

# The Hydroalcoholic Extract of Saffron Protects PC12 Cells against Aluminum-Induced Cell Death and Oxidative Stress *in Vitro*

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## What's Known

- Saffron is a plant with potent radical scavenging and anti-oxidative properties.
- Aluminum causes apoptosis of PC12 cells via inducing oxidative stress.

## What's New

- Hydro-alcoholic extract of Saffron ameliorated aluminum-induced oxidative stress in PC12 cells.
- Saffron extract reduced aluminum-induced apoptosis of PC12 cells.

## Abstract

**Background:** Aluminum (Al) exposure is among the environmental risk factors that may involve in the pathogenesis of neurodegenerative diseases. Oxidative stress has a critical role in the Al-induced toxicity. Saffron is a plant with potent radical scavenging and anti-oxidative properties. This investigation was designed to evaluate the possible protective effects of saffron extract (SE) on aluminum maltolate (Almal)-induced oxidative stress and apoptosis in PC12 cell line.

**Methods:** In this *in vitro* study, PC12 cells were divided into four groups including control, Almal (500  $\mu$ M), Almal+SE (50  $\mu$ g/ml), and Almal+SE (100  $\mu$ g/ml). After 48 hours of treatment with Almal in the absence and presence of SE, cell viability and apoptosis were determined using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay and Annexin V flow cytometry, respectively. Catalase activity was determined as an index of oxidative stress. Statistical analyses were performed using one-way ANOVA (SPSS version 16.0).  $P < 0.05$  was accepted as a statistically significant difference between groups.

**Results:** Almal decreased the PC12 cells viability dose-dependently ( $IC_{50} = 500 \mu$ M). Co-treatment of 50 and 100  $\mu$ g/ml of SE with 500  $\mu$ M of Al increased cell viability to 79% ( $P = 0.04$ ) and 86% ( $P = 0.02$ ) of the control group, respectively. Al also increased PC12 cells apoptosis and catalase activity to 37 and 2.7 folds of those of the control group ( $P < 0.001$  and  $0.001 =$  respectively). 100  $\mu$ g/ml of SE blunted the effects of Al on the increased cell apoptosis ( $P = 0.02$ ) and changes in the catalase activity ( $P = 0.003$ ).

**Conclusion:** SE has protective effects against Al-induced apoptosis and oxidative stress and may possess therapeutic values in the treatment of Al-neurotoxicity.

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**Keywords** • Aluminum maltolate • Crocus • PC12 cells • Apoptosis • Oxidative stress

## Introduction

Due to the high abundance of aluminium (Al) in the earth crust and widespread use in the human daily life, Al toxicity has been the focus of attention for many years. Al enters the human body through inhalation, ingestion of food, drinking water, drugs, and during hemodialysis and vaccination. Al causes toxicity in various organs including liver, bone, urogenital, and most importantly in the

nervous system.<sup>1</sup> Based on the findings obtained from studies on animals and patients, it has been proposed that Al has a role in the initiation and development of neurodegenerative diseases (ND). Using a rodent animal model of ND, *Octodon degus*, Braidy and colleagues showed the accumulation of Al in the brain of animals in an age-dependent manner.<sup>2</sup> Furthermore, alteration in histological characteristics and biochemical functions of the brain of animals treated with Al was demonstrated.<sup>3</sup> In addition, abnormalities in spatial memory and impaired in long-term potentiation of hippocampus were shown in the rodent with chronic exposure to Al.<sup>4</sup> The high incidence of ND in the subjects with chronic occupational exposure to Al and the accumulation of Al in the postmortem brain tissue from ND are among the evidences that suggest the possible role of Al in the development of ND.<sup>5,6</sup>

Oxidative stress appears to play a critical role in Al-induced neurotoxicity.<sup>7,8</sup> *In vivo* and *in vitro* studies have shown that Al was capable to induce the generation of reactive oxygen species (ROS) such as superoxide and hydroxyl radicals. Moreover, it was shown that Al caused a decrease in the cellular concentrations of various antioxidants such as reduced glutathione (GSH), leading to failure in the antioxidant defense mechanisms against free radical-induced cell damage. The important role of oxidative damage in the Al-induced toxicity makes it an important therapeutic target for anti-oxidant therapy against Al-induced toxicity. In this context, it was demonstrated that the administration of antioxidants can reduce the toxic effects of Al, including neurotoxicity.<sup>9</sup>

