

# Neuroprotective and Antiapoptotic Effects of N-acetylcystein and *Crocus sativus* Aqueous Extract on Arsenic-induced Neurotoxicity in SH-SY5Y Human Dopaminergic Neuroblastoma Cells

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## ABSTRACT

**Background:** Parkinson's disease is mainly specified by progressive and selective death of dopaminergic neurons. *Crocus sativus* L. (saffron) has been widely used to cure a diverse range of diseases in herbal medicine. **Aim:** The aim of the present study is to investigate the neuroprotective effects of saffron on arsenic-induced neurotoxicity, which was performed in human neuroblastoma SH-SY5Y cell line as an *in vitro* model of Parkinson's disease and to confirm whether the neuroprotective effects of saffron on Parkinson's disease are mainly due to their interactions with antioxidant systems. Moreover, as the antioxidant effect of N-acetylcysteine and its effectiveness as an antioxidant agent are proven, we compared saffron with NAC to control saffron extract. **Materials and Methods:** The induction of cell damage was done by arsenic and the survival of the cells was measured using the MTT assay. In addition, the assessment of the generation of intracellular reactive oxygen species (ROS) and mitochondrial membrane potential was done using fluorescence spectrophotometry method. Furthermore, immunoblotting analysis was performed to precisely determine the biomarkers level for apoptosis in the cells. **Results:** Our study indicated that arsenic had the ability to decrease cells survival rate, enhance the loss of mitochondrial membrane potential and increase the levels of intracellular ROS, c-Fos ratio and caspase-3. Pretreatment of cells with NAC (5 mM) and saffron aqueous extract (10mg/ml) significantly attenuated the mentioned effects in arsenic-treated cells. **Conclusion:** The outcome of the study has shown that the protective effects of NAC and saffron aqueous extract are produced by their antioxidant and anti-apoptotic properties and their therapeutic potential is demonstrated in the treatment of Parkinson's disease.

**Key words:** Parkinson Disease, N-acetylcysteine, Saffron, Arsenic, SH-SY5Y cells.

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## INTRODUCTION

Among neurological disorders, Parkinson's Disease (PD) is one of the most common in today's world which occurs with the progressive and selective death of dopaminergic neurons in the basal complexes.<sup>1</sup> Parkinson's disease is included in the category of neurodegenerative diseases, while its prominent features include gradual

progressive muscular stiffness, tremor, loss of motor skills and consequently a significant disability, which all lead to a decline in the quality of life. Parkinson's disease is a mostly age-related disorder that threatens about 0.3% of the overall population around the world, 1% of people in their 60's and 4-5% of people over 85.<sup>2</sup>



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One of the most commonly used cell lines for studying PD as an *in vitro* model is human neuroblastoma SH-SY5Y cell. Therefore, we used this cell line in this study as it is a dopaminergic neural cell line and can be used to monitor the protective and therapeutic effects of various drugs.<sup>3</sup>

According to the epidemiological studies, environmental factors have a significant role in neurodegenerative damage. Exposure to pesticides, metals, biphenyl perchlorate, some solvents and some other substances increase the risk of PD.<sup>4</sup> In this regard, arsenic as one of the most toxic environmental stimuli makes millions of people worldwide suffer from PD due to the spread of this toxic metalloid contamination in the environment. Studies on the prevalence of PD in the developing countries have been shown that the contamination of groundwater with this poison has a direct relationship with the recognition of a massive number of people diagnosed with PD.<sup>5</sup> It has been also reported that the penetration of arsenic into agricultural products, fertilizers and pesticides can be the main source of arsenic in the environment. In a study in 2007, it was reported that arsenic can either alone or in combination with dopamine cause a dopaminergic cells death.<sup>6</sup> Therefore, this poison was selected in the present study in order to induce cellular death.

