



Safranal protects against beta-amyloid peptide-induced cell toxicity in PC12 cells via MAPK and PI3 K pathways

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Abstract

Alzheimer's disease is a type of cerebrovascular problem with progressive mental disabilities for the patient. This study aimed to investigate the protective effect of safranal on toxicity and oxidative damage induced by beta-amyloid (A β) and hydrogen peroxide (H₂O₂) in PC12 cells as an appropriate model of Alzheimer's cell damage. PC12 cells pretreated with saffron extract (2.5–40 μ g/ml), essential oil (2.5–40 μ g/ml), safranal (2.5–5–40 μ M) and donepezil (5, 10 and 20 μ M) for 120 min. Then exposed to either A β (25 μ M) for 48 h or H₂O₂ (150 μ M) for 24 h. In the end, the cell survival and intracellular reactive oxygen species (ROS) production analyzed. The anti-apoptotic effects of safranal in PC12 cells were studied using flow cytometry after PI staining. Also, western blot analysis of Cyt c, survivin, p44/42 MAPK (ERK1/2), Phospho-p44/42 MAPK (ERK1/2), PI3 Kinase P85, Phospho-PI3 Kinase P85, phospho SAPK/JNK, SAPK/JNK and caspase 3 performed for detection of apoptosis. Safranal (2.5 and 5 μ M) and donepezil (10 and 20 μ M) significantly decreased the A β toxicity. The ROS significantly attenuated when cells pretreated with essential oil, saffron extract, safranal, and donepezil. Cell apoptosis significantly increased after treatment with A β (25–35) (25 μ M) compared to control. However, after pretreatment with safranal (2.5 μ M) apoptosis was significantly reduced. Western blot analysis of PC12 cells showed that 25 μ M A β (25–35) could increase proteins involved in apoptosis signaling and pretreatment with safranal (2.5 μ M) could decrease the apoptosis. According to the results, safranal showed anti-apoptotic and antioxidant effects and may exert promising potential for the prevention of Alzheimer's disease.

Keywords Alzheimer's disease · Beta-amyloid · Apoptosis · *Crocus sativus*, Safranal, saffron

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease (Colucci et al. 2014) which cause amnesia and behavioral changes in old people. Economically, the cost to Alzheimer's patient is more than that of cancer and heart

disease (Nourhashémi et al. 2000). More important than the financial problem of the disease is the patient suffering. The presence of senile plaques and neurofibrillary tangles are two features present in the brain of the affected person. Neurodegeneration and progress of the Alzheimer's disease correlate with neurotoxic effects of beta-amyloid (A β). β - and γ -secretase cleave the APP which leads to deposition of the resulting A β (Praticò 2008) in neurons. Plaques are the manifestation of the aggregation of A β and tau protein (Jouanne et al. 2017). Interaction of A β with neurons activates local oxidative and inflammatory responses (Praticò 2008).

The presence of a combination of polyunsaturated fatty acids, metals, and ascorbate which is vulnerable to oxidative stress also weak antioxidant defense of the brain, sensitize neural cells to oxidative damage. More important, structural and functional change of bioactive molecules following oxidative damage promote the deposition of A β and enhance the β - and γ -secretase activity (Agis-Torres et al. 2014; Praticò 2008). Consequently, one of the wise strategies to fight against cellular injury of AD is the use of antioxidants which their usefulness both in prevention

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and slowing the progression of Alzheimer's disease are shown in pre-clinical and clinical studies (Kim et al. 2010).

Antioxidants can protect cells from oxidative damage (Jelkmann et al. 1997). Plants and phytochemical as natural antioxidants have been widely used in the treatment and prevention of disease due to low adverse effects (Papandreou et al. 2006) and shown the beneficial effects against Alzheimer's disease (Libro et al. 2016; D'Onofrio et al. 2017; Wightman 2017). Besides conventional treatment for the treatment Alzheimer's disease, the role of phytochemicals in the prevention and progression of the disease has been well documented (Moshiri et al. 2015). Phytochemicals are multi-target agents which act as antioxidant, anti-apoptotic and anti-inflammatory agents. Curcumin, resveratrol, and green tea catechins are successful examples of natural compounds which are not only potent antioxidants but described as novel anti-amyloidogenic, AChE inhibitors and acceptable anti-inflammatory agents (Freitas et al. 2018; Russo et al. 2013).

