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*Full Length Research Paper*

# Antitoxic effect of baicalin and glycyrrhetic acid on PC12 cells induced by *Aconitum brachypodum* Diel

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**The roots of *Aconitum brachypodum* Diel are used clinically in traditional medicine of China. However, it is also found very toxic and to date, few detoxication strategies are available to completely eliminate its toxicity. The present study was conducted to observe the cellular neurotoxicity of *A. brachypodum* Diels and discuss the detoxication effect of baicalin and glycyrrhetic acid, two compounds from Chinese herbal Baical skullcap root and Radix Glycyrrhizae, respectively. The cellular neurotoxicity of *A. brachypodum* Diels on PC12 cell was estimated and assessed. The results showed that low doses of chloroform-soluble fraction (CFA) displayed little toxicity on PC12 cells. However, long term of high doses (300 to 400 μg/ml) exposure to CFA could lead to significant cell damage, mainly including cell apoptosis and necrosis, increasing intracellular reactive oxygen species (ROS), mitochondria dysfunction and redox imbalance. Pre-incubation of baicalin and glycyrrhetic acid could both decrease the cytotoxicity of PC12 induced by CFA. The present study strongly demonstrated the pro-oxidant effects of CFA and suggested that increased intracellular ROS and calcium have mediated a significant time and dose-dependent cytotoxicity in PC12 cells exposed to CFA via a mitochondrial dependent pathway, which could be effectively reduced by baicalin and glycyrrhetic acid.**

**Key words:** *Aconitum brachypodum* Diel, baicalin, glycyrrhetic acid, PC12, detoxication.

## INTRODUCTION

*Aconitum brachypodum* Diels belong to the genus of Aconitum (Family: Ranunculaceae). Its root is used clinically as an anti-rheumatic, anti-inflammatory and anti-nociceptive drug in traditional medicine of China (Ren et al., 2012; Huang et al., 2013). However, it is also very toxic due to aconitine alkaloids, especially aconitine, the main alkaloid of this plant. The toxic effects of aconitine and its structurally related analogs are known to affect mainly the heart and the central nervous system (AMERI, 1998).

In case of overdoses, acute poisoning or intoxication resulting from misuse, instant treatment including the careful monitoring of vital signs (blood pressure, arrhythmia, etc.) is crucial because of the rapid deterioration of the patient’s condition in the first 24 h. However, the treatment can only be supportive since there is no antidote (Chan, 2009). So far, few detoxication strategies are available to completely neutralize the toxicity of *A. brachypodum* Diels. Its neurotoxicity and pharmacological mechanism need further investigation.

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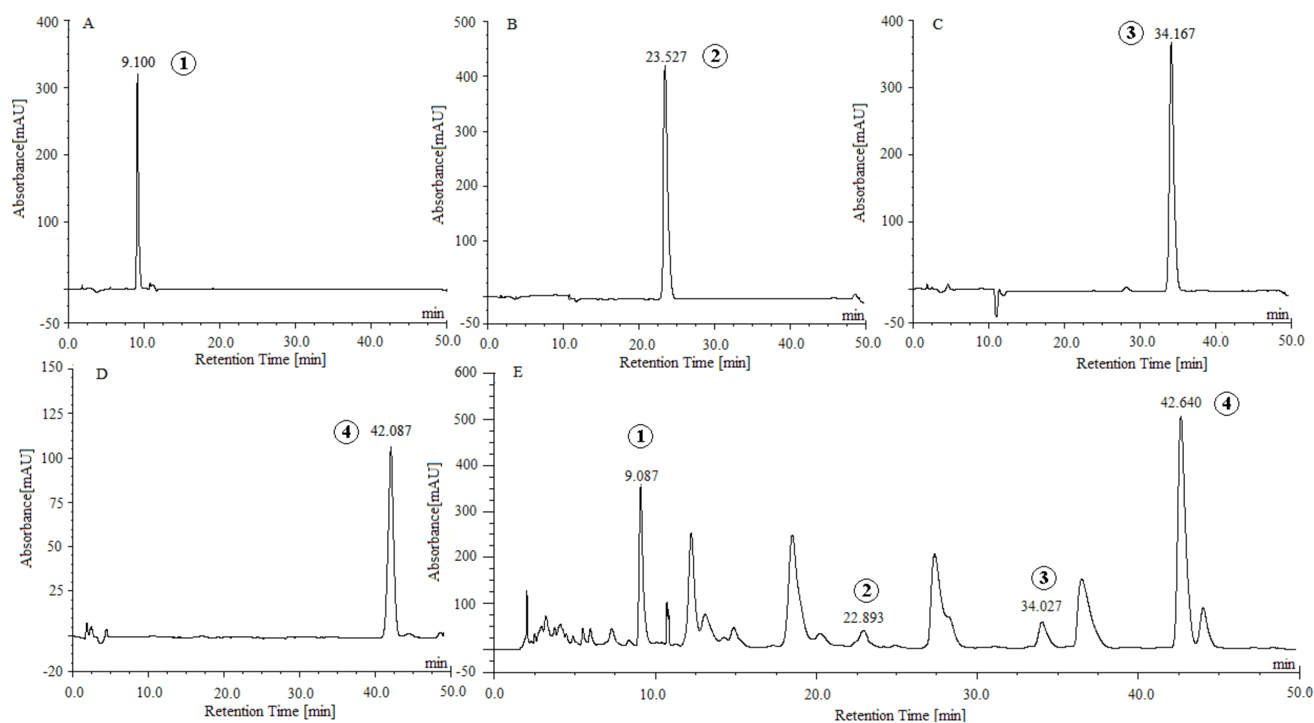


**Figure 1.** The HPLC detection of the chloroform-soluble fraction from *Aconitum brachypodum* diel. (1) benzoyl aconitine; (2) mesaconine; (3) aconitine; (4) bulletine A.

