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Neuroprotective efficacy of naringin on 3-nitropropionic acid-induced mitochondrial dysfunction through the modulation of Nrf2 signaling pathway in PC12 cells

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Abstract Oxidative stress and mitochondrial dysfunction are implicated in neuronal apoptosis associated with Huntington's disease. Naringin is the flavanone present in grapefruit and related citrus species possess diverse pharmacological and therapeutic properties including antioxidant, anti-apoptotic, and neuroprotective properties. The aim of this study was to investigate the protective effect of naringin on 3-nitropropionic acid (3-NP)-induced neurotoxicity in pheochromocytoma cells (PC12) cells and to explore its mechanism of action. Naringin protects PC12 cells from 3-NP neurotoxicity, as evaluated the by cell viability assays. The lactate dehydrogenase release was decreased upon naringin treatment in 3-NP-induced PC12 cells. Naringin treatment enhances the antioxidant defense by increasing the activities of enzymatic antioxidants and the level of reduced glutathione. The increase in levels of reactive oxygen species and lipid peroxidation induced by 3-NP were significantly decreased by naringin. PC12 cells induced with 3-NP showed decrease in the mitochondrial membrane potential and mitochondrial respiratory complex enzymes, succinate dehydrogenase and cytochrome *c* oxidase activities, and it was significantly altered to near normal upon naringin treatment. Naringin reduced the 3-NP-induced apoptosis through the modulation in expressions of B-cell lymphoma 2 and Bcl-2-associated X protein. Further, naringin enhances the nuclear translocation of Nrf2 and induces the NAD(P)H:quinone oxidoreductase-1 and Heme oxygenase-1 expressions through the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway. Taken together, the above findings suggest that naringin augments cellular antioxidant defense capacity and reduces the 3-NP-induced neurotoxicity in PC12 cells through the PI-3K/Akt-dependent Nrf2 activation in PC12 cells.

Keywords Naringin · 3-Nitropropionic acid · Oxidative stress · Mitochondrial dysfunction · Apoptosis · Nrf2 · PI-3K/Akt

Introduction

3-Nitropropionic acid (3-NP) is a neurotoxin produced by numerous fungal species and naturally exists in leguminous plants. 3-NP inhibits the mitochondrial enzyme succinate dehydrogenase, involved in both electron transport chain and the tricarboxylic acid cycle [1]. 3-NP has been shown to be responsible for the neurodegeneration caused in human—by the ingestion of sugar cane, corn, and peanuts contaminated by Arthrinium fungi [2]. Huntington's disease (HD) is an autosomal dominant, inherited neurodegenerative disorder, clinically characterized by involuntary choreic movements, cognitive impairment, and dementia [3]. 3-NP can induce biochemical and pathological changes and also selective striatal lesions in rats and non-human primates, mimicking those in HD [4, 5].

Mitochondria play significant role in the process of apoptosis and also in neurodegeneration [6]. Both HD and 3-NP neurotoxicity are characterized by the functional impairment of mitochondrial complex II [1, 7]. Disruption of mitochondrial activity is associated with the abnormal production of reactive oxidative species (ROS) like superoxide radical, hydrogen peroxide, hydroxyl radical,

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peroxyl radical and peroxynitrite, thereby generating oxidative stress [8]. Oxidative stress has been shown to be associated with the progression of striatal-lesion; a major pathological feature of HD [9]. ROS in turn renders mitochondria susceptible to oxidative damage. Mitochondrial dysfunction caused by ROS-induced oxidative damage has been shown to involve in the development of apoptotic cell death in HD as well as in 3-NP toxicity [10, 11].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor. Nrf2 is a key regulator in the coordinated induction of numerous cytoprotective and endogenous antioxidant genes and controls the physiological and pathophysiological perturbations of oxidants [12]. Under basal conditions, Nrf2 is anchored in the cytoplasm through binding to Kelch-like ECH-associated protein 1 (Keap1), and is maintained at a low level by proteasomal degradation. During oxidative stress, Nrf2 dissociates from Keap1 and translocates into nucleus and elicits the antioxidant response by the induction of target gene products [13]. Nrf2 activation and consecutive cytoprotective gene induction stimulate the restoration of balance between oxidants and antioxidants during oxidative stress. Recently, numerous studies have demonstrated that activation of Nrf2 pathway can intensely attenuate multiple pathophysiological processes, including oxidative stress, mitochondrial dysfunction, and apoptosis [14, 15]. Interestingly, recent studies suggest that Nrf2 is regulated through phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway [16, 17]. Indeed, Nrf2 regulation by this kinase appears to be an important mechanism in Nrf2-mediated ARE activation.

