

Protective effects of peel and seed extracts of *Citrus aurantium* on glutamate-induced cytotoxicity in PC12 cell line

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Abstract

Oxidative stress and apoptosis contribute to neuronal degeneration in many neurodegenerative diseases such as Alzheimer's disease. Glutamate is a major excitatory neurotransmitter in the central nervous system (CNS) and is considered responsible for the pathogenesis of many neurological disorders. Reactive oxygen species (ROS) production is thought to be involved in glutamate-induced apoptosis process. In this study, the neuroprotective effects of *Citrus aurantium* in the glutamate-induced rat's adrenal pheochromocytoma cell line (PC12 cells) were investigated. The cell viability and apoptotic cell death were measured using MTT and propidium iodide (PI)-staining methods, respectively. In addition, intracellular ROS and malondialdehyde (MDA) levels were determined by fluorometric methods. The results showed that glutamate cytotoxicity in PC12 cells was accompanied by an increment of MDA content, ROS generation, and apoptotic induction. However, pretreatment with peel and seed extracts of *C. aurantium* significantly reduced MDA content, ROS generation, and apoptotic cells. All these findings indicated that *C. aurantium* protected PC12 cells against glutamate-induced apoptosis by inhibiting ROS production. Therefore, the present study supports that *C. aurantium* extracts possess neuroprotective effects against glutamate-induced toxicity in PC12 cell line. The protective effect of *C. aurantium* might be attributed to its antioxidant properties.

Key words: apoptosis, *Citrus aurantium*, glutamate cytotoxicity, neuroprotection, PC12.

Introduction

Glutamate is the most excitatory neurotransmitter in the brain. It is an important neurotransmitter for learning and memory in the central nervous system [32]. Excessive amounts of glutamate may act as a potent neurotoxin by activating the proteolytic enzymes [39]. Naturally, glutamate is released into the synaptic cleft and binds to glutamate receptors resulting in the propagation of an action potential

[22]. However, increased amounts of glutamate in the synaptic cleft can lead to neurotoxicity. It has been reported that glutamate increases in patients who suffer from Alzheimer's disease [11]. PC12 cell line is derived from a pheochromocytoma of rat adrenal medulla. This cell model is also widely applied to study cellular glutamate toxicity [38]. Therefore, it is an appropriate model to investigate the protective effect of the compounds on glutamate toxicity. *Citrus aurantium* L. (*Rutaceae*), also called

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Bitter orange, has been used as a traditional medicine for more than 5,000 years [5]. Citrus fruits and their products are rich sources of health-promoting constituents and are widely consumed around the world [5]. They increase serum antioxidant capacity against lipid peroxidation [3] and reduce the elderly oxidative stress. Citrus flavonoids have been shown to prevent oxidative stress, attenuate inflammation and exhibit anti-tumor [31,33] and anti-atherosclerosis properties [27]. In addition, citrus fruits serve as a supplement of chemotherapy [26], diabetic health food [2], and neuroprotection [15]. Furthermore, the aqueous extract from the leaves of the *C. aurantium* have anticonvulsant properties [34]. Considering the antioxidant properties, this research focuses on protective effects of peel and seed extracts of *C. aurantium* against glutamate toxicity in PC12 cells.

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 Eagles Medium (DMEM), penicillin-streptomycin, and fetal bovine serum were purchased from Gibco (Carlsbad, CA, USA). Trichloroacetic acid (TCA), malondialdehyde bis(dimethyl acetal) (MDA) and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). PC12 cells were obtained from Pasteur Institute (Tehran, Iran).

Preparation of the extracts

Peels and seeds of *C. aurantium* were washed, dried, and crushed to powder with an electric micronizer. The peels and seeds were extracted separately in a Soxhlet extractor with ethanol (70%) and the obtained extract was then dried and kept at -20°C until use. The obtained extracts were dissolved in dimethyl sulfoxide and then subjected to cytotoxic and apoptosis assays.