Saffron is derived from the pistils of *Crocus sativus*. Apart from its traditional use as a cooking spice, it is considered as a medicinal plant with a variety of properties such as its anti-inflammatory and anti-oxidant activities. The presence of high concentrations of antioxidant compounds, including crocin, crocetin, and safranal, in the saffron, confers it powerful radical scavenging and antioxidant properties.<sup>10</sup> In this context, the protective effects of saffron against cell toxicity induced by anti-tumor agents such as cyclophosphamide,<sup>11</sup> doxorubicin,<sup>12</sup> and toxic chemicals have been reported.<sup>13</sup> In the experimental animal models, the protective effects of saffron against oxidative stress-related neurological disorders, such as multiple sclerosis,<sup>14</sup> Parkinson's disease,<sup>15</sup> and epilepsy,<sup>16</sup> have also been described.

To the best of our knowledge, the protective effects of saffron against Al-induced cell toxicity, including apoptosis and oxidative stress, have

not been investigated yet. Aluminum maltolate (Almal) is a lipophilic complex of Al and maltolate, which easily passes through the cell membrane and leads to the accumulation of Al in the cells.<sup>17</sup> Therefore, the present study was an effort to explore the possible role of saffron extract (SE) against Almal-induced cell death and apoptosis in PC12 cell line.

## Materials and Methods

### Preparation of Saffron Extract

The stigma of saffron was obtained from a local market in Shiraz (Fars province, Iran). It was authenticated by a taxonomist at the Department of Botany, Faculty of Pharmacy, Shiraz University of Medical Sciences (Shiraz, Iran) and a voucher specimen has been deposited with code "PM 1099". To prepare plant extract, two grams of saffron stigmas were ground and soaked in ethanol: water (70% v/v) for 2 hours at room temperature, in the absence of light and with continuous stirring. Thereafter, the extract was centrifuged, filtered, and evaporated to dryness using a Speed Vac System (Labconco Corp., Kansas City, MO). The final weight of the crude extract was 1.2 g. The dried extract was maintained at -20 °C until further use. A stock solution of SE was prepared by dissolving the dried extract in dimethyl sulfoxide (DMSO). The stock solution was diluted using cell culture medium (DMEM; pH=7.4) to obtain final concentrations of 50 and 100 µg/ml. The final concentration of DMSO in the incubation medium was less than 0.1%, which had no significant effect on the viability of the PC12 cells. Before adding to the culture medium, the SE was sterilized through membrane filtration.

### Preparation of Almal

Almal was prepared as described elsewhere.<sup>17</sup> In brief, a stock solution (80 mM) of aluminum chloride (AlCl<sub>3</sub>·6H<sub>2</sub>O; Sigma) and a stock solution (240 mM) of maltol (3-hydroxy-2-methyl-4-pyrone; Sigma) were prepared. Equal volumes of stock solution were mixed and the pH of the solution was adjusted to 7.4 using NaOH. Stock solution of Almal (25 mM) was freshly prepared in double distilled water and sterilized using a 0.2 µm filter.

### Cell Culture

PC12 cell line was obtained from Pastor institute (Pastor Institute, Iran) and cultured in DMEM (Gibco) with 10% fetal bovine serum (Cinagen, Iran) and 1% penicillin-streptomycin (Cinagen, Iran) at 37 °C and 5% CO<sub>2</sub>. Cells were seeded at 10<sup>5</sup> cells/ml for each experiment and

allowed to grow for 24 hours prior to treatment with Almal.

#### Cell Viability Assay

The effects of Almal on cell viability were measured using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) solution.<sup>17</sup> In brief, PC12 cells were seeded into each well of a 96-well plate and cultured for 24 hours. The cells were then treated with various concentrations of Almal (final concentration 250, 500, 750, and 1000  $\mu$ M) for 48 hours. To evaluate the effects of SE against Almal-induced cell death, PC12 cells were treated with 500  $\mu$ M of Almal in the absence or presence of SE (50 and 100  $\mu$ g/ml) for 48 hours. Next, 20  $\mu$ l of MTT (5 mg/ml) was added to each well and incubated for 4 hours at 37 °C. The culture medium was then replaced with 200  $\mu$ l of DMSO. The absorbance rate at 492 nm was determined using ELISA reader. Cell viability was calculated as the percent of the control group.

