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Phoenix dactylifera L. Pollen and Fluvoxamine Maleate Protect PC12 Cells Against H2O2-Induced Oxidative Stress by Involvement of *Nrf2* and *SIGMAR1* Gene Expression

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Abstract

Background: The association between oxidative stress and the pathogenesis of neurodegenerative diseases has been documented. Besides, there is evidence that antidepressant agents, such as fluvoxamine maleate (Flv), can ameliorate neurotoxicity. The date palm (*Phoenix dactylifera* L.) pollen (DPP) contains various compounds with antioxidant capacity; however, its underlying mechanism of function has not been fully understood.

Objectives: The present study aimed to compare the neuroprotective effects of DPP and Flv on H2O2-induced oxidative damage and their effects on *Nrf2*, *SIGMAR1*, and *Bcl2* gene expression in PC12 cells.

Methods: First, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay examined the toxicities of DPP, Flv, and H2O2 at various concentrations on the PC12 cells. Real-time polymerase chain reaction (PCR) measured the expression of *Nrf2*, *SIGMAR1*, and *Bcl2* in PC12 cells in the presence or absence of DPP or Flv after 4 h of treatment with 100 μM H2O2.

Results: Based on the MTT results, DPP at concentrations of 200 - 1000 μg/mL had no toxic effect on PC12 cells. The IC_{50} was evaluated at 57.26 μM and 109.5 μM under treatment with Flv and H2O2. Real-time PCR analysis showed a decrease in the expression of *Nrf2* and *SIGMAR1* in PC12 cells treated with 100 μM H2O2, an indicator of oxidative stress recruitment in PC12 cells. Pretreatment with DPP and Flv (500 μg/mL and 10 μM, respectively) for 24 h resulted in the upregulation of *Nrf2* relative to the vehicle control. In addition, pretreatment with DPP increased the level of *SIGMAR1* in PC12 cells compared with H2O2-exposed cells. Considering the role of *SIGMAR1* in endoplasmic reticulum oxidative stress, the *SIGMAR1* level should be evaluated at the translational level. Compared to the untreated cells, the expression of *Bcl2* decreased in all the experimental cases, and the difference in *Bcl2* expression was not significant between the co-treatment experimental cases.

Conclusions: Taken together, DPP and Flv have neuroprotective effects against oxidative damage in PC12 cells. Exposure of PC12 cells to 500 μg/mL DPP and 10 μM Flv for 24 h protected the morphology and function of PC12 cells under H2O-induced oxidative stress via regulating oxidative stress-involved genes.

Keywords: Oxidative Stress, Date Palm Pollen, Fluvoxamine Maleate, Hydrogen Peroxide, Sigma-1, Nrf2

1. Background

Cellular damage induced by oxidative stress is a hallmark of neurodegenerative disorders (1). Low concentrations of reactive oxygen species (ROS) are involved in biological processes. In contrast, excessive ROS production and inadequate antioxidant defenses lead to oxidative stress, disrupting various signaling pathways (2). Oxidative stress can lead to various molecular dysfunctions and neuronal apoptosis in the neurodegenerative system. Hence, scavenging free radicals using antioxidant agents is promising for enhancing neuronal antioxidant capacity (3).

Studies have demonstrated that phenolic compounds are natural antioxidants that disrupt cellular redox. Date palm (*Phoenix dactylifera* L.) pollen (DPP), widely used in traditional medicine, has been shown to contain a variety of biochemicals, including carbohydrates, amino acids, fatty acids, vitamins, dietary fiber, estrogenic constituents, hormones, and antioxidant agents (4, 5). Furthermore, DPP has been shown to possess antibiotic, anti-inflammatory, and antioxidant properties, potentially

offering benefits for various human diseases (6).

One of the main challenges of neurodegeneration is the alteration of neuroplasticity features. Studies have shown that antidepressant drugs can influence neuroplasticity and may possess antioxidant properties. Fluvoxamine maleate (Flv) is a selective serotonin reuptake inhibitor (SSRI) used to treat obsessive-compulsive disorder (OCD), a neurodegenerative condition (7, 8). Different molecular pathways can regulate the antioxidant response elements (ARE) pathway. Nuclear factor erythroid 2-related factor 2 (NRF-2; gene name of NFE2L2) is a transcription factor with high expression in the brain. Under normal conditions, *Nrf2* binds to its inhibitor Keap1 and is degraded by proteasomes. During oxidative stress, *Nrf2* forms a heterodimer with Maf protein, binds to its receptor ARE, and activates the transcription of antioxidant enzymes, such as superoxide dismutase, catalase, and heme oxygenase (9, 10).

