

Effects of standardized extract of *Ferula gummosa* root on glutamate-induced neurotoxicity

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Abstract

Glutamate is one of the major excitatory neurotransmitters in the central nervous system. Increasing glutamate leads to neurodegenerative disease. Nowadays, plant medicine plays a role in the treatment of some disorders. In this research, we investigated the neuroprotective effect of *Ferula gummosa* root extract against glutamate-induced oxidative stress in the rat adrenal pheochromocytoma (PC12) and mouse neuroblastoma (N2a) cell lines. The cells were pretreated with extract for 2 h and then exposed to glutamate for 24 h. After 24 h the level of malondialdehyde (MDA), reactive oxygen species (ROS), and apoptotic cells were determined in both cell lines. Glutamate increased lipid peroxidation, ROS, and apoptotic cells in both cell lines. The extract significantly increased the cell viability and decreased the ROS generation under glutamate-induced oxidative stress in these cells. Also, the extract decreased the MDA level and apoptotic cells. The results showed that *Ferula gummosa* root may have a protective effect on glutamate-induced toxicity, suggesting that the extract protects neuronal cells from glutamate-induced oxidative stress.

Key words: PC12, N2a, glutamate, *Ferula gummosa*.

Introduction

Glutamate is released to synaptic spaces as a neurotransmitter in the central nervous system (CNS) and acts via binding to its receptor protein [3,5]. Increasing the glutamate concentration in the brain can lead to a number of neurodegenerative diseases such as stroke, epilepsy, depression, and Huntington's disease [18,28]. *In vitro* studies have shown that high concentrations of glutamate act as a potent neurotoxin that causes the death of neurons by apoptosis [9,10]. Different mechanisms can play role in glutamate

toxicity in neuronal cells. Some of the mechanisms include activation of calcium-dependent enzymes [1], nitric oxide synthase [6], and the production of reactive oxygen species [39]. Recent studies have shown that antioxidant compounds, such as vitamin E [35], curcumin, and epicatechin gallate, protect primary cultured neurons from glutamate-induced cell death [41]. However, excessive accumulation of free radicals is responsible, at least in part, for glutamate-induced neuropathologies. Since plant medicinals contain different active ingredients, such as antioxidants, they

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may represent a viable approach by which glutamate toxicity can be reduced [32]. The present study was designed to evaluate the hypothesis that *Ferula gummosa* extract can protect neuronal cells against glutamate-induced toxicity. *F. gummosa* Boiss is from the *Umbelliferae* (*Apiaceae*) family. *F. gummosa* is known as barijeh in Persian. This plant grows in the northern and western parts of Iran. In traditional medicine, the roots of *F. gummosa* was used as a tonic, emmenagogue, anti-diarrhoea treatment, stomach pain medicine, epilepsy therapy, and as a wound healing remedy [8,25]. *F. gummosa* is composed of coumarin derivatives, such as umbelliprenin and terpenoids, and sesquiterpenes such as linalool, terpinolene, and valencene [30]. *F. gummosa* has different pharmacological effects including anticonvulsant [38], anti-nociceptive and anti-inflammatory [30], antioxidant [7], antimicrobial [37], and antiproliferative effects [19,11,14]. The presence of phenols and flavonoids in the extract can be responsible for the observed effects [7]. In this research, the neuroprotective effect of *F. gummosa* was evaluated on PC12 and N2a cell lines.

Material and methods

Reagents and chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), thiobarbituric acid (TBA), 2,7-dichlorofluorescein diacetate (DCFH-DA), propidium iodide (PI), sodium citrate, and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). High-glucose Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and foetal bovine serum were purchased from Gibco. Trichloroacetic acid (TCA) and malondialdehyde bis-(dimethyl acetal) (MDA) were obtained from Merck (Darmstadt, Germany). Rat pheochromocytoma (PC12, Pasteur Institute, C-1553, Tehran, Iran) and mouse neuroblastoma (N2a, ATCC, CCL-131PC12) cells were obtained from the Pasteur Institute (Tehran, Iran).

