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Isoflavonoids from *Astragalus mongholicus* protect PC12 cells from toxicity induced by L-glutamate

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Abstract

lsoflavonoids, formononetin, 9,10-dimethoxypterocarpan 3-*O*-β-D-glucoside, ononin, calycosin 7-*O*-glc and calycosin, were isolated from the roots of *Astragalus mongholicus* Bunge (Leguminosae). The neuroprotective roles and direct antioxidant effects of these isoflavonoids were investigated by using PC12 cell model and DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. Formononetin, ononin and calycosin were found inhibiting glutamate-induced cell injury, with an estimated 50% effective concentration (EC_{50}) of 0.027 μg/ml, 0.047 μg/ml and 0.031 μg/ml, respectively. Pretreatment with them increased the activities of antioxidant enzymes, including superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and prevented the release of lactate dehydrogenase (LDH) in glutamate-injured PC12 cells. On the other hand, calycosin 7-*O*-glc and calycosin showed more scavenging activity to DPPH radicals than formononetin in the cell-free system. The inconsistency between the neuroprotective capabilities of isoflavonoids and their directly scavenging activity to DPPH radicals indicated that formononetin, ononin and calycosin probably depended on increasing endogenous antioxidant and stabilizing the cells' membrane structures to inhibit the cell damage induced by glutamate.

Keywords: Isoflavonoids; *Astragalus mongholicus*; Neuroprotection; Glutamate; PC12

1. Introduction

Astragalus mongholicus Bunge (Leguminosae), known as Huangqi in China, has been used as one of the primary Chinese tonic herbs with a history of thousands of years (Roy and Cathiroe, 1999). In modern Chinese medicine, Huangqi is widely used as an immune modulator, especially to support immune health for various chronic degenerative diseases. It is also used as an adjunctive therapy to chemo- and radiation-therapy in cancer (Lei et al., 2003; Wang et al., 2003). Recently there are some reports on the neuroprotective activities of the crude extract from Huangqi (He et al., 1998; Yao et al., 2002). In this study we isolated and identified the active components in this plant and characterized their neuroprotective roles.

Glutamate is thought to be the major excitatory neurotransmitter in the central nervous system. Evidence is accumulating that glutamate and relative excitatory amino acid analogs cause a specific pattern of neurodegeneration in the brain of experiment animals, in primary culture of brain neurons and in some cultured neuron cell lines, including PC12 cell line (Atlante et al., 2001; Pereira and Oliveira, 1997; Seyfried et al., 2000). In PC12 cells, a rat pheochromocytoma cell line, lacking ionotropic glutamate receptors, the high concentration glutamate inhibits cystine uptake and depletes intracellular glutathione, which leads to the accumulation of reactive oxygen species (ROS) and ultimately causes cell death (Froissard and Duval, 1994). In the present study, after detecting the antioxidant activities of the five isoflavonoids isolated from Huangqi by DPPH assay in a cell-free system, we investigated whether these compounds were of neuroprotective activity in damaged PC12 induced by L-glutamate in vitro.

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2. Materials and methods

2.1. Chemicals

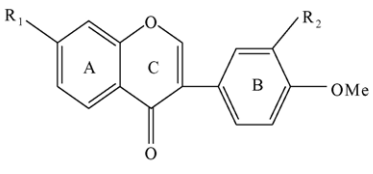
The plant materials used in this study were the dried roots *of Astragalus mongholicus* obtained from Dalian Pharmacy (Dalian, China) and authenticated by Dr. Xian Lan (Pharmacology College, Shandong University). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum were obtained from Gibco. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltera-zolium bromide (MTT), poly-L-lysine, propidium iodine (PI), *N*,*N*-dimethylformamide and sodiumdodecylsulfate (SDS), L-glutamate and DPPH were from Sigma (St. Louis, MO, USA). The reagents used in enzyme assays were provided by Jiancheng Bioengineering Institute (Nanjing, China). All of the solvents used for the isolation and purification were of the highest purity available from Tianjin Kemio Company (Tianjin, China).