Flavonoids are the most abundant polyphenolic substances in our diet, which is present in most of the plant [18]. Recent studies have suggested that the flavonoids exhibit protection against neurodegeneration [19, 20]. Naringin, a well-known flavanone glycoside of grape fruits, e.g., Citrus paradise, Citrus unshiu, and Citrus sinensis, has been reported to attenuate the oxidative stress, apoptosis, and inflammation, demonstrated to possess neuroprotective effect [21, 22]. Further, naringin has been reported to possess various biological and pharmacological properties including antioxidant, anti-carcinogenic, and anti-atherogenic properties [23–25]. In our earlier studies, we have demonstrated the naringin-mediated protection in 3-NP-induced neurodegeneration through the Nrf2 signaling pathway in rats [26, 27]. Further the naringin was previously reported to activate PI-3K/Akt signaling pathway [28]. However, the protective effect of naringin by the PI-3K/Akt-mediated Nrf2 activation in 3-NP-induced neurotoxicity in PC12 cells is not elucidated. The aim of the present study is to investigate the protective effect of naringin on 3-NP-induced neurotoxicity in PC12 cells through the PI-3K/Akt-dependent Nrf2 activation.

Materials and methods

Chemicals and reagents

PC12 cells were purchased from National Centre for Cell Science, Pune, India. Roswell Park Memorial Institute (RPMI) 1640 medium, trypsin–EDTA, L-glutamine, fetal bovine serum (FBS), horse serum, and antibiotics were purchased from Himedia Laboratories, Mumbai, India. Poly-L-lysine solution, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH2-DA), and 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′ tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Sigma-Aldrich Company, St. Louis, MO, USA. LY294002 was purchased from Life Technologies, Inc. All disposable wares used for cell culture including tissue culture flasks, 96 and 6-well plates were purchased from Tarsons Product Pvt. Ltd., Kolkata, India.

Cell culture

The PC12 cells were grown in poly-L-lysine-coated tissue culture flask in RPMI-1640 medium supplemented with 10 % heat-inactivated FBS and 5 % heat-inactivated horse serum, 1 % L-glutamine, and antibiotic solution containing 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. For differentiation, cells were plated on poly-L-lysine-coated (10 μg/ml) dishes. The differentiation was induced 24 h after seeding by adding 50 ng/ml of nerve growth factor (NGF 2.5 S) and incubated for 4 days.

Experimental design

After arriving at a dose and the time-dependent cell death induced by 3-NP and an optimal dose at which naringin protects from cell death, the following experimental design was set up.

Group 1 Control treated with 0.1 % dimethyl sulfoxide (DMSO).
Group 2 3-Nitropropionic acid (15 mM).
Group 3 3-Nitropropionic acid (15 mM) + Naringin (dissolved in 0.1 % DMSO) treated (10 μM).
Group 4 Naringin alone (10 μM).

Cell viability assay

Cell viability was assessed by MTT method by Yuan et al. [29]. Briefly, 5×10^3 cells were plated in 96-well plates. The cells were incubated for 12 h under 5 % CO_2, 95 % O_2 at 37 °C. Then, media were removed and replaced with

serum-free medium containing 1 % BSA for 24 h. After removing the BSA medium, cells were stimulated with 3-NP and were incubated with/without naringin. The control cells received 0.1 % DMSO. After 24 h, 100 µl of 0.5 % MTT stock solution was added to each well and then incubated at 37 °C for 4 h. After incubation, the culture medium was replaced with 100 µl of DMSO to solubilize formazan crystals. Proliferation was expressed as absorbance at 650 nm recorded with a microtiter plate reader. The amount of formazan produced was directly proportional to the number of metabolically active cells. The cell viability was calculated as percentage of viable cells and then plotted on a graph.