In recent years, a great deal of interest has been attracted to the physiological and pharmacological effects of herbal extracts on the treatment of various diseases. Factors such as lesser side effects, various effective compounds, reasonable costs, industrial developments in the cultivation of medicinal plants and recommendations of the World Health Organization for the use of medicinal plants are the reasons for the global approach toward herbal medicine.<sup>7</sup> Many studies have also focused on the effect of herbal extracts on the treatment of neurodegenerative diseases.<sup>8</sup> Recently, there has also been a strong desire to use herbal remedies for pretreatment and treatment of PD.

Saffron (*Crocus sativus* L.) has been widely used as an herbal medicine to promote human health. Four major bioactive compounds of saffron include crocin, crocetin, picrocrocin and safranal which cause its sensory characteristics (in terms of color, taste and aroma) and health improvement properties as well.<sup>9</sup>

Many studies have shown that crocin and crocetin are effective in protecting the brain tissue against several toxic agents, which is thought to be related to their antioxidant capacity. Besides, it has been revealed that saffron extract and its bioactive compounds have anti-inflammatory, anti-atherosclerotic, anti-cancer, anti-genotoxic and cytotoxic effects<sup>10</sup> as well as the

anti-Alzheimer properties.<sup>11</sup> Moreover, its anti-devastating effects have also been demonstrated on some anxiety disorders like the consequence of a seriously traumatic events such as post-traumatic stress disorder.<sup>12-13</sup>

A wide range of reports have suggested that saffron extract and its two main components, crocin and crocetin, can improve memory and learning skills in mice with learning disabilities.<sup>14</sup>

In the present study, the protective effect of saffron aqueous extract was investigated on the neurodegenerative diseases in Parkinson Cell Model, with regard to its antioxidant properties and possible underlying mechanisms. Further, we examined the effectiveness of NAC as an antioxidant agent to control the saffron extract.<sup>15-16</sup>

## MATERIALS AND METHODS

### Cell culture

We purchased human neuroblastoma SH-SY5Y cells from Pasteur Institute of Iran (Tehran, Iran). In order to grow cells, we used Dulbecco's modified Eagle's medium (DMEM) supplemented plus 10% fetal bovine serum (Biosera Co, East Sussex, UK), penicillin (100 U/ml) (Biosera Co, East Sussex, UK) and streptomycin (100 µg/ml) (Biosera Co, East Sussex, UK). To incubate cells at 37°C, a CO<sub>2</sub> incubator (5% CO<sub>2</sub> atmosphere) was used. After two passages, a density of 5000 cells per well was plated in a 96-inch microplate (SPL Lifesciences, Inc, Gyeonggi-Do, South Korea) to carry out the biochemical assays. To extract protein, a 24-hr period of time has been given to cells in order to get attached and grow in a 6-wellplate (SPL Lifesciences Inc, Gyeonggi-Do, South Korea). After that, the cells were incubated with arsenic (Sigma-Aldrich, St Louis, MI, USA) and effective dose of NAC (Sigma-Aldrich, St Louis, MI, USA) and Saffron aqueous extract for 24 hr. Here, 0.5, 1 and 5 mM of NAC and 1, 5 and 10 mg/ml of saffron aqueous extract were selected based on the earlier studies.<sup>16-18</sup> Then, the dose which showed the best protective effect against arsenic was selected as an intervention.

NAC and Saffron aqueous extract were added 3 hr before arsenic.

### Plant material and extract preparation

Saffron (stigmas of *Crocus sativus*) was provided by Talakaran-E- Mazraeh Company (Torbat Heydarieh, Khorasan, Iran) and registered as a voucher specimen (code: P-408) in the Department of Pharmacognosy in the Faculty of Pharmacy in Shahid Beheshti University of Medical Sciences. The extract was obtained as

follows: 100 g of saffron stigmas were dried, milled and then mixed with 1000 ml of distilled water. After that, it was kept for 48 hr at 30-33°C for drying. The yield of extraction was 24 g of dried powder for 100 g of the dried stigma.<sup>19</sup>

### Cell survival analysis

Cell viability was evaluated by the reduction of 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MI, USA) to formazan. After the dissolution of MTT in PBS, it was added to the culture (0.5 mg/ml). Within 2 hr. of extra incubation at 37°C, the media were removed and then DMSO, at the concentration of 100 µl, was added to every single well to determine the absorbance (OD) values at 490 nm with an automatic microplate reader (ELX 808, BioTek, USA). All the experiments were performed at six independent times and the results were demonstrated as percentages of control group.

