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Original Research Article

Safranal induces autophagy by AMPK activation and protects neurons against amyloid beta in Alzheimer's disease

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Abstract

Purpose: *To investigate autophagic induction by safranal and neuroprotection against amyloid beta in Alzheimer's disease.*
Methods: *Primary neurons and SH-SY5Y cells were used in this study. Assessment of cell proliferation and neuroprotection by safranal against amyloid beta was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. AMPK activation and mTOR inhibition were determined by western blot. Changes in intracellular calcium level, reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were assessed by flow cytometry.*
Results: *Safranal protected neurons against amyloid beta toxicity. Furthermore, safranal activated AMPK pathway by activation of calcium/calmodulin-dependent protein kinase (CaMKKβ) to induce autophagy in both cell lines. The toxicity induced by amyloid beta in primary neurons and SH-SY5Y cells were attenuated by safranal. Moreover, amyloid beta-induced calcium levels were significantly decreased by safranal while ROS and MMP loss produced by amyloid beta was attenuated by safranal.*
Conclusion: *These findings suggest that safranal protects neurons against amyloid beta by inducing autophagy via AMPK pathway. Therefore, safranal is a probable therapeutic target for Alzheimer's disease.*

Keywords: *Amyloid beta, Primary neuronal cells, Autophagy, AMPK, LC3-II, Neuroprotection*

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease which happens in old age [1]. The accumulation of amyloid beta and hyperphosphorylation of tau are the main pathological hall markers of AD [2]. Senile plaque comprises amyloid beta protein which plays a role in progression and development of AD [3]. Even though exact mechanism of amyloid beta induced toxicity remains obscure, few studies provide evidence that amyloid beta induced toxicity is mediated by loss of MMP and ROS [4].

Various reports show that oxidative stress is caused by extracellular amyloid beta which leads to the dysfunction of mitochondria [5]. Furthermore, it was shown by in vivo studies that amyloid beta leads to structural abnormalities

that accumulated in the mitochondria of Alzheimer's disease brain [6]. Therefore, these events trigger the activation of apoptotic proteins and cytochrome C release from mitochondria [7]. Although there are four drugs available in the market that give symptomatic benefit, but there is no drug in the market that prevents Alzheimer's disease [8]. Efforts are being made by researchers to discover natural compounds that is potent inhibitors of neuronal loss and provides a neuroprotective role [9,10,14]. Natural products exert antioxidant potentials through various pathways of signal transduction [11,12].

Safranal, a natural compound extracted from *crocus sativas* has an anticancer property [13]. Moreover, safranal has an antioxidant property as it scavenges free radical and protects against gastric cancer induced by indomethacin [14]. Safranal has been found to be cytotoxic against specific cancers and it has also been proved in animal model for anti- depressant property as well as slows down degeneration of photoreceptor [15,16].

In this study, we have screened 500 natural compounds for neuroprotection against amyloid beta in SH-SY5Y cells. Among all the compounds, safranal has been found to be neuroprotective against amyloid beta in SH-SY5Y cells. Interestingly, it has been observed that activation of AMPK leads to the inhibition of mTOR which further leads to the activation of autophagic proteins.

EXPERIMENTAL

Chemicals and reagents

Dulbecco's Minimal Essential medium, trans-retinoic acid, BSA, Penicillin G, Streptomycin sulphate, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), phosphate buffer saline (PBS), RIPA (Radioimmunoprecipitation assay buffer), Sodium pyruvate, safranal, d-glucose, rhodamine-123, amyloid beta Anti-Phospho LKB1(1: 1000) and Anti-Phospho AMPK (1: 1000) were obtained from Sigma-Aldrich Fluo-3AM, HEPES, fetal bovine serum , L-15 media, neurobasal media, Glutamax, B27 was obtained from Invitrogen . Immobilon Western Chemiluminescent HRP substrate and PVDF membrane was obtained fromMillipore Anti-ATG7 (1: 1000), ATG12 (1: 1000), mTOR (1: 1000), LC3-II (1: 1000), SQSTM1 (1: 1000) and Anti-Actin (1: 1000) antibodies was from cell signalling technology (Suite 514 No. 1101 South Pu Dong Road, Shanghai, 200120). In all experiments we have used analytical grade reagents as well as chemicals.