#### Apoptosis Assay

Flow cytometric evaluation using PE Annexin V Apoptosis Detection kit I (BD Pharmingen™) containing Annexin V-PE and 7-aminoactinomycin D (7-AAD) (Dako, cat no: k235011) was performed to quantify the effects of Almal and SE on PC12 cell viability. Briefly, PC12 cells were cultured on T<sub>25</sub> culture flasks for 24 hours. The cells were then treated with Almal (500  $\mu$ M) in the absence or presence of SE (100  $\mu$ g/ml) for 48 hours. The cells were then washed in cold phosphate buffer saline (PBS) and resuspended in 1 ml ice-cold binding buffer. Five  $\mu$ l of Annexin V and five  $\mu$ l of 7-AAD were added to 90  $\mu$ l of cell suspension and gently mixed, followed by keeping the tubes on ice and incubating them for 10 min in the dark. Cells were acquired within 5 min using a BD flow cytometer (BD FACS Calibur, BD Bioscience, USA). A total of 10,000 events were acquired for each sample.

#### Assay of Catalase Activity

Due to the sensitivity of catalase (CAT) activity to Almal-induced oxidative stress, which has been demonstrated in previous studies,<sup>17, 18</sup> the activity of CAT was used as a marker of oxidative stress in this study. CAT activity was measured on the basis of the decomposition of H<sub>2</sub>O<sub>2</sub> at 25 °C by CAT, which resulted in a decrease absorbance at 240 nm. Briefly, the cell lysates were prepared by sonication in ice-cold 50 mM sodium phosphate buffer (pH 7.0). The cell lysates were then centrifuged at 13,000 g for 10 min at 4 °C, and the supernatants were used for the assay of CAT activity. The amount

of protein in the sample was determined by the method of Bradford. CAT activities were calculated as units/mg protein and expressed as a percent of control.

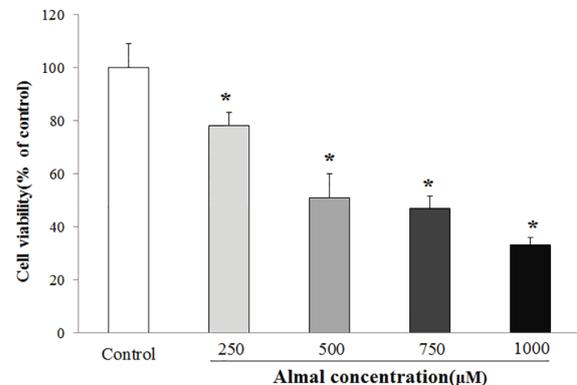
#### Statistical Analysis

SPSS software (version15) was used for statistical analyses. Normal distribution of data was checked using Shapiro-Wilk test (P=0.16). The presence of statistical differences between various groups was checked using one-way of analysis of variance (ANOVA) followed with LSD *post hoc* test for multiple comparisons. Data are expressed as mean $\pm$ SD. P<0.05 was considered statistically significant.

## Results

#### The effects of Almal on the viability of PC12 cells

To evaluate the effects of Almal on PC12 cells viability, the cells were treated with increasing concentrations of Almal (250-1000  $\mu$ M) for 48 hours and the cell viability was determined using MTT assay. The data demonstrated that Almal treatment reduced the viability of PC12 cells in a concentration-dependent manner (figure 1). The IC<sub>50</sub> of Almal in this issue was calculated about 500  $\mu$ M. This concentration of Almal was chosen for subsequent experiments including



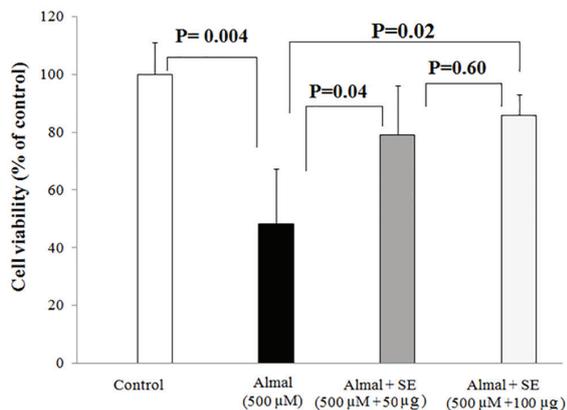
**Figure 1:** Almal exposure reduced the viability of PC12 cells. The cells were treated with different concentrations of Almal (250, 500, 750, 1000  $\mu$ M) for 48 hours and the cell viability was measured using MTT assay. The results illustrate the mean $\pm$ SD of the three independent experiments. \*P<0.001 compared to control group.

apoptosis and oxidative stress assays.