Some Chinese herbal drugs can help to relieve aconite-poisoning (Singhuber et al., 2009). For example, Radix Glycyrrhizae (Gancao) is a commonly used detoxifying herb in traditional Chinese medicine (TCM). The combined use of Radix Glycyrrhizae and Radix Aconiti is a safe pair in TCM formulations (Gu et al., 2008). By combining with Aconitum and Glycyrrhiza (mostly in decoction), the toxicity of Aconitum can be largely reduced (Ma et al., 2006; Noguchi et al., 1978). The decoction with Radix Glycyrrhizae decreases $LD_{50}$ of Fuzi to an undetectable value (Lei et al., 2007).

The concept of “hot/cold” has been used in TCM to describe the “energies” of medicine, foods and drinks. The concept is derived from the Yin/Yang in Daoism: hot being Yang (阳) and cold being Yin (阴). Any Chinese herbs can be roughly classified into hot, cold and neutral categories. Yin/Yang theory also indicates that “cold” medicine can be used to neutralize or decrease the toxicity induced by “hot” medicine, and vice versa.

Since *A. brachypodum* Diels belong to “hot” medicine, “cold” medicine should be used to reduce its toxicity. Baicalin, the major bioactive constituent of the isolated root of *Scutellaria baicalensis*, is also a common “cold” medicine in TCM. It is widely used in China and Southeast Asian countries (Ma et al., 2009). Evidence has indicated that baicalin has anti-apoptotic (Cheng et al., 2012), anti-oxidant (Cao et al., 2011), anti-tumor, anti-ischemic, anti-inflammatory (Guo et al., 2013) and immune system modulatory activities (Li et al., 2010). Baicalin has been reported to play a beneficial role in various experimental models of invasive toxicants by alleviating inflammatory injury (Kim and Lee, 2012) via the involvement of TLR2- or TLR4-mediated innate immune reactions (Hou et al., 2012; Li et al., 2012), and thus was selected to observe the detoxication effect on *A. brachypodum* Diels.

The present study was conducted to observe the cellular neurotoxicity of *A. brachypodum* Diels and discuss its pharmacological mechanism. Moreover, baicalin and glycyrrhetic acid, two compounds from Baical skullcap root and Radix Glycyrrhizae, respectively, were used to observe their detoxication effects.

## MATERIALS AND METHODS

### Extract preparation

The dried roots of *A. brachypodum* Diel were bought from Bozhou city, Anhui province in China. The plant was authenticated by Dr. Xinqiao Liu, Associate Professor in Pharmacognosy at School of Pharmacy, South-central University for Nationalities. The dried roots of *A. brachypodum* Diel were grinded into powder and submerged in 95% ethanol and left to macerate for three times. The combined solution was filtered and evaporated to complete dryness using a standard Buchi rotary-evaporator. The 95% ethanolic extract was dissolved in 2% vitriol and degreased with petroleum ether. Ammonia was used to regulate pH value to 9.0. The majority of the ethanolic extract was suspended with water and successively extracted with chloroform.

In a pilot investigation, the chloroform-soluble fraction (CFA) (Figure 1) was the most toxic fraction among the extractions and was selected for this study. The CFA was subjected to column

chromatography and reversed-phase high performance liquid chromatography (HPLC). The apparatus used for HPLC was Ultimate3000 (Thermo Fisher, USA). The conditions were as follows: column, a YMC-Pack ODS-A column (4.6 × 250 mm, YMC, Japan); mobile phase, methanol - 0.1% triethylamine (0 to 15 min 13:7 [v/v], 16 to 45 min 39:11 [v/v]). Four aconitine alkaloids were identified by comparing their retention time with those of the reference standards (Figure 1).

**Reagents**

3-(4, 5 – Dimethylthiazol -2 – yl ) – 2, 5 –diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemicals Co (St Louis, Missouri, USA). Fluo-3 AM was purchased from Biotium (USA). 7’-dichlorodihydrofluorescin diacetate (DCFH-DA) was purchased from Sigma Chemicals Co (St Louis, Missouri, USA). 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′- tetraethyl benzimidazol carbocyanine iodide (JC-1, Molecular Probes) was purchased from Beyotime Institute of Biotechnology, China. Dopamine (DA) assay was from Shanghai-Jianglai Institute (China). Roswell Park Memorial Institute-1640 (RPMI-1640) was purchased from Hyclone (Logan, UT). Fetal bovine serum (FBS) was purchased from HYCLONE Life Technologies. Baicalin and glycyrrhetic acid were purchased from National Institutes for Food and Drug Control (China). All chemicals were of the highest purity commercially available.

