Rheum turkestanicum reduces glutamate toxicity in PC12 and N2a cell lines

Arezoo Rajabian[1], Hamid-Reza Sadeghnia[1,2,3], Maliheh Moradzadeh[4], Azar Hosseini[1]

[1]Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran, [2]Department of Pharmacology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran, [3]Neurocognitive Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran, [4]Golestan Rheumatology Research Center, Golestan University of Medical Sciences, Gorgan, Iran

Abstract

Glutamate is considered to be responsible for the pathogenesis of many neurodegenerative diseases. Reactive oxygen species (ROS) production is considered to be involved in the glutamate-induced apoptosis process. In this study, we investigated the neuroprotective effects of Rheum turkestanicum in the glutamate-induced rat pheochromocytoma (PC12 cells) and mouse neuroblastoma (N2a) cell lines. Rutin as an antioxidant was used as positive control. Glutamate cytotoxicity was accompanied by an increment of malondialdehyde (MDA) content, ROS generation and apoptosis induction. However, pretreatment with the root extract of R. turkestanicum significantly reduced MDA content, ROS generation and apoptotic cell death. Also rutin at a dose of 100 μM reduced ROS production and protected against glutamate toxicity. Also the quantification of rutin in R. turkestanicum extract was achieved and was about 0.11% ± 0.01 w/w. All these findings indicated that R. turkestanicum protected PC12 and N2a cells against glutamate-induced oxidative cell death and apoptosis and might raise the possibility of R. turkestanicum usage as a neuroprotective agent.

Key words: Rheum turkestanicum, glutamate, PC12, N2a, apoptosis, oxidative damage, rutin.

Introduction

Glutamate, as an important endogenous excitatory neurotransmitter, has major physiological functions in the central nervous system [21]. Nevertheless, glutamate accumulation may result in neuronal damage and cell death under different pathological conditions via various mechanisms, mainly oxidative damage and excitotoxicity. ROS species can trigger protein oxidation, as well as lipid peroxidation during oxidative glutamate toxicity [7,23]. Nervous tissues show great vulnerability to oxidative damage owing to a variety of factors, such as high concentration of polyunsaturated fatty acids, low level of antioxidant enzymes (e.g., superoxide dismutase, catalase, and glutathione peroxidase), and high metabolic activity [27,2]. Recently, application of antioxidant compounds has been highlighted against neurodegenerative diseases [21]. According to clinical studies, occurrence of neurodegenerative disorders can be reduced by using dietary antioxidants [26,30]. The literature suggests that most vegetables and fruits consist of natural anti-

Communicating author

Prof. Azar Hosseini, Pharmacological Research Center of Medicinal Plants, Mashhad University of Mashhad Sciences, Mashhad, Iran, e-mail: hosseiniaz1359@gmail.com

oxidants and flavonoids, which can decrease oxidative stress and cognitive disorders associated with neurodegeneration in animal models [25,14]. Based on recent *in vivo* and *in vitro* studies, natural antioxidant compounds reduce glutamate toxicity [25]. Also, addition of antioxidants to our daily diet can reduce oxidative stress and neurodegeneration. *Rheum turkestanicum* (*Polygonaceae*) is a plant, known to grow widely in Central Asia and Northeast of Iran. *R. turkestanicum* root was traditionally used as an antihypertensive, anticancer, and antidiabetic compound [12]. *R. turkestanicum* reduces doxorubicin toxicity in H9c2 cardiomyocyte cells via reduction of ROS generation [13]. According to recent studies, there are antioxidant compounds in *Rheum* species. *R. undulatum* contains rhapontigenin and rhaponticin, which have been shown to scavenge DPPH radicals, hydrogen peroxide (H_2O_2), and ROS [35]. Also, these compounds can reduce DNA damage, as well as membrane lipid peroxidation, which are recognized as the major targets of cellular damage due to oxidative stress [12]. According to recent research, some antioxidant constituents from *Rheum* species (such as *R. emodi*) could protect H9c2 cells against H_2O_2 [8]. Since *Rheum* genus contains antioxidant compounds, we examined the effects of *R. turkestanicum* root extract against glutamate toxicity in N2a and PC12 cells.

Material and methods

Material

Sigma (St. Louis, MO) supplied 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), 2,7-dichloroflurescin diacetate (DCFH-DA), thiobarbituric acid (TBA), propidium iodide (PI), Triton X-100, and sodium citrate. Gibco (Carlsbad, CA) provided fetal bovine serum (FBS), penicillin-streptomycin, and high-glucose Dulbecco's Modified Eagles Medium (DMEM). In addition, Merck (Darmstadt, Germany) supplied malondialdehyde bis-(dimethyl acetal) (MDA) and trichloroacetic acid (TCA). Finally, Sigma (St Louis, MO, USA) provided rutin hydrate, and the Pasteur Institute Cell Bank (Tehran, Iran) provided PC12 and N2a cells (C-153).