Preparation of extract

Plant material and extraction

The *F. gummosa* roots were collected from Alibolagh Valley (Khorasan Razavi Province, northeast Iran), and identified by Mohammadreza Joharchi, Ferdowsi University of Mashhad Herbarium (Voucher Specimen No. 4577). The hydroalcoholic extract was prepared using the Soxhlet method. The powder of

F. gummosa roots was subjected to extraction with 70% ethanol in a Soxhlet apparatus for 48 h [12], and the hydroalcoholic solvent was then evaporated by drying the extract in a water bath. The solid residue was stored at -20°C until use.

Standardizing the *Ferula gummosa* extract

The hydroalcoholic extract of *F. gummosa* root was standardized by measuring the level of total phenol using the Folin-Ciocalteu reagent. A sample of *F. gummosa* extract (20 μl of 10 mg/ml) or gallic acid standard solutions was mixed with 100 μl Folin-Ciocalteu reagent and 300 μl sodium carbonate solution (1 M), and the volume was adjusted to 2 ml using deionised water. After two hours of incubation at room temperature, the absorbance was measured at 765 nm by spectrometer, and a standard curve was prepared for gallic acid. The total phenol level was expressed as milligrams of gallic acid equivalents per gram of dry *F. gummosa* extract.

Cell culture and treatment

The cells were maintained at 37°C in a humidified atmosphere that contained 5% CO_2 . The cells were cultured in DMEM supplemented with 10% foetal bovine serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. For the experiments, they were seeded in 96-well and 24-well culture plates for MTT/ROS and MDA assays, respectively. For apoptosis assay, cells were seeded at 100,000 cells/well in a 24-well plate. All treatments were carried out in triplicate. The cells were pretreated with extract alone (25 to 200 $\mu\text{g}/\text{ml}$) for 2 h, and then incubation was continued for 24 h in the presence of the extract with or without 8 mM glutamate.

Cell viability assay

The MTT assay was applied for determination of cell viability [17]. The MTT powder was dissolved in phosphate-buffered saline (5 mg/ml) and added to each well at a final concentration of 0.05%. After 3 h, the formazan precipitate was dissolved in DMSO. The absorbance at 570 and 620 nm (background) was measured using a Stat FAX 303 plate reader.

Lipid peroxidation assay

The MDA was measured as lipid peroxidation index [17]. At the end of incubation, the cells were

scraped and centrifuged for 30 min. Then 400 μ l of TCA (15%) and 800 μ l of TBA (0.7%) were added to 500 μ l of cell samples. The mixture was vortexed and heated for 40 min in a boiling water bath. Then, 200 μ l of the sample was transferred to a 96-well plate, and the fluorescence intensity was read with excitation/emission of 480/530 nm. The experiment was carried out in triplicate.

Measurement of reactive oxygen species

The level of ROS was measured by DCFH-DA. At the end of the incubation, the cells were treated (30 min) with DCFH-DA (10 μ M) at 4°C in the dark. The fluorescence intensity was then detected with excitation/emission of 485/530 nm. The experiment was performed in triplicate.

Propidium iodide staining

Propidium iodide (PI) staining was used for detection of apoptotic cells. It has previously been reported that a sub-G1 peak that is reflective of DNA fragmentation can be observed following the incubation of cells in a hypotonic phosphate-citrate buffer containing a quantitative DNA-binding dye such as PI. Apoptotic cells that have lost DNA will take up less stain and appear on the left side of the G1 peak in the histogram. Briefly, the cells were seeded in wells of a 24-well plate and treated according to the mentioned protocol. Floating and adherent cells were then harvested and incubated at 4°C overnight in the dark with 750 μ l of a hypotonic buffer (50 μ g/ml PI in 0.1% sodium citrate with 0.1% Triton X-100). Next, flow cytometry was conducted using a FACScan flow cytometer (Becton Dickinson). A total of 104 events were acquired with FACS.

Statistics

All data were expressed as mean \pm SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tamhane's T2 post-hoc test. Differences were considered significant at $p < 0.05$.

Results

Total phenol content of *Ferula gummosa* extract

The solid residue of Soxhlet hydroalcoholic extract of *F. gummosa* was 19%. Concentration of total phe-

nol was 24 mg gallic acid equivalent per gram of dry *F. gummosa* extract.