### Intracellular reactive oxygen species (ROS) formation measurement

2,7-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St Louis, MI, USA) probe and fluorescence spectrophotometry were used in order to assay the intracellular reactive oxygen species (ROS). DCFH-DA is able to be converted into Dichlorofluorescein which a highly fluorescent substance is, only if an appropriate oxidant would be present. The incubation of cells with 1 mM DCFH-DA was placed in a dark room for 10 mins at 37°C. Then, the cells were immediately analyzed on the fluorescence plate reader (FLX 800, BioTek, USA) and washed (three times) with PBS. An excitation of 485 nm and emission of 538 nm were set to quantify the fluorescence intensity of cells in 96-well microplates. All the experiments were performed at six independent times and the results were expressed as fluorescence percentages of control group.

### Mitochondrial membrane potential measurement

Rhodamine 123 (Sigma-Aldrich, St Louis, MI, USA) was used to determine the potential of mitochondrial membrane. On the highly negative mitochondrial membrane potential, rhodamine 123 can be transferred into the active mitochondria based. The loss of rhodamine 123 from the mitochondria and a reduction in intracellular fluorescence were resulted from mitochondrial membrane potential depolarization. Then rhodamine 123 (10 µM) was added after treating the cells with NAC and saffron aqueous extract (as mentioned above) and incubated for 24 hr at 37°C. After 30 mins of incubation at 37°C, the cells were washed and analyzed on the fluorescence plate reader

(FLX 800, BioTek, USA). Finally, an excitation of 540 nm and emission of 570 nm were set to quantify the fluorescence intensity.<sup>20</sup>

### Immunoblot analysis

An ice-cold buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% SDS, 0.1% Na-deoxycholate, 1% NP-40, protease inhibitors and 1 mM sodium orthovanadate) was used to homogenize the cells. After homogenizing, the cells were centrifuged at 14,000g for 15 mins at 4°C and the supernatant was kept as the whole cell fraction. Bradford method (Bio-Rad Laboratories, Muenchen, Germany) was used to measure the protein concentrations. On a 10% SDS-PAGE gel, equal 40 µg of proteins were resolved electrophoretically and then transferred to PVDF membranes (Roche, Germany). After an overnight blocking at 4°C in blocking buffer (5% non-fat dried milk in Tris-buffered saline with Tween 20, pH 7.5), the primary antibodies (1:1000 overnight at 4°C, Cell Signaling Technology, Inc. Beverly, MA, USA) was added to the membrane.

Furthermore, the blots were immediately incubated for 60 mins at room temperature with a horseradish peroxidase conjugated secondary antibody (1: 15,000, GE Healthcare Bio-Sciences Corp. NJ, USA) after they were being washed in TBS-T (three times, each time 5 min). Using the ECL system and exposing to Lumi-Film chemiluminescent detection film (Roch, Germany), we were able to detect the antibody-antigen complexes. In order to analyze the intensity of the protein expression, Image J analyzing software was used. Finally, as a control for loading, we decided to use Beta-actin immunoblotting (1:1000). For each protein, the immunoblot experiments were performed at 4 independent times.