Furthermore, *Nrf2* is a master regulator that can be targeted for the treatment of various diseases (11-13). The *SIGMAR1* gene encodes sigma-1 receptors, which are primarily located in the endoplasmic reticulum and mitochondria-associated membranes. Sigma-1 receptors are expressed in both the peripheral and central nervous systems to modulate ionic channel functions, neuroprotection, cognition, and substance abuse (14, 15).

In addition, sigma-1 receptors also exhibit a wide range of neuroprotective mechanisms, including the regulation of endoplasmic reticulum (ER) stress via the GRP78-BiP complex and the regulation of *Bcl2*, an anti-apoptotic protein, by NFκB activation (16, 17). These regulatory factors may be affected by antidepressant and antioxidant agents, such as flavonoids and phenolic compounds, which are commonly found in herbal medicines (18).

2. Objectives

This study investigated the protective effects of DPP and Flv on oxidative stress induced by H2O2 in PC12 cells. PC12 cells are a commonly used model in neural biology research to assess oxidative stress (19).

3. Methods

3.1. Chemicals

The antidepressant drug Flv was acquired from Tehran Darou Pharmaceutical Company (Iran). High-glucose Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (100 U/mL), and trypsin-EDTA (0.05%, 1X) were procured from Bio Idea (Iran). Merck (Germany) provided dimethyl sulfoxide (DMSO) and hydrogen peroxide. Moreover, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (USA). The TRIzol® reagent was acquired from GeneAll Biotechnology Co. (South Korea). The DNA synthesis and quantitative real-time polymerase chain reaction (PCR) were carried out using BioFACT™ 2X real-time PCR master mix and pre-mix contents.

3.2. Preparation of DPP and Extraction

Date palm (*Phoenix dactylifera* L.) pollen was collected from Bushehr province (Iran), which lies 18 meters above sea level. It is situated between a longitude of 50° 48' 59.99" E and a latitude of 28° 58' 59.99" N. The identification and confirmation of DPP were conducted by Dr. F. Ghahremaninejad. A voucher specimen of 25556 was deposited at the Herbarium of the Plant Department, Kharazmi University, Tehran, Iran.

For extraction, the grains were rinsed with deionized water, sieved, and dried. The dried powder (10 g) was dissolved in warm deionized water (100 mL at room temperature), shaken for 5 hours, filtered, and evaporated under 55 - 60°C. Finally, the prepared aqueous extract was dissolved in PBS to prepare the required doses. The stock of the DPP extract was stored at 4°C (20).

3.3. Cell Culture, Experimental Design, and Treatment

PC12 cells were cultured in DMEM containing 10% FBS and 1% antibiotic in a humidified incubator (5% CO2 at 37°C). The experimental design was performed using a modified method by Ma et al. (21). Four experimental conditions were investigated. In all cases, untreated cells were considered as the vehicle control. In the first, second, and third conditions, PC12 cells were treated with DPP extract (10 - 1000 μg/mL), H2O2 (10 - 1000 μM), and Flv (10 - 1000 μM) for 24 hours, respectively. In the fourth condition, PC12 cells were pretreated with the desired concentrations of DPP or Flv for 24 hours and then exposed to 100 μM H2O2 for 4 hours.

3.4. Cell Viability Assay

The toxicity of H2O2 (10 - 1000 μM), DPP (10 - 1000 μg), and Flv (10 - 1000 μM) in PC12 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (22). For DPP and Flv exposure, PC12 cells were cultured at a density of 2×104 cells/per well and incubated for 24 hours. The cells were pretreated with DPP (200 and 500 μg/mL) or Flv (10 and 25 μM) for 24 hours and then exposed to 100μM H2O2 to assess the protective effect against oxidative stress. After the treatment, the medium was replaced with 30 μM of MTT (5 mg/mL). After 3 hours, 100 μL DMSO was added to dissolve the formazan crystals. The optical density was measured at 570 nm using an ELISA reader (Epoch, USA).