Ferula gummosa significantly decreased the cell deaths induced by glutamate

To study the possible toxic effects of extracts PC12 and N2a cells were incubated with different concentrations of extract (6-200 μ g/ml), and the viability was determined 24 h after treatment. No significant toxic effect on cell viability was seen subsequent to treatment with extract. Incubation with glutamate significantly decreased cell viability to $51 \pm 0.54\%$ of control ($p < 0.001$). Results showed that treatment of PC12 cells with the extract increased cell viability at concentrations of 12-200 μ g/ml (12 μ g/ml, $68 \pm 1.48\%$, $p < 0.05$, 25 μ g/ml, $72 \pm 2.58\%$, $p < 0.01$; 50 μ g/ml, $78 \pm 2.8\%$, $p < 0.001$; 100 μ g/ml, $82 \pm 4.38\%$, $p < 0.001$; 200 μ g/ml, $87 \pm 2.65\%$, $p < 0.001$), while glutamate decreased cell viability to $50 \pm 2.6\%$, $p < 0.01$ (Fig. 1A). As shown in Figure 1B, treatment of N2a cells with extract increased cell viability subsequent to glutamate insult at concentrations of 25-200 μ g/ml dose dependently (25 μ g/ml, $65 \pm 2.1\%$, $p < 0.05$; 50 μ g/ml, $72 \pm 1.9\%$, $p < 0.001$; 100 μ g/ml, $80 \pm 2.2\%$, $p < 0.001$; 200 μ g/ml, $86 \pm 2.5\%$, $p < 0.001$).

Ferula gummosa significantly decreased ROS content induced by glutamate

As expected, glutamate caused a significant increase in the level of ROS in PC12 cells ($260 \pm 7.8\%$ of control, $p < 0.001$). Extract at concentrations of 25-200 μ g/ml was able to decrease intracellular ROS levels to $227 \pm 6.1\%$ ($p < 0.05$), $211 \pm 7.14\%$ ($p < 0.01$), $187 \pm 6.2\%$ ($p < 0.001$), and $172 \pm 5.7\%$ ($p < 0.001$) of control, respectively (Fig. 2A). Also, the extract decreased ROS levels in N2a cells at concentrations of 50 μ g/ml ($168 \pm 4.5\%$, $p < 0.05$), 100 μ g/ml ($151 \pm 9.1\%$, $p < 0.01$), and 200 μ g/ml ($133 \pm 4.8\%$, $p < 0.001$) against glutamate ($210.7 \pm 11\%$, $p < 0.001$) (Fig. 2B).

Ferula gummosa significantly decreased lipid peroxidation induced by glutamate

The level of lipid peroxidation was evaluated by measuring the level of MDA, which is the end product of lipid peroxidation. As shown in Figure 3, expo-

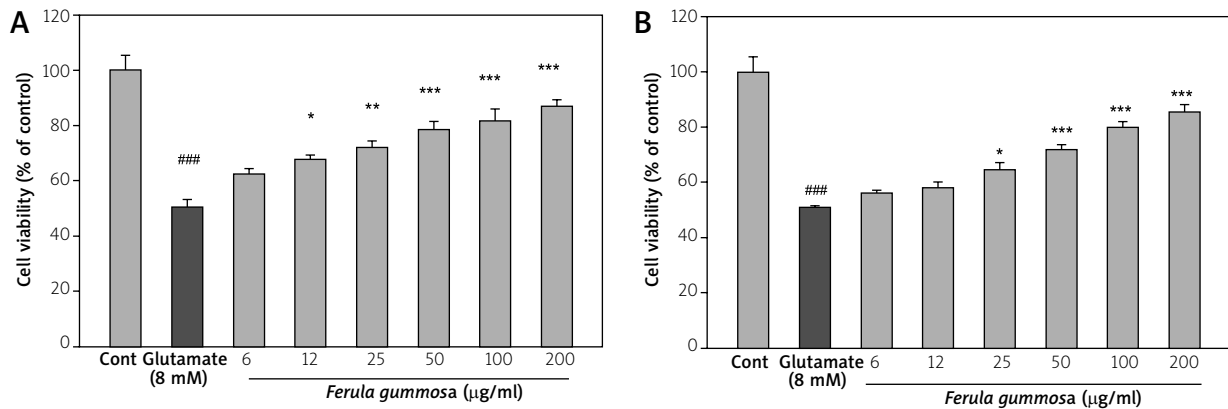


Fig. 1. Effect of *Ferula gummosa* on cell viability of glutamate-injured PC12 (A) and N2a (B) cells. The cells were pretreated with different concentrations of the extract for 2 h and then exposed to glutamate (8 mM) for 24 h. The cell viability was quantitated by MTT assay. Results are mean \pm SEM ($n = 3$). ### $p < 0.001$ versus control, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus glutamate.