**Cell culture and drug treatment**

The rat pheochromocytoma cell line PC12 were obtained from China Center for Type Culture Collection. The cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated horse serum, 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a water-saturated atmosphere of 5% $CO_2$ at 37°C. The culture medium was changed every other day and cells were subcultured about three times a week. All experiments were carried out 12 h after cells were seeded at an appropriate density according to each experimental scale. The CFA was freshly prepared with dimethyl sulfoxide (DMSO) and diluted by culture media at the desired concentrations just before use. The PC12 cells were preincubated with indicated concentration of CFA for various period of time. In a pilot investigation, cells were treated with CFA at concentrations ranging from 0 to 400 µg/ml for various intervals (6, 12, 18, 24 h) and then examined for cell viability. Concentration of 200 µg/ml CFA as control was used in an extensive study of detoxification of baicalin and glycyrrhetic acid. Baicalin (1 to 9 µM) or glycyrrhetic acid (5 to 40 µM) were added 2 h before CFA was added.

**Cell viability assay**

The cell viability was assessed by using the MTT assay as previously described (Li et al., 2013). Briefly, PC12 cells (1 × $10^5$ cells/ml) were treated with CFA and baicalin or glycyrrhetic acid for 24 h at 37°C. After 3 h incubation with MTT (0.5 mg/ml), cells were lysed in DMSO and the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (TECAN A-5082, megllan, AUSTRIA). Cell viability was expressed as a percent of the control value. Meanwhile, the concentration of the CFA used in assays of ROS and mitochondrial membrane potential were based on the results of the MTT test.

**Measurement of DA release**

The supernatant of PC12 cells was collected for the detection of DA release. The production of DA was measured by enzyme linked immunosorbent assay (ELISA) method using commercial kits (Jianglai Co. Ltd, Shanghai, China) according to the manufacturer's instructions.

**Measurement of mitochondrial membrane potential** ($\Delta\Psi_m$)

To assess $\Delta\Psi_m$, a cell-permeable cationic and lipophilic dye, JC-1 was used as previously described (Cossarizza et al., 1993). This probe aggregates within mitochondria and fluoresces red (590 nm) at higher $\Delta\Psi_m$. However, at lower $\Delta\Psi_m$, JC-1 cannot accumulate within the mitochondria and instead remains in the cytosol as monomers, which fluoresce green (490 nm). Therefore, the ratio of red to green fluorescence gives a measure of the transmembrane electrochemical gradient. PC12 ($10^6$ cells) were treated with CFA and baicalin or glycyrrhetic acid for 24 h at 37°C. The cells were then incubated with JC-1 (5 µg/ml) in the dark for 15 min at room temperature. Then, cells were washed in phosphate-buffered saline (PBS), suspended in 400 µl of PBS and analyzed by flow cytometry.

**Measurement of intracellular reactive oxygen species (ROS)**

The generation of ROS for the cells was evaluated by a fluorometry assay using intracellular oxidation of DCFH-DA. The cells in logarithmic growth phase were incubated in a 6-well plates for 24 h for stabilization, then the medium was replaced with medium containing different concentrations (0-400 µg/ml) of CFA for 24 h. Different concentrations of baicalin (2.25, 4.5 µM) and glycyrrhetic acid (10, 20 µM) were pre-incubated with 200 µg/ml of CFA to observe their antitoxic effect. After exposure, the cells were washed with PBS, then they were re-suspended at a concentration of 1 × $10^6$ cells/ml and were stained by the staining solution for 20 min, the cells were detected and analyzed by flow cytometry.

**Measurement of intracellular calcium**

Intracellular calcium level was determined with Fluo-3/AM. The green fluorescent levels reflecting intracellular calcium transient function were determined by flow cytometry. Briefly, the cells were washed with PBS after samples exposure described earlier, then they were re-suspended at a concentration of 1 × $10^6$ cells/ml and were stained with 5 µM Fluo-3AM (Biotium, USA) in RPMI-1640 and incubating at 37°C for 30 min. The fluorescence was measured and analyzed by flow cytometry.

**Statistical analysis**

Statistical analysis was performed using statistical package for social sciences (SPSS) 11.5 for windows. All results were presented as mean ± standard error of mean (SEM). Group differences were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD's) post hoc tests. A probability of $P < 0.05$ was considered significant.

## RESULTS

### The dose- and time- response effect of CFA on PC12 cells

As shown in Figure 2, lower dosage (< 100 µg/ml) of CFA could enhance the proliferation of PC12 cells during a