3.5. Quantitative Real-time PCR

Real-time PCR was used to evaluate gene expression (23). PC12 cells were cultured at a density of 2×10^5 cells/per well. After treatment, the cells were lysed with 500 μL of TRIzol®. After adding 300 μL phenol/chloroform, three phases appeared. The top phase was transferred to another microtube. Finally, after centrifugation, the pellet containing the RNA was mixed with 75% ethanol. A NanoDrop was used to determine the RNA quality. Next, the cDNA was synthesized using BioFACT™ 2X real-time PCR master mix and pre-mix contains. Oligo-DT and random hexamer primers were used for cDNA synthesis. Quantitative real-time PCR was performed in the Qiagen Rotor Gene Corbett RG6000 (Germany). The full sequence of genes *Nrf2*, *Bcl2*, *SIGMAR1*, and *GAPDH* were obtained from the National Center for Biotechnology Information (NCBI) database to design primers using Primer-BLAST. The primer sequences are listed in Table 1. The $\Delta\Delta$Ct method was used to calculate relative gene expression between all experimental groups.

3.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism v. 6 software. The data are presented as mean ± standard deviation (SD). A P-value of less than 0.05 was considered significant.

4. Results

4.1. Evaluation of Cytotoxicity and Morphological Analysis

Based on the findings, DPP at concentrations of 200 - 1000 μg/mL exerted no significant effect on PC12 cells compared to the vehicle control (Figure 1A). As shown in Figure 1B and C, the viability of cells significantly decreased after incubation with H2O2 or Flv (upper dosage of 100 μM H2O2 and 25 μM Flv) in a dose-dependent manner with IC_{50} values of 109.5 μM and 57.26 μM, respectively. Morphological observation, such as cell shrinkage, indicated that 100 μM H2O2 was used to induce cell death and oxidative stress. As shown in Figure 1D, PC12 cells pretreated with 500 μg/mL DPP and 10 μM Flv retained their morphological features (Figure 2) against oxidative stress induced by H2O2.

4.2. Effects of DPP and Flv on SIGMAR1, Nrf2, and Bcl2 gene expression

The results revealed a significant increase in *Nrf2* (P = 0.0011) and *SIGMAR1* (P < 0.0001) mRNA levels in PC12 cells exposed to 500 μg/mL DPP and 10 μM Flv compared to untreated cells (Figure 3A). The *SIGMAR1* expression decreased in cells pretreated with 10 μM Flv (P = 0.0418) compared to cells exposed to H2O2. Therefore, Flv triggers an antioxidant response independently of ER oxidative stress regulation (Figure 3B). *Bcl2* expression was downregulated in cells exposed to H2O2 compared to the vehicle control (P = 0.0294) (Figure 3C). Consequently, pretreatment with DPP and Flv (500 μg/mL and 10 μM) resulted in a small decrease in *Bcl2* expression compared to the control. However, this reduction was not statistically significant compared to H2O2-exposed cells.

5. Discussion

The present study explored the protective effects of DPP and Flv against H2O2-induced oxidative damage in PC12 cells. According to findings, H2O2 below 100 μM is nontoxic. Previous studies have demonstrated that H2O2 can inhibit cell growth and cause cell death; higher doses of H2O2 can lead to oxidative stress (24). In this study, 100 μM H2O2 induced oxidative stress with less severe apoptotic effects.

The results revealed that DPP was nontoxic to PC12 cells and even induced slight cell growth at a concentration of 500 μg/mL compared to the vehicle control. While a previous study has demonstrated the neuroprotective potential of DPP (25), research into its effects on the nervous system and the expression of neural oxidative stress-related genes remains limited. As previously reported, DPP exhibits antioxidant properties owing to the presence of phytochemical agents, including various flavonoids and unsaturated fatty acids (26).

Date palm pollen demonstrated neuroprotection against oxidative stress and neuronal injury induced by bilateral carotid artery occlusion (25). Recently, DPP has been shown to protect against doxorubicin-induced cardiomyopathy and hepatotoxicity, both of which are disorders associated with oxidative stress (4, 27).

Regarding the neuroprotective effect of Flv, doses of 10 μM and 25 μM increased cell growth, but the difference between these doses was not statistically significant. Oxidative stress has been recognized as a crucial factor in depression. However, previous studies have documented the combined antioxidant and antidepressant effects of antidepressants such as fluvoxamine (28, 29). Schlezinger et al. reported that Flv inhibits ROS generation by suppressing the cytochrome P450 family 1 subfamily A member 2 (CYP1A2) enzyme, which is responsible for metabolizing antioxidants in the body (30).

