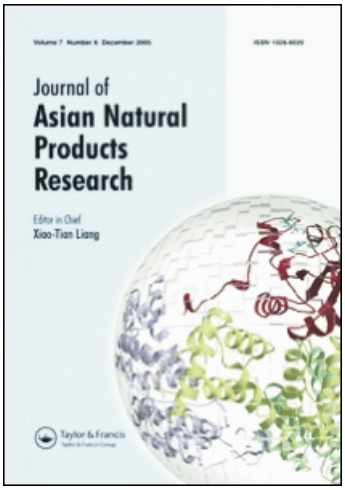

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Scutellarin protects PC12 cells from oxidative stress-induced apoptosis

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Scutellarin protects PC12 cells from oxidative stress-induced apoptosis

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The present study investigated the effects of scutellarin on oxidative stress-induced cell apoptosis in PC12 cells. Exposure of cells to hydrogen peroxide (H_2O_2) triggered a typical apoptosis, as evidenced by DNA fragmentation, DNA loss and externalization of phosphatidylserine (PS). This treatment also caused significant elevation of oxidative stress characterized by intracellular accumulations of reactive oxygen species (ROS) and malondialdehyde (MDA), a product of lipid peroxidation. Preincubation of cells with scutellarin significantly inhibited the fragmentation and loss of DNA, the externalization of PS, and decreased the percentage of cell apoptosis. Also, intracellular accumulations of ROS and MDA resulting from H_2O_2 exposure were significantly reduced by scutellarin. These findings suggest that scutellarin exerts significant protection against oxidative stress-induced apoptosis, which might be beneficial for the prevention and treatment of oxidative stress-mediated disorders.

Keywords: Scutellarin; Apoptosis; Oxidative stress; Reactive oxygen species; Lipid peroxidation; PC12 cells

1. Introduction

Oxidative stress-induced cell apoptosis is involved in the pathogenesis of neurodegenerative disorders such as stroke, Alzheimer's disease (AD), and Parkinson's disease (PD) [1,2]. ROS such as H_2O_2, superoxide and hydroxyl radical, *etc.*, a mediator of oxidative stress, readily damage biological molecules, which can ultimately lead to apoptotic or necrotic cell death [3]. As the major component of reactive oxygen species (ROS), H_2O_2 has been used extensively as an inducer of oxidative stress in *in vitro* models [4]. Freely diffusible H_2O_2, if not degraded by antioxidant enzymes, can damage proteins, lipids, mitochondria, and DNA directly by penetrating the cell nucleus [5]. The delineation of biochemical pathways involved in neuronal cell death due to H_2O_2 may well aid the development of drugs to treat various neurodegenerative diseases.

Scutellarin (β-D-glucopyranosiduronic acid, 5,6-dihydoxy-2-(4-hydroxyphenyl)-4-oxo-4*H*-benzopyran-7-yl) is a primary active ingredient in breviscapine. Breviscapine is a mixture, extracted from the Chinese herb *Erigeron breviscapus* [6]. Breviscapine is effective for ischemic cerebrovascular diseases. *In vivo*, breviscapine and its preparation can protect against cerebral ischemia-reperfusion injury by many pathways of action [7]. In China, breviscapine injection is extensively used to treat ischemic cerebrovascular diseases. During screening of compounds of