2.2. Extraction and isolation of neuroprotective compounds

The potential neuroprotective compounds were isolated as the following way. The powdered materials (10 kg) were extracted with 90% ethanol by maceration at room temperature. The alcoholic solution was concentrated at 60 °C under vacuum. The concentrated extract was diluted with water and successively extracted with petroleum ether, ethyl acetate, and *n*-butanol. The ethyl acetate layer (60 g) and *n*-butanol (25 g) was fractioned by repeated column chromatography over silica gel eluted with a $CHCl_3$–MeOH gradient. Five isoflavonoids were obtained and identified as formononetin (243.7 mg, I), ononin (22.6 mg, II), calycosin (324.0 mg, III), calycosin 7-*O*-glc (39.4 mg, IV) (Fig. 1) and 9,10-dimethoxypterocarpan 3-*O*-3-D-glucoside (76.2 mg, V) (Fig. 2) by matching with spectroscopic data (UR, IR and NMR) previously reported (Dou et al., 2002; Wang et al., 1983).

2.3. Assay for DPPH radical scavenging activity

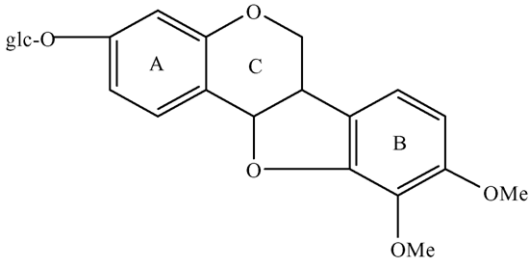
The DPPH radical scavenging activities of the isoflavonoids were examined based on the method previously described (Dok-Go et al., 2003). Isoflovonoids were dissolved



I: Formononetin R_1=OH R_2=H; II: Ononin R_1=Oglc R_2=H;

III: Calycosin R_1=R_2=OH; IV: Calycosin 7-O-glc R_1=Oglc R_2=OH

Fig. 1. Structures of compounds I–IV.



9, 10-dimethoxypterocarpan-3-O-β-D-glucoside

Fig. 2. Structure of compound V.

into DMSO and the concentrations were controlled to be 10, 20, 40, 100 μg/ml, respectively. Then 20 μl isoflavolnoid solution samples were added into 80 μl of 50 μM DPPH ethanol solution. The reaction mixtures were incubated at 37 °C in a 96-well microplate for 30 min and the absorbance was measured at 517 nm using a Sunrise Microplate Reader (TECAN). The ROS scavenging activity, *S*, was calculated according to the equation below.

$$S = \left(1 - \frac{\text{abs}_{\text{sample}}}{\text{abs}_{\text{control}}}\right) \times 100$$

2.4. Cell culture

PC12 cells obtained from the American Type culture Collection (Mannassas) were cultured in DMEM supplemented with 5% fetal bovine serum, 10% horse serum and 50 μg/ml streptomycin in a humidified incubator at 37 °C and 5% CO_2 (Greene and Tischler, 1976). For measurement of viability and intracellular antioxidant, cells were seeded in 6- or 96-well plates coated with poly-L-lysine at $l \times 10^5$ cells/ml and used for experiments after 24 h.

2.5. Cell viability assays

Tested isoflavonoids were dissolved in dimethylsulfoxide (DMSO) (final culture concentration in culture less than 0.1%). For the cell viability assays, 24 h after seeding, the cultures were pretreated with tested isoflavonoids for 12 h before exposure to 10 mM glutamate. After 24 h incubation, cellular viability was evaluated by MTT assay (Abe and Matsuki, 2000), which reflects mitochondrial siccinate dehydrogenase function. A modified MTT assay was used: 10 μl of 5 mg/ml MTT solution in sterile phosphate buffer saline (PBS) was added into 100 μl medium and incubated for 4 h in the incubator chamber at 37 °C. The reaction was stopped by adding 100 μl solution containing 50% (w/v) *N*,*N*-dimethylformamide and 20% SDS (pH 4.8). The plates were maintained overnight in the incubator at 37 °C and the absorption values at 570–630 nm were then detected using an automatic microtiter plate reader. Cell viability was calculated as 100 × (OD of glutamate-drug-treated − OD of glutamate-treated)/(OD of control − OD of glutamate-treated). The

EC_{50} values were denoted as the concentration resulting in 50% protection of control.

