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ORIGINAL ARTICLE

Effect of Gardenia/Acanthopanax-Medicated Serum on High Level Corticosterone-induced Pheochromocytoma Cell Apoptosis and Calcium Ion Concentration

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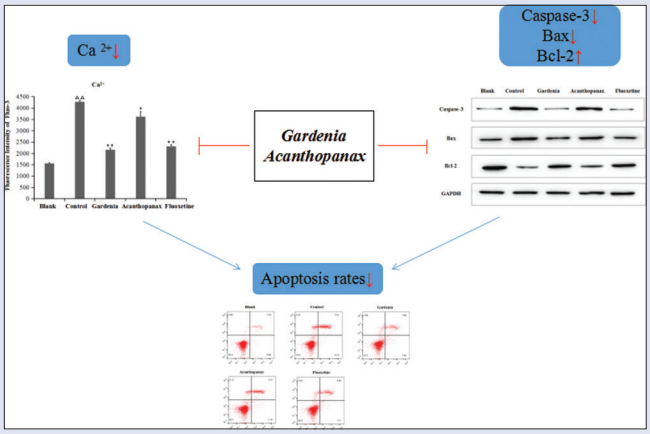
ABSTRACT

Objective: This study aims to investigate the effect of gardenia/acanthopanax-medicated serum on pheochromocytoma (PC12) cell apoptosis and calcium ion concentration, and explore the antidepression mechanism of gardenia/acanthopanax. **Materials and Methods:** In this study, PC12 cells were treated with high concentrations of corticosterone to simulate the effect of high level glucocorticoids in neurons in patients with depression. Next, the apoptosis rate of cells, apoptosis-related proteins, and relative intracellular calcium concentration were detected using flow cytometry. **Results:** Compared with the blank group, the apoptosis rates, pro-apoptotic proteins, and calcium ion concentrations were significantly increased, and the anti-apoptotic proteins decreased in the control groups, and the results were reversed by different drug-medicated serum. Compared with the control groups, the differences in gardenia and fluoxetine groups were statistically significant ($P < 0.01$) and were more significant than acanthopanax group ($P < 0.05$); but the differences between the gardenia and fluoxetine groups were not statistically significant ($P > 0.05$). **Conclusion:** The results of this study suggest that gardenia/acanthopanax-medicated serum can obviously improve the survival rate of neurons under high concentrations of glucocorticoids, downregulate the apoptosis-related proteins, and reduce the intracellular calcium concentration, and gardenia had better effects than acanthopanax.

Key words: Acanthopanax, apoptosis-related proteins, calcium ion concentration, cell apoptosis, gardenia, medicated serum

SUMMARY

- Gardenia and acanthopanax improved the survival rate of neurons under high concentrations of glucocorticoids.
- Gardenia and acanthopanax downregulated the pro-apoptotic proteinss and upregulated the anti-apoptotic proteins.
- Gardenia and acanthopanaxand reduced the intracellular calcium concentration, and gardenia had better effects than acanthopanax.



Abbreviation used: PC12: Pheochromocytoma; GRs: Glucocorticoid receptors; HPLC: High-performance liquid chromatography; DMEM: Dulbecco's modifed eagle medium; HyClone: High glucose medium; PBS: Phosphate buffered saline; SDS: Sodium dodecyl sulfate; PAGE: Polyacrylamide gel electrophoresis; FITC: Fluorescein isothiocyanate; FCM: Flow cytometry; MR: Mineralocorticoid receptor; HPA: Human platelet antigens; NMDA: N-methyl-D-aspartate; nNOS: Nervous nitric oxide synthase; PS: Phosphatidylserine.

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INTRODUCTION

Depression is a group of mood disorders characterized by low mood or emotion, lack of interest, and loss of pleasure. The World Health Organization predicted that depression would become the second major cause of diseases in 2030.[1] The pathogenesis of depression remains unknown. At present, the widespread used antidepressants are based on monoamine hypothesis.[2] The main pharmacological action is inhibiting transportin to block the reuptake of monoamine neurotransmitters, to increase the concentration of transmitters in the synaptic cleft. Although antidepressants can rapidly increase the levels of monoamine neurotransmitters in the central nervous system, its clinical effect requires a few weeks.[3,4] Moreover, the effective rate of antidepressants is only approximately 70%.[5,6] Antidepressants used in the clinic such as fluoxetine, has shown beneficial antidepression effects. However, its clinical use is limited due to side effects such as cardiac toxicity, weight

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gain, sexual dysfunction, and sleep disorders.[7] There is an urgent need to discover the novel potential drug candidate against depression.