Cell culture and treatment

The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 . The cells were then cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal 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, the cells were seeded at 100,000 cell/well in a 24-well plate. All treatments were carried out in triplicate. The cells were pretreated with the extract alone (6 to 200 $\mu\text{g}/\text{ml}$) for 2 h and then incubation was continued in the presence of the extract with or without 8 mM glutamate for 24 h.

Cell viability assay

Cell viability was determined using a modified MTT assay as described previously [13]. Briefly, MTT solution in phosphate-buffered saline (5 mg/ml) was added to each well at a final concentration of 0.05%. After 3 h, the formazan precipitate was dissolved in DMSO. The absorbance of 570 and 620 nm (background) was measured using a StatFAX303 plate reader.

Lipid peroxidation assay

The level of lipid peroxidation was estimated by measuring MDA, which is the end product of lipid peroxidation [13]. At the end of incubation, the cells were scraped and centrifuged for 30 min. Then, 400 μl of TCA (15%) and 800 μl of TBA (0.7%) were added to 500 μl of cell samples. The mixture was vortexed and heated for 40 min in a boiling water bath. Then, 200 μl 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 intracellular ROS level was evaluated using a fluorescent probe, DCFH-DA. At the end of incubation, the cells were treated (30 min) with DCFH-DA (10 μM) at 4°C in the dark. Then, the fluorescence intensity was detected with excitation/emission of 485/530 nm. The experiment was performed in triplicate.

Propidium iodine staining

Apoptotic cells were detected using PI staining of small DNA fragments followed by flow cytometry. In the cells exposed to a hypotonic phosphate-citrate buffer containing PI, a quantitative DNA-binding dye, a sub-G1 peak is reflective of the DNA fragmentation. Apoptotic cells that had 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 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 µl of a hypotonic buffer (50 µg/ml PI in 0.1% sodium citrate with 0.1% Triton X-100). Next, flow cytometry was carried out using a FACS-can flow cytometer (Becton Dickinson). A total of 10⁴ events were achieved with FACS.

Statistics

All the data were expressed as mean ± SEM. Statistical analyses were performed using Prism 6 software. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer post hoc test for comparison between groups. The differences were considered significant at $p < 0.05$.

Results

The effect of the peel and seed extracts on the cell viability

To study the possible toxic effects of the extracts, PC12 cells were incubated with different concentrations of the extracts (12–200 µg/ml), and the viability was determined 24 h after the treatment. No significant toxic effect on the cell viability was seen subsequent to the treatment with the extracts.

Incubation with glutamate significantly decreased cell viability to $43.7 \pm 1.4\%$ of control ($p < 0.001$). As shown in Figure 1A, the treatment with the peel extract increased cell viability following glutamate insult at a concentration of 25–200 µg/ml, in a con-

centration-dependent manner (25 µg/ml, $61.36 \pm 2\%$, $p < 0.05$; 50 µg/ml, $64.62 \pm 1\%$, $p < 0.01$; 100 µg/ml, $69.62 \pm 1.9\%$, $p < 0.001$; 200 µg/ml, $80 \pm 3.7\%$, $p < 0.001$). Moreover, the results showed that the seed extract increased cell viability at the concentrations of 50–200 µg/ml (50 µg/ml, $63 \pm 1.6\%$, $p < 0.01$; 100 µg/ml, $65 \pm 0.98\%$, $p < 0.001$; 200 µg/ml, $70 \pm 1.9\%$, $p < 0.001$) (Fig. 1B).

The effects of the peel and seed extracts on ROS content

As expected, glutamate caused a significant increase in the level of ROS in PC12 cells ($260 \pm 7.8\%$ of control, $p < 0.001$). The peel extract at concentrations of 50, 100 and 200 µg/ml was able to decrease the intracellular ROS level to $219.8 \pm 10.37\%$ ($p < 0.05$), $206 \pm 7.8\%$ ($p < 0.01$) and $152 \pm 6.7\%$ ($p < 0.001$) of control, respectively (Fig. 2A). The seed extract decreased the ROS level at concentrations of 100 µg/ml ($212 \pm 5.2\%$, $p < 0.01$) and 200 µg/ml ($189 \pm 7.6\%$, $p < 0.001$) (Fig. 2B).