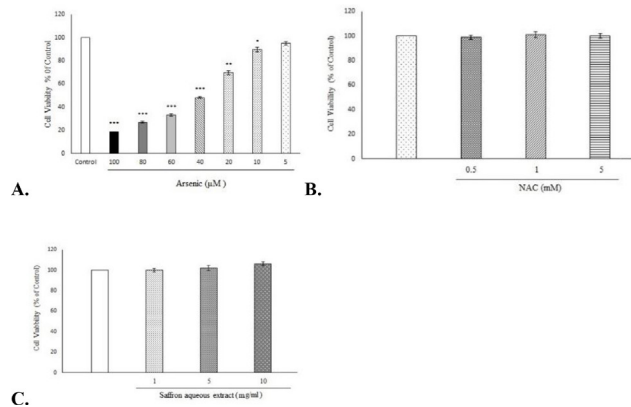
### Statistical analysis

The results were expressed as mean ± SEM. One-way ANOVA was run to indicate the mean differences of MTT, intracellular ROS and mitochondrial membrane potential between experimental groups, followed by Turkey's post hoc tests. Using band densitometry, the values of caspase-3, c-FOS and β-actin band density were obtained, expressed as tested proteins / β -actin ratio for each sample. One-way ANOVA followed by Tukey's post hoc test was also used to compare different groups' averages.  $P < 0.05$  was considered as significant.

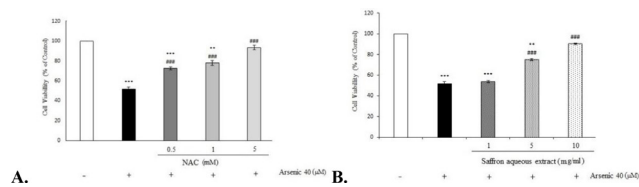
## RESULTS

### Cell viability analysis

To investigate the protective effects of NAC and saffron aqueous extract in SH-SY5Y cells, arsenic was chosen to



**Figure 1: Effects of Arsenic (A), NAC (B) and Saffron aqueous extract (C) on SH-SY5Y cells viability.**



**Figure 2: The protective effects of NAC (A) and Saffron aqueous extract (B) against Arsenic-induced SH-SY5Y cell damage.**

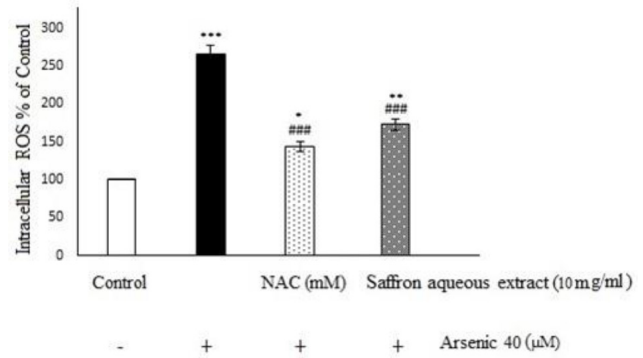
Data are represented as mean ± SEM; \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001 were significantly different versus the control cells.

induce cell damage. According to the result, a significant toxic effect (LD<sub>50</sub>) was observed with 40 µM of arsenic which resulted in 50.11 ± 1.28% of the relative cell viability (Figure 1A). Besides that, it has been revealed that the effect of applying (24 hr) different doses of NAC and saffron aqueous extract on the cultured SH-SY5Y cells survival was not significant (Figure 1B and C). As shown in (Figure 2A and B), 0.5, 1 and 5 mM of NAC and 1, 5 and 10 mg/ml of saffron aqueous extract significantly inhibited arsenic-induced toxicity after 24 h.

Additionally, the results revealed that the administration of NAC at a dose of 5 mM and saffron aqueous extract at a dose of 10 mg had a better effect on protecting cells against arsenic toxicity, compared with the other two doses. Therefore, 5 mM of NAC and 10 mg/ml of saffron aqueous extract were selected to be used in the following phases.