Extract preparation

After collecting *R. turkestanicum* roots from Kalat region (Khorasan Razavi, Iran), they were identified by experts at the herbarium, affiliated to Ferdowsi University of Mashhad (specimen No. 42,082). The roots were dried and then an electric micronizer was used to crush them into a powder. After the hydroalcoholic extract was added to 70% ethanol in a Soxhlet extractor, it was dried and stored at −20°C until further use.

Cell culture and treatment

After storing the cells in a humidified atmosphere (5% CO_2; temperature, 37°C), they were cultured in DMEM medium, consisting of 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 10% FBS. For MTT/ROS and MDA assays, the cells were cultured in 96- and 24-well plates, respectively, and for apoptosis assays, the cells were seeded in 24-well plates (100 000 cells/well); it should be noted that all treatments were performed in triplicate. For two hours, the cells were pretreated with only the extract (6-200 µg/ml). Incubation was carried out with the extract for 24 hours, with or without glutamate (8 mM). Finally, rutin was used to pretreat the cells for two hours, and then, incubation was performed with glutamate for 24 hours.

Cell viability assay

Cell viability was measured using the modified MTT assay, as described in the literature [15]. In each well, MTT solution was added to phosphate-buffered saline (PBS; 5 mg/ml) (final concentration, 0.05%). After three hours, the formazan precipitate was dissolved in DMSO. A StatFAX303 plate reader was employed to determine absorbance at wavelengths of 570 and 620 nm (background).

Lipid peroxidation assay

The MDA level (end product of lipid peroxidation) was measured to determine lipid peroxidation [8]. The cells were scraped, and then, centrifugation was performed for 30 minutes following incubation. TBA (0.7%; 800 µl) and TCA (15%; 400 µl) were added to the cell samples (500 µl). After vortexing the mixture, a boiling water bath was used for heating (40 minutes). The samples (200 µl) were finally moved to a 96-well plate to read fluorescence intensity at 480/530 nm (excitation/emission wavelengths).

ROS measurements

H_2DCF-DA, which was ROS-oxidized and converted to a highly fluorescent compound (DCF),

Arezoo Rajabian, Hamid-Reza Sadeghnia, Maliheh Moradzadeh, Azar Hosseini

was used to determine the intracellular level of ROS. H_2DCF-DA (200 µM, 10 µl) was added to each well before treatment and incubated in darkness at 37ºC for 30 minutes. Following that, PBS buffer was used to wash the cells. The cells were pretreated for two hours with the extract and rutin (100 µM) and exposed to glutamate (8 mM). After incubation for two hours, a fluorescence multi-well plate reader was used to determine the DCF fluorescence intensity (excitation and emission wavelengths, 485 and 530 nm, respectively) [15].

PI staining

Small DNA fragments were PI-stained to identify apoptotic cells, and then, flow cytometry was applied. A sub-G1 peak, indicative of DNA fragmentation, is observed after cell incubation in a hypotonic phosphate-citrate buffer, including a DNA-binding dye (such as PI). In the histogram, DNA-free apoptotic cells absorb less stain and can be seen on the left side of the peak. Based on the described protocol, the cells were treated after seeding in a 24-well plate. Then, adherent and floating cells were harvested, and incubation was performed at 4ºC in darkness overnight, using a hypotonic buffer (750 µl; 50 µg/ml of PI in 0.1% triton X-100 and 0.1% sodium citrate). Finally, a FACScan system (Becton Dickinson) was used to perform flow cytometry, yielding a total of 10^4 events [15].

High-performance liquid chromatography characterization of *R. turkestanicum* extracts

The *R. turkestanicum* extract was standardized based on rutin flavonoid, using the High-performance liquid chromatography (HPLC) method, as described in the literature [1]. Chromatographic separation was performed on a Waters C18 analytical column (particle size, 5 µm; 250 × 4.6 mm). The mobile phase included water and methanol (1 : 1 ratio); pH was adjusted to 2.5 with phosphoric acid. Quantification was carried out based on the standard curve of rutin (0, 5, 10, 25, and 50 µg/ml).

Data analysis

Data are presented as mean ± SEM. One-way ANOVA and Tamhane T2 post-hoc tests were performed for analyses. $P < 0.05$ was considered statistically significant.