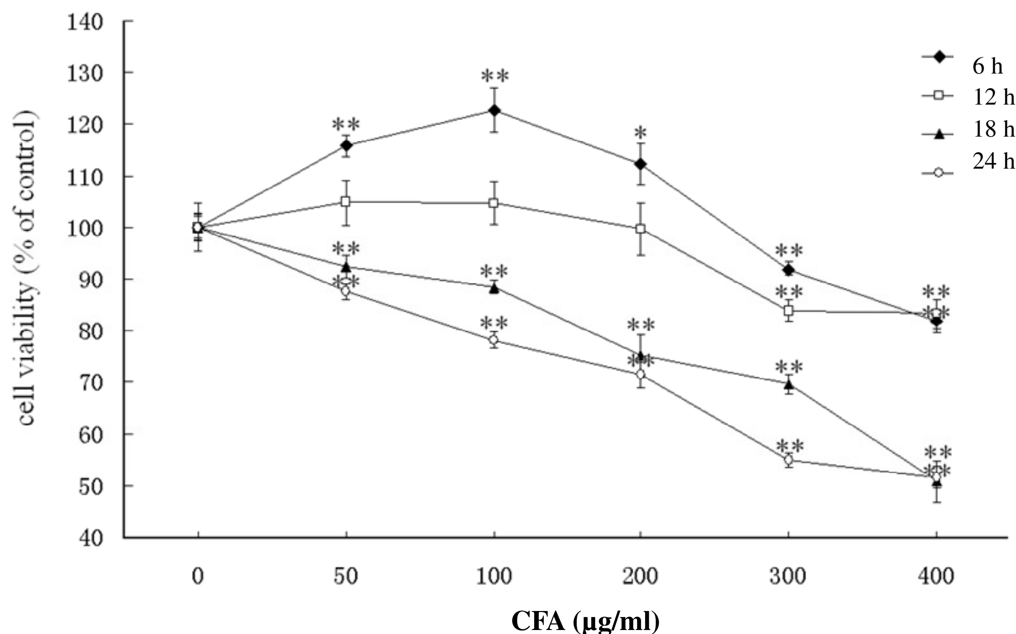


**Figure 2.** Effect of CFA on cell viability of PC12. Cell viability was measured by MTT assay. Control value was taken as 100%. Values are expressed as mean ± SEM, n = 8. Significant statistical difference was indicated by *P < 0.05 **P < 0.01 vs. control group.

short period of 6 h. However, the cell viability was decreased when the concentration and time period increased. After 12 h of incubation, the viability of the cells significantly decreased under the concentration of 300 to 400 µg/ml. As time increased, significant cytotoxicity of CFA was observed at the concentrations of 200 to 400 µg/ml. ANOVA analysis and Dunnett's test revealed that PC12 cells were inhibited both in a dose-dependent and time-dependent manner by CFA.

### Inhibition of baicalin and glycyrrhetic acid on cell viability of CFA-injured PC12 cells

As shown in Figure 3, baicalin and glycyrrhetic acid either for 2 h prior to, or during the CFA insult, prevent the decreased cell viability in a dose-dependent manner, which indicated the neuroprotective effect of baicalin and glycyrrhetic acid on CFA-induced injury. Pre-incubation of baicalin and glycyrrhetic acid prior to CFA insult (Figure 3A) had stronger protective effect than co-incubation during the insult (Figure 3B). The cell viabilities were increased to (82.4 ± 1.6%) and (85.6 ± 2.9%) after co-incubation of baicalin (9 µM) and glycyrrhetic acid (40 µM), respectively. Otherwise, the cell viability were increased to (84.2 ± 3.9%) and (91.8 ± 3.8%), respectively after pre-incubation of them with the same concentration. Based on the results, pre-incubation of baicalin and glycyrrhetic acid were selected for detectation of DA release, $\Delta\Psi m$, intracellular ROS and calcium of insulted PC12 cells.

### The effect of of baicalin and glycyrrhetic acid on DA release

As shown in Figure 4, incubation of CFA (200 µg/ml) for 24 h could significantly evoke DA production from PC12 cells. Pre-incubation of baicalin (1 to 9 µM) and glycyrrhetic acid (5 to 40 µM) could both inhibit CFA-induced DA release of PC12 cells.

### Inhibition of baicalin and glycyrrhetic acid on $\Delta\Psi_m$ induced by CFA

As shown in Figure 5, $\Delta\Psi_m$ decreased after 24 h of CFA treatment. In contrast, pre-treatment of baicalin and glycyrrhetic acid could both partially reverse the decrease of $\Delta\Psi_m$ induced by CFA. These results indicated the implication of mitochondrial dysfunction in the pathogenesis of cell apoptosis and the sensitivity of $\Delta\Psi_m$ towards mitochondrial ROS generated by CFA.

### Inhibition of baicalin and glycyrrhetic acid on generation of intracellular ROS induced by CFA

As shown in Figure 6, there was significant increase of intracellular ROS after incubation of CFA (200 µg/ml) for