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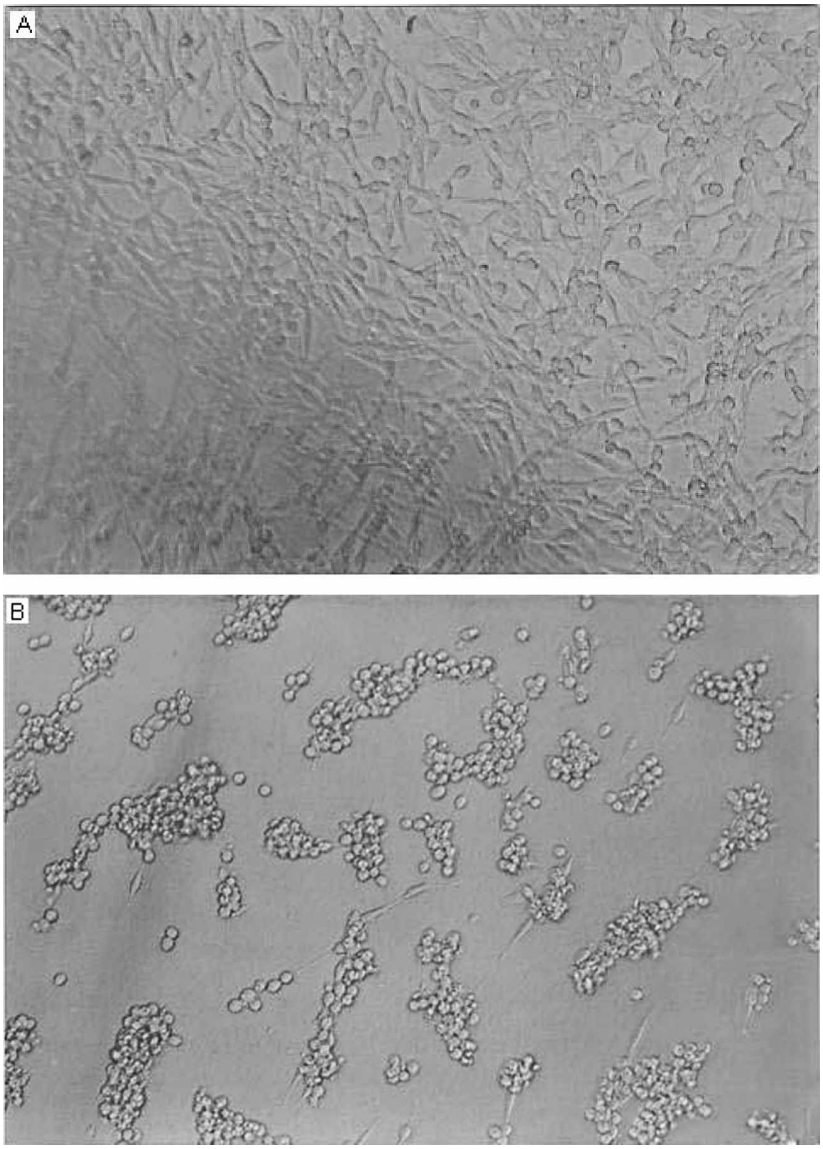
breviscapine, we found that scutellarin showed stronger antioxidative activity. However, there is insufficient information on the neuroprotective properties of scutellarin, especially at cellular level. In this study, we chose PC12 cells to explore the protection of scutellarin against H_2O_2-induced apoptosis.

2. Results and discussion

2.1 Scutellarin protected PC12 cells against H_2O_2-induced cytotoxicity

As shown in figure 1, there was a significant decrease in cell number and most cells lost neurites and became round, some of which were lysed or replaced by debris, following 12 h