2.6. PI staining

PI is a very stable fluorescent dye absorbing blue-green light (493 nm) and emitting red fluorescence (630 nm). As a polar compound, it only enters dead or dying cells with a damage or leaky cell membrane and interacts with DNA to yield a brightly red fluorescence. Due to its properties, PI has been used a marker of cell membrane integrity and cell damage (Kristensen et al., 2003). In the present study, after designated treatment, PI phosphate buffered solution was added to the culture medium (final concentration 10 μg/ml) and incubated 10 min at room temperature. Then cells were fixed by 4% polymerisatum and observed under a fluorescence microscope (Olympus-CK40) (Fig. 5).

2.7. Assays for LDH, GSH-Px and glutathione (GSH)

The medium was collected for LDH assay in order to assess cellular integrity (Lobner, 2000). Cultures were washed twice in ice-cold PBS, pooled in 1 ml 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 30 min at 3000 × *g* at 4 °C and supernatant was used for GSH-Px, SOD enzyme and GSH assays according to the manufacturer instructions.

2.8. Protein assay

Protein content was measure by Bradford method with bovine serum albumin as a standard (Bradford, 1976).

2.9. Statistical analysis

All the experiments were performed in triplicate and data are shown as mean ± S.D. based on three separate experiments. Statistical analysis was performed according to the Student's *t*-test. The probability values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Radical scavenging activity of isoflavonoids from Huangqi

Formononetin, calycosin and calycosin 7-*O*-glc were found to scavenge free radicals generated by DPPH in a dose-dependent manner. Formononetin showed slightly scavenging effect, with 11% clearance rate at the concentration of 100 μg/ml.

Calycosin and calycosin 7-*O*-glc demonstrated more effective than formononetin, with 44.1% and 31.9% clearance rates at 100 μg/ml, respectively. 9,10-dimethoxypterocarpan 3-*O*-β-D-glucoside and ononin were detected to have no roles in scavenging DPPH free radicals at the tested concentrations (Table 1). All of these five compounds did not show obviously scavenging activities for free radicals when their concentrations were lower than 1 μg/ml.

Table 1
The elimination rate of the principles with different concentrations to DPPH free group (% of control)

	10 μg/ml	20 μg/ml	40 μg/ml	100 μg/ml
I	5.5 ± 0.73	6.8 ± 0.68	10.6 ± 0.17	11 ± 0.64
II	ND	ND	ND	ND
III	13.1 ± 0.86	22.9 ± 0.42	34.7 ± 0.78	44.1 ± 0.47
IV	12.3 ± 0.6	17.7 ± 0.27	23.6 ± 0.13	34.5 ± 0.37
V	ND	ND	ND	ND

ND: not detected. (I) formononetin; (II) ononine; (III) calycosin; (IV) calycosin-7-*O*-glc; (V) 9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside.

3.2. Neuronal protections against glutamate-induced toxicity by isoflavonoids, formononetin, ononin and calycosin

The quantitative methods of LDH release, which measures cell lysis and MTT reduction, which measures metabolic activity were used to evaluate cell damage and cellular viability (Lobner, 2000). Among the five isoflavonoids, formononetin, ononin and calycosin could inhibit glutamate-induced oxidant cell injury with MTT reduction increase to 69.5%, 54.69 % and 66.41% at the concentration of 0.05 μg/ml, respectively (Fig. 3). Their estimated EC_{50} were 0.027 μg/ml, 0.047 μg/ml and 0.031 μg/ml, respectively. The results (Fig. 4) also showed that formononetin, ononin and calycosin decreased the LDH release of PC12 injured by 10 mM glutamate, 16.67%, 33.29% and 18.99% at the concentrations 0.05 μg/ml. Formononerin demonstrated the highest protection 85.2% and the lowest LDH release 9.63% at 0.1 μg/ml. When the concentrations of formononetin and calycosin were more than 1 μg/ml, they weakened their protections. The other two isoflavonoids, 9,10-dimethoxypterocarpan 3-*O*-β-D-glucoside and calycosin 7-*O*-glc, demonstrated no protective activities. These results illustrated that formononetin is the best neuronprotective to glutamate-damaged PC12 cells

