2019; Dai et al., 2019). Huang et al. (2015) found that forsythiaside provides protective effects against H_2O_2-induced death of neurons. Divate et al. (2017) demonstrated the neuroprotective effects of Xylaria nigripes mycelia extracts on H_2O_2-induced cytotoxicity in PC12 cells.

In this study, we found that *A. oxyphylla* extracts had no negative effect on the proliferation of PC12 cells, even positively impacting proliferation within a certain range. Among the extracts, EF was particularly effective, closely followed by EE-2. Consistent with previous reports, *A. oxyphylla* extracts possessed significant neuroprotective activity. Wong et al. (2004) reported that the ethanol extract of *A. oxyphylla* fructus improves spatial learning by affecting the serum levels of cytokines. Studies suggest that neuroprotection is achieved via multiple mechanisms, including decreased Bax/Bcl-2 ratio (Peng et al., 2012; Ip et al., 2016; Phatak et al., 2016; Rivero-Segura et ai., 2017; Lima et al., 2018), restored

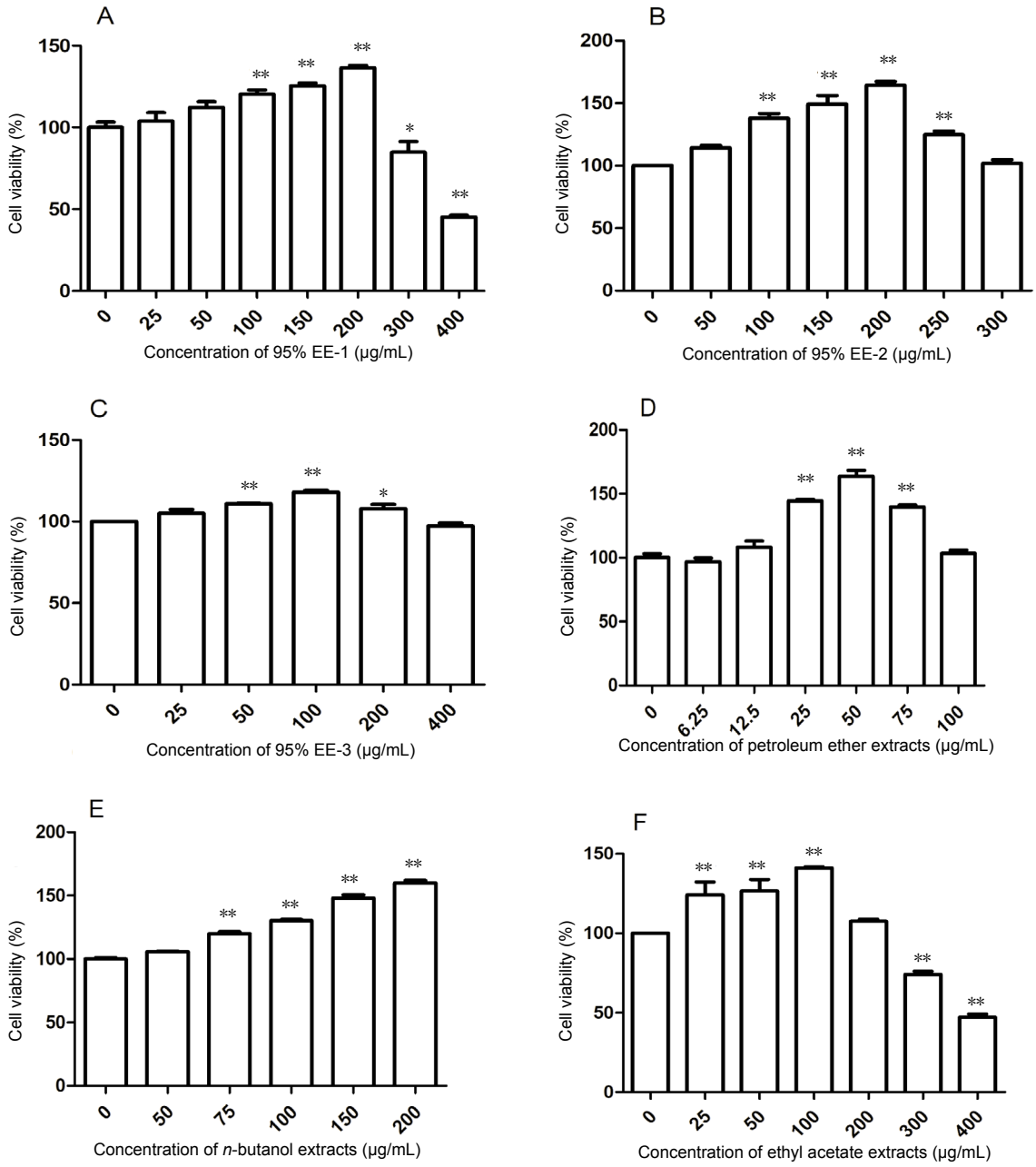

