

## *Rheum turkestanicum* reduces glutamate toxicity in PC12 and N2a cell lines

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### 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  $\mu$ M reduced ROS production and protected against glutamate toxicity. Also the quantification of rutin in *R. turkestanicum* extract was achieved and was about 0.11%  $\pm$  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 oxida-

tive 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-

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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<sub>2</sub>O<sub>2</sub>), 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<sub>2</sub>O<sub>2</sub> [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-dichlorofluorescein 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 Univer-

sity 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<sub>2</sub>; 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<sub>2</sub>DCF-DA, which was ROS-oxidized and converted to a highly fluorescent compound (DCF),

was used to determine the intracellular level of ROS. H<sub>2</sub>DCF-DA (200 μM, 10 μl) was added to each well before treatment and incubated in darkness at 37°C 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 4°C 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<sup>4</sup> 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).

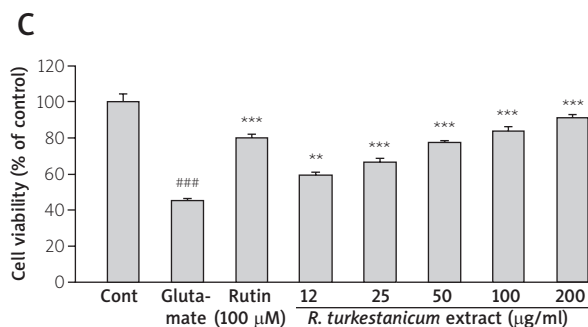
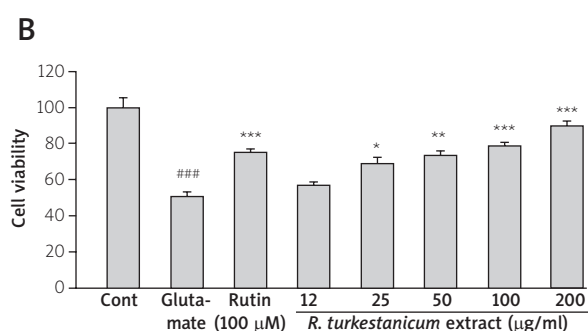
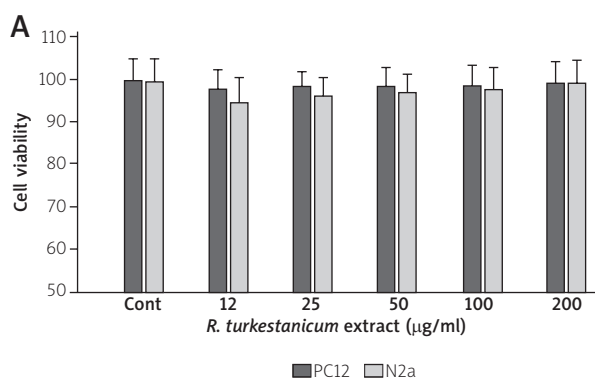
### Data analysis

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.

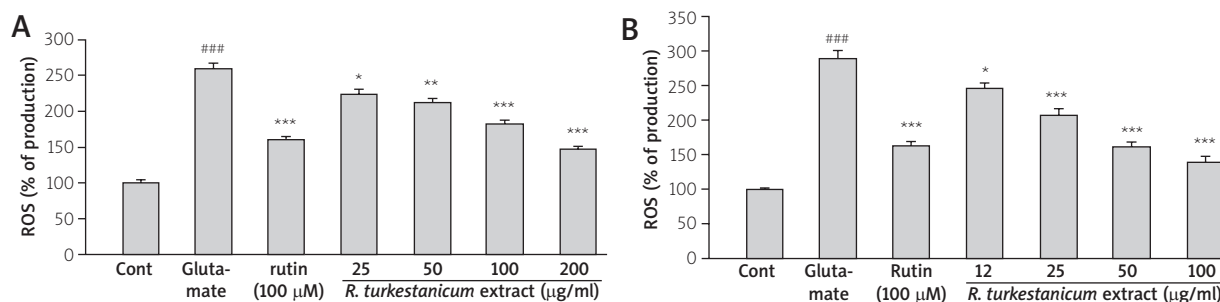
### Results

#### *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 μg/ml, 69 ± 2%, p < 0.05; 50 μg/ml, 73.1 ± 1.8%, p < 0.01; 100 μg/ml, 79.6 ± 2.8%, p < 0.001; 200 μg/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 μg/ml (12 μg/ml, 89.4 ± 1.4%, p < 0.01, 25 μg/ml, 67.2 ± 1.9%, p < 0.001; 50 μg/ml, 77 ± 1%, p < 0.001; 100 μg/ml, 84.1 ± 2.4%, p < 0.001; 200 μg/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 μg/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 μg/ml (245.9 ± 7.8%, p < 0.05), 25 μg/ml (206 ± 9.4%, p < 0.001), 50 μg/ml (161 ± 5.8%, p < 0.001) and 200 μg/ml (138 ± 8.7%, p < 0.001) against glutamate (288 ± 10.5%, p < 0.001). Also rutin