The effect of the peel and seed extracts on lipid peroxidation

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, exposure of the cells to glutamate resulted in a significant increase of MDA level ($269 \pm 7.5\%$, $p < 0.001$) as compared to control cells cultured in the absence of glutamate ($100 \pm 4.5\%$). The content of MDA signifi-

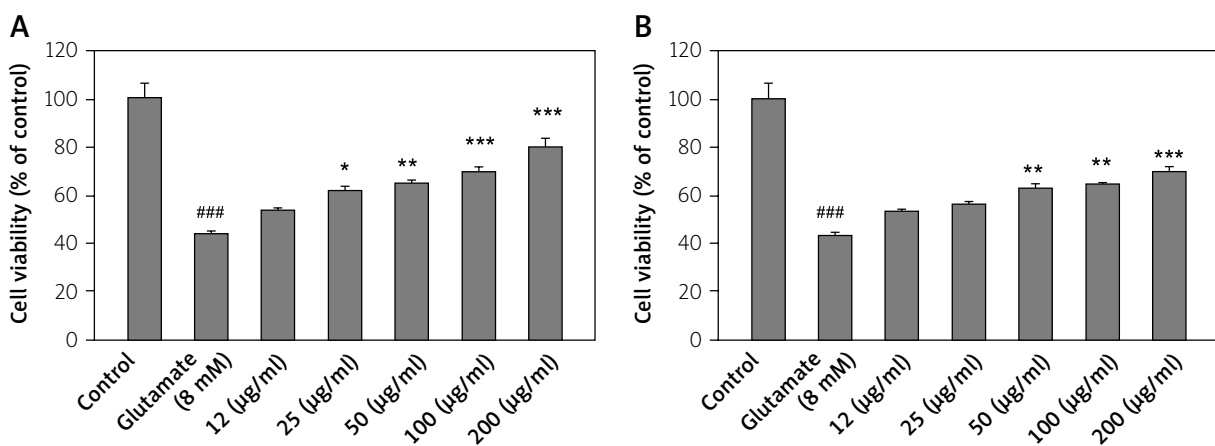


Fig. 1. Effect of *Citrus aurantium* extracts on cell viability of PC12 cells. The cells were pretreated with different concentrations of the peel (A) and seed (B) extracts for 2 h prior to glutamate exposure. Viability was quantitated using MTT assay. Results are mean ± SEM ($n = 3$). ### $p < 0.001$ vs. control, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. glutamate.

cantly decreased in the cells pretreated with 50 $\mu\text{g}/\text{ml}$ ($222.4 \pm 11.3\%$, $p < 0.001$), 100 $\mu\text{g}/\text{ml}$ ($194 \pm 6.86\%$, $p < 0.001$) and 200 $\mu\text{g}/\text{ml}$ ($147 \pm 8.4\%$, $p < 0.001$) of the skin extract (Fig. 3A). The seed extract reduced the level of MDA at 100 $\mu\text{g}/\text{ml}$ ($206 \pm 7.5\%$, $p < 0.05$) and 200 $\mu\text{g}/\text{ml}$ ($160 \pm 8.4\%$, $p < 0.001$) (Fig. 3B).

The effects of the peel and seed extracts on apoptotic cells

Apoptosis in PC12 cell line was detected with flow cytometry using PI staining. Cells were pretreated for 2 h with various concentrations of the *C. aurantium* and exposed to glutamate for 24 h. Analysis of the sub-G1 peak in flow cytometry histograms revealed the induction of apoptosis in the cells treated with

glutamate ($p < 0.001$). As shown in Figure 4A and 4B, the peel and the seed extracts of *C. aurantium* decreased apoptotic induction significantly at the doses of 50, 100 and 200 $\mu\text{g}/\text{ml}$, respectively.