#### The effects of SE on Almal-induced death of PC12 cells

To evaluate the protective effects of SE against Almal-induced cell death, PC12 cells were treated with 500  $\mu$ M of Almal in the absence or presence of SE (50 and 100  $\mu$ g/ml) for 48 hours and the cell viability was determined using MTT assay. The findings revealed that the level of cell viability

decreased in Almal-treated cells to about half of the control group ( $49.0 \pm 8.0\%$ ,  $P=0.004$ ). When SE was added to the medium, the percentage of cell viability increased to 79 and 86 percent by the concentrations of 50 and 100  $\mu\text{g/ml}$ , which was indicative of significant protection against Almal-induced cell death ( $P=0.04$  and  $0.02$ , respectively). No significant difference was observed between the protective effects of 50 and 100  $\mu\text{g/ml}$  of SE ( $P=0.60$ ) (figure 2).



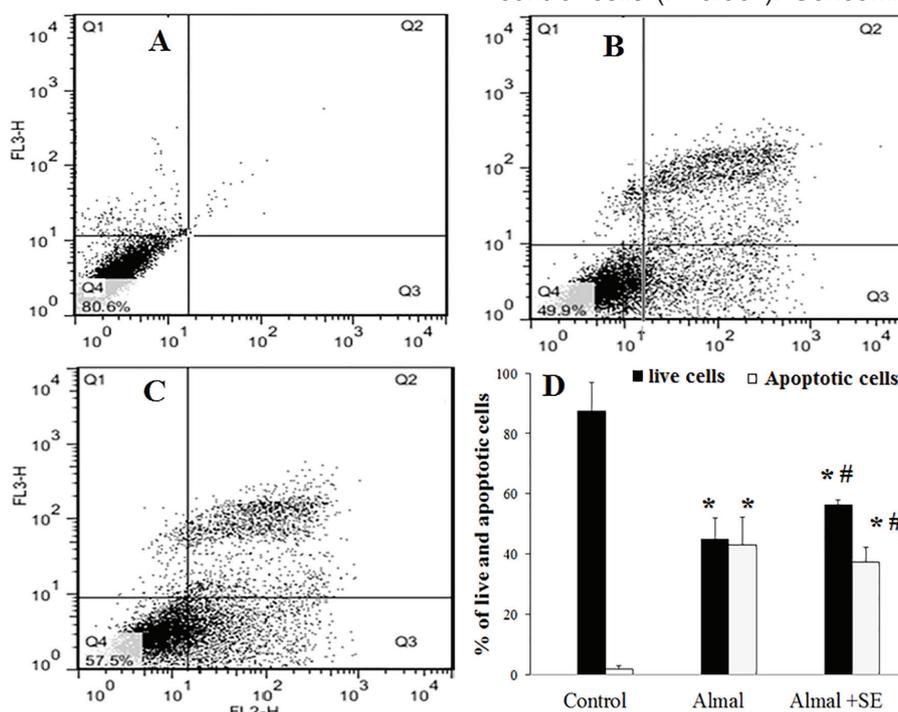
**Figure 2:** Saffron extract (SE) protected PC12 cells from Almal-induced cell death. PC12 cells were treated with 500  $\mu\text{M}$  of Almal in the absence or presence of SE (50 and 100  $\mu\text{g/ml}$ ); cell viability was measured using MTT assay. The results are shown as the mean  $\pm$  SD for at least three independent experiments.

### The effect of SE on Almal-induced apoptosis in PC12 cells

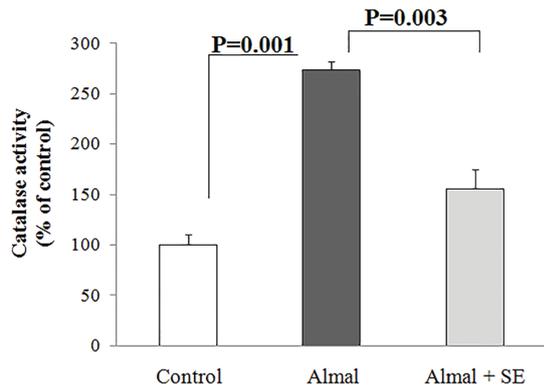
To explore the possible role of apoptosis in Almal-induced cell toxicity, Annexin-V/7-ADD flow cytometric was used. The findings showed that the treatment of PC12 cells with 500  $\mu\text{M}$  of Almal significantly ( $P<0.001$ ) increased the percentage of total apoptotic cells compared to the control cells ( $1.8 \pm 0.23\%$  and  $48.2 \pm 9.0\%$  in the control and Almal-treated cells, respectively). When the cells were treated with Almal and SE (100  $\mu\text{g/ml}$ ) concomitantly, the percent of viable cells increased from  $45.0 \pm 6.9\%$  in Almal-treated cells to  $56.3 \pm 1.8\%$  in Almal+SE-treated cells, while total apoptotic cells decreased from  $48.2 \pm 9.0\%$  in Almal-treated cells to  $37.3 \pm 5.0\%$  in Almal + SE -treated cells. Statistical analyses showed a significant difference between the two groups ( $P=0.02$ ) (figure 3).