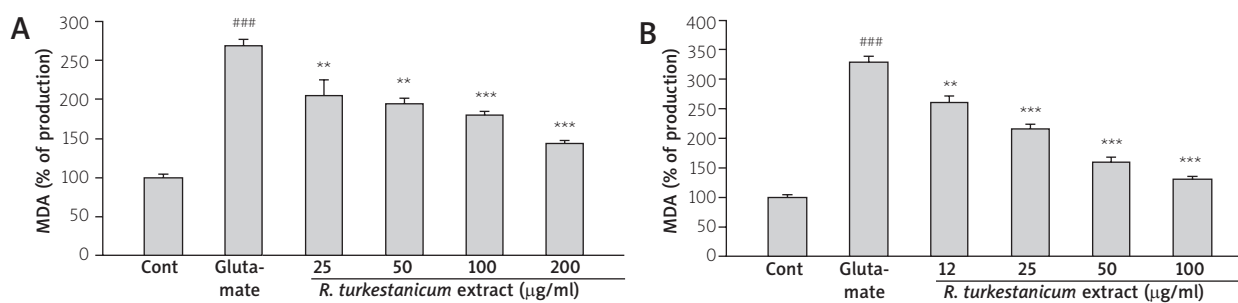
reduced ROS content in N2a cells (100 μM, 163 ± 5%, p < 0.001) (Fig. 2B).

***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 μg/ml (204 ± 11.3%, p < 0.01), 50 μg/ml (194.10 ± 7.18%, p < 0.01), 100 μg/ml (179 ± 4.7%, p < 0.001) and 200 μg/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 μg/ml (259.3 ± 10.3%, p < 0.01), 25 μg/ml (215 ± 9%, p < 0.001), 50 μg/ml (158 ± 8.4%, p < 0.001) and 100 μg/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 μg/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 μg/ml, 28.5 ± 2.1%, p < 0.01; 100 μg/ml, 18.3 ± 1.5%, p < 0.001; 200 μg/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  $\pm$  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  $\mu\text{g/ml}$ ,  $31 \pm 1.8\%$ ,  $p < 0.01$ ; 100  $\mu\text{g/ml}$ ,  $26.8 \pm 1.2\%$ ,  $p < 0.001$ ; 200  $\mu\text{g/ml}$ ,  $13.7 \pm 2.3\%$ ,  $p < 0.001$ ) versus glutamate ( $49 \pm 2\%$ ,  $p < 0.001$ ) (Fig. 4B).