During our clinical practices, we found the Chinese herbs gardenia and acanthopanax with idea antidepression effect. Gardenia has been used as antidepression herb for ages. It appears in many Chinese prescriptions, for the treatment of emotional diseases. Modern researches have revealed that gardenia can increase sucrose intake in rats with chronic unpredictable stress and reduce the immobilizing behavior of rats in a forced swimming test.[8] Furthermore, gardenia and its extract also have a certain neuroprotective effect.[9] Animal experiments reveal that acanthopanax has antidepression effect on desperate animal models.[10] Based on this, we tried to explore the underlying effect mechanisms of the gardenia/acanthopanax.

In recent years, a number of studies have revealed that changes in a variety of neurotransmitters and its receptors in the pathogenesis of depression could lead to changes in hippocampal plasticity, which resulted in apoptosis and loss of neurons in the hippocampus and other limbic areas.[11] Some scholars[12] have proposed that the decrease in the number of neurons is a key link in the occurrence of depression.

Pheochromocytoma (PC12) cells are a common type of nerve cells in which, glucocorticoid receptors (GRs) are highly expressed. High concentrations of corticosterone can induce cell damage response in PC12 cells. Antidepressants can protect PC12 cells from the toxic effects of corticosterone.[13]

In this study, we tried to explore gardenia/acanthopanax effect on cell apoptosis rate, apoptosis-related proteins, and calcium ion concentration of PC12 cells treated with high concentrations of corticosterone to explore their antidepression mechanism.

MATERIALS AND METHODS

Experimental animals

The healthy male Wistar rats (Specific-pathogen-free grade) used in this study were purchased from Shandong Lukang Pharmaceutical Co., Ltd., (License number: SCXY [Lu] 20130001). The body weights of these rats ranged between 300 and 350 g. The feeding environment was clean grade. The Medical Experimental Animal Administrative Committee of Shandong Province, China, approved all the experiments. Adult male standard deviation (SD) rats were bred and maintained in the Experimental Animal Center of Shandong Chinese Medicine University. The protocol was approved by the Institutional Animal Care and Use Committee as well as the Local Experimental Ethics Committee. All rats were allowed free access to food and water under optimal conditions (12 h light/12 h dark, with 60% ± 5% humidity, 22°C ± 3°C).

Cells

Rat PC12 cells were purchased from the Preclinical Medicine Institute of the Chinese Academy of Medical Sciences.

Drugs

Gardenia/acanthopanax was provided by the dispensary of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine, and they were authenticated by Professor Cao Guangshang. The gardenia and acanthopanax were decocted with water. The solution was then dried under a microwave dryer to obtain aqueous extract powder. The aqueous extract powder was then stored at −20°C. We measured the content of geniposide ($C_{17}H_{24}O_{10}$) and syringoside ($C_{17}H_{24}O_{9}$) in the gardenia and canthopanax used the chromatography [Figure 4], the method of content determination was according to "Chinese Pharmacopoeia" 2015 version (Chinese Pharmacopoeia Commission, 2015): The extract powders were dissolved in distilled water (0.115 g/ml for gardenia, and 0.19 g/ml for acanthopanax), and then centrifuged for 10 min at 1500 rpm, an aliquot of the supernatant was filtered through a 0.45 um nylon filter membrane before being injected into the high-performance liquid chromatography (HPLC) system, and the amount of geniposide and syringoside was analyzed by HPLC. The contents of geniposide and syringoside were 2.57% and 0.32%, respectively, higher than Chinese pharmacopoeia standard (1.50% and 0.05%, respectively). Fluoxetine (25 mg/tablet, produced by Patheon France, production batch number: 4533A, imported drug registration certificate No.: H20120468) was used. This drug was dissolved in distilled water, diluted to a concentration of 0.255 mg/ml, and stored in a refrigerator at 4°C. Before intragastric administration, this drug was prewarmed and shaken up.