Figure 1 Effects of different concentrations of the organic extracts of *Alpinia oxyphylla* on the viability of PC12 cells.
(A–F) Effects of 95% ethanol extracts 1–3 (95% EE-1, 95% EE-2, 95% EE-3), petroleum ether extract, ethyl acetate extract and *n*-butanol extract on cultured PC12 cell viability. Cells (4×10^4 cells/mL) were treated with different extracts for 24 hours at 37°C after normal culture for 24 hours. $*P < 0.05$, $**P < 0.01$, *vs.* control group (0 μg/mL). Data are expressed as the mean ± SD ($n = 5$; one-way analysis of variance followed by Dunnett's *post hoc* test). EE: Ethanol extract.

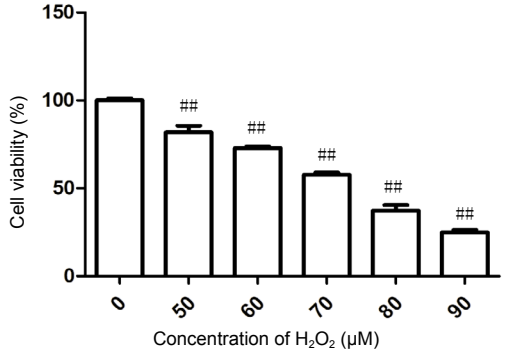

Figure 2 Effects of H_2O_2 on PC12 cell viability.
Cell counting kit-8 assay shows that H_2O_2 decreased cell viability in a concentration-dependent manner. $##P < 0.01$, *vs.* control group (0 μM H_2O_2). Data are expressed as the mean ± SD ($n = 5$; one-way analysis of variance followed by Dunnett's *post hoc* test). Cell viability (%) = (cell viability of drug group – cell viability of model group)/(cell viability of control group – cell viability of model group) × 100.