Saffron, the dried stigma of *Crocus sativus* L. is a well-known medicinal plant used in the folk medicine of Asia, Europe, the Mediterranean countries, and India. Besides traditional use of saffron as the sedative, expectorant, anti-asthma, emmenagogue, and apoptogenic agent multiple pharmacologic properties have been addressed in literature for saffron such as an anti-depressant, anti-cancer, anti-nociceptive, anti-inflammatory and brain performance enhancing (Shafiee et al. 2018; Bathaie and Mousavi 2010). The antioxidant properties of *C. sativus* stigma are due to the presence of phenolic compounds, including safranal, crocin, crocetin, and carotene in the plant (Karimi et al. 2010). The odor of saffron is related to the safranal content, an aldehyde (m.w. = 150) which is the abundant chemical present in the essential oil of saffron (Rezaee and Hosseinzadeh 2013).

There is not any report on the protective activity of safranal in research model of Alzheimer disease. Thus, this study was designed for evaluation of the protective effect of safranal on PC12 cells damage induced by A β and H₂O₂ as an appropriate model of Alzheimer's. For this purpose, we first compared the protective effect of safranal saffron extract, essential oil, safranal and donepezil. Further mechanistic evaluation and the effect safranal on apoptosis related proteins was also explored.

Materials and methods

Materials

Safranal, Resazurin, the fluorescent probe propidium iodide (PI), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), H₂O₂ 33%, A β (25–35) and 1640 RPMI medium and QuantiPro™ BCA Assay Kit from Sigma (Germany); Donepezil from Samisaz (Iran); rabbit anti-serum against survivin, caspase-3, Phospho-p44/42 MAPK (ERK1/2),

Phospho SAPK/JNK, SAPK/JNK and β -Actin (13E5), rabbit polyclonal anti-serum against Cyt c, p44/42 MAPK (ERK1/2), PI3 Kinase P85 and Phospho-PI3 Kinase P85, anti-rabbit IgG HRP-linked Antibody from Cell Signaling Technology (USA); fetal bovine serum (FBS), penicillin-streptomycin (PS) from Gibco (USA); dimethyl sulfoxide (DMSO) from Merk (Germany); *PC12 rat pheochromocytoma cell* line purchased from Institute of Pasteur Institute (Iran).

Cell culture and treatment

A β (25–35) was dissolved in acetic acid 1% to obtain a one mM stock solution. Aliquots stored at –20 °C and incubated at 37 °C for 72 h before use. PC12 pheochromocytoma cells cultured in 25 cm² flasks and maintained in RPMI-1640 medium with 1% penicillin and streptomycin, 10% (v/v) FBS. Cells incubated at 37 °C in 95% humidity and 5% CO₂ environment. RPMI-1640 medium changed every 2 days. Finally, they were seeds in culture plates for experiments. Safranal (100 mM), saffron extract, Saffron essential oil (100 mg/ml), Donepezil (20 mM) dissolved in DMSO to obtain a stock solution. PC12 cells pretreated with saffron extract (2.5–40 μ g/ml), essential oil (2.5–40 μ g/ml), safranal (2.5–5–40 μ M) and donepezil (5, 10 and 20 μ M) for 120 min. Then exposed to either A β (25 μ M) for 48 h or hydrogen peroxide (H₂O₂) (150 μ M) for 24 h. The optimum time and concentration points were used according to the pre-test evaluation with A β (0.04, 0.2, 1, 5, 25 and 50) for 48 h (Data not shown) and literature search (Qu et al. 2011).

Preparation of saffron extract

Dried saffron (spinach and redcurrant saffron) purchased from Qaenat, South Khorasan Province, Iran. After rubbing dried saffron stigma in Chinese moss, 2 g of crushed saffron was dissolved in 20 ml of 75% ethanol and extracted by the ultrasonic apparatus for one hour. Then it was filtered and the solvent evaporated by distillation at reduced pressure. The extract stored at –20 °C overnight. Then it was condensed by the freeze-drying method. The extract again kept at –20 °C.

Preparation of saffron essential oil

The saffron essential oil was extracted as previously explained in our published work using steam distillation-solvent extraction (SDE) (Rahiman et al. 2018).

Analysis of cell viability

Based on cellular metabolic activity, resazurin cell viability assay evaluates the amounts of viable cells. This system has an oxidation-reduction index that is detected both fluorimetric

and colorimetric (O'Brien et al. 2000). 1×10^4 PC12 cells seeded in each well of 96-well culture plates. Cell were pretreated with safranal and donepezil for 2 h then exposed to $A\beta_{(25-35)}$ (25 μ M) for 48 h. 20 μ l resazurin (14 mg/dl) was added to each well and incubated at 37 °C for 4–6 h. The absorbance at 570 and 600 nm was read using Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA) which correlated to the viability of cells.