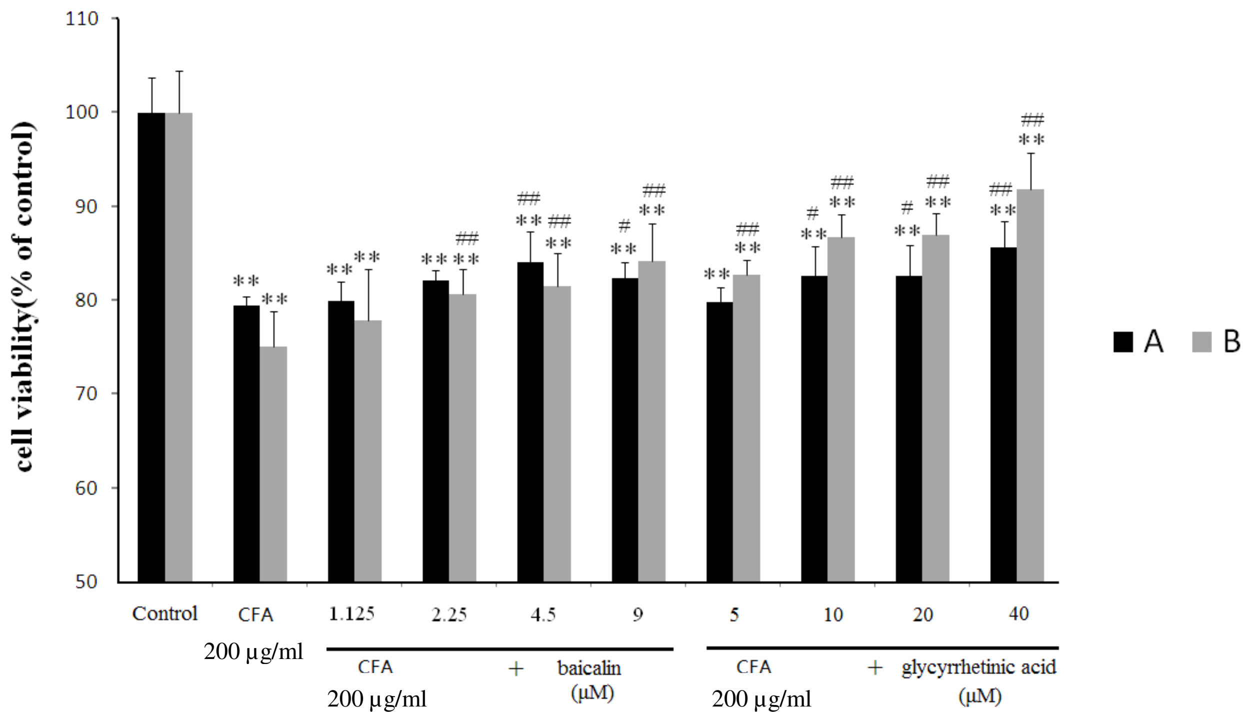


**Figure 3.** Inhibition of baicalin and glycyrrhetic acid on cell viability of CFA-injured PC12 cells. Cells were (A) co-incubation or (B) pre-incubation with baicalin and glycyrrhetic acid for 2 h, then CFA was added for 24 h. Significant statistical difference was indicated by **P<0.01 vs. control group, #P<0.05 ##P<0.01 vs. CFA group.