Evaluation of the protective effects of DPP and Flv showed a significant increase in *Nrf2* expression in DPP and Flv-pretreated cells (500 μg/mL and 10 μM, respectively). H2O2 induces oxidative stress and influences adaptive responses, such as the Nrf2 pathway. Various studies have

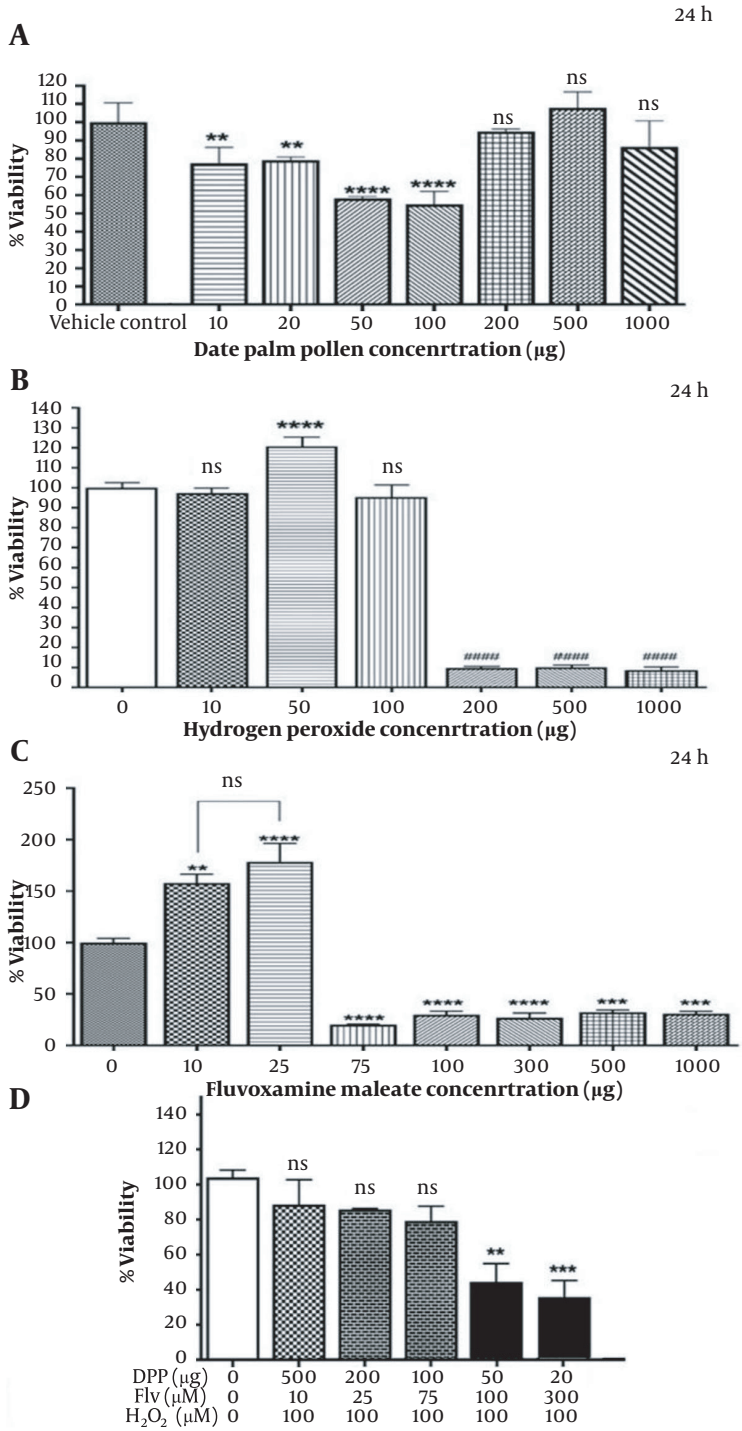

Figure 1. Effects of DPP, Flv, and H2O2 alone or combination of DPP and Flv with H2O2 on the viability of PC12 cells. A, cell viability increased with elevating the concentrations of DPP (200 to 1000 μg/mL). Treatment with 500 μg/mL DPP showed no significant differences with the control; B, H2O2 lower than 100 μM was not toxic, but at 50 μM, it led to significant proliferation ($P < 0.0001$). For oxidative stress induction, the concentration of 100 μM was selected for the subsequent assay; C, Flv caused cell proliferation at doses of 10 μM ($P = 0.0011$) and 25 μM ($P < 0.0001$). In contrast, higher doses of Flv (75 to 1000 μM) significantly reduced PC12 cell survival; D, Effect of the combined treatment of 100 μM H2O2 plus pretreatment with increasing concentration of DPP (20 to 500 μg/mL) or Flv (10 to 300 μM) on PC12 cells. The co-treatment with 