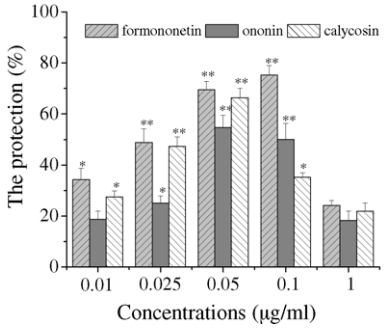


Fig. 3. Protective effects of formononetin, ononin and calycosin on the injured PC12 cells induced by glutamate. Data are presented as mean ± S.D. ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. glutamate-treatment.

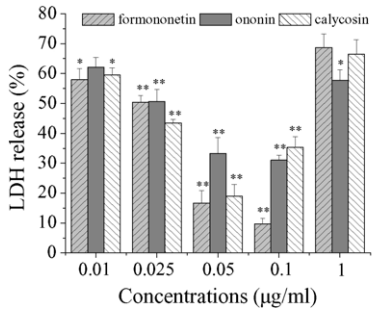


Fig. 4. Inhibition of formononetin, ononin and calycosin on glutamate induced injury. Data were calculated as percentages of glutamate-treatment LDH activity. Each point represents the mean ± S.D. ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. glutamate-treatment.

among the isolated compounds. Microscope observations also supported this conclusion (Fig. 5).

3.3. Roles of Formononetin, calycosin and ononin on preventing the decrease of cellular SOD, GSH-Px and GSH

The antioxidant system includes high levels of antioxidant compounds (e.g., GSH) and antioxidant enzymes (e.g., SOD, GSH-Px). GSH is a major endogenous antioxidant in cells and plays an important role in protecting brain tissue from oxidative stress. We investigated the effect of these isoflavonoids on GSH level after treatment with glutamate. The results showed that these three isoflavonoids can prevent the depletion of GSH in glutamate-induced toxicity. Meanwhile, formononetin, calycosin and ononin significantly preserved the activities of the antioxidant enzymes, SOD and GSH-Px, in glutamate-injured PC12 cells (Table 2).

4. Discussion

Increasing reports have provided evidences implicating oxidative stress as a major pathogenic mechanism in neurodegeneration such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Emerit et al., 2004; Sohal et al., 2002). Oxidant overproduction leads to oxidative molecular damage of the tissue (Zhu et al., 2004). Therefore, protecting neurons from oxidative injuries may provide useful therapeutic potentials for the prevention or treatment of neurodegenerative disorders caused by oxidative stress (Behl and Moosmann, 2002; Bastianetto and Quirion, 2002).

Isoflavonoids are currently receiving much attention due to their various health benefits. It has been suggested that some of these beneficial effects of isoflavonoids might be, at least in part, mediated by their antioxidant activity. The mechanism of antioxidant actions of isoflavonoids mainly conclude (1) suppressing ROS formation either by inhibition of enzymes or chelating trace elements involved in free radical production; (2) scavenging ROS; (3) stabilizing membranes by decreasing membrane fluidity; and (4) up-regulating or protecting the antioxidant defense system (Halliwell and