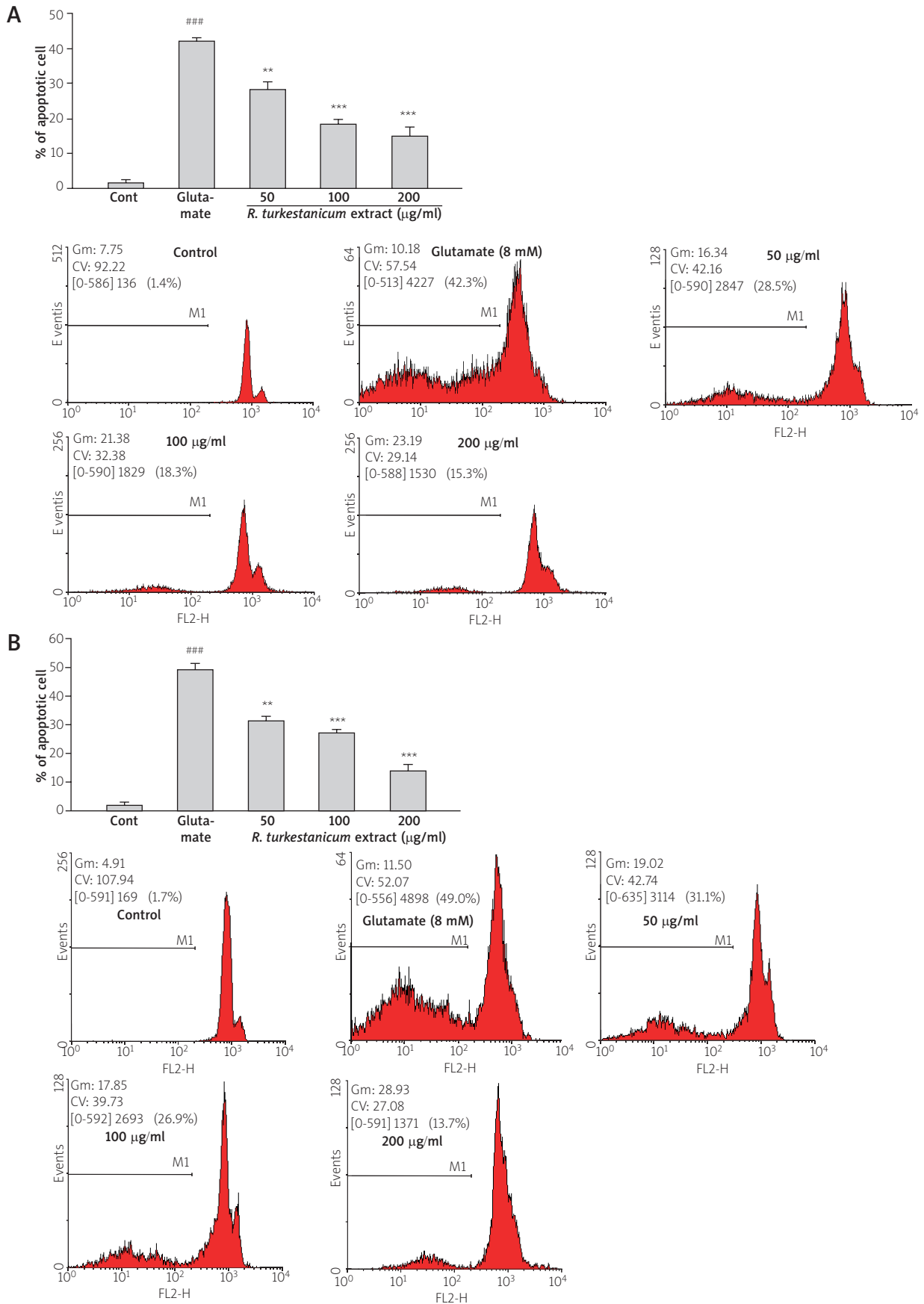
### HPLC characterization of *R. turkestanicum* extract

The chromatograms of standard rutin (10  $\mu\text{g/ml}$ ) and a sample of *R. turkestanicum* extract (1000  $\mu\text{g/ml}$ ) were shown in Figure 5A and 5B. Using the calibration curve, the quantification of rutin in *R. turkestanicum* extract was achieved and was about  $0.11\% \pm 0.01$  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  $\beta$  peptide ( $A\beta$ ) complexes, glutamate,  $A\beta_{25-35}$  fragments, and  $\text{H}_2\text{O}_2$  [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  $\mu\text{g/ml}$  [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.

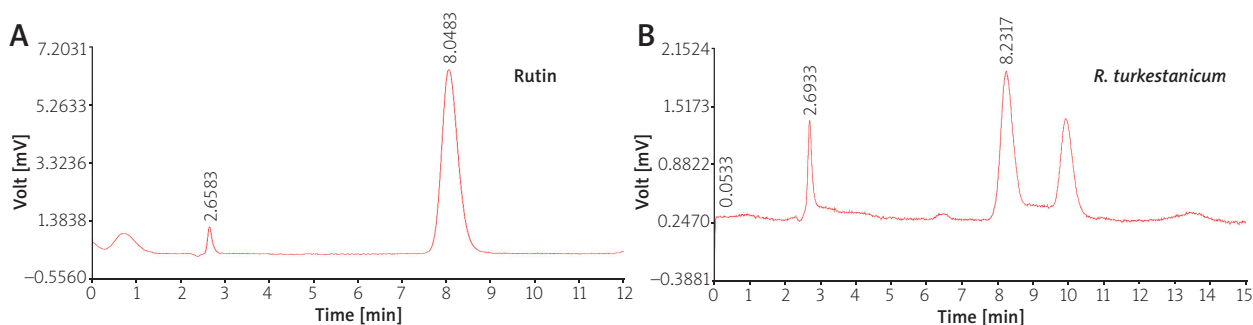


Fig. 5. HPLC fingerprints of the hydro-alcoholic extract of *R. turkestanicum*.

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 dianthrone, 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- $\alpha$  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- $\beta$  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- $\alpha$  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- $\alpha$  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- $\beta$ -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 glu-

tamate 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.

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