Reagents

Dulbecco's Modified Eagle Medium (DMEM) high glucose medium (HyClone); phosphate buffered saline (PBS, Biological Industries); HI horse serum (Glico); fetal calf serum (Zhejiang Tianhang Biotechnology Co., Ltd.); corticosteroids (Sigma); trypsin (0.25%)-EDTA digestive juice (Solarbio); Annexin V/Propidium iodide (PI) Kit (Becton, Dickinson and Company); Fluo3/am kit (Merck Millipore); acrylamide (Shanghai Aladdin Bio-Chem Technology Co., Ltd.); N, N'-methylenediacrylamide (Shanghai Aladdin Bio-Chem Technology Co., Ltd.); Tris Base (Shanghai Aladdin Bio-Chem Technology Co., Ltd.); ammonium persulpfate (Shanghai Aladdin Bio-Chem Technology Co., Ltd.); TEMED (Shanghai Aladdin Bio-Chem Technology Co., Ltd.); glycine (Shanghai Aladdin Bio-Chem technology Co., Ltd.); sodium dodecyl sulfate (SDS) (Shanghai Aladdin Bio-Chem Technology Co., Ltd.); polyvinylidene fluoride (Millipore); ECL Western Blotting Substrate (Thermo Scientific); enhanced BCA Protein Assay Kit (Beyotime); methanol (Analytical Reagent, Nanjing Chemical Reagent CO., Ltd.); and HCl (Analytical Reagent, Nanjing Chemical Reagent CO., Ltd.).

Instruments

CO2 cell incubator (Scientific Forma, model number: 3111); clean bench (Suzhou Antai Air Technology Co Ltd., model number: SW-CJ-1F); high speed centrifuge (Sigma, model number: 1–13); low temperature centrifuge (Thermo IEC, model number: Micromax RF); flow cytometry (Beckman, model number: Epics XL); ND-1000 spectrophotometer (Gene Company, model number: JCS0112); fluorescence inverted microscope (Nikon, model number: Ti-s); ultralow temperature refrigerator (Thermo Forma, model number: 725); pipettor (Eppendorf, model number: 10–1000l); SDS-polyacrylamide gel electrophoresis (PAGE) electrophoresis system (Bio-Rad); wet transfer film (Bio-Rad); decolorization shaker (TY-80R, Medical Instrument CO., Ltd.); and Tanon 5200 chemiluminescence analyzer (Tanon).

Methods

Preparation of medicated serum

Equivalent doses of drugs were calculated according to the body surface areas of humans and animals. Rats were divided into five groups: the gardenia group (0.23 g/day), acanthopanax group (0.38 g/day), fluoxetine group (0.51 mg/day), and control group and blank group ($n = 7$ for each group). Rats in the experimental group, control group, and blank group were lavaged daily with 2 ml of drug solution or distilled water, for 10 consecutive days. 2 h after the last lavage, blood was sampled from the abdominal aorta. After standing for 2 h at room temperature, the blood samples were centrifuged at 2000 rpm for 20 min. Next, the upper serum was obtained, underwent filtration sterilization on a 0.22 μm filter, and were packed in tubes and stored in a refrigerator at −20°C.

Culture of pheochromocytoma cells

The initial medium was sucked out from the culture bottle that contained PC12 cells in a clean bench, a suitable amount of 0.25% trypsin solution was added and digested for 1 min, the complete culture medium was added to terminate digestion, resuspended, and the cell suspension was transferred to a 10 ml centrifuge tube. After centrifugation at 950 rpm for 5 min, the supernatant was discarded, PBS was added to the resuspended cells, and the centrifugation procedure was repeated. The cells were resuspended with complete culture medium containing 5% fetal calf serum, 5% horse serum, and 90% DMEM; then, these were passaged at a ratio of 1:3. Cells were cultured with 5% CO_2 at 37°C and were inoculated on a 24-well plate at a density of 3×10^5 cells/well when the cells fully filled the bottle.

Grouping

The experiment was divided into five groups: the blank group, control group, gardenia group, acanthopanax group, and fluoxetine group, the conventionally cultured PC12 cells were seeded on 24-well plates at a density of 3×10^5 cells/well. Each group has eight duplicated wells. After routine culture for 24 h, the initial medium was removed, and 20% medicated serum or normal rat serum plus 200 μm corticosterone were added (the blank group without corticosterone), and incubated for 48 h.