Results

R. turkestanicum and rutin significantly decreased cells death induced by glutamate

To study the possible toxic effects of the extract, PC12 and N2a cells were incubated with different concentrations of extracts (12-200 µg/ml), and the
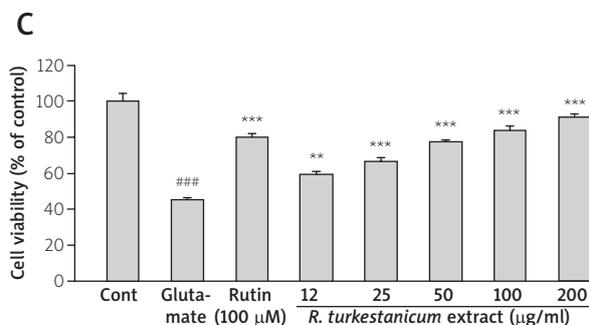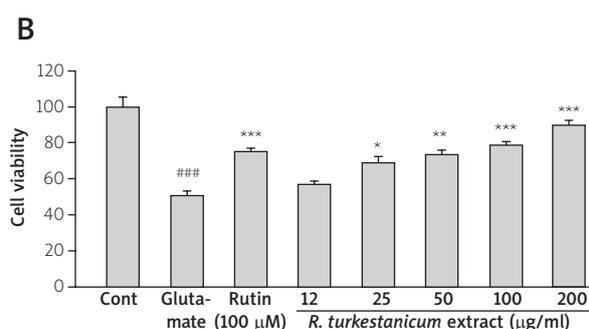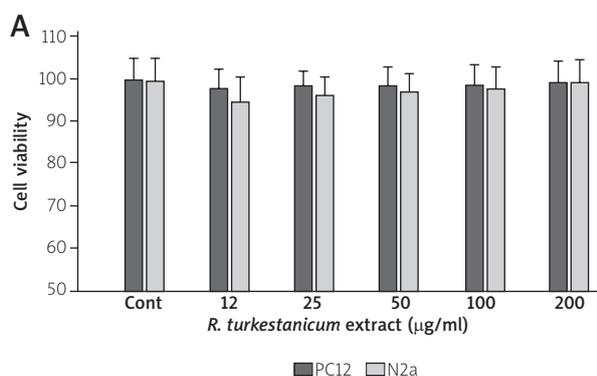
Fig. 1. Effect of *R. turkestanicum* without **(A)** or with glutamate on PC12 **(B)** and N2a **(C)** cells. The cells were pretreated with different concentrations of the extract and rutin for 2 h, then exposed to glutamate (8 mM) for 24 h. The cell viability was quantitated by MTT assay. Results are mean ± SEM (*n* = 9). $^{###}p < 0.001$ vs. control, $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. glutamate.
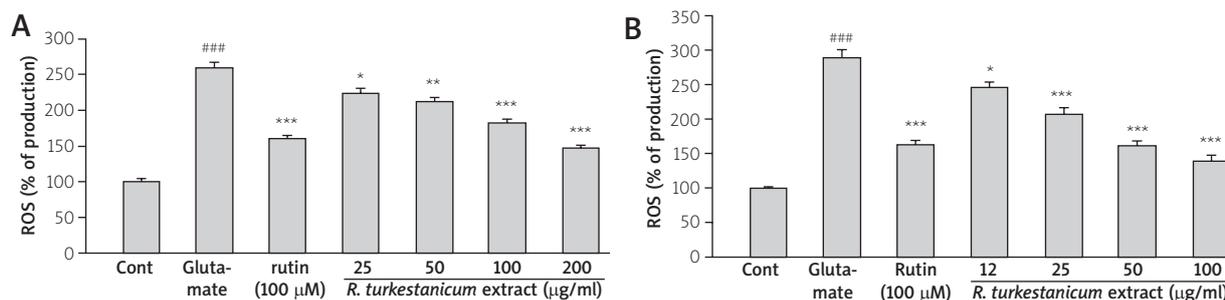
Fig. 2. Effect of *R. turkestanicum* and rutin on ROS generation in glutamate-injured PC12 **(A)** and N2a **(B)** cells. Results are mean ± SEM (n = 3). $^{###}p < 0.001$ vs. control, $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* glutamate.

viability was determined 24 h after treatment. No significant toxic effect on cell viability was seen subsequent to treatment with the extract (Fig. 1A).

Incubation with glutamate significantly decreased cell viability to 50.0 ± 2.6% of control ($p < 0.001$). As shown in Figure 1B, treatment of PC12 cells with the extract increased cell viability subsequent to glutamate insult at a concentration ≥ 25, dose dependently ($25 \mu g/ml$, 69 ± 2%, $p < 0.05$; $50 \mu g/ml$, 73.1 ± 1.8%, $p < 0.01$; $100 \mu g/ml$, 79.6 ± 2.8%, $p < 0.001$; $200 \mu g/ml$, 90.0 ± 2.3%, $p < 0.001$). Results also showed that treatment of N2a cells with the extract increased cell viability at concentrations ≥ 12 $\mu g/ml$ (12 $\mu g/ml$, 89.4 ± 1.4%, $p < 0.01$, 25 $\mu g/ml$, 67.2 ± 1.9%, $p < 0.001$; 50 $\mu g/ml$, 77 ± 1%, $p < 0.001$; 100 $\mu g/ml$, 84.1 ± 2.4%, $p < 0.001$; 200 $\mu g/ml$, 91.2 ± 1.6%, $p < 0.001$). While glutamate decreased cell viability to 45.0 ± 1.2%, $p < 0.01$ (Fig. 1C). Also rutin increased cell viability in PC12 (75 ± 1.8%) and N2a (80 ± 2%).