Discussion

The present study is the first report to evaluate neuroprotective effects of peel and seed extracts of *C. aurantium* against apoptotic cell death induced by glutamate toxicity in PC12 cells. After treating PC12 cells with 8 mM glutamate, a significant decrease in the cell viability and increase in the number of apoptotic cells, ROS and MDA contents were seen, confirming its neurotoxic effect on PC12 cells. PC12 cells were used because they constitute a widely-

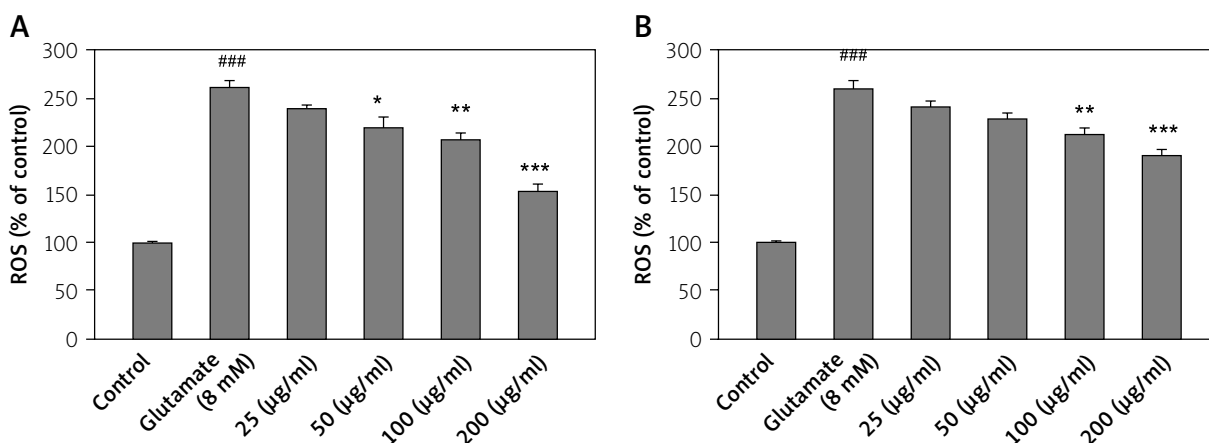


Fig. 2. Effect of *Citrus aurantium* extracts on ROS generation. The cells were pretreated with different concentrations of the peel (A) and seed (B) extracts for 2 h prior to glutamate exposure. Results are mean \pm SEM ($n = 3$). ### $p < 0.001$ vs. control, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. glutamate.

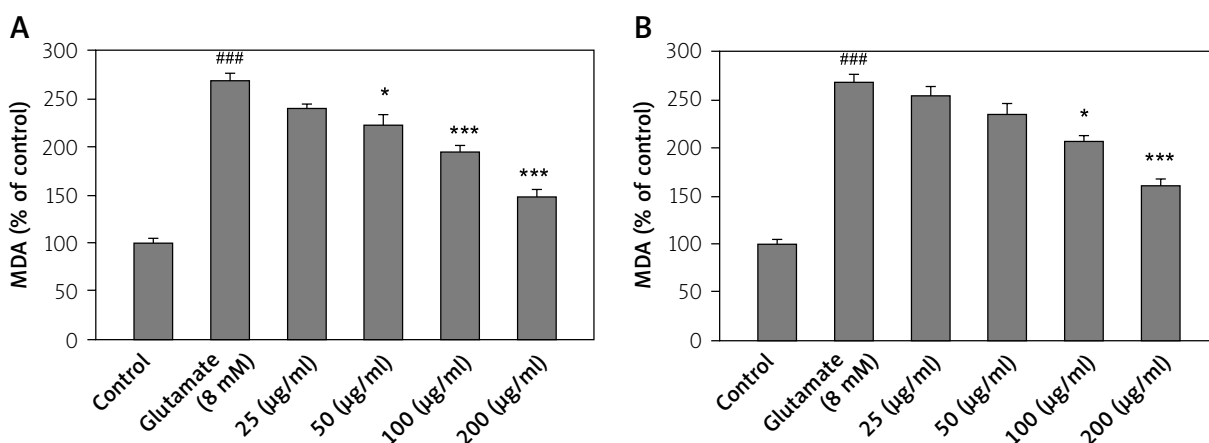


Fig. 3. Effect of *Citrus aurantium* extracts on MDA production. The cells were pretreated with different concentrations of the peel (A) and seed (B) extracts for 2 h then prior to glutamate exposure. Results are mean \pm SEM ($n = 3$). ### $p < 0.001$ vs. control, * $p < 0.05$ and *** $p < 0.001$ vs. glutamate.