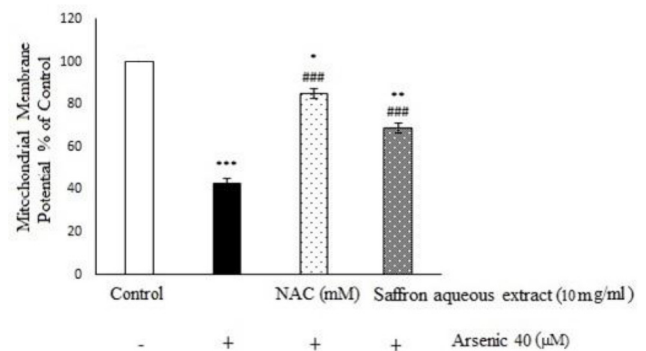
### Intracellular ROS measurement in SH-SY5Y cells

The intracellular ROS levels were measured in control, arsenic-treated and NAC-treated cells and saffron-treated cells. Notably, arsenic exposure of SH-SY5Y cells (2533 ± 95.94) caused an increase in ROS level, comparing to the control group (844 ± 9.73, *P* < 0.001). The very



**Figure 3: The effect of NAC and saffron aqueous extract on ROS level induced by arsenic.**

Data are expressed as mean ± SEM; \*\**P* < 0.001 versus control cells. #*P* < 0.05 and ###*P* < 0.001 versus Arsenic-treated cells.



**Figure 4: The effect of NAC and saffron aqueous extract on the mitochondrial membrane potential depression induced by arsenic.**

Data are expressed as mean ± SEM; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 versus control cells, ####*P* < 0.001 versus Arsenic-treated cells.

increase was significantly attenuated using NAC (1078 ± 16.91) and saffron aqueous extract (1136 ± 13.28) treatment compared to arsenic (*P* < 0.001) (Figure 3).

### Mitochondrial membrane potential determination in SH-SY5Y Cells

As shown in Figure 4, mitochondrial membrane potential dramatically decreased in arsenic-treated SH-SY5Y cells (856 ± 4.19) compared to the control group (2061 ± 5.92, *P* < 0.001). The mitochondrial membrane potential depression was attenuated in NAC (1755 ± 3.92) and Saffron aqueous-treated group (1684 ± 5.66) compared to the arsenic group (*P* < 0.001) (Figure 4).

### Western blot analysis of c-FOS and Caspase-3 in SH-SY5Y cells

As shown in Figure 5. A and B, c-FOS expression level significantly increased in SH-SY5Y cells exposed to arsenic (1.673 ± 0.079) for 24 hr. Comparing with the control group (0.623 ± 0.037, *P* < 0.001). However, the very increase was less in those groups which received



NAC and saffron aqueous extract compared to the arsenic group ( $0.745 \pm 0.063$ ,  $0.768 \pm 0.058$  respectively,  $p < 0.001$ ) (Figure 5 A and B).

In addition, the expression of caspase-3 protein increased in those SH-SY5Y cells with arsenic treatment ( $1.532 \pm 0.084$ ) compared to the control cells ( $0.609 \pm 0.051$ ,  $P < 0.001$ ). Treatment with NAC and saffron aqueous extract ( $0.787 \pm 0.077$ ,  $p < 0.05$  and  $0.804 \pm 0.073$ ,  $P < 0.01$  respectively) significantly reduced the arsenic-induced activation of caspase-3 compared with the arsenic group (Figure 6A and B).

## DISCUSSION

The results of the present study confirm the beneficial effects of the saffron aqueous extract on PD. Since saffron extract constituents are known as a potent antioxidant, it seems that under the oxidative stress conditions, saffron applies its neuroprotective properties on nervous system.<sup>14,21</sup> We examined this hypothesis in the Parkinson's disease cell model and also demonstrated that saffron aqueous extract has the same effects as NAC as an effective antioxidant.

In this study, it has been revealed that arsenic trioxide ( $As_2O_3$ ) at a dose of  $40 \mu M$  can kill nearly 50% of the cells and using NAC and aqueous extract of saffron can significantly increase the cell viability. Various reports suggest that arsenic may cause cell apoptosis via increasing the levels of ROS products, mitochondrial dysfunction and activating caspase-3 in various types of cells including

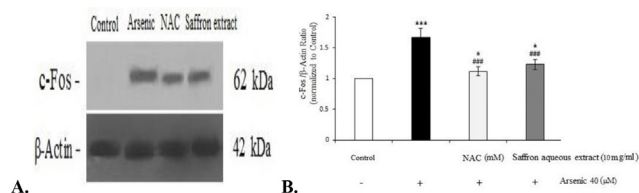
human dopaminergic SH-SY5Y neuroblastoma cells.<sup>19</sup> The results showed that using NAC and aqueous extract of saffron at effective doses can increase cell viability through reducing ROS products and DNA damaging, maintaining mitochondrial membrane potential and inhibiting apoptosis.