ROS generation

Intracellular ROS content compared in PC12 cells after exposure to the oxidation-sensitive fluorescent dye DCFH-DA (Sigma, Germany). DCFH-DA converts into the polar derivative DCFH by intracellular esterase (Liu et al. 2009). 1×10^4 PC12 cells per well were seeded in 96-well culture plates. Cell were pretreated with safranal, donepezil, saffron extract and saffron essential oil for 2 h then exposed to $A\beta_{(25-35)}$ (25 μ M) or H_2O_2 for 24 h. After 24 h, DCFH-DA (10 μ M) added to each well and the fluorescent emission at 525 nm was measured after excitation at 488 nm by Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA). The intracellular ROS level expressed as a percentage of the control.

Flow cytometry apoptosis assay

For the detection of apoptosis, treated cells stained with PI and then the so-called sub-G1 peak detected by flow cytometry (Zhang et al. 1999; Zaker et al. 2017). 10^5 PC12 seeded in each well of a 12-well plate. After 24 h, PC12 cells pretreated with safranal (2.5 μ M) for 2 h then exposed to $A\beta_{(25-35)}$ (25 μ M). 48 h later, cells harvested by trypsin and centrifuged for 5 min at 6000 rpm. Then, 400 μ l of a hypotonic buffer contains 50 μ g/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100 was added to each sample before flow cytometric analysis using a FAC Scan flow cytometer (BD Biosciences, CA, USA).

Western blotting

For the experiment, about 10^6 PC12 cells cultured in three 25 cm² flasks. After 24 h, PC12 cells were pretreated with safranal (2.5 μ M) for 2 h then exposed to $A\beta_{(25-35)}$ (25 μ M). Two days later, cells were rinsed and harvested with cold PBS in triplicate. Western protocol done according to our previously published work (Tayarani-Najaran et al. 2017). The membrane exposed to rabbit monoclonal survivin, polyclonal Cyt c, polyclonal p44/42 MAPK (ERK1/2), monoclonal Phospho-p44/42 MAPK (ERK1/2), polyclonal PI3 Kinase P85, polyclonal Phospho-PI3 Kinase P85, polyclonal Phospho SAPK/JNK, SAPK/JNK, polyclonal caspase-3 and β -Actin (13E5) as primary antibodies and anti-rabbit IgG, a HRP-linked antibody as secondary antibody. The density of each band was divided into

the related β -Actin and compared with the corresponding control using Gel-pro Analyzer V.6.0 Gel Analysis Software (Media Cybernetics, InG, Bethesda, MD).

Statistical analysis

All experiments were done in triplicate, and each experiment was repeated three times. Mean \pm SEM of data were compared with the related control using one-way ANOVA, followed by Dunnett's post hoc test in Graph Pad Prism 5 software.

Results

Effects of safranal on cell viability by resazurin assay

To choose the optimal protective concentration of safranal the cytotoxicity of the compound was determined. Safranal up to 40 μ M does not show any cytotoxicity compared to the control group (Fig. 1).

Effects of safranal and donepezil on $A\beta_{(25-35)}$ induced PC12 on cell viability determined by resazurin assay

As shown in Fig. 2, $A\beta_{(25-35)}$ significantly reduced the cell viability compared with the control group ($P < 0.05$) while pretreatment with safranal (2.5 μ M) and donepezil (10 and