### The Effect of SE on Almal-Induced Catalase Activity in PC12 Cells

Due to the sensitivity of CAT activity to Almal-induced oxidative stress, the possible protective effects of SE against Almal-induced oxidative stress was evaluated using CAT assay. As can be seen in figure 4, Almal treatment increased CAT activity in the PC12 cells compared to the control cells ( $P=0.001$ ). Concomitant treatment



**Figure 3:** Saffron extract (SE) ameliorated Almal-induced apoptosis of PC12 cells. PC12 cells were treated with vehicle (Control group; A), 500  $\mu\text{M}$  of Almal (B) or 500  $\mu\text{M}$  of Almal and 100  $\mu\text{g/ml}$  of SE (C) for 48 hr. The percentage of apoptotic cells were then quantified using Annexin V/7AAD flow cytometric method. Data were expressed as percentage of apoptotic cells in  $10^5$  events per sample. Q1, Q2, Q3, and Q4 are necrotic (Annexin V-/7AAD+), late apoptotic (Annexin V+/7AAD+), early apoptotic (Annexin V+ and 7-AAD-), and viable cells (Annexin V- and 7-AAD-), respectively. Represented data are mean  $\pm$  SD of total apoptotic cells. The total apoptotic cells were calculated using Q2+Q3. Data were analyzed using one-way ANOVA followed with LSD *post hoc* test for multiple comparisons. \* $P<0.001$  compared to control group; # $P=0.02$  compared to Almal-treated group.



**Figure 4:** Saffron extract (SE) decreased Almal-induced catalase activity in PC12 cells. PC12 cells were treated with 500  $\mu$ M of Almal in the absence or presence of SE (100  $\mu$ g/ml) for 48 hr. Catalase activity was then measured and expressed as a percent of control. Data are mean $\pm$ SD of three independent experiments.

with SE (100  $\mu$ g/ml) reduced CAT activity compared to Almal-treated cells ( $P=0.003$ ) and reached approximately to the same values that had been observed in the control cells.

## Discussion

The data obtained from the current study revealed that Almal increased cell death and apoptosis of PC12 cells through increasing the oxidative stress, as evidenced by the increase in the catalase activity following the exposure of PC12 cells with Almal. Our findings also revealed that the treatment of PC12 cells with SE significantly prevented Al-induced cell death, apoptosis, and oxidative stress, suggesting possible beneficial effects of SE against Al-induced cell toxicity.

Al is known as a neurotoxic agent that is implicated in the pathogenesis of ND. Necrosis, apoptosis, and autophagy are among the most important causes of cell death. Previous studies have addressed the role of all these mechanisms individually or in combination with Al-induced cytotoxicity. For example, Zhang and colleagues revealed that a combination of apoptosis and necrosis were involved in neuroblastoma (SH-SY5Y) cell death following exposure to AlCl<sub>3</sub>.<sup>19</sup> Similar results have obtained from animal studies, in which apoptosis has been reported as the main cause of rat brain cell death following exposure to AlCl<sub>3</sub>.<sup>20</sup> In the primary culture of rat astrocyte, both autophagy and apoptosis have been introduced as the causes of cell death after treatment with Almal.<sup>21</sup> In the present study, we applied Annexin V/7AAD autophagy method as a reliable method to determine the role of apoptosis in Almal-induced cell death. Consistent with the results of previous studies,<sup>17,</sup>

<sup>18</sup> the results of the current study indicated the role of apoptosis as the leading cause of Al-induced cell death of PC12 cells.