R. turkestanicum and rutin significantly decreased ROS content induced by glutamate

As expected, glutamate caused a significant increase in the level of ROS in PC12 cells (260 ± 7.8% of control, $p < 0.001$). The extract at concentrations of 25-200 $\mu g/ml$ and rutin (100 μM, 160 ± 4.5%, $p < 0.001$) were able to decrease intracellular ROS level to 223 ± 7.6% ($p < 0.05$), 210.0 ± 6.8% ($p < 0.01$), 181 ± 5% ($p < 0.001$) and 146 ± 3.6% ($p < 0.001$) of control value, respectively (Fig. 2A). Also, the extract decreased the ROS level in N2a cells at concentrations of 12 $\mu g/ml$ (245.9 ± 7.8%, $p < 0.05$), 25 $\mu g/ml$ (206 ± 9.4%, $p < 0.001$), 50 $\mu g/ml$ (161 ± 5.8%, $p < 0.001$) and 200 $\mu g/ml$ (138 ± 8.7%, $p < 0.001$) against glutamate (288 ± 10.5%, $p < 0.001$). Also rutin

reduced ROS content in N2a cells (100 μM, 163 ± 5%, $p < 0.001$) (Fig. 2B).

R. turkestanicum significantly decreased lipid peroxidation induced by glutamate

As shown in Figure 3, exposure of the cells to glutamate resulted in a significant increase of MDA level in PC12 cells (269 ± 7.5%, $p < 0.001$) as compared to control cells cultured in the absence of glutamate (100 ± 4.5%). The content of MDA was significantly decreased in the cells pretreated with 25 $\mu g/ml$ (204 ± 11.3%, $p < 0.01$), 50 $\mu g/ml$ (194.10 ± 7.18%, $p < 0.01$), 100 $\mu g/ml$ (179 ± 4.7%, $p < 0.001$) and 200 $\mu g/ml$ (143.3 ± 3.3%, $p < 0.001$) of the extract (Fig. 3A). In N2a cells, the extract also reduced the level of MDA at 12 $\mu g/ml$ (259.3 ± 10.3%, $p < 0.01$), 25 $\mu g/ml$ (215 ± 9%, $p < 0.001$), 50 $\mu g/ml$ (158 ± 8.4%, $p < 0.001$) and 100 $\mu g/ml$ (130 ± 4.7%, $p < 0.001$) against glutamate (329 ± 9.6%, $p < 0.001$) (Fig. 3B).

R. turkestanicum significantly decreased apoptotic cell death induced by glutamate

Apoptosis in PC12 and N2a cell lines was detected with flow cytometry using PI staining. Cells were pretreated for 2 h with various concentrations of *R. turkestanicum* (50-200 $\mu g/ml$) and exposed to glutamate for 24 h. Analysis of the sub G1 peaks in flow cytometer histograms revealed the induction of apoptosis in PC12 treated with glutamate (42.3 ± 1.2%, $p < 0.001$). On the other hand, the extract reduced apoptotic PC12 cells induced by glutamate (50 $\mu g/ml$, 28.5 ± 2.1%, $p < 0.01$; 100 $\mu g/ml$, 18.3 ± 1.5%, $p < 0.001$; 200 $\mu g/ml$, 15.5 ± 2.5%, $p < 0.001$)
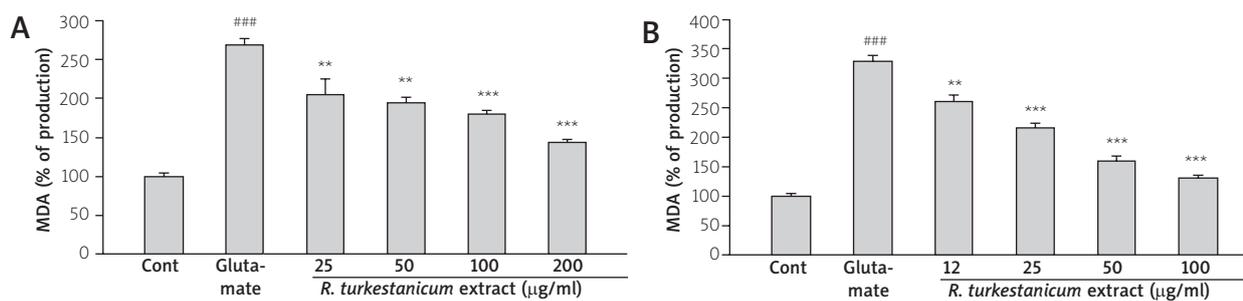
Fig. 3. Effect of *R. turkestanicum* on MDA production in glutamate-injured PC12 **(A)** and N2a **(B)** cells. The cells were pretreated with different concentrations of the extract for 2 h, then exposed to glutamate (8 mM) for 24 h. Results are mean ± SEM (n = 3). ### $p < 0.001$ vs. control, * $p < 0.05$ and *** $p < 0.001$ vs. glutamate.