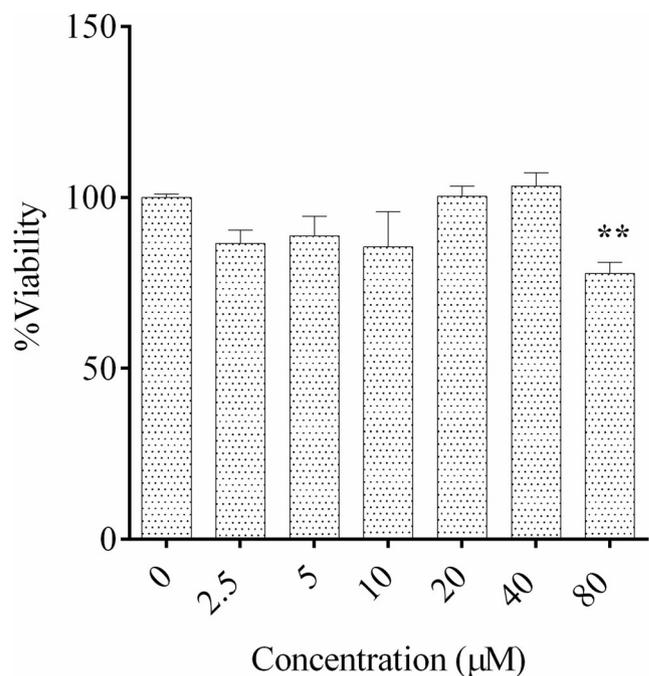


Fig. 1 Effects of safranal on cell viability by resazurin assay. The viability of PC12 cells determined after treatment with safranal (2.5–80 μ M) for 24 h. The data presented as the mean \pm SEM of three independent experiments ($n = 9$). *** $P < 0.001$ compared with the control group

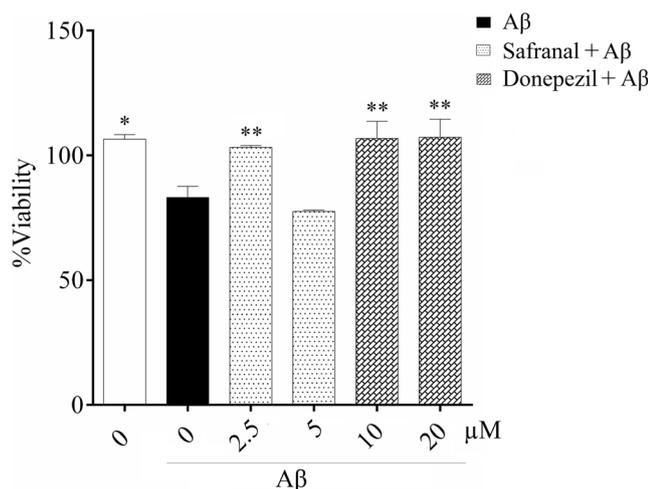


Fig. 2 Effects of safranal and donepezil on A β (25–35)-induced PC12 cell viability by resazurin assay. PC12 cells were pretreated with safranal (2.5 and 5 μ M) and donepezil (10 and 20 μ M) for 2 h then exposed to A β (25–35) (25 μ M) for 48 h, and cellular viability detected by resazurin assay. The data presented as the mean \pm SEM of three independent experiments (n = 9). * P < 0.05 compared with 25 μ M A β (25–35)

20 μ M) exhibited significantly higher cell survival rates compared with the 25 μ M A β (25–35) (P < 0.05). The results revealed that safranal (2.5 μ M) and donepezil could protect PC12 cells from A β (25–35) induced cell death (Fig. 2).

Effects of saffron extract, essential oil, safranal and donepezil on H₂O₂ induced ROS production

As shown in Fig. 3, treatment with H₂O₂ (150 μ M) and A β (25–35) (25 μ M) for 24 h induced a significant increase in the cell fluorescence intensity compared with the control group (P < 0.001). After pretreatment with saffron extract (2.5–40 μ g/ml), safranal (2.5, 5, 20, 40 μ M) and donepezil (5, 10, 20 μ M); however, the fluorescence intensity decreased significantly. The results indicated that A β (25–35) (25 μ M) increased ROS level which attenuated with donepezil (10, 20 μ M), also safranal and donepezil could attenuate H₂O₂ induced ROS production (Fig. 3).

Effects of safranal on A β (25–35) induced apoptosis by flow cytometry

The anti-apoptotic effects of safranal in PC12 cells were assessed using flow cytometry after PI staining. As shown in Fig. 4, cell apoptosis was significantly increased to 63.7% after treatment with A β (25–35) (25 μ M) compared to control (19.8%). After pretreatment with safranal (2.5 μ M); however, apoptosis was significantly reduced to 48.8%. The sub-G1 peak of treated cells in flow cytometry histograms compared to treated A β (25–35) (25 μ M) cells revealed the decrease in the amount of apoptosis in safranal treated cells (Fig. 4).