Cell culture

Human neuroblastoma SH-SY5Y cells were purchased from ATCC and were cultured in DMEM with sodium pyruvate, glutamine, streptomycin, penicillin, 10% FBS maintained with a humidified condition 95 % O_2 with 5 % CO_2 at 37 °C. Retinoic (10 µM) with 5 % FBS were used for differentiation of SH-SY5Y cells.. For every two days media was decant and replaced with fresh media supplemented with 5 % FBS and 10 µM retinoic acid. For drug treatments in case of neuroprotection assay, cells at a confluence of 70 % were pre-treated with safranal followed by amyloid beta treatment at indicated time periods and concentrations.

Isolation of primary neuronal cells

For isolation of primary neuronal cells *Balb/c* female mice were sacrificed after 18 days of gestation, decapitated and whole brain was isolated in ice-cold L15 media with 0.75 % glucose, HEPES 25 mM, 100 mg/L streptomycin and 100 mg/L penicillin. The dissociation of brain was done enzymatically with papain (1 mg/mL) in hibernated media in an incubator at 37 °C for 30 min. The brain sample was tapped every 10 min. The cells were passed through fire coated pipette to make cell suspension. Cells were resuspended into neurobasal media and were passed through cell strainer 40-µm in order to remove cell debris. Cells at a density of ~106 were seeded into each 6 well of poly-l-lysine coated 6 well plates. For every day half of the media was decanted and replaced with fresh neurobasal media until the culture period ends.

Neuroprotection and cell viability assay

Neuroprotection assay was performed by MTT assay in which differentiated SH-SY5Y cells and primary neurons were grown in 96 well plates and were pre-treated with safranal for 24 h at a concentration of 5, 10, 20, 40, 60, 80 and 100 µM followed by amyloid beta (20 µM). Therefore the total treatment is for 48 h. Before 4 h of termination of experiment MTT was added and media were decanted followed by DMSO (150 µL) into each well. Absorbance was taken on a plate reader at 570 nM. Cell viability assay was performed by MTT assay, in which differentiated SH-SY5Y cells and primary neurons were grown in 96 well plates. These cells were treated with safranal at a concentration of 1, 5, 10, 20, 40, 60, 80 and 100 µM for 48 h and 72 h. Before 4 h of termination of experiment, MTT were added as above in neuroprotection assay.

Western blot

Differentiated SH-SY5Y cells and primary neurons were treated with safranal at a concentration of 5, 10 and 20 µM for 48 h. These Cells were lysed with RIPA buffer containing 2 % protease inhibitor cock tail, 150 mM NaCl 1mM Na3VO4, 5 mM EDTA, 1mM PMSF for 45 min in ice and vortexed for every 10 min. Lysed cells were centrifuged at 1600 g for 15 min and supernatant containing proteins were collected followed by protein estimation by Bradford method. Protein loading dye (2X) was added to each protein sample.

Protein samples of 70 µg were loaded into each well of SDS PAGE and were run for 3 h at 85 V; the gels were transferred into PVDF membranes for 2 h at 100 V. Protein membranes were blocked with skimmed milk for 1 h at room temperature.. Primary antibodies were added overnight to each protein membrane at 4 °C followed by three times TBST washing for 5 min and secondary antibodies were added into membrane at room temperature for 1 h. Blots were incubated with Millipore Immobilon western chemiluminescent HRP substrate, before being analyzed for signal on either x-ray film or chemidoc system.

Determination of intracellular calcium level

Human neuroblastoma SH-SY5Y cells were grown for indicated time periods in well plates. Safranal were pre-treated to cells a concentration of 5, 10 and 20 µM for 24 h. After 24 h, amyloid beta (30 µM) was added cells for 24 h. i.e. total treatment is for 48 h. Cells were treated with FLU 3A dye (5 µM) for 30 min into each well at 37 °C, followed by centrifugation. Unbound dye were removed by three times washed with incomplete DMEM media and these cells were re-suspended in DMEM incomplete media and subjected to flow cytometer (BD FACS Calibur BD Biosciences, San Jose, California) to measure FLU 3A dye fluorescence intensity bound to calcium.