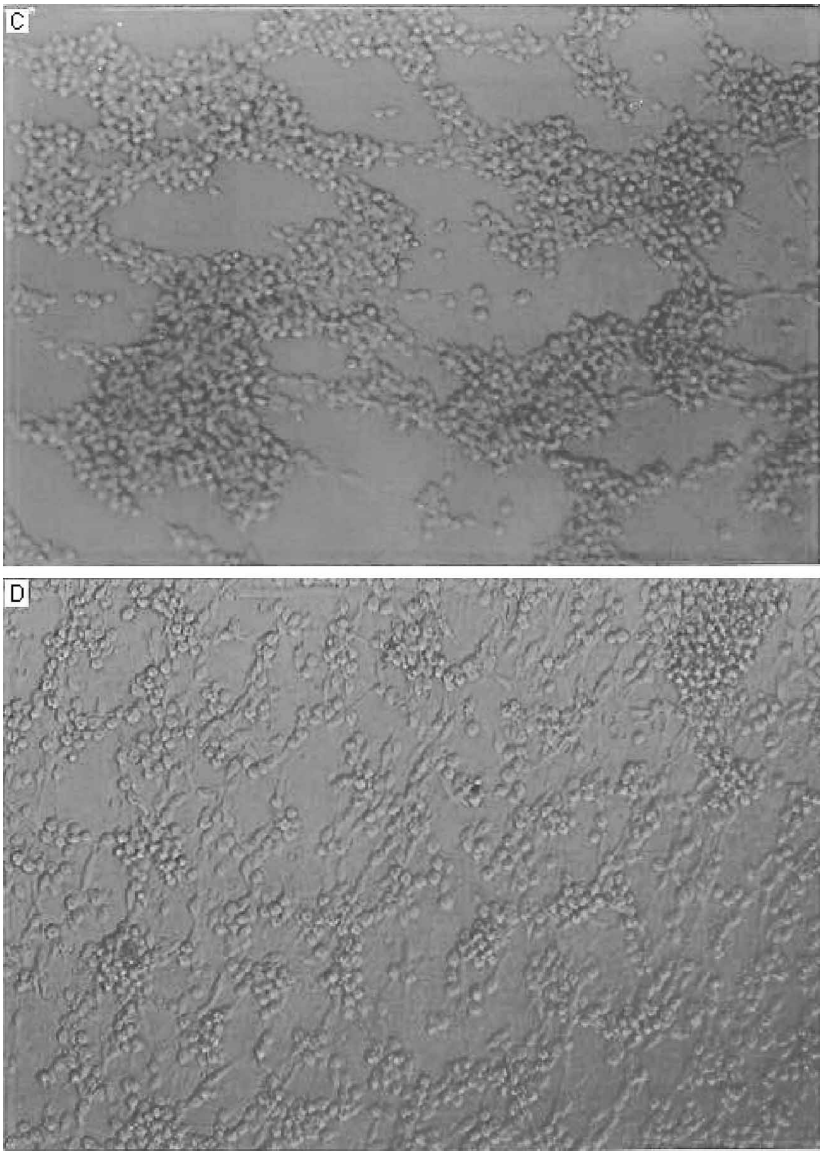


Figure 1. Effects of scutellarin on PC12 cell injury by H_2O_2. (A) PC12 control cells. (B) PC12 cells exposed to 200 μmol L^{-1} H_2O_2 for 12 h. There is a significant decrease in cell number and most of the cells lose neurites and are round in shape. (C and D) PC12 cells preincubated with 1 and 10 μmol L^{-1} scutellarin, respectively.

exposure of the cells to 200 μmol L^{-1} H_2O_2. In contrast, cultures exposed to the same amount of H_2O_2 in the presence of scutellarin appeared remarkably preserved, indicating scutellarin protects against H_2O_2. Mitochondria of living cells can reduce MTT to produce formazan, the amount of which is directly related to the living cell number [8]. Cell viability as determined by MTT reduction was decreased from $100 \pm 4.26\%$ in controls to $38.10 \pm 2.49\%$ after PC12 cells were exposed to H_2O_2 (Table 1), suggesting that PC12 cells were very sensitive to H_2O_2-induced cell injury. When the cells were preincubated with 0.1, 1 and 10 μmol L^{-1} scutellarin, cell viability was increased from $38.10 \pm 2.49\%$ to $53.4 \pm 3.52\%$, $61.76 \pm 4.78\%$ and $78.33 \pm 4.66\%$, respectively.