Detection of apoptosis rate

Apoptosis was detected using Annexin V/PI double staining flow cytometry. Cells were collected, washed twice with PBS, resuspended after the addition of 1 ml of binding buffer, and diluted until a cell density of 1×10^6 cells/ml was achieved. Next, 100 μl of cell suspension was obtained, 5 μl of PI and 5 μl of fluorescein isothiocyanate (FITC) were added, resuspended, incubated at 25°C in the dark for 20 min, centrifuged at 950 rpm for 5 min, and resuspended after the addition of 1 ml of binding buffer. Cells were detected using a flow cytometer. When the staining result was Annexin V+/PI-, the cell was determined as an early apoptotic cell, and Annexin V+/PI+ as a late apoptotic cell. The total percentage of early apoptotic cells and the late apoptotic cells was calculated.

Western blotting test

PC12 cells were treated with different drugs. Harvested cell were washed two times with ice-cold PBS and disrupted in cell lysates. Proteins were extracted and measured using BCA Protein Assay Kit. The cell samples were applied to SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Next, the membranes were incubated with specific primary antibodies overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies. After being incubated with secondary antibodies, the reactive bands were identified using a commercially available ECL Kit. The image processing software was used to analyze the gray values and their ratios of the target strip and the inner reference strip, and the results of the experiment were repeated three times for statistical analysis.

Determination of calcium ion concentration in pheochromocytoma cells

Intracellular calcium concentration was detected by flow cytometry and calcium ion fluorescence probe Fluo3/am. Cells were collected, washed once with D-Hanks liquid, and diluted until a cell density of 1×10^6 cells/ml was achieved. For each 250 μL of cell suspension, 250 μL of Fluo3/am containing 10 μm of D-Hanks liquid was added and incubated at 37°C for 45 min. After the end of the incubation, the cells were washed twice with D-Hanks liquid, and 1 ml of cell suspension was prepared and detected. The excitation wavelength was set at 519 nm, and the emission wavelength was set at 488 nm.

Statistical methods

Data were statistically analyzed using statistical software SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as mean ± SD (x^- ± SD). Intergroup comparison was conducted using one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

The effect of gardenia/acanthopanax-medicated serum on PC12 cell apoptosis induced by corticosterone. PC12 cells were analyzed by FCM using the Annexin V/PI dual-labeling technique. Cells were simultaneously stained for FITC- Annexin V and PI, and then, the cell apoptosis was analyzed by flow cytometry, with results displayed as a bivariate distribution of annexin V and PI fluorescent intensity. The apoptosis rate of PC12 cells was greatly increased in the control group, and significantly decreased in groups treated with medicated serum. The apoptosis rate of PC12 cells decreased more significantly in the fluoxetine and gardenia groups ($P < 0.01$, vs. control group) than in acanthopanax group ($P < 0.05$, vs. control group, fluoxetine and gardenia group, respectively). The difference in apoptosis rate between gardenia and fluoxetine was not statistically significant ($P > 0.05$) [Figure 1].

Effects of gardenia/acanthopanax on apoptosis-related proteins in PC12 cells, the effect of gardenia/acanthopanax on Caspase-3, Bax (pro-apoptotic proteins), and Bcl-2 (anti-apoptotic protein) were estimated by Western blot. Pro-apoptotic proteins, Caspase-3 and

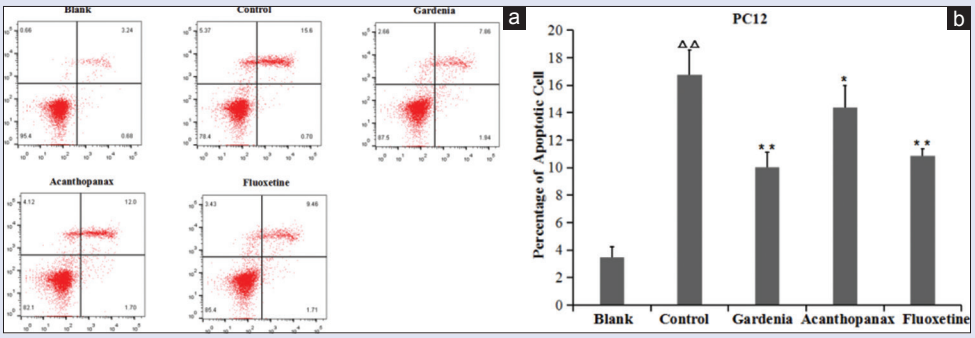

Figure 1: The effectsof (a) High level corticosterone-induced apoptotic pheochromocytoma cells. In each plot, the lower left quadrant represents the normal cells, the lower right quadrant denotes late apoptotic cells, the upper right quadrant represents the early apoptotic cells and the upper left quadrant denotes necrosis cells. (b) Percentage of early and late apoptotic cells by quantitative analysis. Data are expressed as mean ± standard deviation. Presented in the bar graphs, $n = 8$, $^*P < 0.05$, and $^{**}P < 0.01$ versus control group; $^{\Delta\Delta}P < 0.01$ versus blank group

