

Neuroprotective effects of Asiaticoside

Feng-yan Qi¹, Le Yang¹, Zhen Tian¹, Ming-gao Zhao¹, Shui-bing Liu¹, Jia-ze An²

1 Department of Pharmacology, School of Pharmacy, Fourth Military Medical University of Chinese PLA, Xi'an, Shaanxi Province, China
2 Department of Hepatobiliary Surgery, Xijing Hospital, Fourth Military Medical University of Chinese PLA, Xi'an, Shaanxi Province, China

Fengyan Qi and Le Yang contributed equally to this work.

Corresponding author:

Jiaze An, Department of Hepatobiliary Surgery, Xijing Hospital, Fourth Military Medical University of Chinese PLA, Xi'an 710032, Shaanxi Province, China, anchen@fmmu.edu.cn.
Shuibing Liu, Department of Pharmacology, School of Pharmacy, Fourth Military Medical University of Chinese PLA, Xi'an 710032, Shaanxi Province, China, liushb1974@aliyun.com.

doi:10.4103/1673-5374.137574

<http://www.nrronline.org/>

Accepted: 2014-05-17

Abstract

In the central nervous system, Asiaticoside has been shown to attenuate *in vitro* neuronal damage caused by exposure to β -amyloid. *In vivo* studies demonstrated that Asiaticoside could attenuate neurobehavioral, neurochemical and histological changes in transient focal middle cerebral artery occlusion animals. In addition, Asiaticoside showed anxiolytic effects in acute and chronic stress animals. However, its potential neuroprotective properties in glutamate-induced excitotoxicity have not been fully studied. We investigated the neuroprotective effects of Asiaticoside in primary cultured mouse cortical neurons exposed to glutamate-induced excitotoxicity invoked by N-methyl-D-aspartate. Pretreatment with Asiaticoside decreased neuronal cell loss in a concentration-dependent manner and restored changes in expression of apoptotic-related proteins Bcl-2 and Bax. Asiaticoside pretreatment also attenuated the upregulation of NR2B expression, a subunit of N-methyl-D-aspartate receptors, but did not affect expression of NR2A subunits. Additionally, in cultured neurons, Asiaticoside significantly inhibited Ca^{2+} influx induced by N-methyl-D-aspartate. These experimental findings provide preliminary evidence that during excitotoxicity induced by N-methyl-D-aspartate exposure in cultured cortical neurons, the neuroprotective effects of Asiaticoside are mediated through inhibition of calcium influx. Aside from its anti-oxidant activity, down-regulation of NR2B-containing N-methyl-D-aspartate receptors may be one of the underlying mechanisms in Asiaticoside neuroprotection.

Key Words: nerve regeneration; brain injury; Asiaticoside; apoptosis; N-methyl-D-aspartate; glutamate; neurotransmitter; neurotoxicity; calcium imaging; Bcl-2; Bax; NSFC grant; neural regeneration

Funding: This study was supported by the National Natural Science Foundation of China, No. 31271126, 81372606.

Qi FY, Yang L, Tian Z, Zhao MG, Liu SB, An JZ. Neuroprotective effects of Asiaticoside. *Neural Regen Res.* 2014;9(13):1275-1282.

Introduction

Glutamate plays a critical role in synaptic transmission and plasticity of the central nervous system. Excessive glutamate, however, is the main cause of excitotoxicity and leads to neuronal damage as seen in stroke, spinal cord injury, traumatic brain injury, and neurodegenerative diseases (Mattson, 2003). The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor is a calcium permeable ion channel which mediates glutamate excitotoxicity (Hardingham and Bading, 2003). The receptor is composed of different subunits that link to different intracellular cascades, which subsequently determine synaptic plasticity and cytotoxic potential. NR2B-containing NMDA receptors are reported to selectively trigger excitotoxicity (Krapivinsky et al., 2003; Liu et al., 2007) and our previous studies are consistent with these findings (Liu et al., 2012; Zhang et al., 2013). Ca^{2+} influx from NMDA receptors activates reactive oxygen species which are the cause of excitotoxic neuronal death (Carriedo et al., 2000; Stanciu et al., 2000; Zhang et al., 2013).

Asiaticoside (**Figure 1**) is a triterpenoid product derived from the plant *Centella asiatica*. It has been shown to have wound healing effects (Lee et al., 2012; Somboonwong et al., 2012), anti-inflammatory effects (Wan et al., 2013), and promotes liver protective activity (Zhang et al., 2010) *in vivo*.

Previous studies have also shown Asiaticoside to ameliorate cognitive impairment and neurotoxicity in animal models (Defillipo et al., 2012; Xu et al., 2012). However, the neuroprotective activity of Asiaticoside and the underlying pathways that inhibit neuronal apoptosis caused by glutamate are not clear. In this study, we used an NMDA-induced injury model to investigate the protective effects of Asiaticoside in cultured mouse cortical neurons with an attempt to uncover the underlying mechanisms involved.

Materials and Methods

Primary culture of neurons

All animal protocols used were approved by the Animal Care and Use Committee of the Fourth Military Medical University of Chinese PLA, Xi'an, Shaanxi Province, China. Primary cortical neuronal cultures were harvested from brains of E15-E16 C57BL/6 mouse embryos (the Animal Center of the Fourth Military Medical University of Chinese PLA). Briefly, dissociated cortex tissues were incubated with 0.125% trypsin (Sigma, St Louis, MO, USA) in Ca^{2+} and Mg^{2+} -free Hank's balanced salt solution for 10 minutes at 37°C. Trypsin was inactivated by washing with DMEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cortices were then further