2.2 Scutellarin attenuated H_2O_2-induced intracellular accumulation of ROS and lipid peroxidation

To determine whether the cytoprotective effect of scutellarin is related to its antioxidant properties, we analyzed its ability to prevent intracellular accumulation of ROS and malondialdehyde (MDA), which is the most important indicator for lipid peroxidation. The treatment of cells with H_2O_2 induced lasting increases in the fluorescence inside cell. This continuous increase was suppressed by scutellarin (data not shown). Table 1 shows that intracellular ROS accumulation 12 h after H_2O_2 exposure was significantly reduced in the presence of scutellarin. The resultant oxidative stress assay showed that H_2O_2 exposure produced a significant increase in MDA levels, representing a 73% increase above untreated control levels. When the cells were pretreated with 0.1, 1, and 10 $\mu mol\,L^{-1}$ of scutellarin, H_2O_2-induced accumulation of MDA was reduced from $173.54 \pm 5.88\%$ to $148.50 \pm 4.70\%$, $132.27 \pm 10.34\%$ and $122.17 \pm 7.82\%$, respectively.

2.3 Scutellarin protected PC12 cells against H_2O_2-induced apoptosis

To test whether H_2O_2 induced cell death *via* apoptosis, three different indices were applied: apoptotic cell count with flow cytometry, DNA fragmentation analysis by agarose gel electrophoresis and the externalization of phosphatidylserine (PS) assay by Annexin V staining. Staining with propidium iodide revealed that ca. 30% of H_2O_2 exposed cells died *via* apoptosis (figure 2). However, pretreatment of cells with 1 and 10 $\mu mol\,L^{-1}$ of scutellarin was very effective in attenuating H_2O_2-induced apoptotic cell death, reducing the apoptotic cell count to 5–8%. To confirm apoptosis further, we analyzed genomic DNA for the presence of a typical fragmentation pattern of apoptosis and processed cellular DNA for agarose gel electrophoresis. A ladder pattern representing fragmentation of DNA into oligonucleosome length fragments is observed after H_2O_2 treatment for 12 h (figure 3). Pretreatment of cells with 1 and 10 $\mu mol\,L^{-1}$ of scutellarin inhibited remarkably the H_2O_2-induced DNA fragmentation. To discriminate between apoptotic and necrotic cells, the Annexin V-FITC binding assay was combined with PI vital staining. As shown in figure 4, PC12 cells showed increased Annexin V staining 6 h after H_2O_2 exposure in the absence of scutellarin (16.65%), indicating rapid onset of apoptosis, as the increased cell population stained PI negative, excluding the possibility of necrosis. Pretreatment of the cells with

Table 1. Effects of scutellarin on cell viability, levels of ROS and MDA in PC12 cells.

	% of control		
	Cell viability	*ROS*	*MDA*
Control	100 ± 4.26	100 ± 4.71	100 ± 8.86
H_2O_2	38.10 ± 2.94^a	334.59 ± 23.50^a	173.54 ± 5.88^a
H_2O_2 + scutellarin (μM)			
0.1	53.40 ± 3.52^b	205.57 ± 3.84^c	148.50 ± 4.70^b
1	61.76 ± 4.78^c	153.94 ± 5.36^c	132.27 ± 10.34^b
10	78.33 ± 4.66^c	126.70 ± 7.92^c	122.17 ± 7.82^c

Cells were incubated with 200 $\mu mol\,L^{-1}$ H_2O_2 for 12 h. Scutellarin was added to the culture 30 min prior to H_2O_2 addition. Cell viability, ROS and MDA were assayed 12 h after exposure of PC12 cells to H_2O_2. For ROS assay, 10,000 cells per sample were collected and analyzed with a flow cytometer ($n = 3$). Data are means ± SEM expressed as percentages of the corresponding untreated control ($n = 3$–4). [a]$p < 0.01$ *vs.* control; [b]$p < 0.05$, [c]$p < 0.01$ *vs.* H_2O_2 group.