The data obtained from this study indicated the suppressor effect of NAC and aqueous extract of saffron against the production of arsenic-induced intracellular ROS in SH-SY5Y cells. The results of another study investigating the effects of saffron extract and its active ingredient, crocin, on the induced oxidative stress in the brain, liver and kidneys of rats indicated that saffron extract can reduce the oxidative stress in rats' mentioned organs by decreasing the levels of ROS, as well as increasing the Total Antioxidant Reactivity (TAR) and improving the activity of antioxidant enzymes such as superoxide dismutase.<sup>22</sup> According to another study investigating the effect of crocin on acrylamide-induced toxicity in PC12 cell lines, this substance could prevent intracellular ROS production as well as the Bcl-2 down-regulation and Bax up-regulation to preserve cell survival, which clearly indicates the antioxidant properties of crocin.<sup>23</sup> The data obtained from our study was also consistent with the results of the mentioned studies.

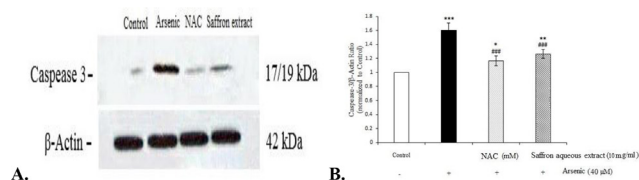
The present study demonstrated that arsenic-induced cytotoxicity caused a high loss of mitochondrial membrane potential and then the use of NAC and saffron aqueous extract at effective doses significantly improved the mitochondrial membrane potential. Mitochondrial dysfunction has been recognized in many studies, on the neuroendocrine diseases, as one of the involved factors in neuronal cell deaths.<sup>24</sup> Recent studies on the involved mechanisms of arsenic cytotoxicity mention the loss of mitochondrial membrane potential as one of the main mechanisms.<sup>21</sup>

One study showed that treatment of rat with saffron can increase synaptosomal survival by reducing the lipid peroxidation and maintaining mitochondrial survival against the toxic effects of 3-NPA.<sup>25</sup> In another study on the neuroprotective effects of crocin on PC-12 damaged cells by 1-methyl-4-phenylpyridinium (MPP+) as an *in vitro* model of PD, prevention of mitochondrial impairment was achieved through the treatment of cells with crocin due to preserving the mitochondrial membrane potential.<sup>26</sup> The data obtained from the present study showed similarities with the previous studies.

Different studies demonstrated the role of c-Fos as a proto-oncogene factor in the nervous system.<sup>27</sup> There are a lot of questions on the role of arsenic in the incidence and even treatment of various cancers, which is a dose-dependent feature. The results of these studies



**Figure 5: The effect of NAC and saffron aqueous extract on the c-Fos/β-Actin ratio reduction resulted from arsenic (A, B).** Data are expressed as mean ± SEM; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control cells, ### $P < 0.001$  versus Arsenic treated cells.



**Figure 6: The block of arsenic-induced caspase 3 activation by NAC and saffron aqueous extract (A, B).** Each value expresses the mean ± SEM band density ratio \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$  significantly different versus control cells. ### $P < 0.001$  versus Arsenic-treated cells.