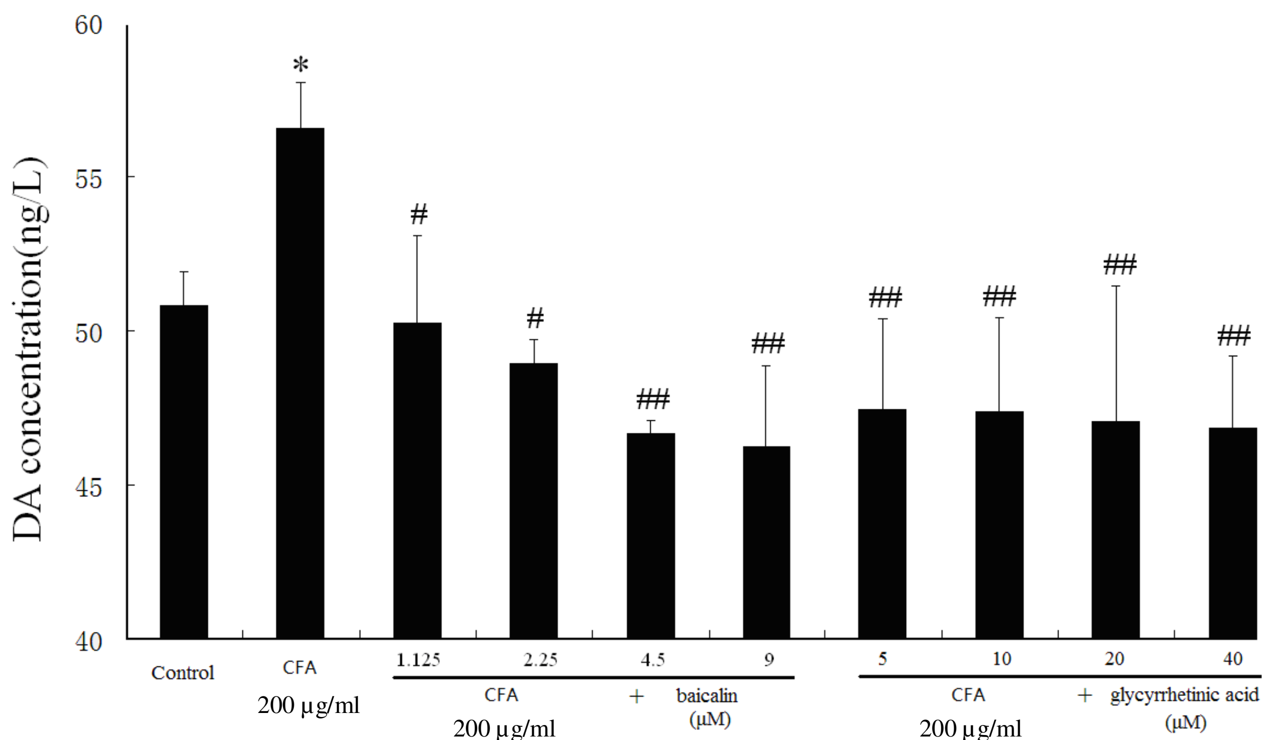


**Figure 4.** The effect of baicalin and glycyrrhetic acid on DA release induced by CFA. Cells were pre-incubation with baicalin or glycyrrhetic acid for 2 h, then CFA was added for 24 h. Significant statistical difference was indicated by *P<0.05 vs. Control group, #P<0.05 ##P<0.01 vs. CFA group.

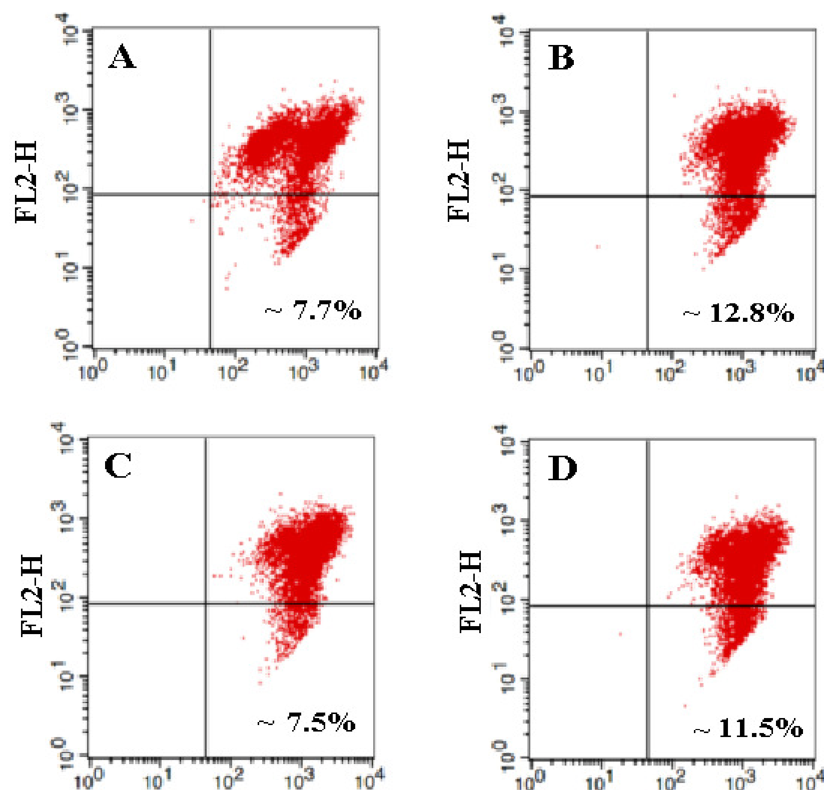


**Figure 5.** Inhibition of baicalin and glycyrrhetic acid on $\Delta\Psi_m$ induced by CFA. Cells were pre-incubation with baicalin or glycyrrhetic acid for 2 h, then CFA was added for 24 h. (A) Control; (B) CFA 200 µg/ml; (C) baicalin 4.5 µM; (D) glycyrrhetic acid 20 µM. The figure shows an average of three experiments done independently.