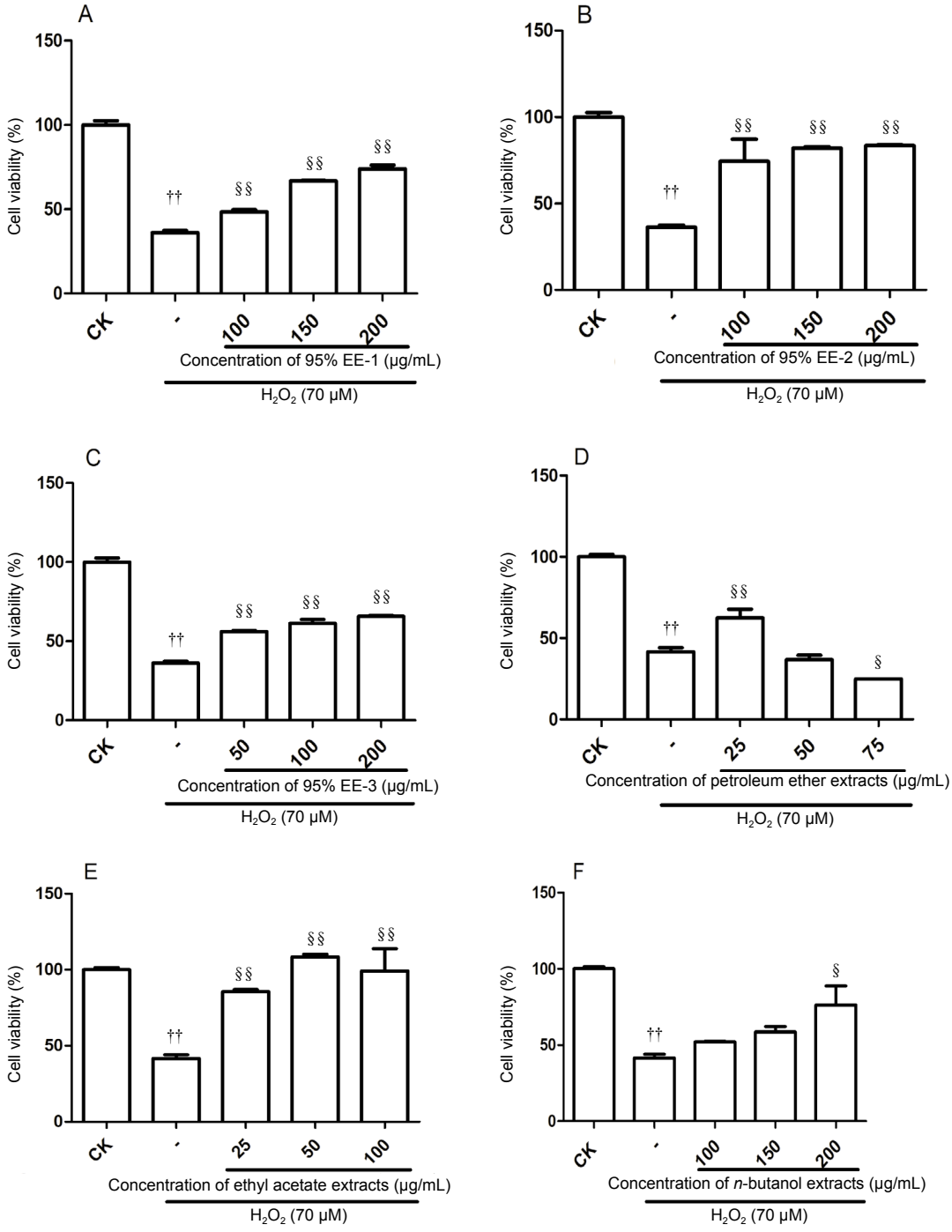

Figure 3 Effects of the three sequential 95% ethanol extracts (EE-1–3), petroleum ether extract, ethyl acetate extract and *n*-butanol extract on H_2O_2-induced PC12 cell damage.

(A) EE-1; (B) EE-2; (C) EE-3. (D–F) Effects of petroleum ether, ethyl acetate and *n*-butanol extracts, respectively. PC12 cells (4×10^4 cells/mL) were treated with 70 μM H_2O_2 in the absence or presence of the extracts. Viability is calculated as the percentage of living cells in treated cultures compared with control cultures (CK). Data are expressed as the mean ± SD ($n = 5$; one-way analysis of variance followed by Dunnett's *post hoc* test). ††P < 0.05, *vs.* CK; §$P < 0.05$, § §$P < 0.01$, *vs.* cells exposed to H_2O_2 alone. Cell viability (%) = (cell viability of drug group – cell viability of model group)/(cell viability of control group – cell viability of model group) × 100. EE: Ethanol extract.

mitochondrial membrane potential (Chtourou et al., 2015; Chiang et al., 2016; Chen et al., 2018; Tian et al., 2018; Wang et al., 2019), and downregulated caspase-3 (Chen et al., 2016; Zhang et al., 2016; Zhou et al., 2016; Ding et al., 2017; Rivero-Segura et al., 2017; Lima et al., 2018). Furthermore, studies show that Xylaria nigripes mycelia extracts inhibit the release of lactate dehydrogenase and decrease DNA damage (Divate et al., 2017). Huang et al. (2015) found that forsythiaside decreased reactive oxygen species levels and lipid peroxidation. Thus, the neuroprotective effect of nat-