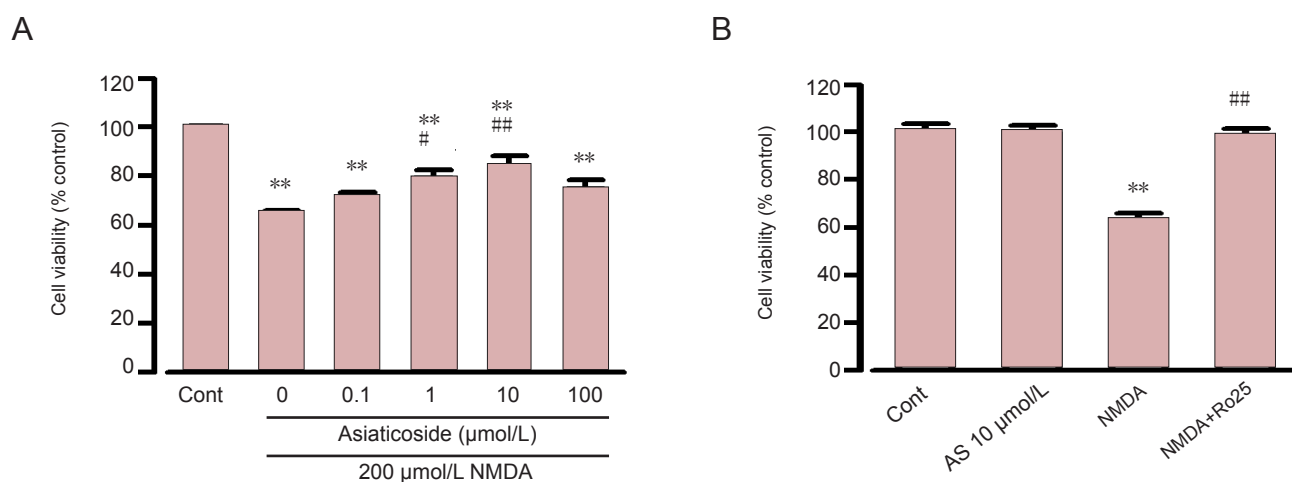


Figure 2 Effects of Asiaticoside (AS) on cell viability in cultured cortical neurons.

Mouse cortical neurons cultured for 7 days *in vitro* were pretreated with Asiaticoside (0.1, 1, 10, 100 $\mu\text{mol/L}$) for 24 hours, and then with N-methyl-D-aspartate (NMDA) for 30 minutes. The cells were returned to the original culture medium containing Asiaticoside for 24 hours, and cell viability was assessed with MTT assay. (A) Exposure to NMDA caused a significant reduction in the viability of cultured neurons. Pretreatment with Asiaticoside dose-dependently attenuated the cell loss. (B) 10 $\mu\text{mol/L}$ Asiaticoside alone had no effect on the cell viability. Ro25-6981 (Ro25, NR2B receptor specific antagonist) totally reversed the loss in cell viability induced by NMDA exposure. All data are expressed as mean \pm SEM of three experiments. One-way analysis of variance was used for comparison among multiple groups followed by Tukey's multiple comparison tests as a *post hoc* comparison. ** $P < 0.01$, vs. control group (Cont); # $P < 0.05$, ## $P < 0.01$, vs. NMDA treatment group.

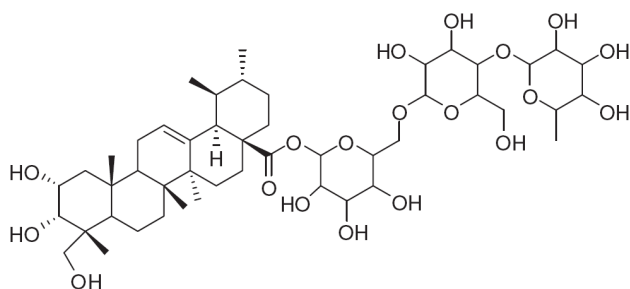


Figure 1 Chemical structure of Asiaticoside.

dissociated by trituration. The single cell suspension was cultured on poly-D-lysine coated plates in Neurobasal media supplemented with 2% B27 (Invitrogen), 0.5 mmol/L glutamine (Sigma), 100 U/mL penicillin (Sigma), and 100 U/mL streptomycin (Sigma). Maturation of cortical neurons took 7 days and half of the medium was changed every 2 days. Anti-MAP2 antibody (Sigma) staining revealed that more than 95% cells were neurons obtained by this culture procedure (Yang et al., 2012). All plates were pre-coated with 50 $\mu\text{g/mL}$ poly-D-lysine (Sigma) in water.

Treatment of the neurons

Asiaticoside was purchased from the Shanghai PureOne Biotechnology (Shanghai, China). Its purity was 98%, detected by high-performance liquid chromatography. Asiaticoside was dissolved in ethanol with the final ethanol concentration less than 1%.

Cortical neurons cultured for 7 days *in vitro* were divided into three groups: control, NMDA treatment (200 $\mu\text{mol/L}$ NMDA and 10 $\mu\text{mol/L}$ glycine, NMDA receptor co-activator) and Asiaticoside pretreatment (Asiaticoside (less than

1%) pretreatment for 24 hours, then NMDA (200 $\mu\text{mol/L}$) and glycine (10 $\mu\text{mol/L}$) treatment for another 30 minutes). The NR2B receptor specific antagonist, RO-25-6981 (Sigma), was used as a positive control. The cells were then returned to the original culture medium for further 24 hours.

Cell viability analysis

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay was used to detect cell viability, as previously described (Yang et al., 2010). Neurons used for the experiments were cultured *in vitro* for 7 days (DIV 7). Cells were incubated with MTT (0.5 mg/mL) at 37°C for 4 hours on day 9. Cells were then washed and incubated in 150 μL dimethyl sulfoxide. The absorbance value was read on a Universal Microplate Reader (Elx800, Bio-TEK instruments Inc., Winooski, VT, USA) at 570 nm (taking 630 nm as a reference). Cell viability was expressed as a percentage of control value (%). All data were collected from three independent experiments.