Measurement of reactive oxygen species level

Differentiated SH-SY5Y cells were pre-treated with safranal at a 5, 10 and 20 µM followed by amyloid beta at a concentration of 30 µM for a further 24 h. DCFHDA at a concentration of 10 µM was added into each well for 30 min before termination of experiment. Cells were washed and trypsinized and centrifuged at 400 g for 5 min. Further, cells were re-suspended into PBS and were subjected to flow cytometer (BD FACS Calibur *BD* Biosciences, San Jose, California) to measure florescence intensity of DCFHDA dye.

Analysis of mitochondrial membrane potential

MMP was monitored by flow cytometer by using JC-1. Briefly, differentiated SH-SY5Y cells were treated with safranal at a concentration of 5, 10 and 20 µM were added into each well for 48 h and amyloid beta for 24 h in presence and absence of safranal. JC-1 dye was added was added 30 min before termination of the experiment. Cells were washed with PBS and were analyzed by flow cytometry (BD FACS Calibur).

Statistical analysis

Data are presented as mean ± SD (n = 3). Statistical analysis was done by Students' t test and differences were considered statistically significant at $*p < 0.05$, $**p < 0.01$ or $***p < 0.005$. Origin 8.1 and SPSS 16.0 (SPSS, Inc. Chicago, IL, USA) were used for statistical analysis.

RESULTS

Safranal protects SH-SY5Y cells and primary neuronal cells against amyloid beta

Furthermore, safranal protected SH-SY5Y cells and primary neuronal cells at concentrations of 5, 10, 20, 40 and 60 µM against amyloid beta induced toxicity as the viability of cells treated with safranal only increased for 48 h (Figure 1 A). Moreover, the viability of cells remains constant after 20 µM. Furthermore, toxicity of safranal was not found at 1, 5, 10, 20, 40, 60, 80 and100 µM concentrations at 48 h in differentiated SH-SY5Y and primary neuronal cells (Figure 1 B).

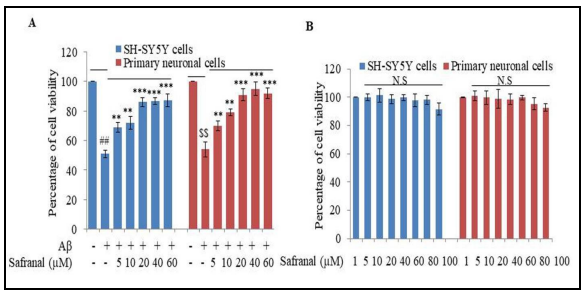

Figure 1: Effect of safranal on amyloid beta induced toxicity in SHSY5Y cells and primary neuronal cells. (A-B) Differentiated SH-SY5Y cells and primary neurons were treated with safranal at a concentration of 5, 10, 20, 40 and 60 µM followed by amyloid beta treatment for 48 h. MTT assay was determination for cell proliferation; n = 3

Safranal induces autophagy by activating AMPK pathway

Differentiated SH-SY5Y cells and primary neurons were treated with safranal at 5, 10 and 20 µM for 48 h. The expression level of LKB1 remained constant in safranal (5, 10 and 20 µM) treated cells compared with untreated cells. Interestingly, the expression of CaMKKβ was gradually increased with increased concentration of safranal (5, 10 and 20 µM) for 48 h. Furthermore, CaMKKβ promoted phosphorylation of AMPK at thr-172 which led to the inhibition of mTOR. Therefore, inhibition of mTOR leads to the activation of autophagy which was evident by the expression of LC3-11 level and down regulation of SQSTM1. The expression of other autophagic proteins like ATG7 and ATG12 were increased in cells by safranal at 5, 10 and 20 µM for 48 h (Figure 2 A, B). Thus, safranal activates AMPK by CaMKKβ which further induces autophagy through mTOR inhibition.

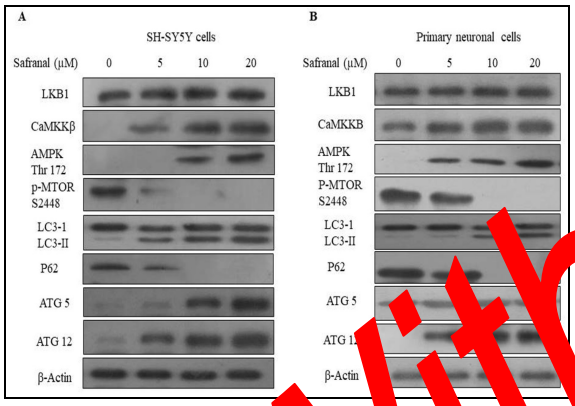

Figure 2: Safranal induces autophagy by AMPK activation. (A, B) Differentiated SH-SY5Y and primary neuronal cells were treated with 5, 10 and 20 µM for 48 h in which the expression of CaMKKβ increased which leads to the activation of p-AMPK (Thr 172) and inhibition of p-MTOR (S2448) while the autophagic marker LC3-II lipidation is observed and P62 degradation takes place in SH-SY5Y cells and primary neuronal cells