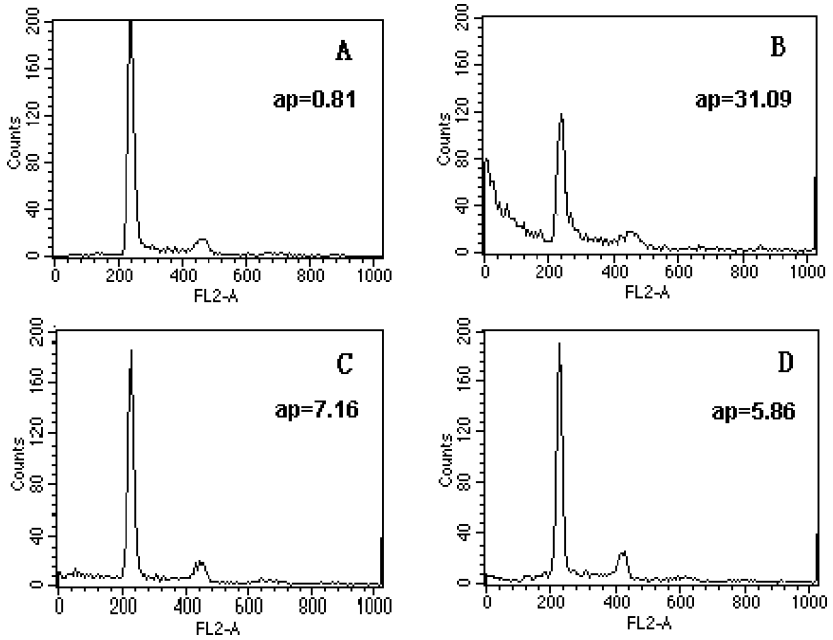


Figure 2. Reduction of H_2O_2-induced apoptosis in PC12 cells by scutellarin assessed by flow cytometric assay. Cells pretreated with 1 and 10 μmol L^{-1} of scutellarin for 30 min followed by exposure to 200 μmol L^{-1} H_2O_2 for an additional 12 h. Cells were collected and examined by flow cytometry. The presence of hypodiploid peak reflects the number of cells suffering from DNA loss. Apoptotic rate is designated by 'ap'. (A) Control PC12 cells; (B) PC12 cells exposed to 200 μmol L^{-1} H_2O_2; (C and D) PC12 cells exposed to 200 μmol L^{-1} H_2O_2 in the presence of 1 and 10 μM scutellarin, respectively. Figure is representative of three experiments with similar results.

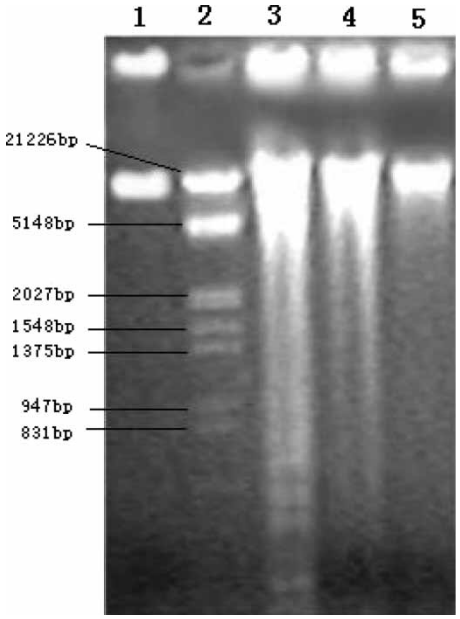


Figure 3. Inhibition of H_2O_2-induced DNA fragmentation in PC12 cells by scutellarin. Cells were exposed to 200 μmol L^{-1} H_2O_2. Scutellarin (1 and 10 μmol L^{-1}) was added to the culture 30 min prior to H_2O_2 addition. Cells were collected at 12 h after H_2O_2 exposure. Genomic DNA was subjected to agarose gel electrophoresis. Lane: (1) control, (2) 200 bp DNA marker, (3) 12 h after H_2O_2 treatment in the absence of scutellarin, (4 and 5) 12 h after H_2O_2 treatment in the presence of 1 and 10 μmol L^{-1} scutellarin, respectively.

scutellarin was also very effective for inhibiting this increased Annexin V staining, reducing the population of Annexin V-positive cells to 3–5%.