20, 50 μg/mL DPP and 100, 300 μM Flv caused significant cell toxicity ($P = 0.0007$). Data are presented as mean ± standard deviation. **$P \leq 0.01$, ***$P \leq 0.001$ were considered statistically significant. Abbreviations: DPP, date palm pollen; Flv, fluvoxamine maleate.

Table1. Designed Primers Used in Real-time PCR

Genes	Primer Sequences (5'-3')	Product Size (bp)	Tm (˚C)
Nrf2		166 bp	
F	GGACATGGAGCAAGTTTGGC		59.75
R	TCCAGCGAGGAGATCGATGA		60.18
Bcl2		120 bp	
F	ATGCCTTTGTGGAACTATATGGC		59.11
R	GGTATGCACCCAGAGTGATGC		61.08
SIGMAR1		146bp	
F	AGGGCACCACAAAAAGTGAG		60.1
R	AAGTGCAAATGCCAGGGTAG		60.1
GAPDH		68 bp	
F	AGGTCGGTGTGAACGGATTTG		61
R	TGTAGACCATGTAGTTGAGGTCA		61

Abbreviation: PCR, polymerase chain reaction.

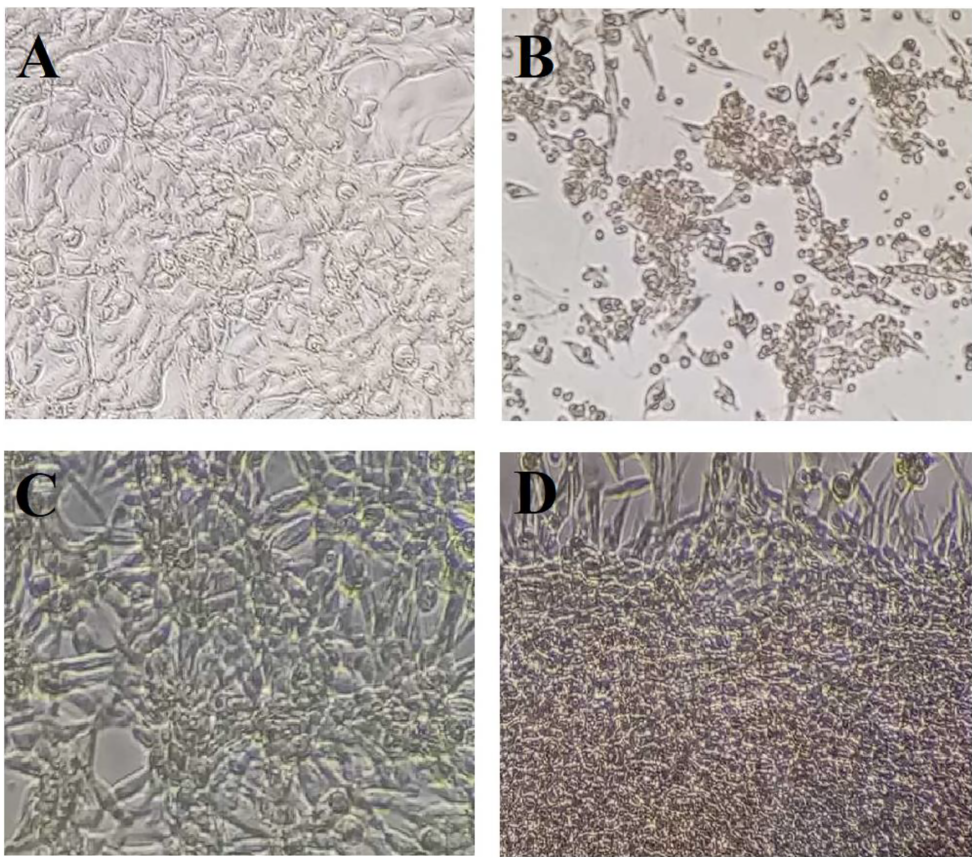

Figure 2. The microscopic images of PC12 cells were treated with different concentrations of DPP, Flv, and H2O2. (A) Vehicle control. (B) As shown, treatment with 100 μM H2O2 resulted in cell shrinkage after 4 h of exposure. (C) DPP exposure (500 μg/mL) suppressed morphological alteration induced by H2O2 on PC12 cells. (D) PC12 cells treated with 10 μM Flv inhibited cell death induced by H2O2. Scale bar, 100 μm.