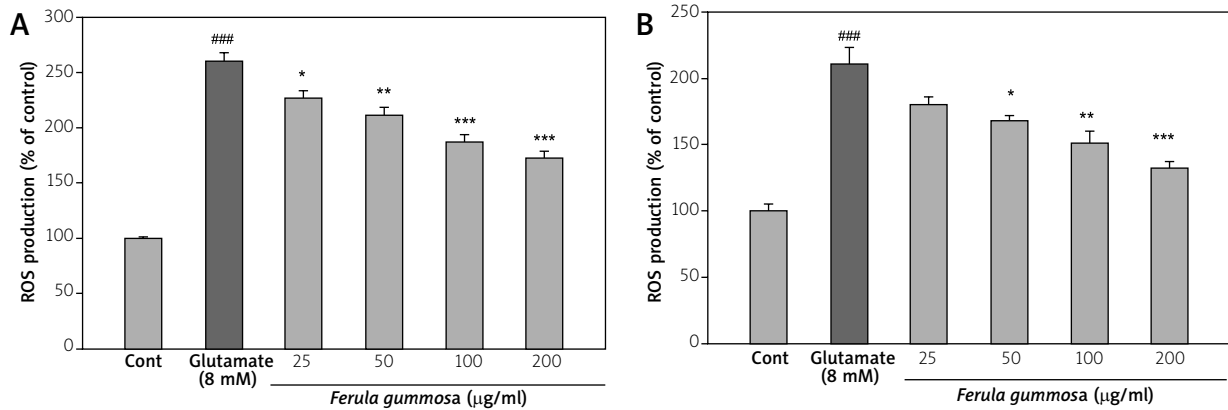


Fig. 2. Effect of *Ferula gummosa* on ROS generation in glutamate-injured PC12 (A) and N2a (B) cells. Results are mean \pm SEM ($n = 3$). ### $p < 0.001$ versus control, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus glutamate.

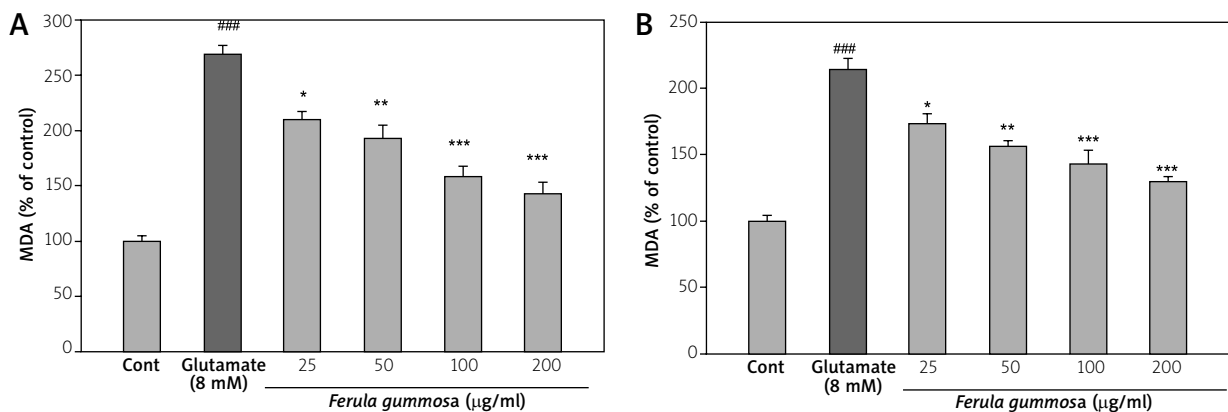


Fig. 3. Effect of *Ferula gummosa* on MDA production in glutamate-injured PC12 (A) and N2a (B) cells. The cells were pretreated with different concentrations of the extract for 2 h then exposed to glutamate (8 mM) for 24 h. Results are mean \pm SEM ($n = 3$). ### $p < 0.001$ versus control, * $p < 0.05$ and *** $p < 0.001$ versus glutamate.