Hoechst/propidium iodide double staining

Cortical neurons were cultured in 24-well plates at a density of 3×10^5 cells per well. Propidium iodide (Sigma) and Hoechst 33258 (Sigma) double fluorescent staining was used to determine either cell death or apoptosis as described previously (Liu et al., 2012). Neurons were incubated with propidium iodide (10 $\mu\text{g/mL}$) and Hoechst 33258 (10 $\mu\text{g/mL}$) for 15 minutes, and then fixed in 4% formaldehyde for 20 minutes. Imaging was detected under a fluorescence microscope (Olympus BX61, Tokyo, Japan) at 340 and 620 nm, respectively. Six visual fields were selected randomly from each well and data were collected from three independent experiments. The percentage of propidium iodide positive neurons compared with total Hoechst stained neurons

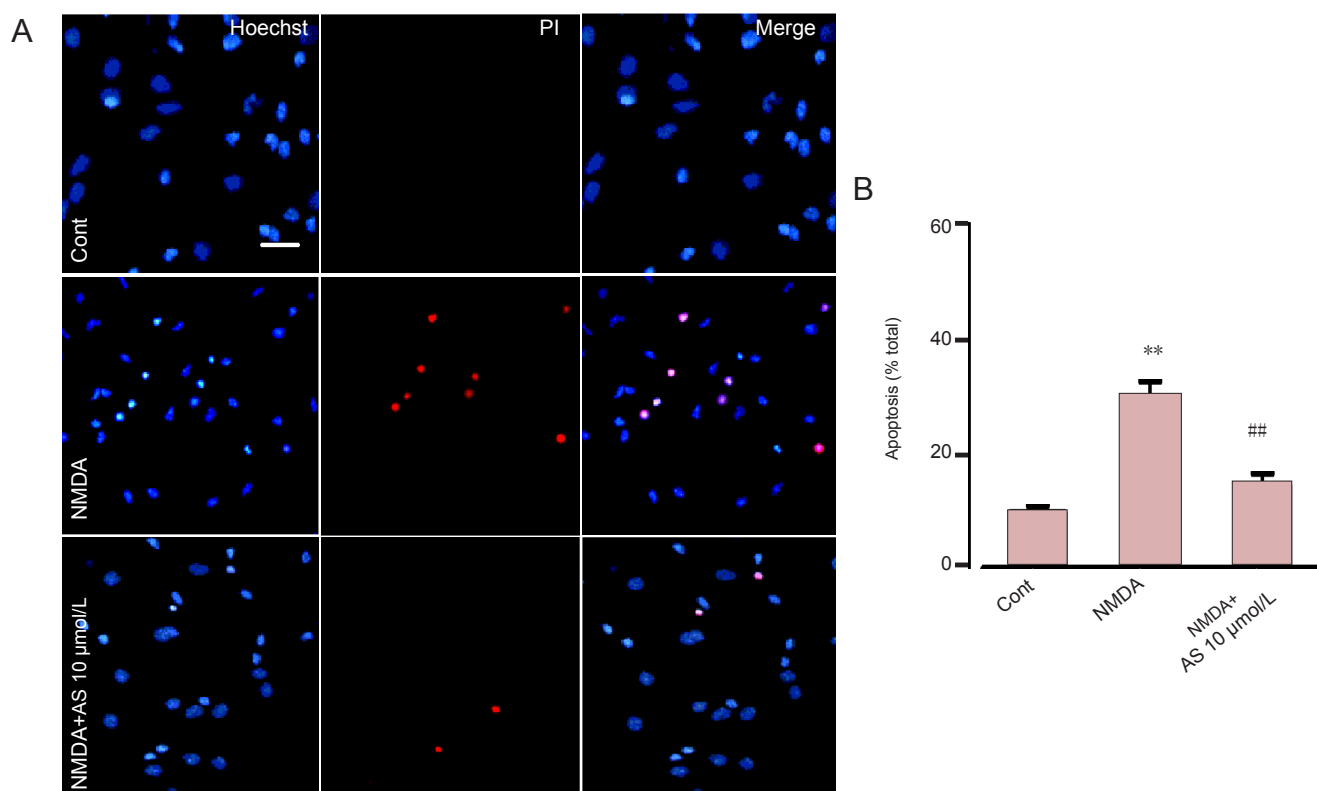


Figure 3 Neuroprotective effect of Asiaticoside (10 $\mu\text{mol/L}$) in cultured cortical neurons treated with N-methyl-D-aspartate (NMDA) (Hoechst 33258 and propidium iodide double staining).

(A) Representative fluorescence images obtained after Hoechst staining, propidium iodide staining, and double staining in control, and NMDA + 10 $\mu\text{mol/L}$ Asiaticoside (AS) treated groups. NMDA treatment increased the number of propidium iodide positive cells that was decreased in neurons pretreated with Asiaticoside. Scale bar: 20 μm . (B) The percentage of apoptotic neurons compared with total neurons for control, NMDA, and NMDA + 10 $\mu\text{mol/L}$ Asiaticoside treated groups. Apoptotic or necrotic cells were calculated from three independent experiments. All data are expressed as mean \pm SEM. One-way analysis of variance was used for comparison among multiple groups followed by Tukey's multiple comparison tests as a *post hoc* comparison. ** $P < 0.01$, vs. control group (Cont); ## $P < 0.01$, vs. NMDA treatment group.

was used to indicate cell death or apoptosis.

Western blot analysis

For western blot analysis, neurons were cultured in 6-well plates at a density of 2×10^6 cells/well. After pretreatment with Asiaticoside for 24 hours, cells were treated with NMDA (200 $\mu\text{mol/L}$) and glycine (10 $\mu\text{mol/L}$) for another 30 minutes. The next day, cells were rinsed twice with PBS and lysed using M-PER Protein Extraction Buffer containing $1 \times$ protease inhibitor cocktail. Equal amounts of protein (50 μg) were separated on 10% polyacrylamide gel and then transferred onto an Immun-Blot polyvinylidene difluoride membrane. To block the membrane for 1 hour, 5% non-fat milk in Tris-Phosphate buffer containing 0.05% Tween 20 was used. Membranes were incubated with the appropriate antibody overnight at 4°C; either mouse anti-NR2A (1:200), anti-NR2B (1:1,000), anti-Bax (1:1,000), or anti-Bcl-2 (1:1,000), with β -actin (1:10,000) as a loading control. Bands were visualized using an ECL system (Bio-Rad, Hercules, CA, USA) after incubation with the appropriate secondary antibody (goat anti-mouse immunoglobulin; Boster, Wuhan, Hubei Province, China). Anti-NR2A was purchased from Millipore (Billerica, MA, USA). Anti-NR2B, anti-Bax,

and anti-Bcl-2 antibodies were purchased from Chemicon (Temecula, CA, USA). β -Actin antibody was purchased from Sigma. Levels of protein were expressed as the percentage of control (β -actin).