(Fig. 4A). Also, the extract decreased apoptotic N2a cells induced by glutamate (50 µg/ml, 31 ± 1.8%, $p < 0.01$; 100 µg/ml, 26.8 ± 1.2%, $p < 0.001$; 200 µg/ml, 13.7 ± 2.3%, $p < 0.001$) versus glutamate (49 ± 2%, $p < 0.001$) (Fig. 4B).

HPLC characterization of *R. turkestanicum* extract

The chromatograms of standard rutin (10 µg/ml) and a sample of *R. turkestanicum* extract (1000 µg/ml) were shown in Figure 5A and 5B. Using the calibration curve, the quantification of rutin in *R. turkestanicum* extract was achieved and was about 0.11% ± 0.01 w/w.

Discussion

To date, the neuroprotective effects of *R. turkestanicum* have not been investigated in any models of neurodegenerative disorders. Therefore, in our study, the protective effects of *R. turkestanicum* against glutamate toxicity were investigated for the first time in PC12 and N2a cells. These cell lines were selected, as they are neuronal model systems and show different behaviors in exposure to different stimuli. Considering the higher intracellular level of antioxidant defense factors, especially glutathione, N2a cells, in comparison with PC12 cells, are less vulnerable to acetylcholinesterase-amyloid β peptide (Aβ) complexes, glutamate, Aβ$_{25-35}$ fragments, and H$_2$O$_2$ [6]. Glutamate, which is described as a major excitatory amino acid neurotransmitter in the brain, is involved in synaptic transmission and plasticity, related to cognitive processes [5]. However, excessive accumulation of glutamate causes neuronal apoptotic death both *in vivo* and *in vitro* [22]. The protective effects of pretreatment with *R. turkestanicum* before glutamate insult were investigated in our study. Following cell treatment with glutamate (8 mM), cell viability significantly decreased, while apoptotic cell count increased, suggesting its neurotoxic activities. Also, glutamate increased oxidative stress by improving ROS and MDA production. The results were consistent with the findings observed in other studies [24]. The compounds with potential antioxidant activity have been suggested to inhibit oxidative stress and resultant toxicity of many chemicals [3,4]. On the other hand, *R. turkestanicum* pretreatment improved the viability of glutamate-exposed cells and reduced the apoptotic cell count concentration accordingly. Also, it decreased glutamate-induced toxicity by reduction of ROS and MDA. Therefore, *R. turkestanicum* can be a potential contributor to neurodegenerative disorders. Rutin as a polyphenolic flavonoid shows antioxidant activities [18]. Fruits and some foods contain rutin [28]. Studies have reported the antibacterial, anti-inflammatory, antiulcer, antidiarrheal, immune modulating, hepatoprotective, and vasodilator effects of fruits [18]. Different *in vitro* and *in vivo* studies have used rutin as an antioxidant. This study showed that rutin increased cell viability and decreased ROS generation following glutamate insult. The extract functioned similarly to rutin, especially at 100 and 200 µg/ml [16,17]. Herbal plants and medicines are commonly used in traditional medicine and are growing in popularity as natural alternatives [29]. Today, different studies have shown that natural antioxidant compounds may be suitable drugs for degenerative disorders (such as Parkinson's and Alzheimer's diseases).
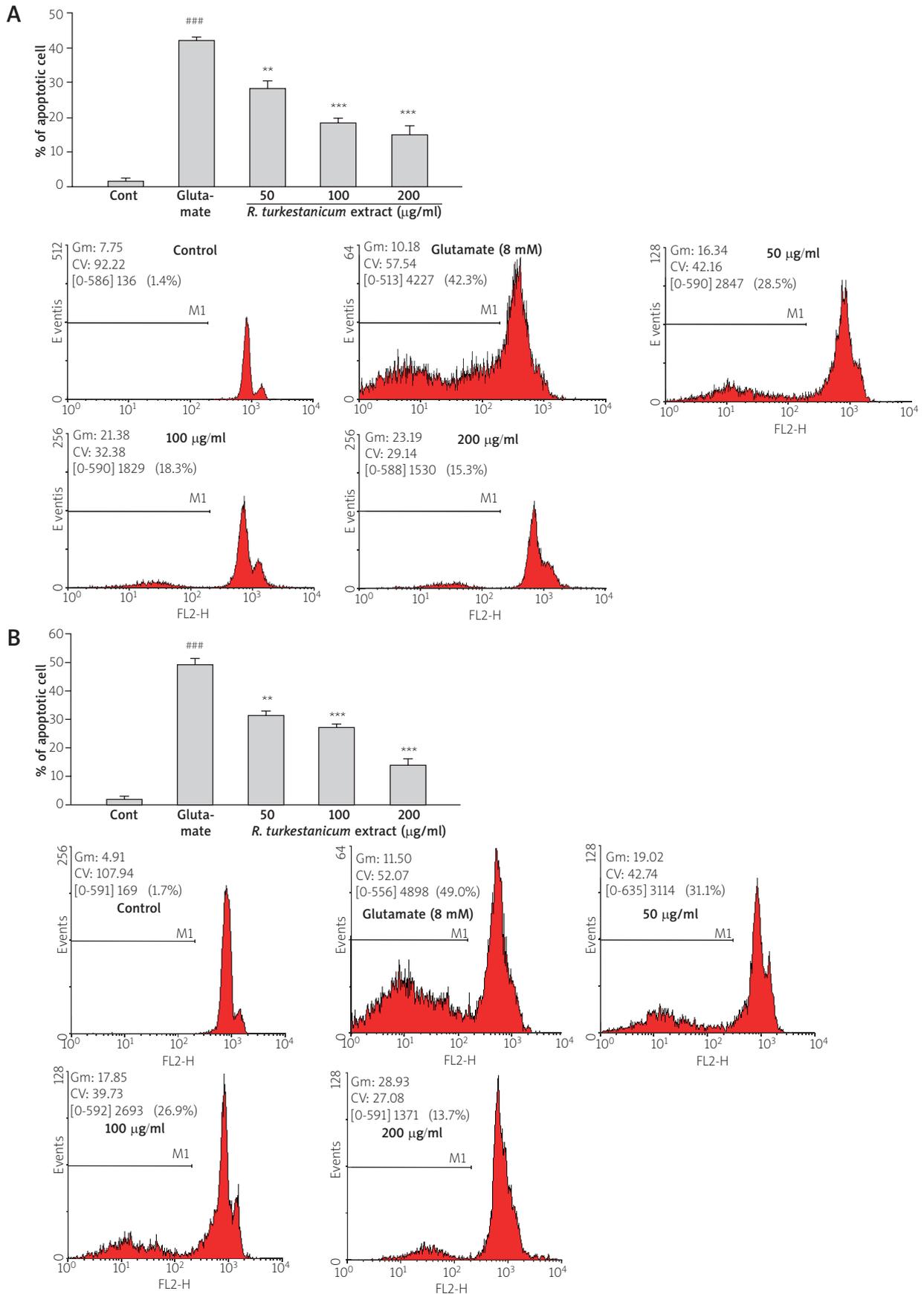
Fig. 4. The effects of the extract of *R. turkestanicum* on apoptosis in PC12 **(A)** and N2a **(B)** cells using PI staining and flow cytometry. The cells were pretreated with different concentrations of the extract for 2 h, then exposed to glutamate (8 mM) for 24 h. Results are mean ± SEM (n = 3). ###p < 0.001 versus control, **p < 0.01 and ***p < 0.001 versus glutamate.