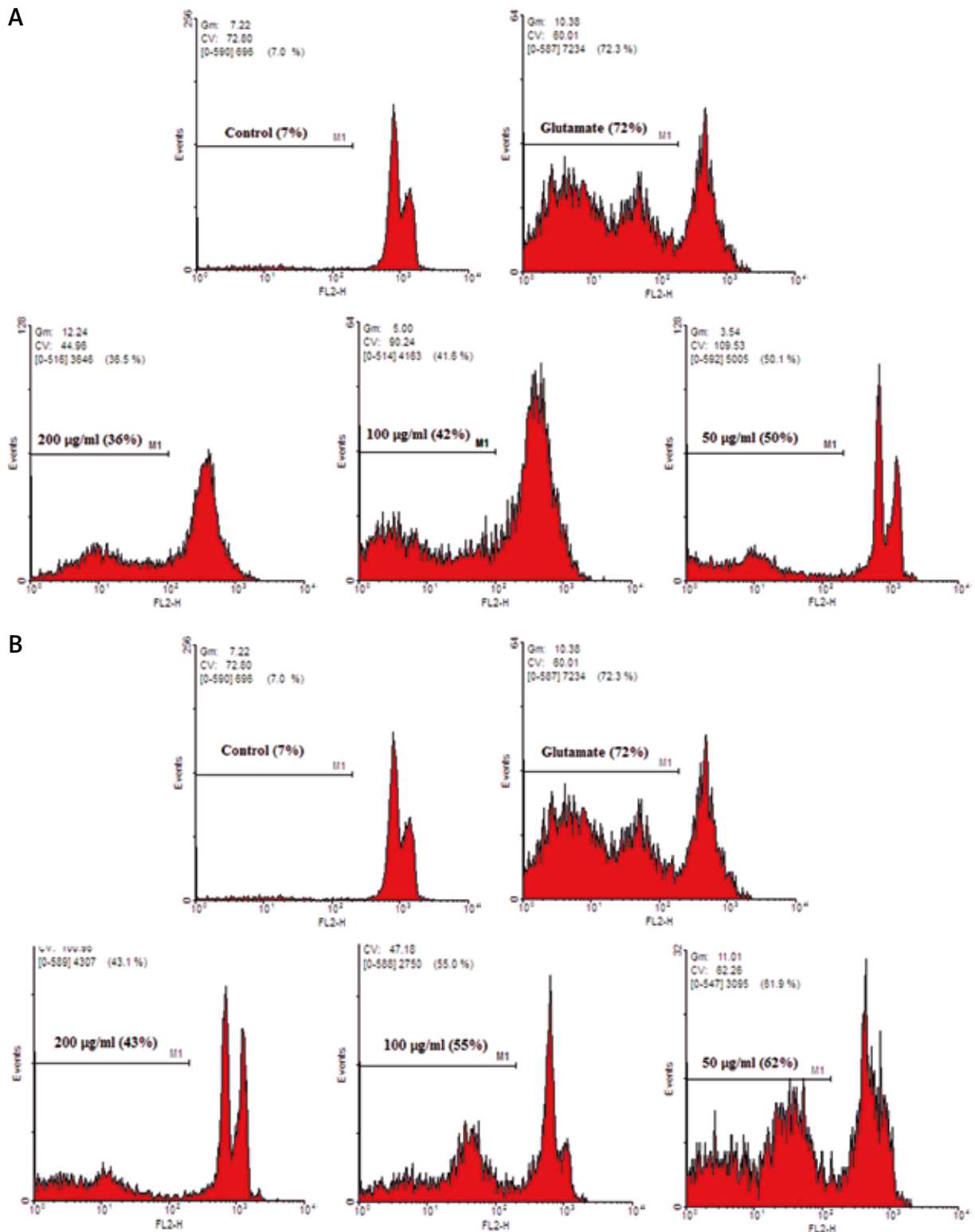


Fig. 4. The effects of the peel (A) and seed (B) extracts of *Citrus aurantium* extracts on apoptosis in PC12 cells using propidium iodide staining and flow cytometry.