Calcium imaging

Calcium imaging was performed as previously described (Yang et al., 2013). Neurons were cultured in 3.5 mm plates made especially for laser scanning microscope at a density of 3×10^5 per plate. Neurons were washed twice using Mg^{2+} -free extracellular solution. The extracellular solution contained NaCl (140 mmol/L), KCl (3 mmol/L), CaCl_2 (2 mmol/L), HEPES (10 mmol/L), and glucose (10 mmol/L). To the extracellular solution, 2.5 $\mu\text{mol/L}$ fluo-3/AM was added and neurons were incubated for 30 minutes at 37°C to load the dye. The neurons were washed and incubated in the culture medium for another 30 minutes. Calcium fluorescence curves were detected by a confocal laser scanning microscope (Olympus). Basal calcium levels were scanned for approximately 1 minute before NMDA (200 $\mu\text{mol/L}$) was added to the cultures. Calcium fluorescence curves were compared between control neurons and Asiaticoside pretreated neurons. The results show the fluorescence intensity

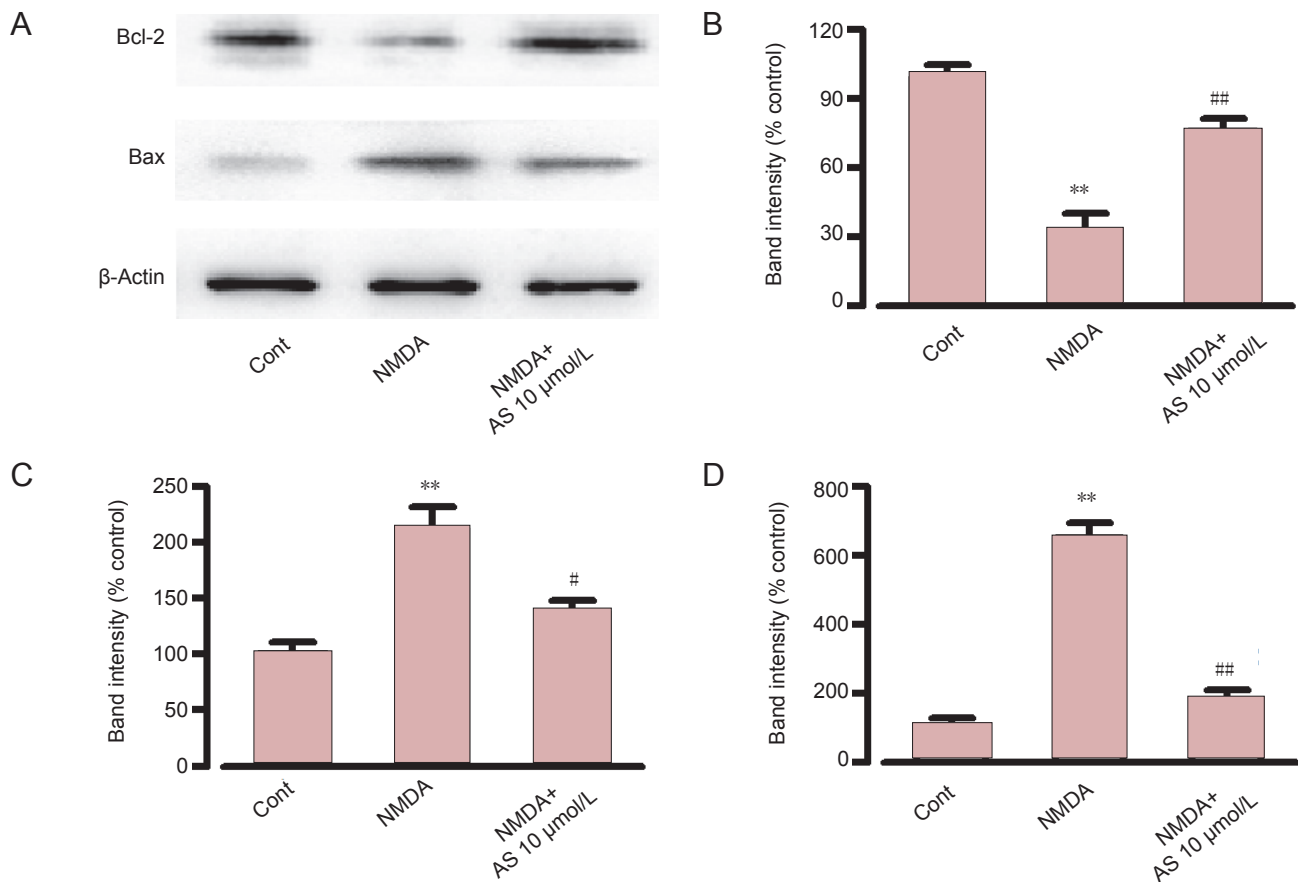


Figure 4 Effects of Asiaticoside (AS; 10 μmol/L) on the expression levels of Bcl-2 and Bax in cultured cortical neurons.

(A) Expression levels of Bax and Bcl-2 proteins were detected by western blot analysis. (B) The levels of Bcl-2 significantly decreased after exposure to N-methyl-D-aspartate (NMDA) in cultured cortical neurons. Pretreatment with 10 μmol/L AS evidently increased levels of Bcl-2. (C) The levels of Bax markedly increased after exposure to NMDA in cultured cortical neurons. Pretreatment with 10 μmol/L AS significantly decreased the levels of Bax. (D) The ratio of Bax/Bcl-2 was notably increased after NMDA exposure; however, 10 μmol/L AS reversed the ratio. Data are expressed as mean ± SEM from three independent experiments. One-way analysis of variance was used for comparison among multiple groups followed by Tukey’s multiple comparison tests as a *post hoc* comparison. ** $P < 0.01$, vs. control group (Cont); # $P < 0.05$, ## $P < 0.01$, vs. NMDA treatment.

was expressed as a change relative to basal levels.

Statistical analysis

Data were presented as mean ± SEM. Data analysis was performed using SPSS 10.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used for comparison among multiple groups followed by Tukey’s multiple comparison tests as a *post hoc* comparison. In all cases, $P < 0.05$ was considered statistically significant.