In the present study, exposure of PC12 cells to H_2O_2 triggered cell apoptosis, characterized by DNA damage and the loss of plasma membrane phospholipid asymmetry. Scutellarin pretreatment mitigated this DNA damage, as assessed by a DNA sensitive dye with flow cytometry. Scutellarin also reduced H_2O_2-induced DNA laddering, resulting from fragmentation of internucleosomal DNA, which has been observed in many types of cells undergoing apoptotic death and hence is considered as a biochemical hallmark of apoptosis. Alternatively, Annexin V was utilized to confirm the presence of an apoptotic process in H_2O_2-treated cells. Annexin V has a strong binding affinity for PS in cellular membranes. In apoptotic cell, PS loses its normal location in the inner plasma membrane and translocates to the outer layer of the membrane where it is exposed to the extracellular environment and can bind Annexin V [9]. There was a significant decrease in the proportion of Annexin V-positive (*i.e.* early apoptotic) cells with scutellarin treatment, indicating scutellarin did rescue PC12 cells treated with H_2O_2 from apoptotic death. Scutellarin, presumably *via* high catalytic activity or greater membrane permeability, exhibited a higher potency against oxidative stress induced by extracellular H_2O_2, as identified by blocking intracellular of ROS and lipid peroxidation in the present work. Thus the mechanisms of scutellarin action against oxidative stress-induced apoptosis appear to result from the above-described antioxidant effect. Future experiments using primary cultured neurons and animal models would be worthwhile and might provide new insights.

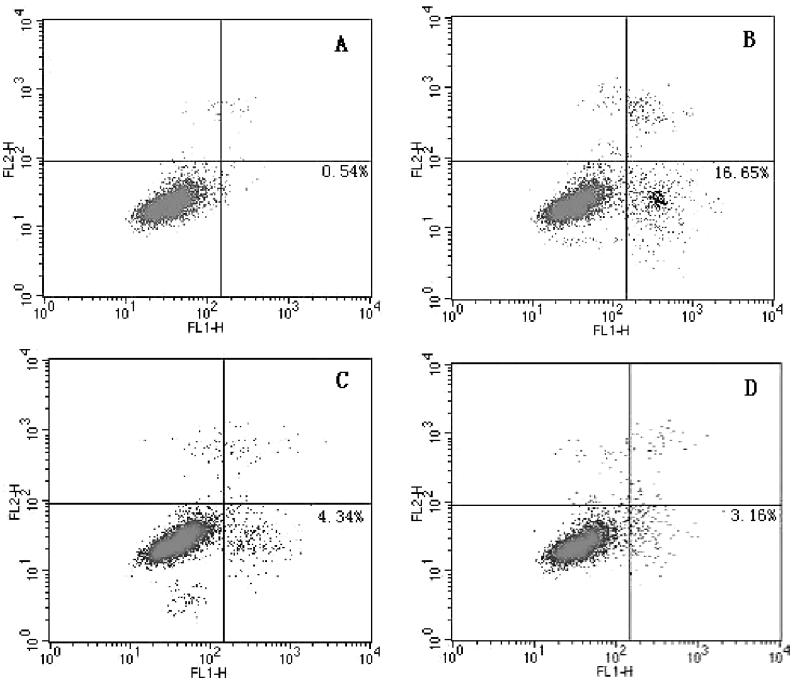


Figure 4. Flow cytometric analysis of apoptotic cells using Annexin V-FITC. Cells were left untreated (A) or treated for 6 h (B) with 200 μmol L^{-1} of H_2O_2. Cells pretreated with 1 and 10 μmol L^{-1} of scutellarin for 30 min were followed by exposure to H_2O_2 for an additional 6 h (C and D), respectively. Cells were incubated with Annexin V-FITC in a buffer containing PI and analyzed by flow cytometry. Ten thousand cells in each sample were analyzed.