indicate that arsenic can induce gene expression of proteins such as p53, c-jun, c-fos, etc. in different types of tumors such as leukemia (HL-60) and hepatocellular carcinoma cells (HepG<sup>2</sup>).<sup>28</sup> Our study also found that SH-SY5Y arsenic-induced cell toxicity could significantly increase the expression of c-Fos, which is in consistent with the results of other studies. The results of a study which was designed to determine the effects of NAC on hallucinogenic 5-HT (2A) receptor agonist DOI ( $\pm$ 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) in mice suggest that NAC can significantly reduce the expression of c-Fos in the infralimbic of mice.<sup>29</sup> Recently, the therapeutic effect of crocin was investigated in an *in vivo* amyloid beta model of the Alzheimer's disease. The outcome of this study documented that crocin can significantly decrease the c-Fos levels in the hippocampus compared to A $\beta$ -group of rats.<sup>11</sup> According to the above-mentioned studies, probably NAC and aqueous extract of saffron can apply their protective effects in cellular model of PD by reducing the expression of arsenic-induced c-Fos protein.

Caspase-3 belongs to caspases family which are a group of protease enzymes, having a crucial role in apoptosis and inflammation. Therefore, we decided to investigate the effect of NAC and saffron aqueous extract on caspase-3 activation in arsenic-treated SH-SY5Y cells. Our data showed that arsenic-induced cell damage in SH-SY5Y cells increased caspase-3 levels and treatment of cells with effective doses of NAC and saffron aqueous extract could decline the mentioned apoptotic factor. A previous report documented that the activation of caspase-3 is highly related to the programmed death of dopaminergic neurons in PD.<sup>30</sup> And, a mitochondrial damage leads to the release of cytochrome c and other apoptotic proteins. This chain of events can promote the activation of caspase-3.<sup>31</sup> Based on the recent studies, cytotoxic drugs such as arsenic can activate the mentioned chain of events.<sup>6</sup> In an earlier study to investigate the effects of NAC's long-term treatment in a spinal cord injury model of rats, it was demonstrated that within two weeks long-term NAC treatment can decrease the death of motoneurons in the rubrospinal tract through the reduction of caspase-3 activation.<sup>32</sup> It has been also shown that saffron and its derivatives can reduce apoptosis markers such as caspase-3. A previous study aimed at comparing the effect of saffron and crocetin on SH-SY5Y cells H<sub>2</sub>O<sub>2</sub>-induced toxicity suggest that treatment of cells with both saffron and crocetin can afford a strong protection against H<sub>2</sub>O<sub>2</sub>-induced cell death through an increase in cell survival, a repression in ROS levels and a reduction

in caspase-3 expression.<sup>33</sup> Our results were also similar to the outcome of the mentioned cases.

## CONCLUSION

Our results indicate that both NAC and saffron aqueous extract can protect SH-SY5Y cells against apoptosis induced by arsenic, although NAC showed a little more protective effect compared to saffron but not significant. The outcome of the current study also suggests that saffron's protective ability might be mediated via its antioxidant properties and apoptosis pathway modulation. Finally, it can be concluded that saffron can be considered as a herbal medicine in the pretreatment of PD, but yet it needs more clinical trials.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

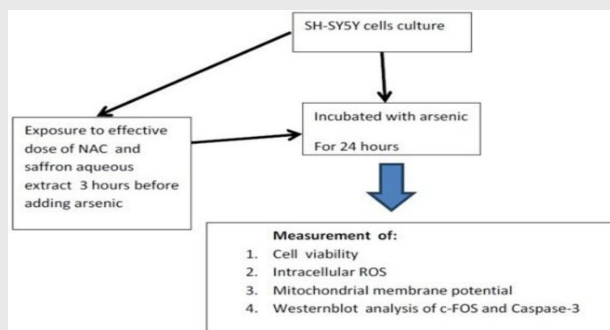
**PD:** Parkinson's disease; **NAC:** N-acetylcysteine; **MTT:** 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide; **ROS:** Reactive oxygen species.

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### PICTORIAL ABSTRACT



### SUMMARY

N-acetylcysteine and *Crocus sativus* aqueous extract have neuroprotective and antiapoptotic effects on Arsenic-induced neurotoxicity in SH-SY5Y human dopaminergic neuroblastoma cells as an *in vitro* model of Parkinson disease.

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