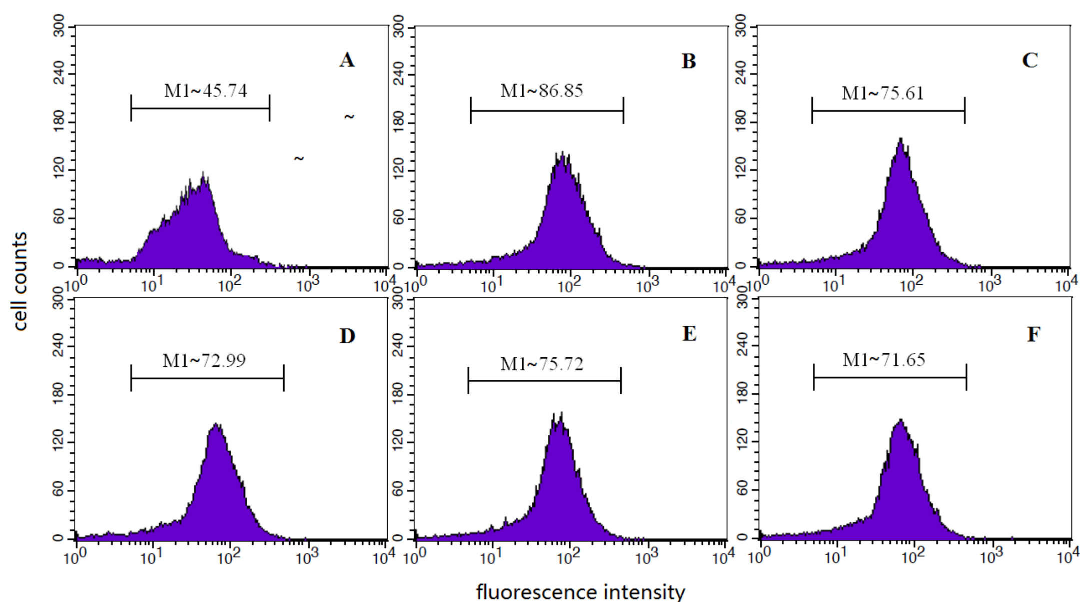


**Figure 6.** Inhibition of baicalin and glycyrrhetic acid on generation of intracellular ROS induced by CFA. Cells were pre-incubation with baicalin or glycyrrhetic acid for 2 h, then CFA was added for 24 h. (A) control; (B) CFA 200 µg/ml; (C) CFA 200 µg/ml + baicalin 2.25 µM; (D) CFA 200 µg/ml + baicalin 4.5 µM; (E) CFA 200 µg/ml + glycyrrhetic acid 10 µM; (F) CFA 200 µg/ml + glycyrrhetic acid 20 µM. The figure shows an average of three experiments done independently.

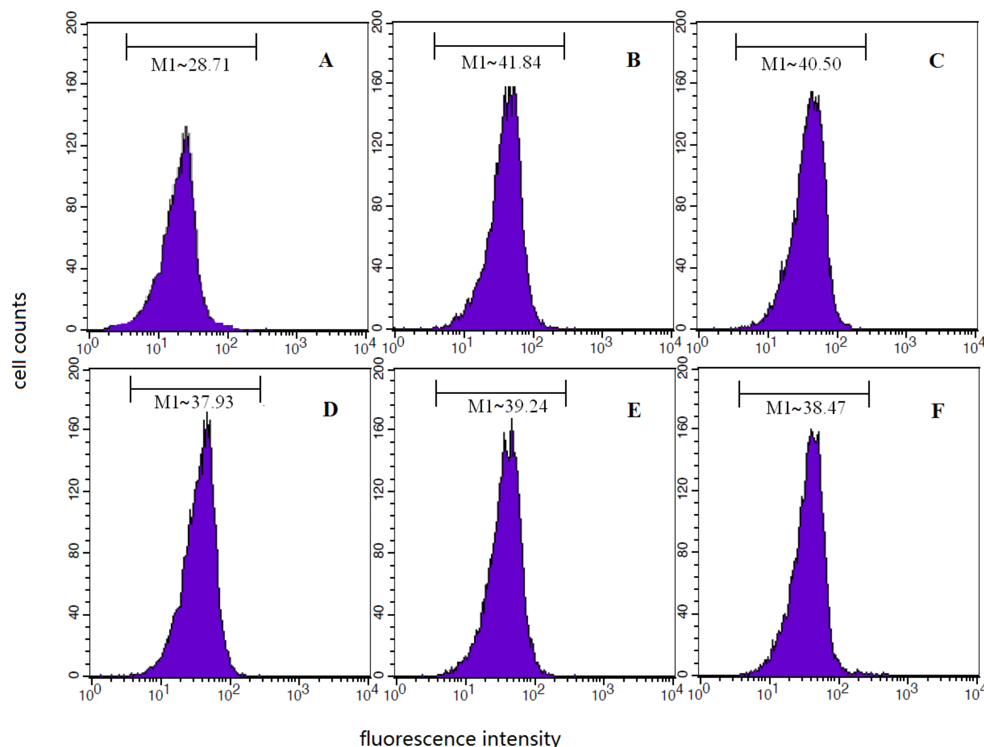


**Figure 7.** The effect of of baicalin and glycyrrhetic acid on intracellular calcium of PC12 induced by CFA. Cells were pre-incubation with baicalin or glycyrrhetic acid for 2 h, then CFA was added for 24 h. (A) control; (B) CFA 200 μg/ml; (C) CFA 200 μg/ml + baicalin 2.25 μM; (D) CFA 200 μg/ml + baicalin 4.5 μM; (E) CFA 200 μg/ml + glycyrrhetic acid 10 μM; (F) CFA 200 μg/ml + glycyrrhetic acid 20 μM. The figure shows an average of three experiments done independently.

24 h. Pre-incubation of baicalin and glycyrrhetic acid at different concentrations could both decrease the intracellular ROS induced by CFA to some extent.

## The effect of of baicalin and glycyrrhetic acid on intracellular calcium

As shown in Figure 7, there was significant increase of intracellular calcium after incubation of CFA (200 μg/ml) for 24 h. Pre-incubation of baicalin and glycyrrhetic acid at different concentration could both decreased the intracellular calcium induced by CFA to some extent.

## DISCUSSION

The present results showed that lower dose of CFA displayed little toxicity on PC12 cells. However, long term exposure to 300 to 400 μg/ml of CFA could lead to cell apoptosis and necrosis, increasing DA production, increasing intracellular ROS, mitochondria dysfunction and redox imbalance (Figure 8).