Biochemical assays

The activity of lactate dehydrogenase (LDH) was assayed by the method of King [30]. Superoxide dismutase (SOD) was assayed by the method of Marklund and Marklund [31]. Catalase (CAT) activity was assayed by the method of Sinha [32]. Glutathione peroxidase (GPx) was assayed by the method of Rotruck et al. [33]. Glutathione reductase (GR) was assayed by the method of Staal et al. [34]. Reduced glutathione (GSH) was determined by the method of Moron et al. [35]. The level of lipid peroxidation (LPO) was assayed by the method of Ohkawa et al. [36]. Mitochondrial fractions were isolated by the method of Moreadith and Fiskum [37]. The Complex II (succinate dehydrogenase) activity was assayed by Slater and Borner [38]. The Complex IV (cytochrome *c* oxidase) activity was assayed by Mutisya et al. [39].

Measurement of ROS

ROS was measured using the fluorescent probe H_2DCF-DA. The PC12 cells (5.0×10^3 cells/well) were exposed to 3-NP (15 mM) in the presence or absence of naringin (10 µM) and incubated for 16 h. Briefly, after the corresponding treatments, cells were incubated with 5 µM H_2DCF-DA for 30 min in dark. Fluorescence measurement was performed at the excitation wavelength of 495 nm and the emission wavelength of 530 nm in a spectrofluorometer. For each experiment, fluorometric measurements were performed in triplicate and expressed as fluorescence intensity units [40].

Measurement of mitochondrial membrane potential changes

Mitochondrial membrane potential determination ($\Delta\psi_m$) was measured using JC-1 as a probe according to the method of Dey and Moraes [41]. The PC12 cells were exposed to 3-NP (15 mM) in the presence or absence of naringin (10 µM) and incubated for 16 h. Briefly, after the corresponding treatments, cells were incubated with 10 µM JC-1 for 6 min at 37 °C. The fluorescence was measured at two different wavelengths. The ratio of the reading at 590 nm to the reading at 530 nm was considered as the relative $\Delta\psi_m$ value.

Cytosolic and nuclear protein extraction

The extraction of cytosolic and nuclear fractions was performed in PC12 cells to assess the Nrf2 expression. Briefly, PC12 cells (8×10^6) were suspended in hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol] containing protease inhibitors, and their outer membranes were disrupted by homogenization, and then centrifuged at $10{,}000\times g$ for 20 min to collect the supernatant containing cytosolic fraction. The resultant nuclear pellet was resuspended, homogenized, and incubated in nuclear extraction buffer [20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 25 % glycerol] containing the protease inhibitors and finally, centrifuged at $20{,}000\times g$ for 5 min to collect supernatant as nuclear fraction [42].

Western blot analyses

Cytosolic or nuclear protein (50 µg) was separated by 12 % SDS–polyacrylamide gel and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked with 5 % BSA and 0.1 % Tween-20 in Tris-buffered saline at room temperature for 2 h. The membrane was then incubated overnight with the rabbit polyclonal IgG Nrf2 (Santa Cruz Biotech, CA, USA) at 4 °C. Then, the membrane was washed thrice for 15 min and incubated with the secondary horseradish peroxidase-linked antibody (Bangalore GeNei, India) for 2 h. Membrane was then washed thrice for 15 min, and then the protein-antibody complexes were detected by the addition of diaminobenzidine as a substrate.

Reverse transcriptase polymerase chain reaction

Briefly, after treatment of PC12 cells with 3-NP and naringin for desired time period, the medium was removed. The cells were washed with 1 ml of PBS, and then 700 µl of Trizol reagent was added to harvest the cells. After harvesting, 200 µl of chloroform was added; the samples were covered tightly and shaken vigorously for 15 s and allowed to stay on ice for 15 min. Then, the complementary DNAs (cDNA) were synthesized using AuPreP TM Gold cDNA (Life Technologies Pvt. Ltd., Delhi, India) according to the manufacturer's instructions. The

polymerase chain reactions (PCR) were performed using PCR Master Mix (GeNet Bio, Chungnam, Korea) with specific primers listed in Table 1. The PCR products were resolved in 1.4 % agarose gel. The gene expressions were shown as the ratio of densitometric value between target mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical methods

All the data were statistically evaluated with Statistical Package for Social Sciences (SPSS.10) software. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. All the results were expressed as mean ± SD. The 'p' value of less than 0.05 was considered to indicate statistical significance.