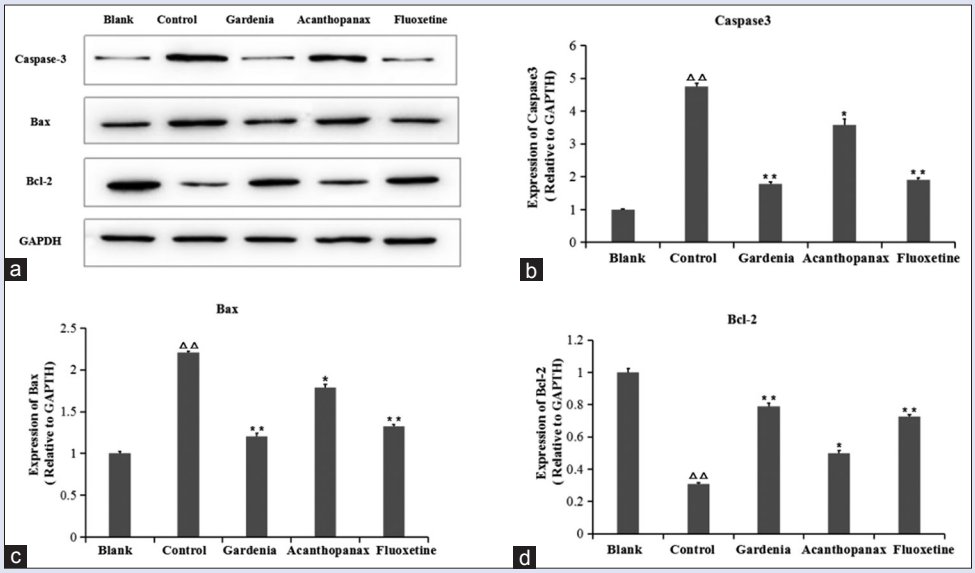

Figure 2: The effects of gardenia/acanthopanax-Medicated serum on high level corticosterone-induced pheochromocytoma cell apoptotic proteins. (a) The expressions of caspase-3, Bcl-2, and Bax by Western blot with glyceraldehyde 3-phosphate dehydrogenase as a standard. (b-d) Densitometric uantification of caspase-3, Bcl-2, and Bax of Western blot using Image Studio software (LI-COR). Data are presented as mean ± standard deviation ($n = 8$) *$P < 0.05$, and **$P < 0.01$ versus control group; $^{\Delta\Delta}P < 0.01$ versus blank group

Bax were found to be overexpressed, and anti-apoptotic protein Bcl-2 downexpressed in PC12 cells treated with high level glucocorticoids compared with the blank group ($P < 0.01$), which had the lowest Caspase-3 and Bax expressions and highest Bcl-2 expression. Compared with control group, Caspase-3 and Bax expressions in PC12 Cells treated with different drug-medicated serum were significantly reduced, whereas, Bcl-2 expression was increased. The gardenia and fluoxetine had better effect ($P < 0.01$ vs. control group and $P < 0.05$ vs. acanthopanax group) than acanthopanax ($P < 0.05$ vs. control group), there was no significant difference between gardenia and fluoxetine group ($P > 0.05$). These results indicated high level glucocorticoids might lead to significant overexpression of pro-apoptotic proteins and downexpression of anti-apoptotic protein in PC12 cells, that can be reversed by the gardenia/acanthopanax, and gardenia had better effect than acanthopanax [Figure 2].

The effect of gardenia/acanthopanax medicated serum on calcium concentration under high concentrations of corticosterone. High level glucocorticoid-induced PC12 cells had increased calcium concentration compared with the blank group ($P < 0.01$), and the result significantly decreased in the gardenia and fluoxetine groups ($P < 0.01$). However, there was no significant difference in intracellular calcium concentration in PC12 cells between the two groups ($P > 0.05$). Compared with the control group, gardenia and fluoxetine groups, the differences in intracellular calcium concentration in PC12 cells in the acanthopanax group were statistically significant separately ($P < 0.05$). The gardenia has shown better effect on downregulating calcium concentration than acanthopanax [Figure 3].