Safranal attenuates intracellular calcium level induced by amyloid beta in SH-SY5Y cells

Amyloid beta increases the intracellular calcium level of the cells which further leads cells towards death. Based on then neuroprotection data, we hypothesised that safranal can reduce the intracellular calcium level induced by amyloid beta. Cells were pre-treated with safranal at 5, 10 and 20 µM for 24 h and after 24 h amyloid beta was added in the presence and absence of safranal at 5, 10 and 20 µM. Surprisingly, intracellular calcium levels were decreased with co-treated cells (amyloid beta and safranal) compared with amyloid beta alone as shown through flow cytometer (Figure 3). Safranal at a concentration of 5, 10 and 20 µM decreased relative fluorescence intensity which was increased by amyloid beta alone.

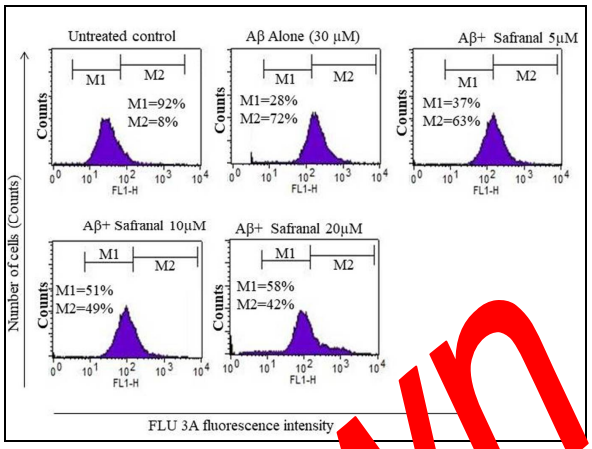

Figure 3: Effect of safranal on intracellular calcium level in SH-SY5Y cells. Intracellular calcium is elevated by amyloid beta which is decreased by safranal at a concentration dependent manner for 48 h. Intracellular calcium level was detected by FLU-3A dye at a concentration of 5 µM; n = 3

Reactive oxygen species elevated by amyloid beta is attenuated by safranal in SH-SY5Y cells

Amyloid beta elevated the reactive oxygen species at a concentration of 30 µM which was decreased by safranal at a concentration of 5, 10 and 20 µM for 48 h in SH-SY5Y cells (Figure 4). Safranal decreases the relative fluorescence intensity of DCFHDA dye which was increased by amyloid beta as observed by flow cytometer. Therefore it is clearly evident from the results that reactive oxygen species generated by amyloid beta is attenuated by safranal.

Safranal increased mitochondrial membrane potential after exposure of Amyloid beta in SH-SY5Y cells

Amyloid beta decreased the mitochondrial membrane potential of SH-SY5Y and primary brain neuronal cells which was attenuated by safranal at concentrations of 5 µM, 10 µM and 20 µM. The relative fluorescence intensity ofrhodamine 123 was decreased in amyloid beta treated cells which were further increased by safranal as evaluated by flow cytometer (Figure 5). Therefore, these results are clear evidence that mitochondrial membrane potential loss by amyloid beta is attenuated by safranal.

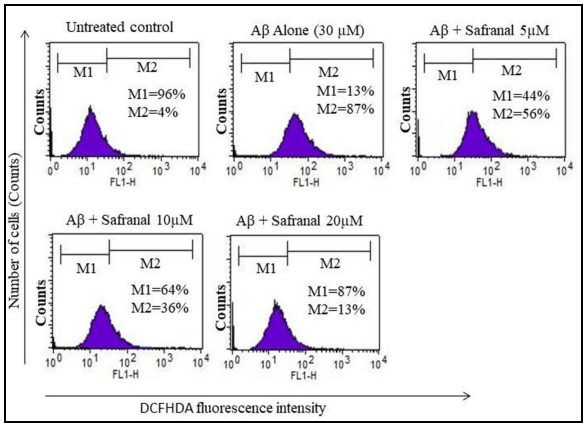

Figure 4: Effect of safranal on reactive oxygen species induced by amyloid beta in SH-SY5Y cells. The reactive oxygen species generated by amyloid beta was reduced by safranal at a concentration of 5, 10 and 20 μM for 48 h. DCFDA at a concentration of 20 μM was used to capture the fluorescence through flow cytometer; n = 3