Results

Effects of Asiaticoside on cell viability in cultured cortical neurons

Exogenous NMDA was used to induce excitotoxicity in cultured mouse cortical neurons and cell viability was subsequently determined using an MTT assay. Neurons were cultured for 7 days *in vitro* and then pretreated with Asiaticoside for 24 hours. On day 8, NMDA (200 μmol/L) and glycine (10 μmol/L) were added to the medium containing Asiaticoside and the cells were incubated for 30 minutes. Cells were then

washed and cultured in the original culture medium for further 24 hours. As shown in **Figure 2A**, high concentrations of NMDA led to decreased cell viability measured by MTT assay (63%, $P < 0.01$ compared with control group). However, pretreatment with Asiaticoside significantly increased the cell viability of neurons in a dose-dependent manner. Neuroprotection was most effective at 10 μmol/L Asiaticoside (84%, $P < 0.01$ compared with NMDA treatment group), and 100 μmol/L Asiaticoside did not improve the viability of the neurons. Therefore, 10 μmol/L Asiaticoside was used in subsequent experiments. Asiaticoside (10 μmol/L) itself did affect the cell viability but Ro 25-6981 (0.3 μmol/L, added with the NMDA), an NR2B-selective antagonist, completely blocked the injury induced by NMDA (98%, $P < 0.01$ compared with NMDA treatment group; **Figure 2B**).

Effects of Asiaticoside on NMDA-induced excitotoxicity in cultured cortical neurons

Hoechst and propidium iodide double staining were used to determine the neuroprotective effects of Asiaticoside.

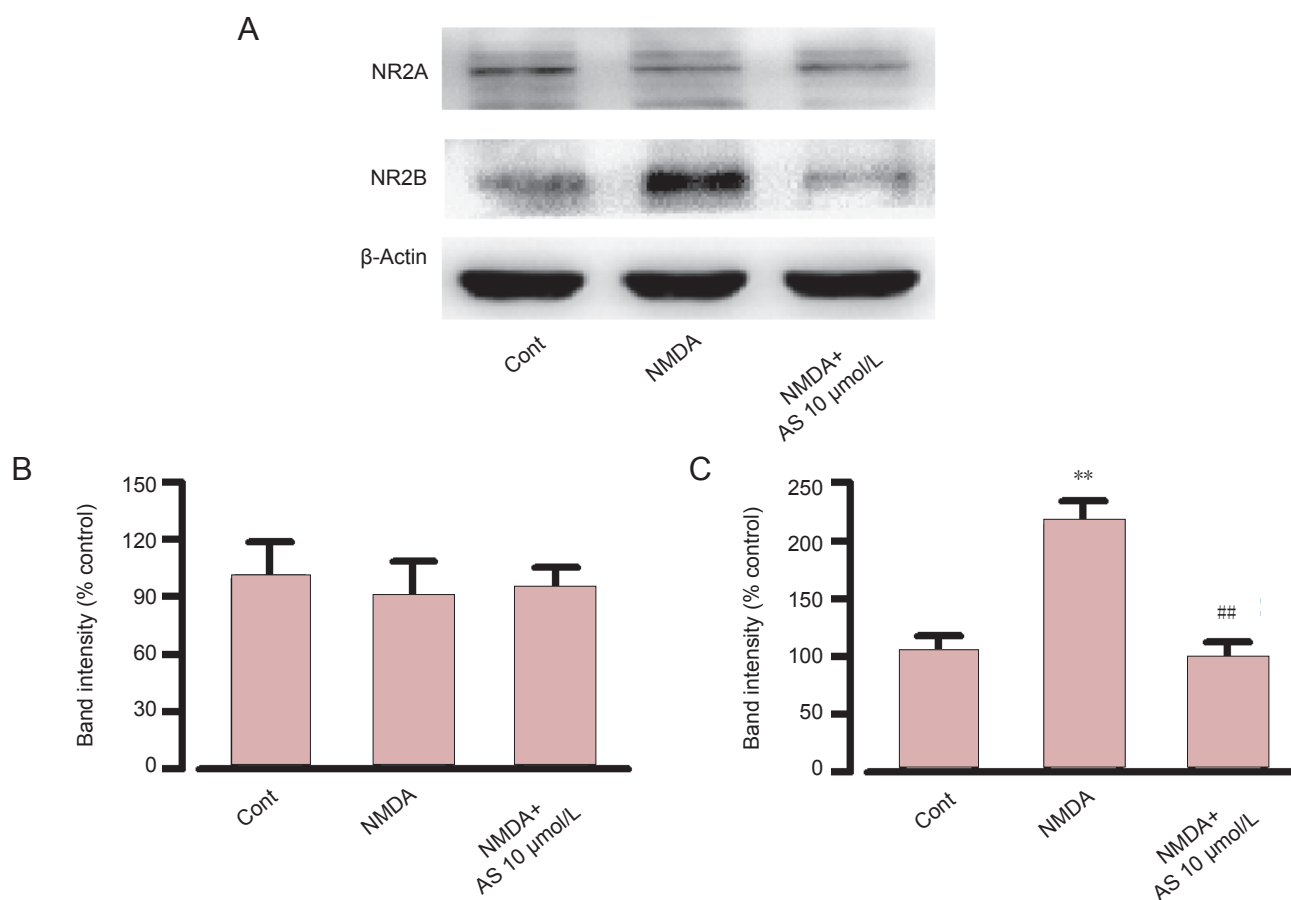


Figure 5 Effects of Asiaticoside (AS; 10 $\mu\text{mol/L}$) on the protein expression levels of N-methyl-D-aspartate (NMDA) receptor subunits, NR2A and NR2B.

(A) Expression levels of NR2A and NR2B proteins were detected by western blot analysis. (B) NMDA and AS treatment did not influence the levels of NR2A. (C) The levels of NR2B were markedly increased after exposure to NMDA in cultured cortical neurons. Pretreatment of 10 $\mu\text{mol/L}$ AS significantly decreased the levels of NR2B. Data are expressed as mean \pm SEM from three independent experiments. One-way analysis of variance was used for comparison among multiple groups followed by Tukey's multiple comparison tests as a *post hoc* comparison. ** $P < 0.01$, vs. control group (Cont); ## $P < 0.01$, vs. NMDA treatment group.