sure of the cells to glutamate resulted in a significant increase of MDA level in PC12 cells ($269 \pm 7.5\%$, $p < 0.001$) as compared to control cells cultured in the absence of glutamate ($100 \pm 4.5\%$). The content of MDA was significantly decreased in the cells pretreated with 25 $\mu\text{g/ml}$ ($209 \pm 7.6\%$, $p < 0.05$), 50 $\mu\text{g/ml}$ ($193 \pm 11.6\%$, $p < 0.01$), 100 $\mu\text{g/ml}$ ($158 \pm 9.7\%$, $p < 0.001$), and 200 $\mu\text{g/ml}$ ($142 \pm 10.9\%$, $p < 0.001$) of extract (Fig. 3A). The extract reduced the level of MDA at 25 $\mu\text{g/ml}$ ($173 \pm 7\%$, $p < 0.05$), 50 $\mu\text{g/ml}$ ($156 \pm$

4.5%, $p < 0.01$), 100 $\mu\text{g/ml}$ ($143 \pm 10\%$, $p < 0.001$), and 200 $\mu\text{g/ml}$ ($130 \pm 3.7\%$, $p < 0.001$) against glutamate ($214 \pm 8.3\%$, $p < 0.001$) (Fig. 3B).

Ferula gummosa 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

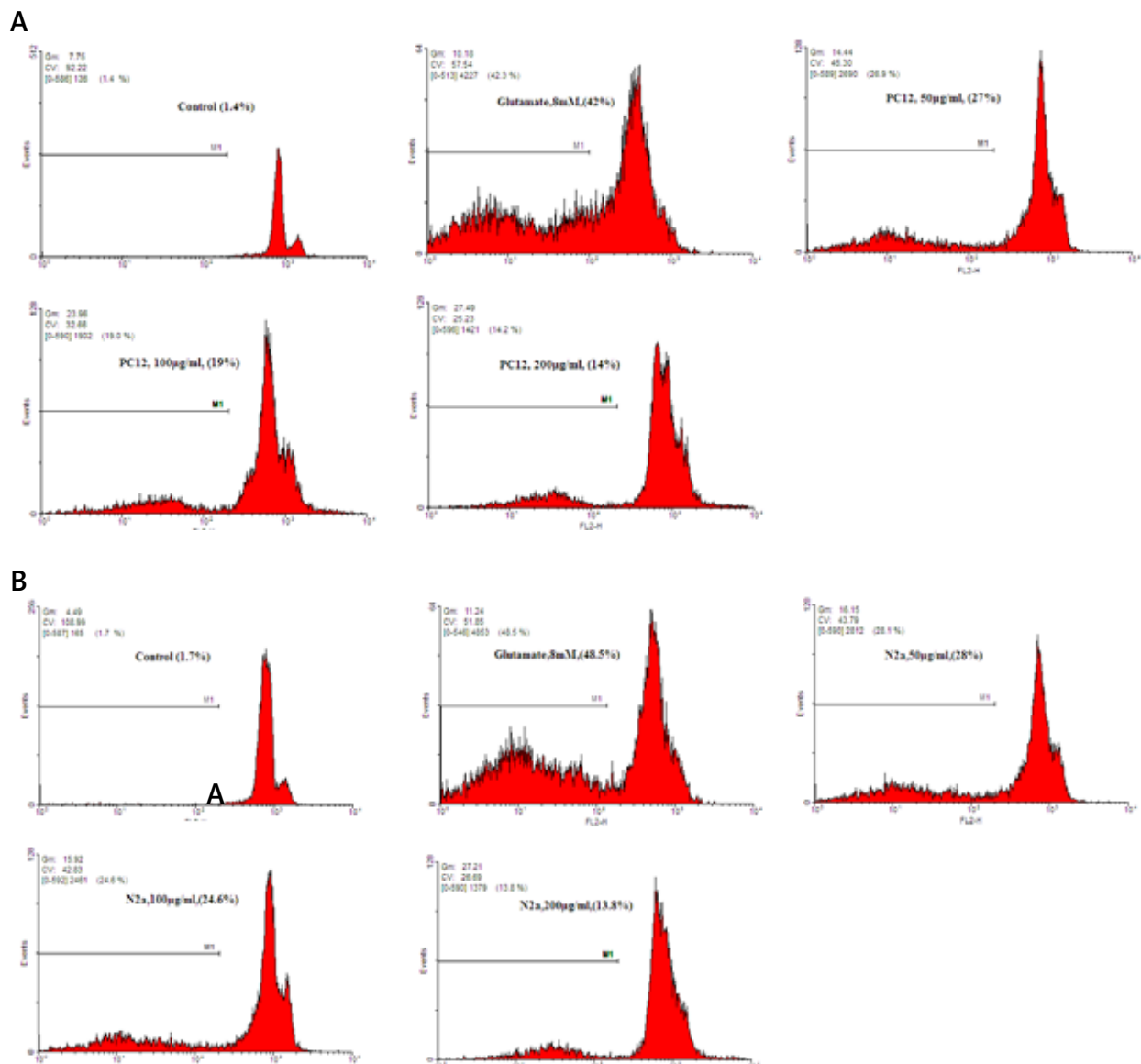


Fig. 4. The effects of extract of *Ferula gummosa* on apoptosis in PC12 (A) and N2a (B) cells using PI staining and flow cytometry. The cells were pretreated with different concentrations of the extract for 2 h then exposed to glutamate (8 mM) for 24 h.

the *F. gummosa* and exposed to glutamate for 24 h. Analysis of the sub G1 peak in flow cytometry histograms revealed the induction of apoptosis in cells treated with glutamate ($p < 0.001$).

Discussion

This study showed, for the first time, that *F. gummosa* has significant neuroprotective effects against glutamate-induced cytotoxicity in PC12 and N2a cells in a dose-dependent manner. Whereas, dopamine and glutamate are synthesised in PC12 cell line [13]; however, this cell line can be appropriate for some studies, such as function of neurons, neuronal differentiation, and neurotoxicity [25]. PC12 cells are sensitive to glutamate toxicity; therefore, they can be used as a suitable model for investigating whether an extract has protective effect against glutamate-induced cytotoxicity. Increasing of glutamate can lead to neuronal death in cerebral ischaemia [23] via glutamate receptor-mediated [21] and oxidative