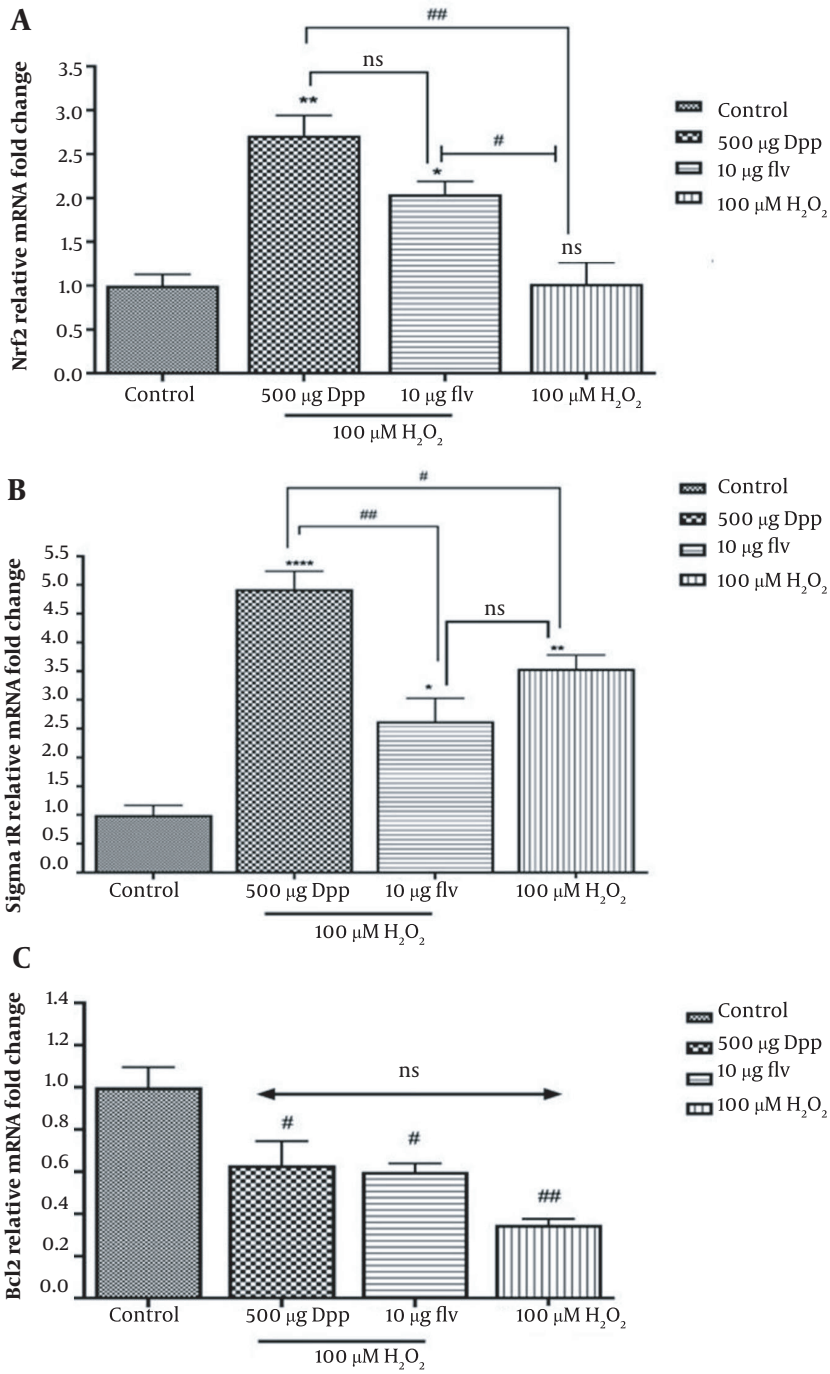

Figure 3. Effects of DPP and Flv on the expression of *Nrf2*, *SIGMAR1*, and *Bcl2* at the transcriptional level. A, DPP pretreatment (500 μg/mL) elevated the level of *Nrf2* rather than Flv (10 μM); B, The Flv at a concentration of 10 μM prevented PC12 cells from *SIGMAR1* upregulation, and the P-value was 0.0185 compared with vehicle control. In contrast, DPP at the 500 μg/mL dose increased *SIGMAR1* expression; C, DPP and Flv (500 μg/mL, 10 μM respectively) nonsignificantly elevated the *Bcl2* downregulation induced by H2O2 in PC12 cells.

identified *Nrf2* as a guardian of redox homeostasis (9). The expression level of *Nrf2* in the DPP group was higher than that in the H2O2 group, indicating that DPP and Flv prevent the initiation of oxidative stress primarily through the Nrf2 pathway.

Previous studies have shown that forsythiaside provided protection against neurotoxicity through the elevation of *Nrf2* levels and the upregulation of SOD and CAT (31). In 2018, it was reported that duloxetine could protect neuroblastoma cells primarily through *Nrf2* upregulation and the expression of HO-1, a target gene of *Nrf2* (32). Kolla et al. reported findings that amitriptyline and fluoxetine exerted neuroprotective effects on PC12 cells exposed to H2O2, which was associated with upregulation in SOD activity (7). The sigma-1 receptor was chosen because it has been shown that fluvoxamine maleate (Flv) binds to it, so being an agonist of the sigma-1 receptor would be congruent with our antidepressant selection (33). The sigma-1 receptor agonist's fluvoxamine, fluoxetine, and citalopram bind to the ER sigma-1 receptors and cause them to separate from the complex with BiP (GRP78). Further, dissociating the sigma-1 receptor will allow it to generate chaperone activity, resulting in neuroprotection (15).

According to our findings, *SIGMAR1* expression increased in all experimental states compared with the control. Treatment with DPP (at 500 μg/mL) significantly impacted *SIGMAR1* expression compared to H2O2-exposed cells. This finding suggested that DPP could have a more significant impact on the Nrf2 pathway than the ER pathway. In contrast, Flv slightly attenuated the expression of *SIGMAR1* in H2O2-exposed cells, indicating the role of Flv via regulation of ER oxidative stress.