Hoechst and propidium iodide are dyes that stain nucleic acids. Propidium iodide, however, cannot pass through living cells, and therefore only stains apoptotic and necrotic cells. In normal culture conditions, only $9.7 \pm 1.5\%$ of cells were positive for propidium iodide staining. NMDA treatment for 30 minutes induced significant cell death or apoptosis, as $29.58 \pm 2.41\%$ of the cells were propidium iodide positive ($P < 0.01$ compared with control group; **Figure 3**). Asiaticoside pretreatment (10 $\mu\text{mol/L}$) prevented cell damage caused by NMDA treatment as fewer cells were positive for propidium iodide staining ($14.61 \pm 2.14\%$; $P < 0.01$ compared with NMDA treatment group; **Figure 3**). These results demonstrate that Asiaticoside is protective against injury induced by NMDA.

Anti-apoptotic effects of Asiaticoside

Western blot analysis was used to detect the levels of apoptosis-related proteins, anti-apoptotic Bcl-2 and pro-apoptotic Bax. The ratio of Bax/Bcl-2 reflects the susceptibility of cells to harmful response (Liu et al., 2012). Treatment with 200

$\mu\text{mol/L}$ NMDA markedly decreased levels of Bcl-2 (32% of control; $P < 0.01$ compared with control group; **Figure 4A, B**) and increased the levels of Bax (211% of control; $P < 0.01$, compared with control group; **Figure 4A, C**) and the ratio of Bax/Bcl-2 (652% of control; $P < 0.01$ compared with control group; **Figure 4A, D**). However, pretreatment of Asiaticoside (10 $\mu\text{mol/L}$) prior to administration of NMDA significantly changed the levels of Bcl-2 (76% of control; $P < 0.01$ compared with NMDA treatment group; **Figure 4A, B**) and Bax (137% of control; $P < 0.05$ compared with NMDA treatment; **Figure 4A, C**), and the ratio of Bax/Bcl-2 (180% of control; $P < 0.01$ compared with NMDA treatment group; **Figure 4A, D**).

Effects of Asiaticoside on the levels of NMDA receptors

NMDA receptor subunit composition (NR2A or NR2B) and NMDA receptor localization (intrasynapse or extrasynapse) determine the intracellular cascades and functions of the receptor in neurons (Liu et al., 2007). NR2B-containing NMDA receptors, particularly those located extrasynaptically, contribute to neuronal damage (Liu et al., 2007; Shen

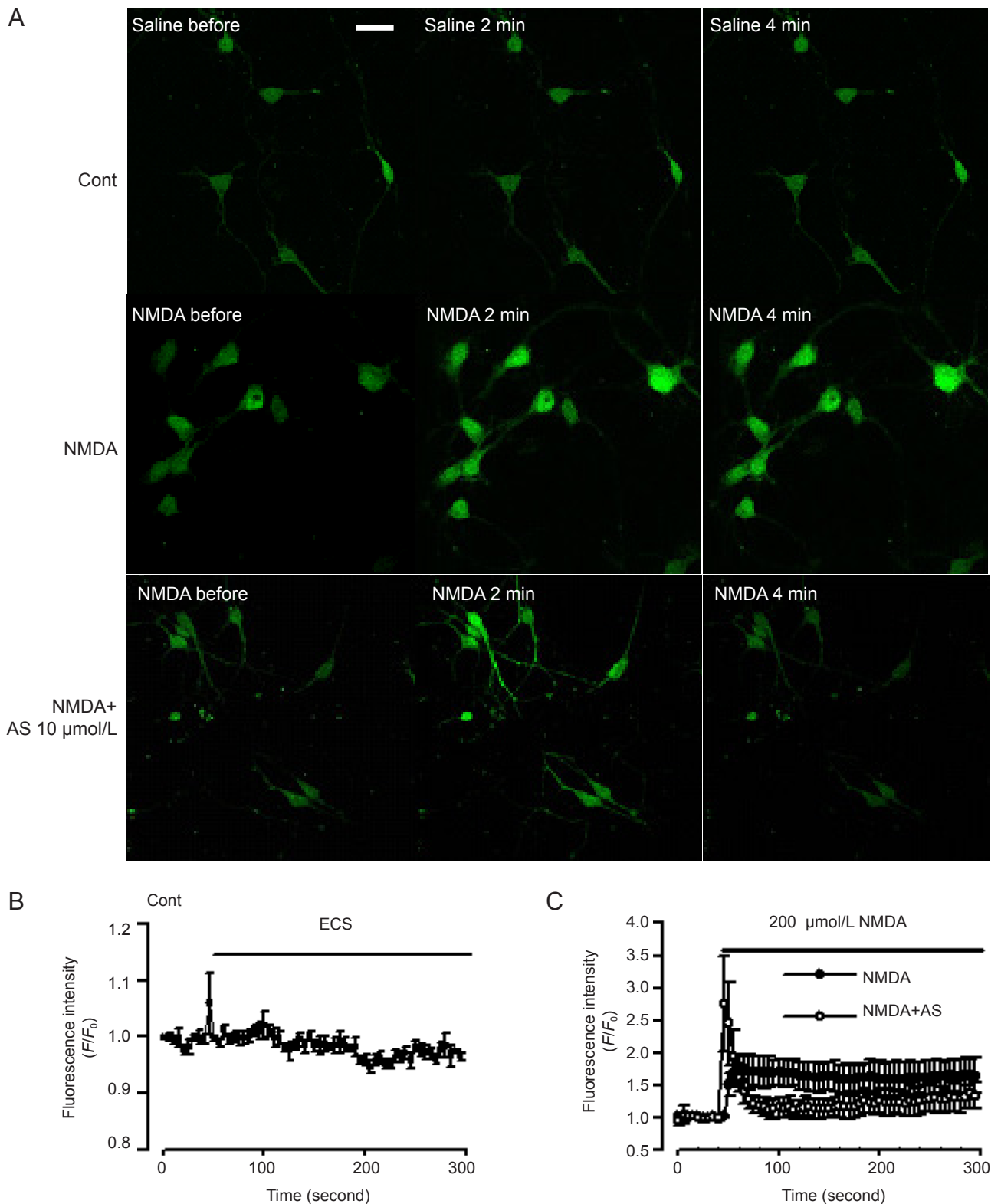


Figure 6 Effect of Asiaticoside (AS) on Ca^{2+} levels mediated by N-methyl-D-aspartate (NMDA) receptors.