Results

Dose- and time-dependent effect of 3-NP on cell viability

The dose- and time-dependent 3-NP toxicity was evaluated by determining the reduction of MTT upon incubation of differentiated PC12 cells for 0–40 h with increasing 3-NP concentrations (1–25 mM). As shown in Fig. 1, increasing the 3-NP concentration decreases cell viability in a dose- and time-dependent manner. The viability of PC12 cells was observed to be reduced to 60 % by 15 mM of 3-NP, 16 h after treatment and more drastically at 24 h. Further, increasing the concentration of 3-NP also reduces the cell viability, but there was no significant difference compared to 15 mM concentration. Hence, the induction of PC12 cells with 15 mM of 3-NP for 24 h was followed for further studies.

Table 1 RT-PCR primer pairs for amplification of mRNA

Genes	Primer sequence
Bax	Sense: CGAGTGTCTCCGGCGAATTG
	Antisense: CACCCTGGTCTTGGATCCAG
Bcl-2	Sense: GTACCTGCAGCTTCTTTCCCC
	Antisense: AAGAAGGCCACAATCCTCCCC
GAPDH	Sense: TCTGCTCCTCCCTGTTCTAGAGACA
	Antisense: TCAGGTGAGCCCCAGCCTTCT
HO-1	Sense: ACTTTCAGAAGGGTCAGGTGTCC
	Antisense: TTGAGCAGGAAGGCGGTCTTAG
NQO-1	Sense: CATTCCAGCCGACAACCAGA
	Antisense: ATTCCCTCCTGCCCTAAACC

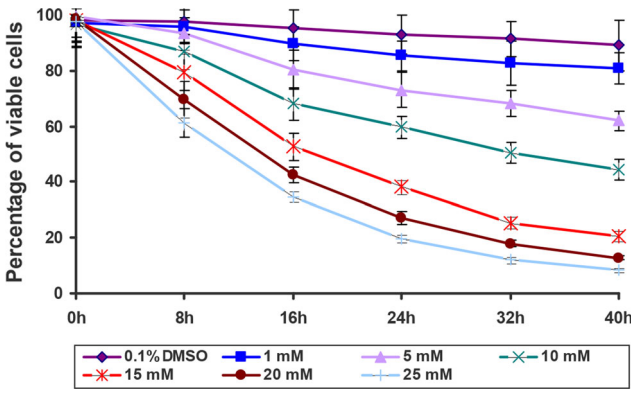

Fig. 1 Dose- and time-dependent effect of 3-nitropropionic acid on PC12 cells. MTT cell viability assay was performed in PC12 cells exposed to 3-NP at the concentrations of 1–25 mM for 0–40 h. Results are expressed as the percentage of viable cells. Values are represented as the mean ± SD of three independent experiments

Protective effect of naringin on 3-NP-induced neurotoxicity

To test whether naringin could reduce the cell death in PC12 cells during 3-NP administration, MTT reduction assay was performed. The cells were treated with 1–20 μM of naringin 1 h prior to the 3-NP exposure and incubated for 24 h. As shown in Fig. 2, naringin at the concentration of 10 μM protected the PC12 cells from 3-NP toxicity ($p < 0.05$).

Effect of 3-NP and naringin on LDH activity

LDH is an enzyme found in the cells of many tissues, including brain, which catalyzes the interconversion of