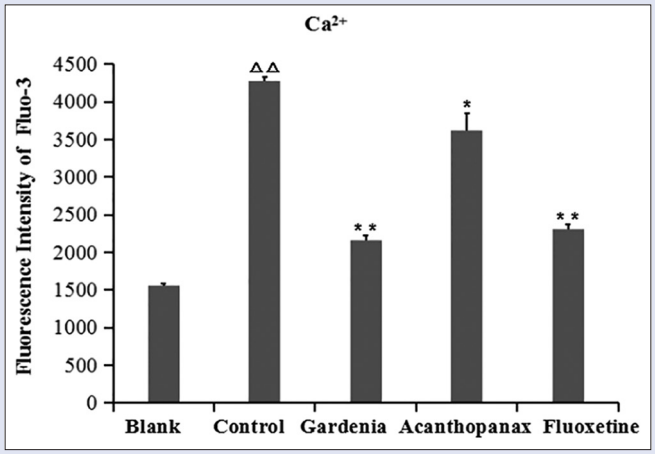

Figure 3: The effects of gardenia/acanthopanax-Medicated serum on high level corticosterone-induced pheochromocytoma cell Calcium Ion Concentration. Data are presented as mean ± standard deviation ($n = 8$) *$P < 0.05$, and **$P < 0.01$ versus control group; $^{\Delta\Delta}P < 0.01$ versus blank group

DISCUSSION

Proper stress helps improve the ability of the body to adapt to the environment, while excessive and long-term stress can lead to excessive active HPA axis function. Glucocorticoid levels beyond the bearing ability of the body may cause a series of damage to the body, especially to the hippocampus. The hippocampus has two main types of steroid receptors: mineralocorticoid receptor (MR) and GR. MRs mainly participates in the negative feedback regulation of the HPA axis at the basic level, and GRs mainly participate in the negative feedback regulation of the HPA axis at high levels of glucocorticoids. Therefore, MRs are the main receptors responsible for regulating the HPA axis function under a stress state.[14] Sustained high levels of glucocorticoids can impair the negative feedback regulation of GRs to the HPA axis function. Increased glucocorticoid level and excessive activation of GRs can cause neuronal apoptosis and degeneration in the central nervous system.[2] Persistent hyperfunction of the HPA axis promotes the release of excitatory amino acids, and excitatory amino acids can further aggravate hyperfunction of the HPA axis.[15]

Excitatory amino acids are the most extensive and strongest excitatory neurotransmitter that has the highest level in the human body.[16] These induce the apoptosis or death of neurons by exerting on receptors to

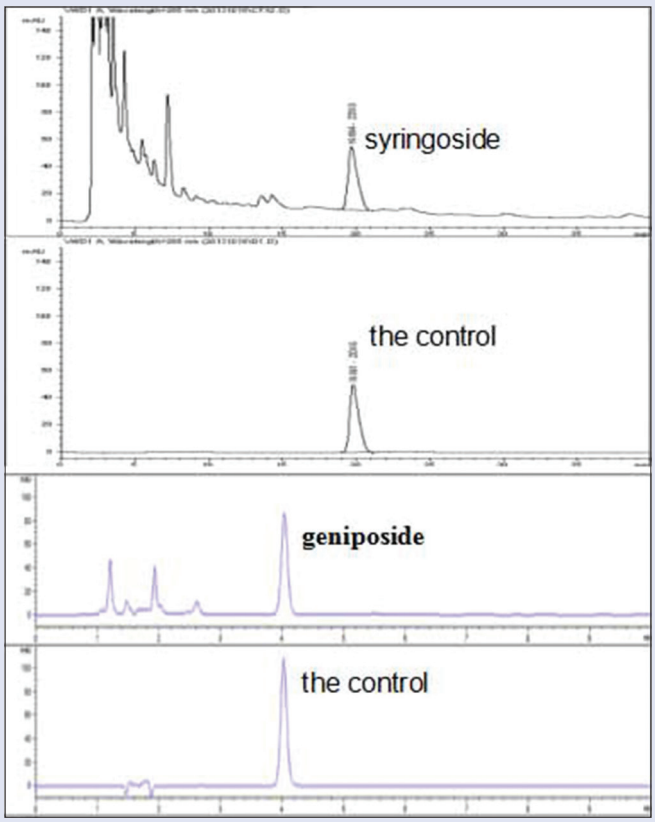

Figure 4: High performance liquid chromatography of syringoside and geniposidethe. Amount of geniposide and syringoside was analyzed by HPLC. The contents of geniposide and syringoside were 2.57% and 0.32%, respectively, higher than Chinese pharmacopoeia standard (1.50% and 0.05%, respectively)