3. Experimental

3.1 Materials

Scutellarin (figure 5) was isolated as described by Zhang *et al.* [6]. Its purity, determined by HPLC, was 98.06%. It was dissolved and diluted with phosphate-buffered saline (PBS) before experiments. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Fluka, and Dulbecoo's Modified Eagles Medium (DMEM) was the product of Gibco. Propidium diiodide (PI), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), thiobarbituric acid, and tetraethoxypropane were purchased from Sigma. The Annexin V-FITC apoptosis detection kit was from BD Pharmingen. All other chemicals were of analytical grade.

3.2 Cell culture and experimental treatment

PC12 cell line was obtained from the Shanghai Institute of Cell Biology and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in high glucose DMEM supplemented with 15% (v/v) heat-inactivated fetal bovine serum (FBS), 100 ku L^{-1} penicillin and 100 mg L^{-1} streptomycin. Experiments were carried out 24–48 h after the cells were seeded into plates or dishes. To produce an oxidative stress, H_2O_2 was freshly prepared from a 30% stock solution prior to each experiment. Preincubation of cells with scutellarin was conducted for 30 min before H_2O_2 was added. Assays for cell viability, ROS, lipid peroxide, and apoptosis were performed at 6 or 12 h after addition of H_2O_2.

3.3 Viability assay

Cell survival was evaluated by two different methods: morphological observation with a phase-contrast microscope and MTT reduction [8]. PC12 cells exposed to H_2O_2 or scutellarin for 12 h were viewed on the microscope and photographed using 10 × or 20 × objectives. For assay of MTT reduction, after 12 h H_2O_2 exposure, MTT solution in PBS was added, with a final concentration of 0.5 mg mL^{-1}, and the incubation continued for 4 h. Finally, an equal volume of a lysis buffer containing 50% dimethylformamide and 20% SDS (pH 4.8) was added. The mixtures were kept overnight and then the amount of formazan was quantified by determining the absorbance at 570 nm using an ELISA plate reader (Hua Dong Electronic Co, Nanjing, China).

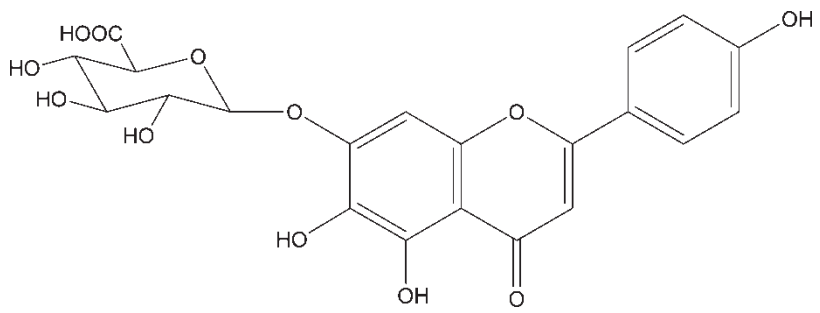


Figure 5. Chemical structure of scutellarin.

3.4 Measurement of ROS

The intracellular accumulation of ROS in PC12 cells was determined with DCFH-DA [10]. This nonfluorescent compound accumulates within cells upon deacetylation. DCFH then reacts with ROS to form fluorescent dichlorofluorescein (DCF). PC12 cells were dissociated from culture dishes with pancreatin in DMEM in the presence of 10 μmol L^{-1} DCFH-DA for 10 min at 37°C, washed once using DMEM (without phenol red) supplemented with 2% dialyzed FBS, and resuspended in 750 μl of the same solution containing 2 μg PI. Flow cytometric analysis was performed using a FACScan instrument (BD Pharmingen) with an excitation wavelength (λ_{ex}) of 475 nm and emission wavelength (λ_{em}) of 525 nm. Data were collected in list mode on 10,000 cells after gating only for characteristic forward *versus* orthogonal light scatter and low PI fluorescence to exclude death cells. Median fluorescence intensities of control and test samples were determined with CellQuest™ software (BD Pharmingen).