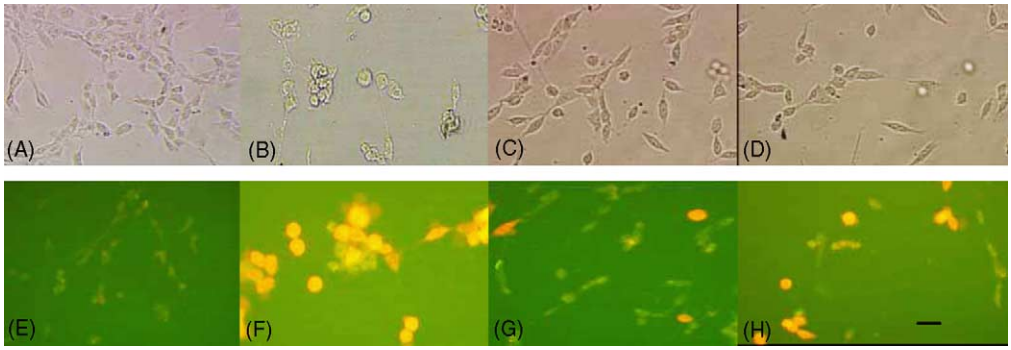


Fig. 5. Protective effects of formononetin and calycosin on PC12 cells injuries induced by glutamate in morphology. A/E: control; B/F: glutamate; C/G: 0.1 μg/ml formononetin + glutamate; D/H: 0.05 μg/ml calycosin + glutamate. The first line: microscope view. The second line: PI fluorescence. Scale bar: 10 μm.

Table 2
Effects of formononetin, ononin and calycosin on the activities of SOD, GSH-Px and GSH in glutamate-injured PC12cells

	SOD (U/ml)	GSH-Px (μmol/mg pro.)	GSH (μmol/mg pro.)
Control	15.58 ± 0.18	110.6 ± 1.85	8.57 ± 1.29
Glutamate-treatment	9.65 ± 0.5	81.87 ± 5.3	5.45 ± 0.79
Glu + I (0.1 μg/ml)	14.85 ± 0.62*	119.44 ± 3.7**	8.43 ± 0.33**
Glu + II (0.05 μg/ml)	14.33 ± 1.04*	111.43 ± 2.42*	7.72 ± 0.45
Glu + III (0.05 μg/ml)	15.65 ± 0.32**	111.20 ± 4.05*	8.16 ± 0.47*

Values given represent the mean ± S.D. ($n = 3$).
* $p < 0.05$.
** $p < 0.01$ vs. glutamate-treatment.

Gutteridge, 1998; Arora et al., 2000; Mira et al., 2002; Guo et al., 2002).

In the present study, we damaged the PC12 cells with high concentration of L-glutamate and assessed the protective actions of five isoflavonoids isolated from Huangqi. The experimental results demonstrated that three of them, formononetin, nononin and calycosin could protect PC12 cells from oxidative stress induced by glutamate. Pretreatment with them could prevent the decrease in activity of antioxidant enzymes (SOD and GSH-Px), and the depletion of GSH in glutamate-damaged PC12 cells, indicating that antioxidant capacity of cells had been improved. Moreover, the findings that they decreased the LDH release illustrated that they stabilized membrane structures and improved the membrane integrity. It has been suggested that isoflavonoids penetrate into the hydrophobic core of the membrane and cause a dramatic decrease in lipid fluidity in this region of membranes, which could sterically hinder the diffusions of free radicals, decrease the kinetic rate of free radical reactions and thereby lessen the cellular oxidative damage (Guo et al., 2002).

Experimental results also suggested that 7-hydroxyl group in the A ring and 3′-hydroxyl group in the B ring be the crucial moiety possessing the antioxidant activity. Calycosin has the two groups and showed to be the most effective compound among the five isoflavonoids. 9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside and ononin are lack of the groups and demonstrated no activities in scavenging the free radicals. That calycosin-7-*O*-glc had greater antioxidant activity than formononetin illustrated that 3′-hydroxyl group in the B ring is more powerful than 7-hydroxyl group in the A ring. In addition, the protective actions of these three isoflavonoids were not concordance with their scavenging activities to organic free radicals. Calycosin 7-*O*-β-D-glucoside that has the same aglycone as calycosin showed no neuroprotective activity to glutamate-damaged PC12 cells despite their better antioxidant activities measured in the DPPH assay. In contrast, ononin, which have no antioxidant activity, exhibited neuroprotective capability, but is less efficient than formononetin.

In short, we could draw these conclusions: the aglycones possess better biological activities due to the more lipophilic than its glycosides. The similar results were obtained by Kim et al. (2002). The free radical-scavenging activities of these isoflavonoids, formononetin, ononin and calycosin might not significantly contribute to their antioxidant properties since their bioavailable dose in cell system is far less than in cell-free system. They probably depend on increasing endogenous antioxidant and stabilizing the cells' membrane structures to inhibit the cell damage induced by glutamate.

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