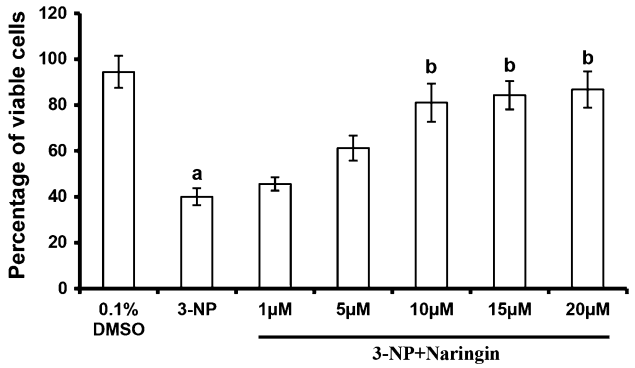

Fig. 2 Dose-dependent effect of naringin on 3-nitropropionic acid-induced cell death in PC12 cells. Naringin inhibits 3-NP-induced cytotoxicity in PC12 cells; induced with 15 mM of 3-NP for 24 h. Naringin (1–20 μM) was added 1 h before 3-NP administration. Results are expressed as the percentage of viable cells. Values are represented as the mean ± SD of three independent experiments. Values are statistically significant at $p < 0.05$, [a]3-NP versus control, [b]3-NP + Naringin versus 3-NP

lactate to pyruvate. LDH is often used as a marker to detect cellular damage during toxic insults [43]. Figure 3 shows the effect of naringin on 3-NP-induced LDH release from PC12 cells. Increased LDH release was observed in 3-NP-induced PC12 cells ($p < 0.05$) as compared to control. Treatment with naringin significantly decreased the LDH activity towards normal ($p < 0.05$) as compared to 3-NP-induced cells.

Naringin enhances the antioxidant status

The activation of endogenous antioxidant defense system by flavonoids has attracted special attentions, besides the direct quenching effect on ROS. The activities/level of enzymatic and non-enzymatic antioxidant in 3-NP and naringin-treated PC12 cells are shown in Table 2. The activities of SOD, CAT, GPx, and GR, and the level of GSH were decreased in 3-NP-induced cells ($p < 0.05$) as compared to control. Treatment with naringin significantly ameliorated these antioxidants in 3-NP-induced cells ($p < 0.05$) and exhibits its protective effect.

Naringin decreases the lipid peroxidation in 3-NP-induced PC12 cells

One of the consequences of oxidative stress is increase in LPO, which is caused by an attack of free radicals on cell membrane lipids. LPO is known to exert deleterious effects on neuronal membrane integrity and fluidity [44]. 3-NP caused an intense oxidative stress characterized by increase in LPO product. Figure 4 shows the effect of naringin on 3-NP-induced LPO in PC12 cells. Treatment with naringin renders protection to PC12 cells by reducing the level of LPO ($p < 0.05$), which was elevated upon 3-NP administration. Naringin-alone treated cells show similar effect as of control.

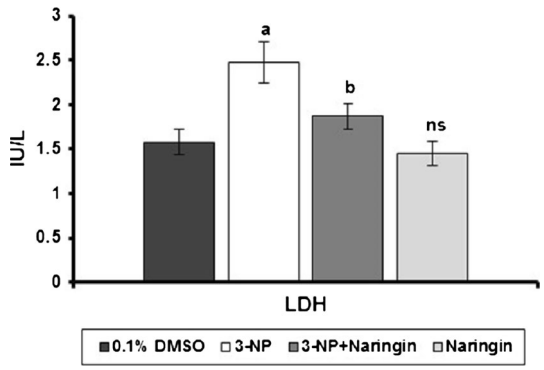

Fig. 3 Effect of naringin on 3-nitropropionic acid-induced lactate dehydrogenase release from PC12 cells. Values are represented as the mean ± SD of three independent experiments. Values are statistically significant at $p < 0.05$, [a]3-NP versus control, [b]3-NP + Naringin versus 3-NP, *ns* non-significant as compared to control

Naringin reduces the 3-NP-induced ROS generation

Oxidative stress refers to an imbalance between the production of free radicals and the ability of cells to defend against them. There is growing evidence implicating oxidative stress as the major cause of cellular injury in variety of human diseases including neurodegenerative disease [45]. Figure 5A shows the ROS generation in 3-NP- and naringin-treated PC12 cells. Administration of PC12 cells with 3-NP resulted in an elevated ROS production ($p < 0.05$) compared to control. Treatment with naringin defended the PC12 cells by decreasing the 3-NP-induced ROS accumulation ($p < 0.05$) through its antioxidant property.