Arezoo Rajabian, Hamid-Reza Sadeghnia, Maliheh Moradzadeh, Azar Hosseini

A

B
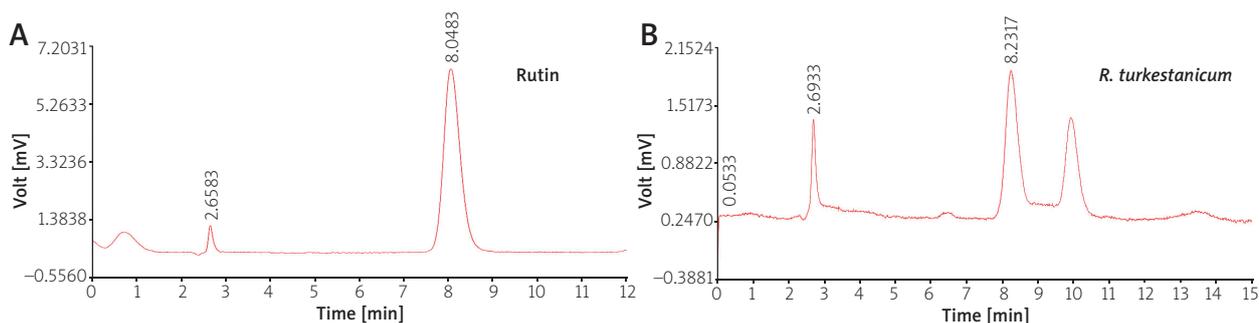
Fig. 5. HPLC fingerprints of the hydro-alcoholic extract of *R. turkestanicum*.

Since oxidative stress is involved in the pathogenesis of neurodegenerative diseases, natural antioxidants may open new doors against these diseases [38]. *Rheum* species mainly consist of dianthrones, glycosides, anthraquinones, and tannins. Rhein, emodin, chrysophanol, alizarin, aloe-emodin, physcion, and citreorosein are the anthraquinone derivatives [12]. According to recent studies, anthraquinone glycosides and double anthrone glycoside, chrysophanol, emodin, aloe-emodin, physcion, and rhein have potentially neuroprotective functions against cerebral ischemic injury [3,9,10,29,30,38].