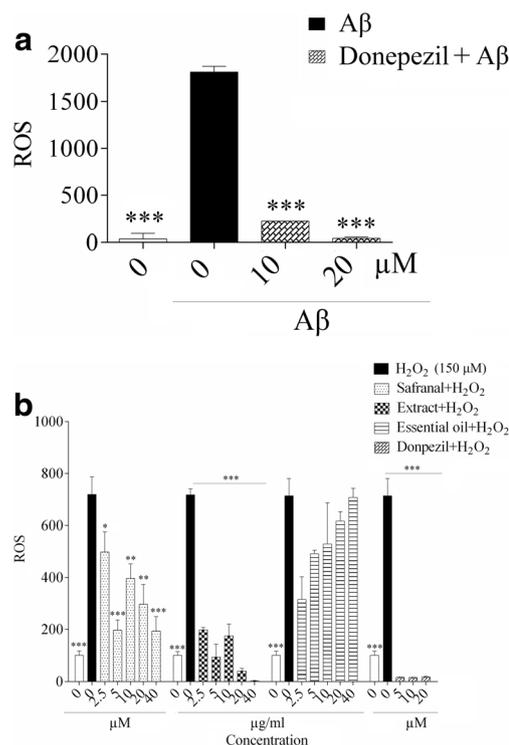


Fig. 3 Effects of safranal, donepezil, saffron extract and saffron essential oil on H₂O₂; and donepezil on A β (25–35) (25 μ M) induced ROS production. PC12 cells pretreated with saffron extract (2.5–40 μ g/ml), essential oil (2.5–40 μ g/ml), safranal (2.5–40 μ M) and donepezil (5, 10, 20 μ M) for 2 h then exposed to A β (25–35) (25 μ M) or H₂O₂ (150 μ M) for 24 h. The data presented as the mean \pm SEM of three independent experiments (n = 9). *** P < 0.001, ** P < 0.01 and * P < 0.05 compared with A β (25–35) (25 μ M) or H₂O₂ (150 μ M)

Effect of amyloid beta and safranal on apoptosis signaling proteins

To determine the whether the protective effect of safranal mediated through inhibition of apoptosis, the amounts of Cyt c, survivin, p44/42 MAPK (ERK1/2), Phospho-p44/42 MAPK (ERK1/2), PI3 Kinase P85, Phospho-PI3 Kinase P85, phospho SAPK/JNK, SAPK/JNK and caspase 3 were compared in PC12 cells treated with A β (25–35) or safranal. In the end, the amount of each protein compared with the related control after western blot analysis.

The results showed that treatment with A β (25–35) (25 μ M) for 48 h significantly increased Cyt c (P < 0.001), and cleaved caspase 3 (P < 0.001) whereas pretreatment with safranal (2.5 μ M) markedly decreased Cyt c (P < 0.001), and cleaved caspase 3 (P < 0.01 and P < 0.001). Also, A β (25–35) (25 μ M) increased phospho SAPK/JNK to SAPK/JNK compared with the control group (P < 0.05), and pretreatment with safranal (2.5 μ M) inverted the A β (25–35) induced apoptosis. A β (25–35) (25 μ M) reduced survivin and the ratio of Phospho-p44/42 MAPK (ERK1/2) to p44/42 MAPK (ERK1/2) and increased Phospho-PI3 kinase p85/p55 to PI3 kinase p85/p55 compared

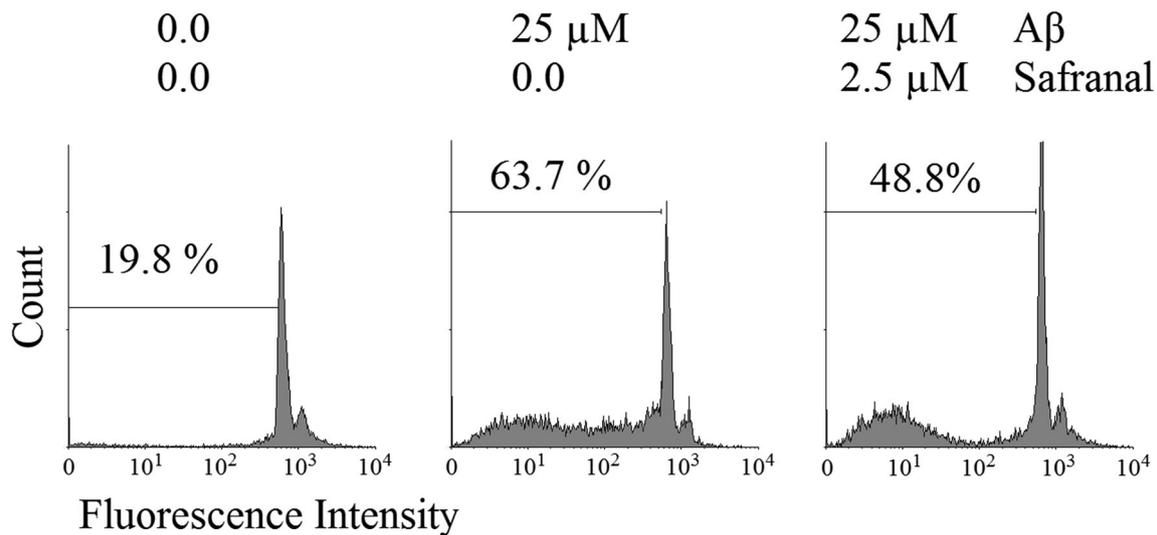


Fig. 4 PI staining and flow cytometry analysis of protective activity of safranal (2.5 μM) on Aβ_(25–35) (25 μM) induced apoptosis in PC12 cells. PC12 cells pretreated with safranal (2.5 μM) then exposed to Aβ_(25–35) for 48 h