Naringin protects against 3-NP-induced mitochondrial dysfunction

Mitochondria are the vulnerable target for various toxicants because of their important role in maintaining cellular integrity and function. The functional alterations occur in mitochondria are due to the changes in mitochondrial membrane potential. Disruption of the mitochondrial membrane potential (i.e., depolarization) is one of the earliest indicators of cellular disturbance. Protective effect of naringin was examined on 3-NP-induced mitochondrial depolarization in PC12 cells. As shown in Fig. 5B, the administration of PC12 cells with 3-NP significantly depolarizes mitochondria ($p < 0.05$) compared to control. Naringin treatment augmented the mitochondrial membrane polarization in the cells exposed to 3-NP ($p < 0.05$) and exhibits its protective effect against 3-NP-induced mitochondrial dysfunction.

The activities of mitochondrial respiratory chain enzymes are presented in Fig. 6. The activities of succinate dehydrogenase and cytochrome *c* oxidase were decreased in 3-NP-induced cells compared to control. Naringin treatment ameliorated the activities of these enzymes in 3-NP-induced PC12 cells.

Anti-apoptotic effect of naringin on 3-NP-induced PC12 cells

Apoptosis is a highly organized form of cell death that is common in variety of biological processes and pathological conditions. Aberrant apoptosis has been implicated in the pathogenesis of neurodegenerative diseases caused by protein misfolding [46]. There is rising evidence suggesting the role of apoptosis in 3-NP-induced HD [47]. To study the anti-apoptotic effect of naringin, RT-PCR analyses of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) were performed in PC12 cells. Figure 7 shows that 3-NP exposure increased the mRNA expression

Table 2 Effect of 3-nitropropionic acid and naringin on antioxidants in PC12 cells

Antioxidants	Control	3-NP	3-NP + Naringin	Naringin
SOD	7.14 ± 0.64	3.74 ± 0.42[a]	5.39 ± 0.36[b]	7.24 ± 0.57[ns]
CAT	2.67 ± 0.25	1.25 ± 0.13[a]	2.14 ± 0.16[b]	2.81 ± 0.23[ns]
GPx	0.19 ± 0.02	0.11 ± 0.01[a]	0.16 ± 0.01[b]	0.20 ± 0.02[ns]
GR	0.10 ± 0.007	0.06 ± 0.005[a]	0.08 ± 0.009[b]	0.10 ± 0.009[ns]
GSH	46.23 ± 3.60	20.55 ± 2.10[a]	33.24 ± 2.68[b]	49.46 ± 4.40[ns]

Values are represented as the mean ± SD of three independent experiments. Values are statistically significant at $p < 0.05$

Enzyme activities are expressed as SOD: units/min/mg protein. CAT: μmoles of H_2O_2 hydrolyzed/min/mg protein. GPx: μmoles of NADPH oxidized/min/mg protein. GR: μmoles of NADPH oxidized/min/mg protein. GSH: μmoles of GSH/mg protein

ns non-significant as compared to control

[a] 3-NP versus control

[b] 3-NP + Naringin versus 3-NP

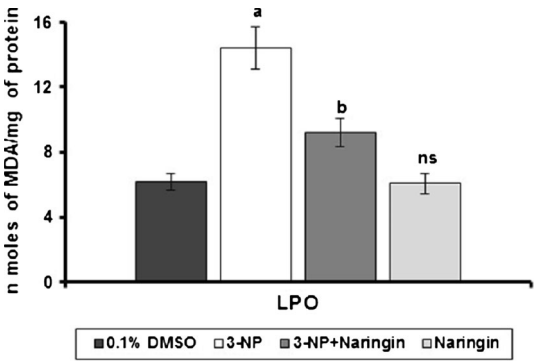

Fig. 4 Effect of naringin on 3-nitropropionic acid-induced lipid peroxidation in PC12 cells. Values are represented as the mean ± SD of three independent experiments. Values are statistically significant at $p < 0.05$, [a]3-NP versus control, [b]3-NP + Naringin versus 3-NP, *ns* non-significant as compared to control

of Bax with decrease in Bcl-2 expression ($p < 0.05$) as compared to control. Naringin modulated the expressions of Bcl-2 and Bax towards normal level in the cells exposed to 3-NP ($p < 0.05$), showing its anti-apoptotic effect.