(A) Fluo-3/AM (2.5 μmol/L) was added to the extracellular solution and neurons were incubated for 30 minutes at 37°C to load the dye. The green fluorescence under the laser scanning microscope at different time points reflected the concentration of Ca^{2+} in neurons, which was stable during the experiment. Before: No NMDA perfusion; 2 min: 2 minutes after NMDA perfusion; 4 min: 4 minutes after NMDA perfusion. Scale bar: 20 μm. (B) The fluorescence intensity (F/F_0 : the ratio of the timing fluorescence to the basal calcium fluorescence intensity before NMDA perfusion) reflecting Ca^{2+} concentration was stable during detection (recording time: seconds) by a laser scanning microscope ($n = 32$ neurons). (C) 200 μmol/L NMDA evoked strong fluorescence intensity ($n = 41$ neurons) and 10 μmol/L AS significantly reduced fluorescence intensity in neurons ($n = 36$ neurons). Cont: Control.

et al., 2008; Stanika et al., 2009). Western blot analysis indicated that NMDA treatment caused an increase of NR2B expression (214% of control; $P < 0.01$ compared with control group; **Figure 5A, C**) but expression of NR2A subtype did not change (**Figure 5A, B**). Asiaticoside (10 $\mu\text{mol/L}$) pretreatment notably reduced the up-regulation of NR2B levels by NMDA exposure (95% of control; $P < 0.01$ compared with NMDA treatment group; **Figure 5A, C**). Therefore, down-regulation of NR2B levels may be the major mechanism underlying the neuroprotective effects of Asiaticoside.

Asiaticoside decreases NMDA-induced Ca^{2+} overload

Following excessive activation of NMDA receptors, Ca^{2+} influx from NMDA receptors and sustained intracellular Ca^{2+} overload contribute to neuronal injury (Vander Jagt et al., 2009). Cytoplasmic Ca^{2+} concentration was marked by fluorescence intensity under the laser confocal microscopy (Matsumoto et al., 2004). Under control conditions, a stable recording of Ca^{2+} levels was observed in neurons (**Figure 6A, B**). NMDA (200 $\mu\text{mol/L}$) evoked a rapid increase in cytoplasmic Ca^{2+} which stayed at a high concentration for the next 4 minutes (**Figure 6C**). In neurons pretreated with Asiaticoside (10 $\mu\text{mol/L}$) Ca^{2+} levels were markedly attenuated in response to NMDA exposure (**Figure 6A, C**).

Discussion

Glutamate plays a key role in neural transmission, development and synaptic plasticity. Glutamate binds to ionotropic receptors including NMDA, kainate, and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Watkins and Jane, 2006). In pathological conditions such as stroke, traumatic damage and inflammation, excessive accumulation of cytoplasmic Ca^{2+} influx through NMDA receptors triggers excitotoxicity and neuronal damage (Jayaraman et al., 2013). In this study, elevation of Ca^{2+} stimulated by NMDA was inhibited by Asiaticoside, suggesting inhibition of calcium influx through NMDA receptors contributes, at least partly, to neuroprotection by Asiaticoside.

Asiaticoside exhibits antipyretic, anti-inflammatory and antioxidant activity (Tabassum et al., 2013; Wan et al., 2013). In the central nervous system, Asiaticoside could attenuate neurobehavioral, neurochemical and histological changes in transient focal middle cerebral artery occlusion rats (Tabassum et al., 2013). Another study demonstrated anxiolytic effects of Asiaticoside in both acutely and chronically stressed animals (Wanasuntronwong et al., 2012). In this study, we found that Asiaticoside pretreatment dose-dependently attenuated reduction in cell viability by exposure to high concentration of NMDA. Hoechst and propidium iodide double staining further verified the neuroprotective effects of Asiaticoside, although the staining could not discriminate between apoptosis and necrosis. Western blot analysis suggested Asiaticoside has anti-apoptotic activities as it affected the expression of apoptotic proteins, Bcl-2 and Bax. Further experiments showed that Asiaticoside attenuated expression of NR2B-containing NMDA receptors, but not

NR2A-containing NMDA receptors. This is consistent with previous findings that suggest NR2B is responsible for mediating excitotoxicity (Liu et al., 2007). NMDA receptors that have more NR2B than NR2A subunits (a low NR2A:NR2B ratio) open for a longer length of time. Thus, neurons with a low NR2A:NR2B ratio are more sensitive to glutamate (Yamakura and Shimoji, 1999). Pretreatment with Asiaticoside decreased the level of NR2B receptors. It is possible that lowered NR2B expression resulted in a shorter opening time of NMDA receptors and subsequently less calcium influx into neurons, and consequently a neuroprotective effect.

In conclusion, our results provide a new insight into the neuroprotective effects of Asiaticoside. Aside from its anti-oxidant activity, down-regulation of NR2B-containing NMDA receptors may be one of the underlying mechanisms in Asiaticoside neuroprotection. Our results suggest that Asiaticoside protects neurons from excitotoxicity induced by NMDA exposure by inhibiting cell apoptosis and calcium overload.

Acknowledgments: We would like to thank Dr. Wu YM from the Fourth Military Medical University of Chinese PLA for her helpful comments for the discussion.