stress-mediated [4,15]. Recent studies have shown that glutamate exerts its toxic effects on PC12 in a dose- and time-dependent manner. Its toxic concentration varies between 0.01 and 10 mM [29,34], while incubation time varies from 30 min to 3-12-24-48 h. The cytotoxicity effect of glutamate at very high doses [33] (5-10 mM) is independent of receptors and is mediated through the depletion of GSH and oxidative glutamate toxicity [25]. One way to reduce glutamate toxicity is to use antioxidant compounds such as medicinal plants. Recent studies have shown some herbal medicines have protective effects against glutamate such as peel and seed extract of *Citrus aurantium* [16], *Persea major* [8], *Uncaria sinensis* [20], *Scrophularia striata* Boiss [36], and *Withania somnifera* [22]. In this research, we used *Ferula gummosa* extract against glutamate toxicity in two cell lines: PC12 and N2a. The results revealed that the extract reduced ROS, lipid peroxidation, and apoptotic cells in treated cells in comparison with the glutamate group. There are different compounds in *F. gummosa* such as coumarin derivatives (umbelliprenin and terpenoids) and sesquiterpenes (linalool, terpinolene, and valencene) [30]. Polyphenols are found in vegetables, fruits, grains, bark, roots, and tea [26]. These compounds are important antioxidants [40] that have a neuroprotection effect against glutamate-induced excitotoxicity and ischaemia-induced neurodegeneration [27,31]. The studies have shown that linalool and eugenol,

which are found in *F. gummosa*, have neuroprotective effects [2,24]. Eugenol is an agonist on γ -aminobutyric acid [23] (GABA) and an antagonist on NMDA (N-methyl-D-aspartate) glutamate receptors, which play an important role in pain transmission [2]. Also, linalool inhibits glutamate release *in vitro* and *in vivo* [24]. However, the results observed in this study may be related to the active compounds of *F. gummosa*; more studies are required to understand the accuracy mechanism in more detail.

Disclosure

Authors report no conflict of interest.

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