persistently activate neurons.[17] Glutamate is an important excitatory amino acid in the mammalian brain.[18] In general, glutamate is stored in the synapse in the form of vesicles which is released into the synaptic cleft at the depolarization of the nerve endings, and binds with receptors in the postsynaptic membrane to complete an excitatory synaptic transmission. A study[19] revealed that under acute stress, the glutamate efflux was found in the hippocampus, amygdala, and prefrontal synaptic cleft in rodents. Tetrodotoxin could block changes in glutamate, which suggests that the increase in glutamate level in the synaptic cleft was caused by the increase in its release. Ca^{2+} influx occurs after excessive glutamate binds with N-methyl-D-aspartate (NMDA) receptors.[20] Furthermore, cell depolarization can open voltage-dependent calcium channels, and further aggravate calcium influx. Choi confirmed that the long-term high concentration of glutamate could cause the calcium-dependent death of neurons.[21] Calcium ion plays an important role in cell apoptosis. First, Ca^{2+} binds with calmodulin and connects the reductase region and oxidase region in the nervous nitric oxide synthase (nNOS), when the influx of Ca^{2+} is induced by opening the NMDAR channels; thus, nNOS is activated to produce NO.[22] In hippocampal neurons, nNOS anchors with NMDARR through the PSD95 protein;[23] thus, this greatly improves the efficiency of the excitatory amino acid signal to activate NOS.[24] NO could induce the decrease in mitochondrial membrane potential or the upregulation of Bax and Bcl-XL proteins, and guide in the release of cytochrome C in the mitochondria; thus, the apoptotic signal pathway in caspase cells initiates.[25]

Phosphatidylserine (PS) is located on the inner side of the cell membrane under normal conditions, and this can be transferred to the external side of the cell membrane when the cell is in the early stage of apoptosis. Annexin V is a kind of Ca^{2+} dependent phospholipid binding protein which can specifically binds with PS. Fluorescein-labeled Annexin V can be detected by flow cytometry. PI can pass through the cell membrane of apoptotic cells, and stain the cell nucleus to red. The combination of Annexin V and PI can distinguish cells in early apoptosis from necrotic cells. Fluo3/am is a fluorescent probe to detect intracellular calcium. Fluorescence intensity can be elevated by 70–80 times when Fluo3/am passes through the cell membrane and binds with the intracellular calcium. The average fluorescence intensity represents the concentration of calcium ion.

Apoptosis is an active suicidal machinery or programmed cell death, and Bcl-2 family proteins are recognized as major regulators of the mitochondrial pathway of apoptosis, including anti-apoptotic proteins (such as Bcl-2) and pro-apoptotic proteins (such as Caspase-3 and Bax).[26] Hence, in the present study, we selected the three indices as apoptotic makers to investigate the effects of the Chinese herbs gardenia/acanthopanax on high level glucocorticoid-induced PC12 cells apoptosis.

CONCLUSION

The results of this study suggest that gardenia/acanthopanax-medicated serum can obviously improve the survival rate of neurons under high concentrations of glucocorticoids, downregulate the apoptosis-related proteins, and reduce the intracellular calcium concentration, and gardenia had better effects than acanthopanax. The previous animal experiments revealed gardenia and fluoxetine can effectively improve depression-like behavior in rats with chronic stress. Related pharmacological researches confirmed that the extract of gardenia has the effect of inhibiting the activation of the toxic components in the microglia[27] and antioxidative stress.[28] The results of this experiment revealed that high concentrations of corticosterone could increase the apoptosis of PC12 cells, pro-apoptotic proteins, and intracellular calcium concentration, and decrease the anti-apoptotic proteins. Gardenia/acanthopanax medicated serum has anti-apoptotic effect in PC12 cells. The neuroprotective effect may be related to its ability to downregulate the pro-apoptotic proteins, upregulate the anti-apoptotic proteins, and recover the balance of calcium concentration. At the same time, we found that gardenia had similar effects on high level glucocorticoids-induced PC12 cells apoptosis to fluoxetine, however with rare toxicity and side effects. We expect the study may provide a new substitute for the therapy of depression.

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Conflicts of interest

There are no conflicts of interest.

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