Naringin activates Nrf2 signaling pathway

The facts that ROS lead to neurodegeneration and antioxidant therapy has neuroprotective effects in HD may point to a potential beneficial effect of the Nrf2 pathway. To investigate whether naringin treatment in PC12 cells regulates key proteins involved in the cellular response to oxidative stress, Nrf2 activation was analyzed. Figure 8 shows that 3-NP-induced PC12 cells had minor increase in the Nrf2 activation as compared to control. Naringin treatment exhibited significant increase in the nuclear accumulation of Nrf2 in 3-NP-induced cells ($p < 0.05$).

Figure 9 shows the mRNA expressions of NAD(P)H:-quinone oxidoreductase-1 (NQO-1) and heme oxygenase-1 (HO-1) in 3-NP- and naringin-treated PC12 cells. Minor increase in the expressions of NQO-1 and HO-1 was observed in 3-NP-induced cells compared to control. Administration of naringin significantly increased the expressions of these genes and renders protection against 3-NP-induced neurotoxicity ($p < 0.05$), which shows that naringin activates the Nrf2 signaling pathway and exhibits protection to 3-NP-induced cells.

PI-3K/Akt signaling was reported to involved in the activation of Nrf2 and up-regulation of several antioxidant gene expression [16]. Further, the activation of Akt by naringin was well documented in earlier study [28]. In this study, we investigated the involvement of PI-3K/Akt signaling in naringin-driven activation of Nrf2 using LY294002, the pharmacological inhibitor of PI-3K/Akt signaling cascade. As shown in Figs. 10 and 11, naringin-induced Nrf2 nuclear translocation and its target genes NQO-1 and HO-1 expressions were significantly reduced by LY294002 suggesting that PI-3K/Akt signaling is involved in the activation of Nrf2 in PC12 cells.

Discussion

Attenuation of oxidative stress and mitochondrial dysfunction protects against neurodegenerative disease [48]. Induction of endogenous antioxidant genes by the transcription factor Nrf2 is considered as the major regulator of redox homeostasis. This is a promising strategy to combat oxidative stress [49]. In this study, the protective effect of naringin on 3-NP-induced neurotoxicity through the PI-3K/Akt-mediated Nrf2 activation in PC12 cells was investigated.

LDH release is an index of cell membrane integrity. LDH activity was measured to determine the protective effect of naringin on 3-NP-induced neurotoxicity in PC12