Previous studies have demonstrated the association between sigma-1 receptor dysfunction and depression, suggesting its potential as an antidepressant target (17). Additionally, studies have shown that sigma-1 receptor agonists can exert neuroprotection against neurotoxicity in neuronal cell lines (34). Recently, the antifibrotic efficacy of fluvoxamine has been confirmed in a vascular disorder associated with oxidative damage (35).

The role of oxidative stress and mitochondrial dysfunction through apoptosis in neurodegenerative disease has been studied previously (35). It was elucidated that oxidative stress exerts ferroptosis and mitochondrial dysregulation, which lead to neuronal cell death and apoptosis (2). The expression of *Bcl2*, an antiapoptotic factor from the Bcl2 family, was found to be significantly attenuated in H2O2-treated cells compared to the control. However, pretreatment with DPP and Flv reduced *Bcl2* gene expression, but this downregulation did not show any significant difference between the experimental groups.

Nakayama et al. demonstrated the neuroprotection of ferulic acid as a phytochemical against H2O2-induced apoptosis. Ferulic acid increased the expression of BDNF, a neuroprotective factor, and regulated the activity of phosphokinases and apoptosis-related proteins (23). Additionally, quercetin has been shown to protect PC12 cells from H2O2-induced neurotoxicity by elevating the SOD and CAT level, reducing the apoptosis markers, such as Bax and caspase-3, and upregulating the *Bcl2* (22).

The combined suppression of oxidative stress induced by H2O2 by DPP and Flv through the Nrf2 and sigma-1 signaling pathways suggests that these pretreatment packages could be an effective modality for preventing neurodegenerative damage in an animal neurotoxicity model.

Footnotes

Authors' Contribution: MN: Supervising the research; EA: Designing study and analyzing the MTT data and editing the manuscript; EM: Performing the cell culture protocols, real-time PCR, and writing the manuscript; AN: Writing and editing the manuscript and performing gene expression analysis.

Conflict of Interests: The authors have no conflict of interest to declare.

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