with the control group ($P < 0.05$ and $P < 0.001$), and safranal (2.5 μM) protect against apoptosis ($P < 0.05$ and $P < 0.01$) (Figs. 5 and 6).

Discussion

In the present study, we have investigated the protective effect of extract, essential oil and safranal as one of the most effective components of saffron on Aβ-induced cytotoxicity in

PC12 cells. To clarify the hypothesis, the protective activity of extract, essential oil and safranal on cell survival, cell apoptosis, and the ROS level evaluated. PC12 neuronal cells originate from a rat pheochromocytoma are extensively used as suitable in vitro model of neural injuries which has the similar morphologic and physiologic features of neural cells (Westerink and Ewing 2008).

In this study, safranal up to 40 μM does not show any toxicity compared to the control group. While Aβ_(25–35) decreased the cellular survival, safranal (2.5 μM) and donepezil

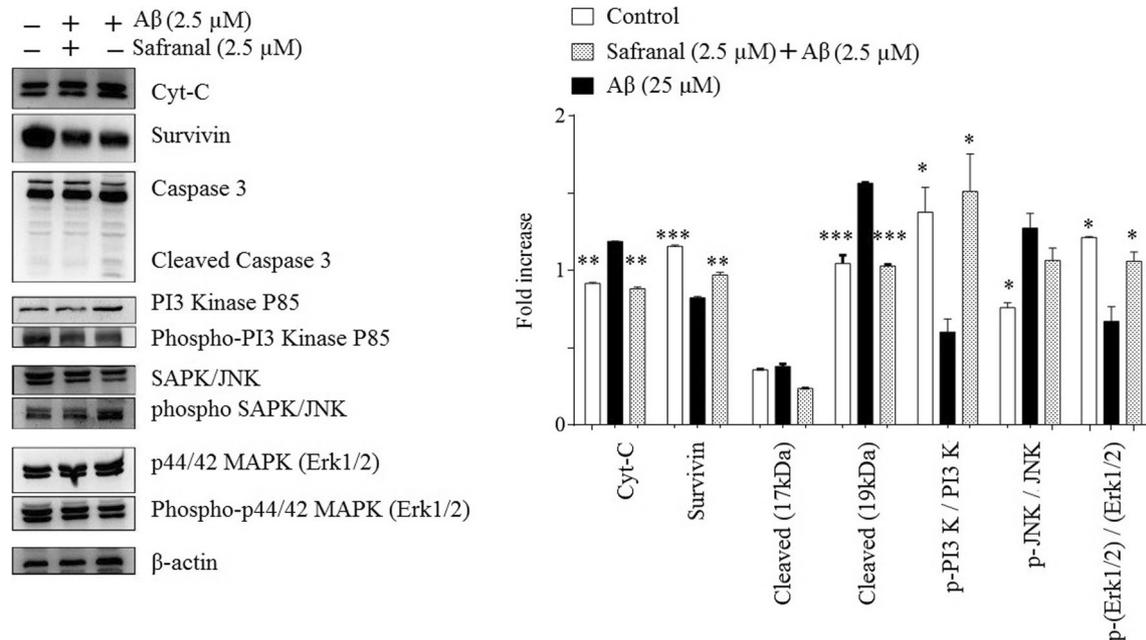


Fig. 5 Western blot analysis of apoptosis signaling proteins on PC12 cells. About 10⁶ PC12 cells were treated with safranal (2.5 μM) for 2 h then exposed to Aβ_(25–35) (25 μM) for 48 h. β-actin used as a control. Images were quantified using Gel-pro Analyzer V.6.0 Gel Analysis

Software. The data presented as the mean ± SEM of three independent experiments ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with Aβ_(25–35)