Chrysophanol can reduce TNF-α in the mouse brain and ameliorate cerebral ischemia/reperfusion injury [37]. Chrysophanol liposome shows beneficial effects on the neurobehavioral score and hippocampal pathological damage in ischemic mice [3]. Emodin can reduce the inflammatory cascade, increase TGF-β level [20], and inhibit activation of caspase-3 in the cerebral ischemic model [32]. Aloe-emodin can provide neuroprotection against cerebral ischemic injury by reduction of TNF-α level [3,9]. Physcion can enhance ischemic tolerance, induced by brain ischemic preconditioning in focal cerebral ischemia of rats [36]. Rhein exerts neuroprotective effects through reduction of nitric oxide (NO) and TNF-α level in ischemic brain tissues of mice [33]. Clinical application of rhubarb (*R. rhabarbarum*) root and rhizome for stroke dates back to at least 2000 years ago. Rhubarb is still widely used in China and other parts of the world. The neuroprotective effects of active compounds of rhubarb root and rhizome on experimental ischemic stroke have been reported [19]. Also, emodin-8-O-β-D-glucoside, isolated from *Polygonum cuspidatum* (*Polygonaceae* family), can have neuroprotective effects on glutamate-induced neuronal damage and cerebral ischemia-reperfusion injury due to its antioxidative effects and inhibition of glutamate neurotoxicity [34]. According to the findings, the protective effects *of R. turkestanicum* may be mediated by its active compounds. More studies are needed to understand its protective mechanisms.

Disclosure

The authors report no conflict of interest.

References
1. Ashok PK, Saini B. HPLC Analysis and Isolation of Rutin from Stem Bark of Ginkgo biloba L. J Pharmacog Phytochem 2013; 2: 68-71.
2. Bleich S, Romer K, Wiltfang J, Kornhuber J. Glutamate and the glutamate receptor system: a target for drug action. Int J Geriatr Psychiatry 2003; 18: S33-40.
3. Boroushaki MT, Asadpour E, Sadeghnia HR, Dolati K. Effect of pomegranate seed oil against gentamicin-induced nephrotoxicity in rat. J Food Sci Technol 2014; 51: 3510-3514.
4. Bouroshaki MT, Sadeghnia HR, Banihasan M, Yavari S. Protective effect of pomegranate seed oil on hexachlorobutadiene-induced nephrotoxicity in rat kidneys. Ren Fail 2010; 32: 612-617.
5. Burnett EJ, Chandler LJ, Trantham-Davidson H. Glutamatergic plasticity and alcohol dependence-induced alterations in reward, affect and cognition. Prog Neuropsychopharmacol Biol Psychiatry 2016; 65: 309-320.
6. Calderon FH, Bonnefont A, Munoz FJ, Fernández V, Videla LA, Inestrosa NC. PC12 and neuro 2a cells have different susceptibilities to acetylcholinesterase-amyloid complexes, amyloid 25-35 fragment, glutamate, and hydrogen peroxide. J Neurosci Res 1999; 56: 620-631.
7. Cassano T, Pace L, Bedse G, Lavecchia AM, De Marco F, Gaetani S, Serviddio G. Glutamate and mitochondria: Two prominent players in the oxidative stress-induced neurodegeneration. Curr Alzheimer Res 2016; 13: 185-197.
8. Chai Yy, Wang F, Li Yl, Liu K, Xu H. Antioxidant Activities of Stilbenoids from Rheum emodi Wall. Evid Based Complement Alternat Med 2012; 2012: 603678.
9. Chen LY, Su LK, Liao RH, Wang J. Active Compounds of Rhubarb root and rhizome in animal model experiments of focal cerebral ischemia. Med Recapitulate 2015; 2015: 210546.

10. Chen LY, Wang, HJ, Tao XX. Systems pharmacology for traditional Chinese medicine with application to cardio-cerebrovascular diseases. Chinese J Integ Med Cardio/Cerebrovascular Dis 2014; 2: 84-91.

11. Dong W, Jian F, Jiansheng L, Weiyu Z, Jingxia L. Shengwang L. The Geriatric Institute of Henan College of TCM; A Comparison study on treating cerebral ischemia of rats with emodin and Aloe-emodin. J Tradit Chin Med 2005; 5.

12. Dorsey J F, Kao GD. Aloe (-emodin) for cancer? More than just a comforting salve. Cancer Biol Ther 2007; 6: 89-90.

13. Hosseini A, Mahdian D. Protective effect of *Lactuca serriola* on doxorubicin-induced toxicity in H9c2 cells. Acta Pol Pharm 2016; 73: 659-666.

14. Hosseini A, Sadeghnia HR, Rajabian A. Protective effects of peel and seed extracts of Citrus aurantium on glutamate-induced cytotoxicity in PC12 cell line. Folia Neuropathol 2016; 54: 262-272.

15. Hosseini A, Shafiee-Nick R, Mousavi SH. Combination of *Nigella sativa* with *Glycyrrhiza glabra* and *Zingiber officinale* augments their protective effects on doxorubicin-induced toxicity in h9c2 cells. Iran J Basic Med Sci 2014; 17: 993-1000.

16. Jakobek L, Seruga M, Seruga B, Novak I, Medvidovic-Kosanovic M. Phenolic compound composition and antioxidant activity of fruits of Rubus and Prunus species from Croatia. Int J Food Sci Technol 2009; 44: 860-868.