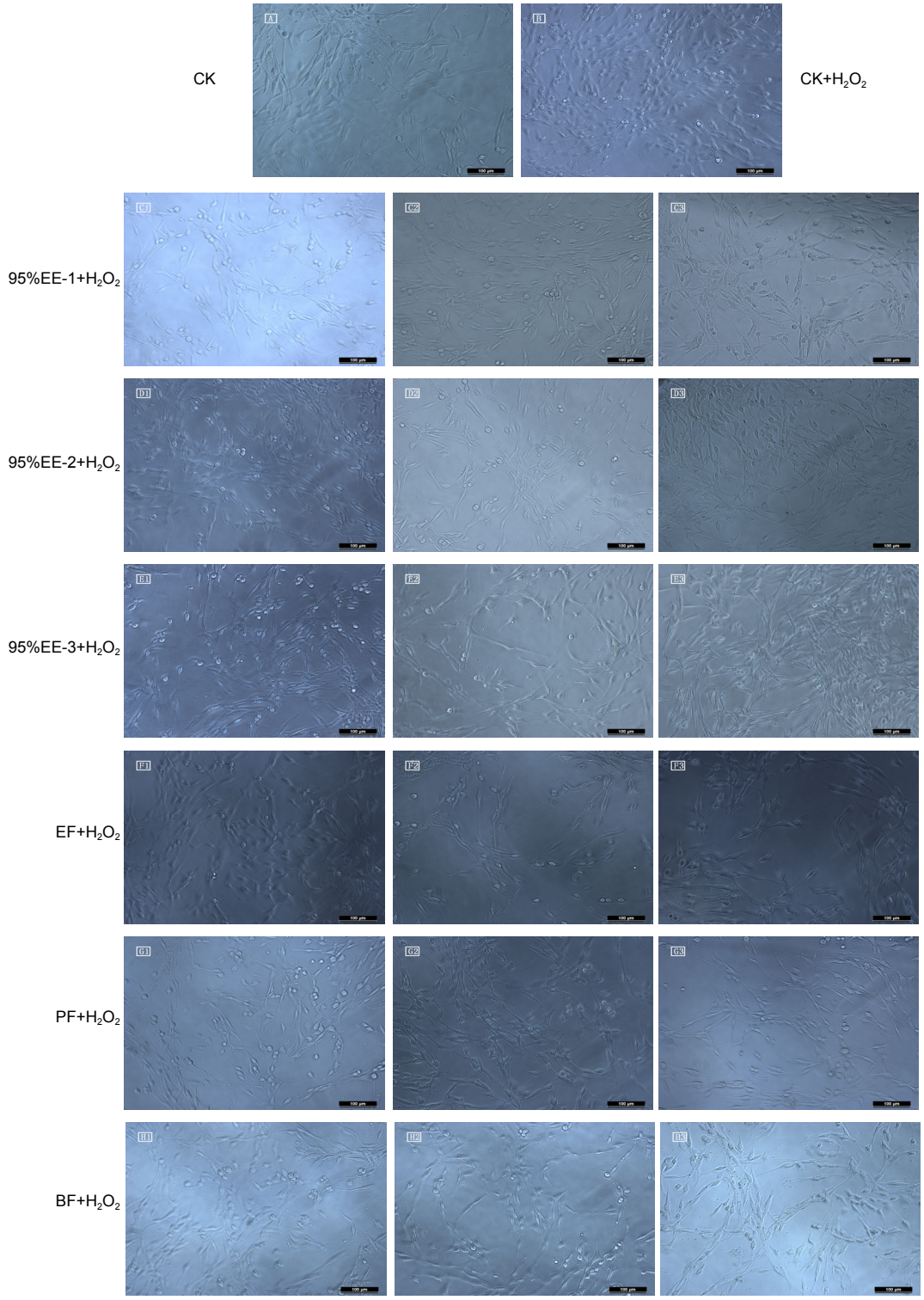

Figure 4 Morphology of PC12 cells pretreated with different concentrations of the various organic extracts of *Alpinia oxyphylla* and exposed to H_2O_2.

Control group (CK) (A); model group (CK + H_2O_2) (B); 95% EE-1 + H_2O_2 (C1, 100 μg/mL; C2, 150 μg/mL; C3, 200 μg/mL); 95% EE-2 + H_2O_2 (D1, 100 μg/mL; D2, 150 μg/mL; D3, 200 μg/mL); 95% EE-3 + H_2O_2 (E1, 50 μg/mL; E2, 100 μg/mL; E3, 200 μg/mL); ethyl acetate fraction (EF) + H_2O_2 (F1, 25 μg/mL; F2, 50 μg/mL; F3, 75 μg/mL); petroleum ether fraction (PF) + H_2O_2 (G1, 25 μg/mL; G2, 50 μg/mL; G3, 100 μg/mL); *n*-butanol fraction (BF) + H_2O_2 (H1, 100 μg/mL; H2, 150 μg/mL; H3, 200 μg/mL). The cells were not stained. Original magnification: 20×. Scale bars: 100 μm.

ural substances have some common mechanisms as well as some unique ones. Therefore, the neuroprotection afforded by *A. oxyphylla* extracts against H_2O_2-induced apoptosis in PC12 cells may involve unique mechanisms. A shortcoming of this study is that the underlying neuroprotective mechanisms were not investigated. In future studies, we will focus on identifying the bioactive components in the EF extract and on elucidating the cell and molecular pathways involved in neuroprotection.

In summary, we investigated the neuroprotective effects of six organic extracts of *A. oxyphylla* on apoptosis in PC12 cells induced by H_2O_2. The EF extract had the best neuroprotective effect. In a following study, we will aim to isolate the bioactive components in this extract and clarify their mechanisms of action. Despite the shortcomings of this study, our findings provide substantial insight into the neuroprotective action of *A. oxyphylla* and its therapeutic potential for the treatment of neurodegenerative disorders.

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