Author contributions: An JZ and Zhao MG designed the study. Qi FY, Yang L and Tian Z performed the experiments. Liu SB analyzed data. An JZ wrote the paper. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

References

- Carriedo SG, Sensi SL, Yin HZ, Weiss JH (2000) AMPA exposures induce mitochondrial Ca^{2+} overload and ROS generation in spinal motor neurons in vitro. *J Neurosci* 20:240-250.
- Defillipo PP, Raposo AH, Fedoce AG, Ferreira AS, Polonini HC, Gattaz WF, Raposo NR (2012) Inhibition of cPLA2 and sPLA2 activities in primary cultures of rat cortical neurons by Centella asiatica water extract. *Nat Prod Commun* 7:841-843.
- Hardingham GE, Bading H (2003) The Yin and Yang of NMDA receptor signalling. *Trends Neurosci* 26:81-89.
- Jayanarayanan S, Smijin S, Peeyush KT, Anju TR, Paulose CS (2013) NMDA and AMPA receptor mediated excitotoxicity in cerebral cortex of streptozotocin induced diabetic rat: ameliorating effects of curcumin. *Chem Biol Interact* 201:39-48.
- Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, Ben-Ari Y, Clapham DE, Medina I (2003) The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. *Neuron* 40:775-784.
- Lee JH, Kim HL, Lee MH, You KE, Kwon BJ, Seo HJ, Park JC (2012) Asiaticoside enhances normal human skin cell migration, attachment and growth in vitro wound healing model. *Phytomedicine* 19:1223-1227.
- Liu SB, Zhang N, Guo YY, Zhao R, Shi TY, Feng SF, Wang SQ, Yang Q, Li XQ, Wu YM, Ma L, Hou Y, Xiong LZ, Zhang W, Zhao MG (2012) G-protein-coupled receptor 30 mediates rapid neuroprotective effects of estrogen via depression of NR2B-containing NMDA receptors. *J Neurosci* 32:4887-4900.
- Liu Y, Wong TP, Aarts M, Rooyackers A, Liu L, Lai TW, Wu DC, Lu J, Tymianski M, Craig AM, Wang YT (2007) NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *J Neurosci* 27:2846-2857.
- Matsumoto Y, Yamamoto S, Suzuki Y, Tsuboi T, Terakawa S, Ohashi N, Umemura K (2004) Na^+/H^+ exchanger inhibitor, SM-20220, is protective against excitotoxicity in cultured cortical neurons. *Stroke* 35:185-190.

- Mattson MP (2003) Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. *Neuromolecular Med* 3:65-94.
- Shen H, Yuan Y, Ding F, Liu J, Gu X (2008) The protective effects of *Achyranthes bidentata* polypeptides against NMDA-induced cell apoptosis in cultured hippocampal neurons through differential modulation of NR2A- and NR2B-containing NMDA receptors. *Brain Res Bull* 77:274-281.
- Somboonwong J, Kankaisre M, Tantisira B, Tantisira MH (2012) Wound healing activities of different extracts of *Centella asiatica* in incision and burn wound models: an experimental animal study. *BMC Complement Altern Med* 12:103.
- Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klann E, Angiolieri MR, Johnson JW, DeFranco DB (2000) Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J Biol Chem* 275:12200-12206.
- Stanika RI, Pivovarova NB, Brantner CA, Watts CA, Winters CA, Andrews SB (2009) Coupling diverse routes of calcium entry to mitochondrial dysfunction and glutamate excitotoxicity. *Proc Natl Acad Sci U S A* 106:9854-9859.
- Tabassum R, Vaibhav K, Shrivastava P, Khan A, Ejaz Ahmed M, Javed H, Islam F, Ahmad S, Saeed Siddiqui M, Safhi MM (2013) *Centella asiatica* attenuates the neurobehavioral, neurochemical and histological changes in transient focal middle cerebral artery occlusion rats. *Neurol Sci* 34:925-933.
- Vander Jagt TA, Connor JA, Weiss JH, Shuttleworth CW (2009) Intracellular Zn²⁺ increases contribute to the progression of excitotoxic Ca²⁺ increases in apical dendrites of CA1 pyramidal neurons. *Neuroscience* 159:104-114.
- Wan J, Gong X, Jiang R, Zhang Z, Zhang L (2013) Antipyretic and anti-inflammatory effects of asiaticoside in lipopolysaccharide-treated rat through up-regulation of heme oxygenase-1. *Phytother Res* 27:1136-1142.
- Wanasuntronwong A, Tantisira MH, Tantisira B, Watanabe H (2012) Anxiolytic effects of standardized extract of *Centella asiatica* (ECA 233) after chronic immobilization stress in mice. *J Ethnopharmacol* 143:579-585.
- Watkins JC, Jane DE (2006) The glutamate story. *Br J Pharmacol* 147 Suppl 1:S100-108.
- Xu CL, Wang QZ, Sun LM, Li XM, Deng JM, Li LF, Zhang J, Xu R, Ma SP (2012) Asiaticoside: attenuation of neurotoxicity induced by MPTP in a rat model of Parkinsonism via maintaining redox balance and up-regulating the ratio of Bcl-2/Bax. *Pharmacol Biochem Behav* 100:413-418.
- Yamakura T, Shimoji K (1999) Subunit- and site-specific pharmacology of the NMDA receptor channel. *Prog Neurobiol* 59:279-298.
- Yang L, Li XB, Yang Q, Zhang K, Zhang N, Guo YY, Feng B, Zhao MG, Wu YM (2013) The neuroprotective effect of praeruptorin C against NMDA-induced apoptosis through down-regulating of GluN2B-containing NMDA receptors. *Toxicol In Vitro* 27:908-914.
- Yang Q, Feng B, Zhang K, Guo YY, Liu SB, Wu YM, Li XQ, Zhao MG (2012) Excessive astrocyte-derived neurotrophin-3 contributes to the abnormal neuronal dendritic development in a mouse model of fragile X syndrome. *PLoS Genet* 8:e1003172.
- Yang Q, Yang ZF, Liu SB, Zhang XN, Hou Y, Li XQ, Wu YM, Wen AD, Zhao MG (2010) Neuroprotective effects of hydroxysafflor yellow A against excitotoxic neuronal death partially through down-regulation of NR2B-containing NMDA receptors. *Neurochem Res* 35:1353-1360.
- Zhang K, Li YJ, Yang Q, Gerile O, Yang L, Li XB, Guo YY, Zhang N, Feng B, Liu SB, Zhao MG (2013) Neuroprotective effects of oxymatrine against excitotoxicity partially through down-regulation of NR2B-containing NMDA receptors. *Phytomedicine* 20:343-350.
- Zhang L, Li HZ, Gong X, Luo FL, Wang B, Hu N, Wang CD, Zhang Z, Wan JY (2010) Protective effects of Asiaticoside on acute liver injury induced by lipopolysaccharide/D-galactosamine in mice. *Phytomedicine* 17:811-819.

Copyedited by Paul P, Norman C, Wang J, Yang Y, Li CH, Song LP, Zhao M