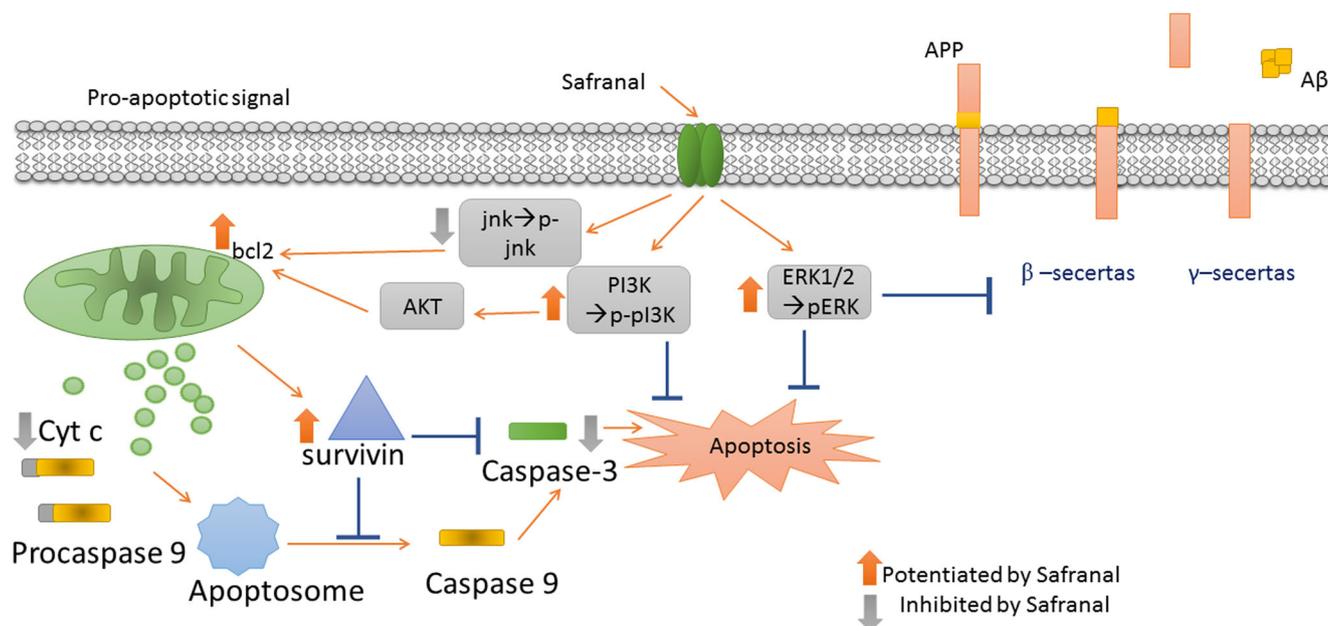


Fig. 6 Schematic representation of the protective role of safranal on A β -induced cytotoxicity. A β (25–35) induce apoptosis through PI3K/AKT and the MAPK/ERK pathway. Also, A β (25–35) activated the intrinsic pathway of apoptosis

(10 and 20 μ M) increased the cell viability. Treatment with H₂O₂ (150 μ M) and A β (25–35) for 24 h elevated the fluorescence intensity of DCFH-DA in cells compared with the control group which inhibited with safranal (2.5–40 μ M) and donepezil (5, 10, 20 μ M). A similar effect was seen when cells pretreated with saffron essential oil (2.5 μ g/ml) and saffron extract (2.5–40 μ g/ml).

ROS begins the cascade of cell death in a pathway of oxidative stress. One of most common injuries of the neurons in AD is oxidation of macromolecules with progressing peroxidation of lipids. Lack of controlling anti-oxidative system promote the oxidation cascade in the brain and sensitize the cell to oxidative stress. Also, the decrease in the level of antioxidant enzymes has also reported in the brain of the AD patients. Factors such as the accumulation of metal, dysfunction of mitochondria, the hyper phosphorylation of tau, deposition of A β and inflammation proposed as the inducers for oxidative stress. Notably, increase in ROS level stimulates the hyperphosphorylation of tau and deposition of A β (Chen and Zhong 2014). Phytochemicals target multiple pathways and act as the potent antioxidant. Beside antioxidative agents, curcumin, resveratrol, and green tea catechins serve as anti-apoptotic, anti-inflammatory, anti-amyloidogenic, and AChE inhibitors in the treatment AD (Freitas et al. 2018; Russo et al. 2013). The presence of phenolics and terpenoids as frequently used antioxidant in saffron increased the antioxidant properties of the plant. Extensive researches both in vitro and in vivo have been proved the antioxidant properties of saffron extract and its active ingredients such as safranal, crocin, crocetin, and carotene (Rahaiee et al. 2015; Hashemi-Shahri et al. 2017). In particular, Safranal reduces oxidative damage in the hippocampus of the ischemic rats and has protective effects against