3.5 MDA assay

Cells were homogenized, and the homogenates used to perform the thiobarbituric acid (TBA) test as previously described [11]. Briefly, aliquots of cell homogenates was resuspended in 0.5 mL of 20% trichloroacetic acid (TCA), then microfuged at 12,000*g* to remove precipitated proteins. Supernatant was then mixed with 0.5 mL of TBA reaction mixture (0.67% w/v TBA in H_2O mixed with an equal volume of glacial acetic acid) and boiled for 60 min, then cooled on ice. The samples were microfuged for 3 min, and the OD_{530} of the supernatant read. Samples of homogenates from each cell type were saved for protein analysis, using the Coomassie blue method [12]. The concentrations of MDA were calculated using tetraethoxypropane as a reference standard. The quantities of MDA were expressed in terms of amount (nmol) per 100 mg protein and converted into percentage of control values to compensate for variations of absolute weights of lipid oxides among different experiments.

3.6 Flow cytometric detection of apoptotic cells

The percentage of late apoptosis was determined by assay for hypodiploid [13], using a FACS 440 flow cytometer. Cells were collected 12 h after H_2O_2 exposure by centrifugation (350*g*) and washed with PBS. The pellets were resuspended in ice cold 70% ethanol and fixed at 4°C for 24–48 h. Cells were then centrifuged, and ethanol was removed by washing thoroughly with PBS. The cell pellets were resuspended in 1 mL of DNA staining reagent containing 50 μg mL^{-1} RNase, 0.1% Triton X-100, 0.1 mmol L^{-1} EDTA (pH 7.4), and 50 μg mL^{-1} PI. The staining was stable at 4°C for 30 min. Red fluorescence (DNA) was detected through a 563–607 nm bandpass filter by using a FACS flow cytometer. In flow cytometric histograms, apoptotic cells will give DNA fluorescence in subdiploid regions, which are well separated from the normal G1 peak. Ten thousand cells in each sample were analyzed.

3.7 Analysis of DNA fragmentation by gel electrophoresis

Fragmented DNA was isolated as described by Li *et al.* [14]. DNA pellets were dissolved in 20 μl TE buffer (Tris 10 mmol L^{-1}, EDTA 1 mmol L^{-1}, pH 7.6). Ten microlitres of DNA

sample were loaded on 1.5% horizontal agarose gels with bromphenol blue/cyanol xylene tracking dyes. Gels were run at 70 V for 45 min submerged in TAE buffer (Tris 40 mmol L^{-1}, acetic acid 20 mmol L^{-1}, EDTA 1 mmol L^{-1}), stained with ethidium bromide (0.5 mg mL^{-1}), and photographed with an ultraviolet gel documentation system.

3.8 Annexin V binding assay

The movement of PS to the extracellular surface was determined by Annexin V binding using a commercially available kit (BD Pharmingen Annexin V-FITC) [15]. Briefly, cells were harvested with trypsin (0.025%)/EDTA (1 mmol L^{-1}) and suspended in binding buffer at a final cell concentration of 10^6 cells mL^{-1}. Approximately 10^5 cells were transferred to a flow cytometric vial, and Annexin V-FITC and propidium diiodide were added to the suspension. The suspension was then vortexed and incubated in the dark for 15 min and subsequently analyzed in the flow cytometer.

3.9 Statistics analysis

Data were expressed as means ± SEM and evaluated for statistical significance with one-way ANOVA followed by Duncan's multiple range.

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