The protective effects of SE against cell death induced by several cytotoxic compounds, including glucose-induced PC12 cell death,<sup>22</sup> doxorubicin-induced cardiomyocytes injury,<sup>23</sup> and acetaminophen-induced hepatotoxicity,<sup>24</sup> have been documented in several studies. To explore the possible impact of SE on Al-induced apoptosis, PC12 cells were treated with Almal and SE concomitantly. Findings from Annexin V/7AAD flow cytometric analysis showed that SE treatment had a significant inhibitory effect on Almal-induced apoptosis and increased cell viability in Almal-treated cells. These data indicate that the inhibition of apoptosis may be involved in SE-protective effects against Al-induced cytotoxicity in PC12 cells. Although the mechanism responsible for Al-induced apoptosis was not investigated in this study, a number of mechanisms, including the activation of caspase-3 activity, release of cytochrome c from mitochondria, up-regulation of apoptotic Bax protein, down-regulation of anti-apoptotic Bcl2 protein, and induction of DNA fragmentation, have been proposed for Al-induced cell apoptosis.<sup>25</sup> On the other hand, the protective effects of corcic, the main constituent of SE, against cell apoptosis, which was mediated through inhibition of caspase-3 activity, suppression of cytochrome c release, down-regulation of Bax, up-regulation of Bcl2, and prevention of DNA damage have been reported in several studies.<sup>26-29</sup> Whether such mechanisms are involved in the anti-apoptotic effects of SE observed in the current study warrants further investigation.

Several mechanisms have been described for Al-induced cytotoxic effects whose ability in inducing oxidative stress is the most considered one. On the basis of previous studies, Al could enhance oxidative stress by decreasing antioxidants, such as GSH, and causing changes in the activity of antioxidant enzymes such as glutathione reductase, glutathione peroxidase, and catalase. In accordance with previous studies,<sup>17, 18</sup> our data showed an increase in the activity of CAT following the treatment of PC12 cells with Almal, indicating the effect of Al in changing the oxidative balance of the cells. Antioxidant and neuroprotective effects of saffron main constituents, including crocin, crocetin, and safranal, when used individually, have been demonstrated in many *in vitro* and *in vivo* studies.<sup>30</sup> In the current study, we evaluated the impact of crude hydro-alcoholic extract of saffron against Al-induced cell toxicity because

a potent synergistic effect between the main components of saffron has been proposed in previous studies.<sup>31</sup> Treatment with 100 µM/ml of SE reduced the level of CAT activity to approximately 60 percent of that present in Almal-treated PC12 cells. This finding implies that the observed SE impact on Al-induced toxicity, at least in part, is mediated through ameliorating the oxidative stress. The neuroprotective effects of SE, which are attributed to its antioxidant activity, have also been supported by previous studies. For example, in the fly model of Parkinson's disease, the protective effects of SE against rotenone-induced neurotoxicity and oxidative stress were demonstrated.<sup>32</sup> Furthermore, in the brain of mice, SE was found to reverse the Al-induced elevation of lipid peroxidation and caused a reduction of GSH.<sup>33</sup> In a recent study, Samarghandian and colleagues showed that SE could protect rat hippocampus against age-related oxidative damage including a reduction in the activities of antioxidant enzymes and an increase in the levels of MDA and nitric oxide.<sup>34</sup>

Alpha-synuclein is a cytoplasmic protein that is involved in the pathogenesis of neurodegenerative diseases such as Parkinson's disease. Previous research from our laboratory revealed the role of  $\alpha$ -synuclein protein in the mediation of Al cytotoxicity including apoptosis and oxidative stress.<sup>17</sup> Moreover, it has been demonstrated that some plant extracts with antioxidant properties could prevent aggregation of  $\alpha$ -synuclein protein in PC12 cells and primary culture of neurons.<sup>35, 36</sup> Further investigation is needed to determine the possible effects of SE on the expression and aggregation of  $\alpha$ -synuclein.

A combination of various technical approaches, including quantification of GSH, measurement of fatty acids, nucleic acids, protein oxidation products, and direct measurement of ROS generation, are usually performed to confirm the occurrence of cell oxidative stress.<sup>37</sup> A limitation in our study is the use of only one method, i.e. assay for catalase activity, to detect the role of oxidative stress in Almal-induced cytotoxicity in PC12 cells. However, due to the close relationship between CAT activity and Al-induced oxidative stress reported in previous studies as well as the presence of an available, simple,<sup>17, 18</sup> and sensitive procedure for the assay of CAT activity, CAT activity was considered a reliable marker for the cell response to Almal-induced oxidative stress in this study.

## Conclusion

Taken together, the findings of this investigation suggest that SE can inhibit Al-induced cell death

and apoptosis probably through ameliorating oxidative stress. Due to the beneficial protective effects of SE on other models of ND, it could be considered as a promising therapeutic agent in the prevention or treatment of ND.

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**Conflict of Interest:** None declared.

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