various oxidative markers (Hosseinzadeh and Sadeghnia 2005). In another study, supplementation with saffron (100 mg/kg) in people with metabolic syndrome was shown to be effective in modifying the serum levels of pro-oxidant-antioxidant balance (PAB) patients and the use of saffron for 12 weeks led to an equilibrium between oxidants and the antioxidant (Kermani et al. 2015). Additional to antioxidant activity, it has been shown that saffron and crocin could decrease the deposition and fibrillation of A β and prevent the toxicity of A β (Papandreou et al. 2006). Similarly, in the present study safranal, saffron essential oil and its extract suppress free radicals and, protected PC12 cells against H₂O₂. Additionally, cell apoptosis induced by A β decreased following treatment of cells with 2.5 μ M safranal which shows the anti-apoptotic properties of safranal.

There is a report on the protective activity of donepezil as a cholinesterase inhibitor on PC12 cells which helps in the effectiveness of the drug in the treatment of Alzheimer's disease (Cai et al. 2017). Donepezil has been accepted as a standard treatment of Alzheimer's disease to prevent memory impairment (Yamada et al. 2005) which may prevent the free radical-mediated neuroinflammation (Umukoro et al. 2014). In this study, donepezil as a positive control reversed the toxicity of A β (25–35) also decreased the ROS level in A β (25–35) exposed cells.

A β , has been proved to induce apoptosis through multiple pathways (Stadelmann et al. 1999). Considering the crucial role of mitogen-activated protein kinase (MAPK) pathways (the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 pathways) in the pathogenesis of AD, implicating of chemicals to regulate the MAPK pathways has been suggested for the treatment of disease (Zhu et al.

2002). Strong evidences support the role of JNK and the entire ERK Pathway in neural cell death occurred in the brain of AD patients (Zhu et al. 2002). Our results showed that A β (25 μ M) activated JNK, while safranal decreased the ratio of Phospho SAPK/JNK to SAPK/JNK and reduced cell death. In spite of the large number of studies on the anti-apoptotic effects of activation and phosphorylation of ERK1/2, there is some evidence that constituent activation of ERK1/2 leads to cell death (Zhang and Liu 2002). Our results showed that A β (25 μ M) reduced Phospho-p44/42 MAPK (ERK1/2) to p44/42 MAPK (ERK1/2), while safranal increased the ratio of Phospho-p44/42 MAPK (ERK1/2) to p44/42 MAPK (ERK1/2) and significantly reduced cell death (Figs. 5 and 6). The expression of the enzyme β -secretase regulated by ERK1/2 and controls the A β production (Tamagno et al. 2009). It seems that when the cascade of neural degeneration by A β initiated, inhibition of ERK1/2, increases the activity of β -Secretase and induction of apoptosis promote cells to death. The PI3K pathway, activated by many survival factors and activate the Akt. Once activated, Akt phosphorylates and inhibits the activity of Bcl-2 family members such as Bax as pro-apoptotic factor and caspase-9. The role of Bcl-2 family on the outer membrane of mitochondria is to protect the organelle against apoptosis and the release of Cyt c and activation of caspase 9 (Griffin et al. 2005). Our results showed that A β (25 μ M) reduced the ratio of Phospho-PI3 kinase p85/p55 to PI3 kinase p85/p55 while safranal (2.5 μ M) protected against apoptosis. Cyt c is a pro-apoptotic protein which released from mitochondrial. The release of Cyt c, in turn, activates cysteine protease caspase 9 and caspase 3 and caspase 7, which are responsible for promoting apoptosis (Brentnall et al. 2013). The results of this study show that A β (25 μ M) increased the amount of Cyt c protein while safranal (2.5 μ M) had the inhibitory effect on the release of Cyt c. Also A β (25 μ M) could increase the cleaved form of caspase-3 and safranal (2.5 μ M) could attenuate these changes. Beside the mentioned changes safranal (2.5 μ M) increased survivin level relative to the A β treated cells and function as a protective agent in A β induced-toxicity (Fig. 6).

Conclusions

In summary, our study revealed the A β (25–35) induce apoptosis through PI3K/AKT and the MAPK/ERK pathway. Also, A β (25–35) activated the intrinsic pathway of apoptosis. Safranal protects against A β (25–35)-induced apoptosis in PC12 cells and may exert promising potential for the treatment of Alzheimer's disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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