17. Jo S, Ka E, Lee H, Apostolidis E, Jang H, Kwon Y. Comparison of Antioxidant Potential and Rat intestinal a-Glucosidases inhibitory Activities of Quercetin, Rutin, and Isoquercetin. Int J Applied Res Nat Prod 2009; 4: 52-60.

18. Kamalakkannan N, Prince PSM. Antihyperglycaemic and antioxidant effect of rutin, a polyphenolic flavonoid, in streptozotocin-induced diabetic wistar rats. Basic Clin Pharmacol Toxicol 2006; 98: 97-103.

19. Liu JX, Li JS, Liang SW, Gao JF, Zhao JM, Zhang WH. Protective effects of rhubarb aglycone and its monomers on rats with cerebral ischemia. J Chinese Med 2004; 19: 23-25.

20. Lu L, Li HQ, Fu DL, Zheng GQ, Fan JP. Rhubarb root and rhizome-based Chinese herbal prescriptions for acute ischemic stroke: a systematic review and meta-analysis. Complement Ther Med 2014; 22: 1060-1070.

21. Mao QQ, Zhong XM, Feng CR, Pan AJ, Li ZY, Huang Z. Protective effects of paeoniflorin against glutamate-induced neurotoxicity in PC12 cells via antioxidant mechanisms and $Ca^{(2+)}$ antagonism. Cell Mol Neurobiol 2010; 30: 1059-1066.

22. Nishizawa Y. Glutamate release and neuronal damage in ischemia. Life Sci 2001; 69: 369-381.

23. Olatunji OJ, Feng Y, Olatunji OO, Tang J, Wei Y, Ouyang Z, Su Z. Polysaccharides purified from *Cordyceps cicadae* protects PC12 cells against glutamate-induced oxidative damage. Carbohydr Polym 2016; 153: 187-195.

24. Rajabian A, Boroushaki MT, Hayatdavoudi P, Sadeghnia HR. *Boswellia serrata* protects against glutamate-induced oxidative stress and apoptosis in PC12 and N2a cells. DNA Cell Biol 2016; 35: 666-679.

25. Ramassamy C. Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets. Eur J Pharmacol 2006; 545: 51-64.

26. Rao A, Balachandran B. Role of oxidative stress and antioxidants in neurodegenerative diseases. Nutr Neurosci 2002; 5: 291-309.

27. Salim S. Oxidative stress and the central nervous system. J Pharmacol Exp Ther 2017; 360: 201-205.

28. Sánchez-Salcedo EN, Mena P, García-Viguera C, Martínez JJ, Hernández F. Phytochemical evaluation of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry fruits, a starting point for the assessment of their beneficial properties. J Functional Foods 2015; 12: 399-408.

29. Shoeb M. Anti-cancer agents from medicinal plants. Bangladesh J Pharmacol 2006; 1: 35-41.

30. Singh RP, Sharad S, Kapur S. Free radicals and oxidative stress in neurodegenerative diseases: Relevance of dietary antioxidants. J Indian Acad Clin Med 2004; 5: 218-225.

31. Song JY, Zhang L, Zhao XQ, Song ZB. Effects of chrysophanol liposomes on apoptosis of the hippocampal neurons in mice after cerebral ischemia and reperfusion injury. Acta Neuropharmacol 2011; 1: 7-13.

32. Tan L, Wang LY, Xiang HY, Li M. Study on the protective effects and mechanisms of emodin on cerebral ischemia/reperfusion injury in rats. Chinese J Integ Med Cardio/Cerebrovascular Dis 2010; 8: 1100-1101.

33. Wang XR, Hou Y, Xue GP. Influence of rhein on NO in cerebrum of cerebral ischemia reperfusion mice. J Hebei North Univ 2012; 28: 73-75.

34. Wang C, Zhang D, Ma H, Liu J. Neuroprotective effects of emodin-8-O-β-D-glucoside in vivo and in vitro. Eur J Pharmacol 2017; 577: 58-63.

35. Zhang R, Kang KA, Piao MJ, Lee KH, Jang HS, Park MJ, Kim BJ, Kim JS, Kim YS, Ryu SY, Hyun JW. Rhapontigenin from *Rheum undulatum* protects against oxidative-stress-induced cell damage through antioxidant activity. J Toxicol Environ Health Part A 2007; 70: 1155-1166.

36. Zhang P, Su LK, Li HM, Zhao YC, Yang ZQ, Cui XY. Protective effects of physcion against cerebral injury induced by ischemia-reperfusion in rats. Chin J Pathophysiol 2005; 21: 1829-1833.

37. Zhang N, Zhang X, Liu X, Wang H, Xue J, Yu J, Kang N, Wang X. Chrysophanol inhibits NALP3 inflammasome activation and ameliorates cerebral ischemia/reperfusion in mice. Med Inflam 2014; 2014: 12.

38. Zhao B. Natural antioxidants protect neurons in Alzheimer's disease and Parkinson's disease. Neurochem Res 2009; 34: 630-638.