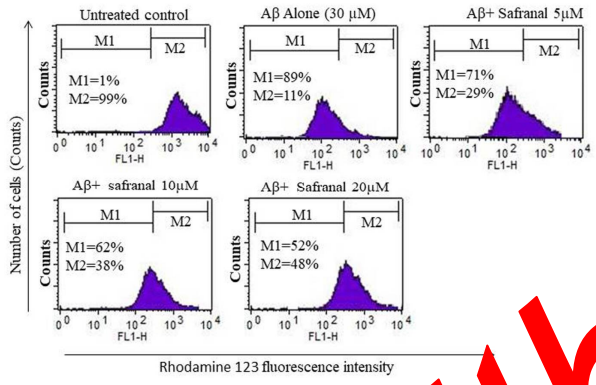

Figure 5: Mitochondrial membrane potential loss by amyloid beta is attenuated by safranal in SH-SY5Y cells**.** Loss of mitochondrial membrane potential by amyloid beta was attenuated by safranal at 10 and 20 μM for 48 h. Rhodamine 123 at a concentration of 500 nM was used for 45 min and fluorescence intensity was captured by flow cytometer; n = 3

DISCUSSION

Crocus sativus (saffron) is used for colour and taste as a food additive across the world [17]. It is a well-established fact that saffron has anticancer activity *in vitro* and *in vivo* against various cancers [18]. It has been shown that saffron has neuroprotective effect in various neurodegenerative diseases [19]. Safranal, a natural compound derived from *Crocus sativus*, has shown notable anti-cancer activity both *in vivo* and *in vitro* in tissue cultures [18]. Over the past years, it has been shown that safranal plays various roles in neurodegenerative diseases by providing neuroprotection via various mechanisms such as anti-apoptotic, edema attenuating and anti-inflammatory activities in a rat model of traumatic injury [20,21].

Safranal has been proposed as a potent antioxidant agent that affords cytoprotection [22]. It has been reported for the first time in this study that AMPK is activated by CaMKKβ in safranal treated SH-SY5Y and primary cells. Furthermore, AMPK activation leads to the inhibition of mTOR which further leads to autophagic induction as shown by the autophagic marker, LC3-II and P62. The results also showed that safranal attenuates the mitochondrial membrane potential loss induced by amyloid beta but decreases the reactive oxygen species induced by amyloid beta. Furthermore, safranal protected SH-SY5Y cells against amyloid beta toxicity. Therefore, safranal should be further investigated as a potent therapeutic target in Alzheimer's di[illegible].

CONCLUSION

Amyloid beta is a well-established neurotoxin which generates reactive oxygen species, and increases calcium level and loss of mitochondrial membrane potential, ultimately leading to neuronal cell death. Safranal protects neuronal cells against amyloid beta toxicity which ultimately reduces ROS, calcium level and mitochondrial membrane potential loss. The neuroprotection provided by safranal occurs via AMPK pathway against beta amyloid. Therefore, safranal may be suitable for use in clinical practice to provide protection against amyloid beta in Alzheimer's disease.

DECLARATIONS

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Conflicts of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaocheng Huang designed all the experiments and revised the manuscript. Zanlian Zhu and Ying Hua performed the experiments, Xiumei Yan

designed flow cytometer experiments. Ruilai Jiang designed and wrote the manuscript.