used neuronal model system [17]. PC12 cells are more sensitive to glutamate injury; therefore, this cell model is also widely applied to study cellular glutamate toxicity [38]. Results showed that the pretreatment with *C. aurantium* peel and seed extracts can decrease glutamate-toxicity with different potencies. Our findings indicated that the peel extract has a more protective effect than the seed extract. Glutamate, a major excitatory neurotransmitter, plays an important role in synaptic transmission, formation of neuronal circuit, and neuronal development in the nervous system [37]. The elevated level of glutamate due to excess release or/and uptake disorder led to induce extensive neuronal damage and cell loss in brain tissue. Glutamate cytotoxicity has been associated with the activation of glutamate receptors and non-receptor-mediated oxidative glutamate toxicity [4,35]. Recent studies have shown that glutamate-induced cytotoxicity could be mediated by oxidative stress, depletion of GSH, and down regulation of SOD activity that leads to apoptosis [18]. Reactive oxygen species are typically defined as molecules or ions formed by the incomplete one-electron reduction of oxygen. Elevated levels of ROS are well-known etiological factors associated with oxidative stress leading to cell death via apoptosis in a variety of cell types [20,36], and such effects can be blocked or delayed by a wide variety of antioxidants [30]. Such antioxidants are reported to scavenge free radicals by raising the levels of endogenous antioxidant defense systems such as glutathione peroxidase and glutathione reductase [8,28]. Consequently, pretreatment of the cells with *C. aurantium* extracts prior to glutamate exposure resulted in a significant decrease in ROS and MDA content as well as apoptotic cells. *Citrus aurantium* peel contains citral, limonene, and several citrus bioflavonoids, including hesperidin, neohesperidin, naringin, and rutin [29]. These compounds have been attributed with a range of properties. For example, naringin is believed to cross the blood brain barrier [1,40] and to have antioxidant, anti-inflammatory, anti-hypercholesterolemic, anti-hypertensive, neuroprotective [9,6,16], and anticonvulsant properties. It also increases the seizure latency to kainic acid administration [9]. Naringenin and hesperetin exhibit antiatherogenic properties by activating the peroxisome proliferator-activated receptor (PPAR) and up-regulating adiponectin expression [23]. Hesperidin, neohesperidin, and neohesperidin dihydro-

chalcone, the most abundant species detected, are attributed antioxidant [14,19], anti-inflammatory [10,12], and vasopressive and antiplatelet properties [24] and may be responsible for the anticonvulsant properties of the *C. aurantium* extract. However, the peels and seeds of citrus fruits are rich in nutrients and contain many phytochemicals with strong potential to be used in drug production or as food supplements [7,21,25]. Some studies showed that *C. aurantium* has direct antioxidant activity. For example, Karimi *et al.* (2012) reported that *C. aurantium* extract scavenges free radicals and exhibits ferric reducing potential, with values of 55.3% and 51.7%, respectively [16]. The obtained results from pretreatment of cells with *C. aurantium* extracts demonstrated increased antioxidant capacity of cells prior to exposure to glutamate may be involved in cytoprotective properties of the extracts. In consistent with our results, other investigations also showed antioxidative properties of its bioactive constituents [6,9].

In conclusion, our study demonstrated that *C. aurantium* has an apparently neuroprotective effect against glutamate oxidative damage through its antioxidant activity. However, further studies are required to elucidate its molecular mechanism before the clinical therapeutic application of these extracts.

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Disclosure

Authors report no conflict of interest.

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