DA, the neurotransmitter in DAergic neurons, can be spontaneously oxidized into electron-deficient DA quinone, which readily forms a covalent bond with nucleophiles, such as the thiol group on the amino acid cysteine (Graham et al., 1978). DA can be oxidized to generate semiquinones, quinones, oxygen radicals and other reactive oxygen species. These toxic molecules ultimately contribute to the inhibition of mitochondrial respiration and lipid peroxidation, which may play an important role in neuronal cell death (Chen et al., 2003; Zhao et al., 2010). Irreversible modification of cysteine residues on proteins can alter the function of the protein, potentially jeopardizing the health of the cell. Covalent modification of cysteinyl residues forming 5-cysteinyl-dopamine in both proteins and glutathione side effects (GSH) is thought to be the mechanism underlying the toxicity of DA to the neurons (Hastings et al., 1996).

It is possible that free radical metabolites are responsible for the cytotoxicity observed in PC12 cells. Mitochondria are important producers of ROS, which can be highly damaging and inhibitory to cardiomyocyte function. Mitochondria also serve as calcium buffers, protecting neurons from excitotoxic cell death (Duchen, 2000). At the same time, mitochondria are the main targets of ROS-induced oxidative damage (Starkov, 2008; Saretzki, 2009). The mitochondrion-to mitochondrion ROS-induced ROS release constitutes a positive feedback mechanism for enhanced ROS production leading to potentially significant mitochondrial

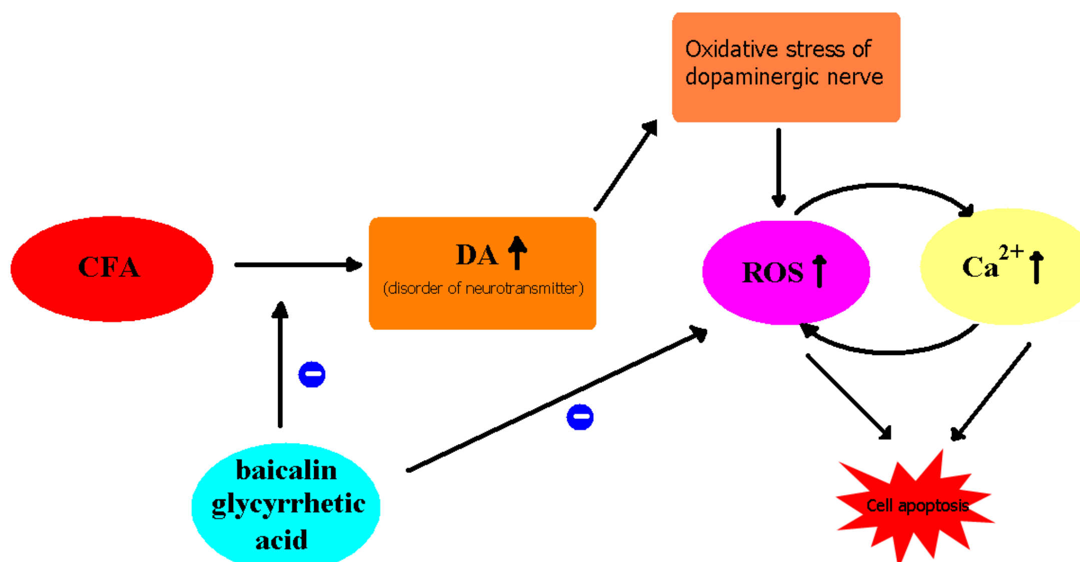


**Figure 8.** The neurotoxicity of CFA and the target of baicalin and glycyrrhetic acid.

and cellular injury (Zorov et al., 2006). The present study demonstrated that CFA-induced alterations in mitochondrial redox functions were critical in promoting ROS-mediated cell death. The decrease of $\Delta\Psi_m$ induced by CFA showed a ROS-mediated mitochondrial dysfunction in the cells. Thus, the CFA-induced alterations in mitochondrial function in PC12 cells may be a major cause of apoptosis and cell death. The results showed that CFA-induced cell death was an effect of increased oxidative stress signaling and alterations in the mitochondrial redox. Baicalin and glycyrrhetic acid might improve mitochondrial function through eliminating the CFA-induced overproduction of ROS.

Calcium plays a pivotal role in many inter- and intraneuronal processes, including (dopaminergic) neurotransmission (Westerink, 2006), gene transcription (Carrasco and Hidalgo, 2006), neurodegeneration (Mattson, 2012), and neurodevelopment (Pravettoni et al., 2000). Arrhythmogenic toxicity of aconitine is related to intracellular $Ca^{2+}$ signals (Zhou et al., 2013). Neuronal cells rely heavily on strict regulation of their intracellular calcium concentration ($[Ca^{2+}]_i$). Increased mitochondrial oxidative stress was dependent on mitochondrial $Ca^{2+}$ overload in PC12 cells, because blocking mitochondrial $Ca^{2+}$ uptake prevented elevated superoxide production. In this study, the results also showed that in PC12 cells, CFA increased $[Ca^{2+}]_i$ and induced triggered activities such as ROS.

The present study demonstrated the pro-oxidant effects of CFA and suggested that increased intracellular ROS and calcium have mediated time and dose-dependent cytotoxicity in PC12 cells exposed to CFA via a mitochondrial dependent pathway. Baicalin and glycyrrhetic acid could effectively reduced CFA-induced neurotoxicity.

## Conflict of interest

The authors declare that there is no conflict of interest.

## ACKNOWLEDGMENTS

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