REFERENCES

1. Johnson IP. Age-related neurodegenerative disease research needs aging models. Front Aging Neurosci. 2015; 7: 168.
2. Huang HC, Jiang ZF. Accumulated amyloid-β peptide and hyperphosphorylated tau protein: relationship and links in Alzheimer's disease. J Alzheimers Dis. 2009; 16(1): 15-27.
3. Sadigh-Eteghad S, Sabermarouf B, Majdi A, Talebi M, Farhoudi M, Mahmoudi J. Amyloid-beta: a crucial factor in Alzheimer's disease. Med Princ Pract. 2015; 24(1): 1-10.
4. Du H, Guo L, Yan S, Sosunov AA, McKhann GM, Yan SS. Early deficits in synaptic mitochondria in an Alzheimer's disease mouse model. Proc Natl Acad Sci USA. 2010; 107(43): 18670-18675.
5. Mao P, Reddy PH. Aging and amyloid beta-induced oxidative DNA damage and mitochondrial dysfunction in Alzheimer's disease: implications for early intervention and therapeutics. Biochim Biophys Acta. 2011; 1812(11): 1359-1370.
6. Reddy PH. Amyloid beta, mitochondrial structural and functional dynamics in Alzheimer's disease. Exp Neurol. 2009; 218(2): 286-292.
7. Santos RX, Correia SC, Wang X, Perry G, Smith MA, Moreira PI, Zhu X. Alzheimer's disease: diverse aspects of mitochondrial malfunctioning. Int J Clin Exp Pathol. 2010; 3(6): 570.
8. Cummings J, Lee G, Mortsdorf T, Ritter A, Zhong K. Alzheimer's disease drug development pipeline. Alzheimers Dement. 2017; 3(3): 367-384.
9. Bagli E, Goussia A, Moschos MM, Agnantis N, Kitsos G. Natural compounds and neuroprotection: mechanisms of action and novel delivery systems. In Vivo. 2016; 30(5): 535-547.
10. Essa MM, Vijayan RK, Castellano-Gonzalez G, Memon MA, Braidy N, Guillemin GJ. Neuroprotective effect of natural products against Alzheimer's disease. Neurochem Res. 2012; 37(9): 1829-1842.
11. Ng TB, Liu F, Wang ZT. Antioxidative activity of natural products from plants. Life Sci. 2000; 66(8): 709-723.
12. Nogueira CC, Paixão IC, Teixeira VL. Antioxidant activity of natural products isolated from red seaweeds. Nat Prod Commun. 2014; 9(7): 1031-1036.
13. Mardani H, Sekine T, Azizi M, Mishyna M, Fujii Y. Identification of safranal as the main allelochemical from saffron (Crocus sativus). Nat Prod Commun. 2015; 10(5): 775-777.
14. Samarghandian S, Azimi-Nezhad M, Samini F. Preventive effect of safranal against oxidative damage in aged male rat brain. Exp Anim. 2015; 64(1): 65-71.
15. Khazdair MR, Boskabady MH, Hosseini M, Rezaee R, Tsatsakis AM. The effects of Crocus sativus (saffron) and its constituents on nervous system: A review. 2015; Avicenna J Phytomed. 5(5): 376.
16. Fernandez-Sánchez L, Lax P, Esquiva G, Martín-Nieto J, Pinilla I, Cuenca N. Safranal, a saffron constituent, attenuates retinal degeneration in P23H rats. PLoS One. 2012; 7(8): e43074.
17. Milajerdi A, Djafarian K, Hosseini B. The toxic[illegible] saffron (Crocus sativus L.) and its constituents against normal and cancer cells. J Cell Biochem. 2016; 3: 23-32.
18. Samarghandian S, Shoshtari MB, Sargolzaei J, Hossinimoghadam H, Farahzad JA. Anticancer activity of safranal against neuroblastoma cells. Pharmacogn Mag. 2014; 10(2): S415.
19. Fernandez-Sánchez L, Lax P, Noailles A, Angulo A, Maneu V, Cuenca N. Natural compounds from saffron and their preventive visual loss and retinal degeneration. Molecules. 2010; 15(8): 13875-13893.
20. Samarghandian S, Samini F, Azimi-Nezhad M, Farkhondeh T. Antioxidative effects of safranal on immobilization induced oxidative damage in rat brain. Neurosci Lett. 2017; 659: 26-32.
21. Zhang C, Fan L, Zou Y, Dang X, Wang K, Song J. Neuroprotective effects of safranal in a rat model of traumatic injury to the spinal cord by anti-apoptotic, anti-inflammatory and edema-attenuating. Tissue Cell. 2015; 47(3); 291-300.
22. Nassiri-Asl M, Hosseinzadeh H. Neuropharmacology effects of saffron (Crocus sativus) and its active constituents. In: Bioactive Nutraceuticals and Dietary Supplements in Neurological and Brain Disease; 2015; pp 29-39.