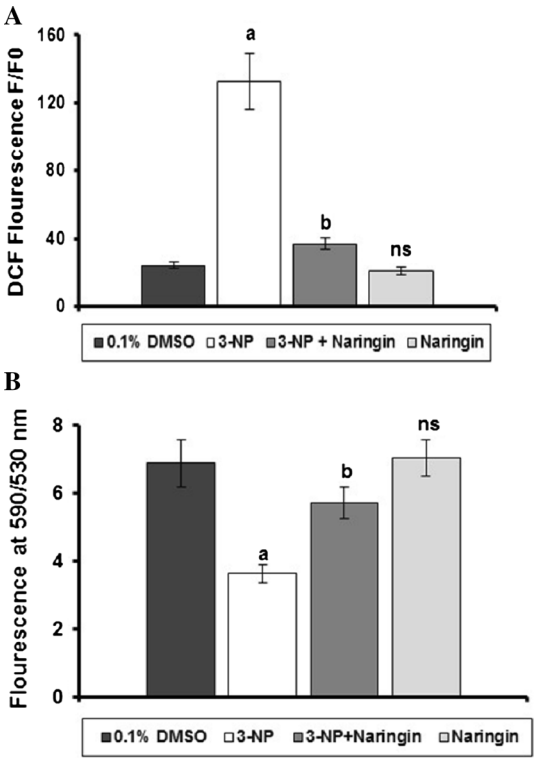

Fig. 5 Effect of naringin on 3-nitropropionic acid-induced reactive oxygen species and mitochondrial membrane potential in PC12 cells. **A** ROS measurement: PC12 cells were exposed to 3-NP (15 mM) in the presence or absence of naringin (10 μM) and incubated for 16 h. The cells were incubated with 5 mM H2DCF-DA at 37 °C for 20 min and then after washing, fluorescence was measured at 505 nm excitation and 550 nm emission in spectroflourometer. **B** Mitochondrial membrane potential: PC12 cells were exposed to 3-NP (15 mM) in the presence or absence of naringin (10 μM) and incubated for 16 h. Then, the cells were subsequently stained with JC-1. Relative mitochondrial membrane potential values are expressed as the ratio of reading at 590 nm to the reading at 530 nm. ROS and mitochondrial membrane potential values are represented as the mean ± SD of three independent experiments. Values are statistically significant at $p < 0.05$, [a]3-NP versus control, [b]3-NP + Naringin versus 3-NP, *ns* non-significant as compared to control

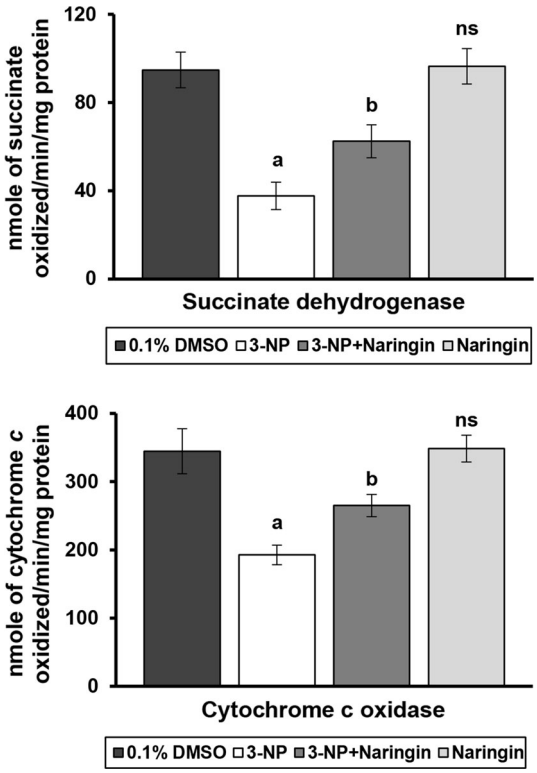

Fig. 6 Effect of naringin on succinate dehydrogenase and cytochrome *c* oxidase activities in 3-nitropropionic acid-induced PC12 cells. Values are represented as the mean ± SD of three independent experiments. Values are statistically significant at $p < 0.05$, [a]3-NP versus control, [b]3-NP + Naringin versus 3-NP, *ns* non-significant as compared to control

cells. 3-NP-induced oxidative damage has been characterized by detrimental changes in the LDH activity, resulting in reduction of cell viability. The LDH activity was found to be increased after 3-NP administration. The loss of intracellular LDH and its release into the culture medium is an indicator of cell death due to cell membrane damage induced by 3-NP [50]. Naringin decreased the LDH release and indicates its protective effect through the maintenance of plasma membrane integrity.

Oxidative stress has been reported in 3-NP-induced neurotoxicity through depleted GSH level, and altered profiles of antioxidant enzymes with elevated levels of ROS [51]. It is essential to scavenge free radicals produced during the metabolism of toxic substances and protect cells from oxidative injuries. Therefore, the effect of naringin on antioxidants was examined. The activities of enzymatic antioxidants and level of GSH were found to be decreased in the cells exposed to 3-NP, whereas treatment with naringin significantly augmented the antioxidants in 3-NP exposed cells. The results of this study demonstrate that naringin reduces oxidative damage in 3-NP-induced neurotoxicity. The protective efficacy of naringin also partly attributed to its ability to enhance the antioxidant status in brain [52].

An increase in the lipid peroxidation is one of the consequences of oxidative stress. Measurement of malondialdehyde (MDA), the most abundant product arising from LPO, has been extensively used as an index of oxidative stress. In this study, 3-NP-induced PC12 cells showed increased level of LPO as similar to the previous report [53]. Naringin treatment decreased the LPO in PC12 cells through its ability to scavenge free radicals produced by the 3-NP.

Oxidative stress is a major deleterious event observed in HD [54]. Excessive generation of ROS induces mitochondrial membrane depolarization, damages the respiratory chain, and induces apoptosis in neurons. ROS overproduction is one of the possible mechanisms for

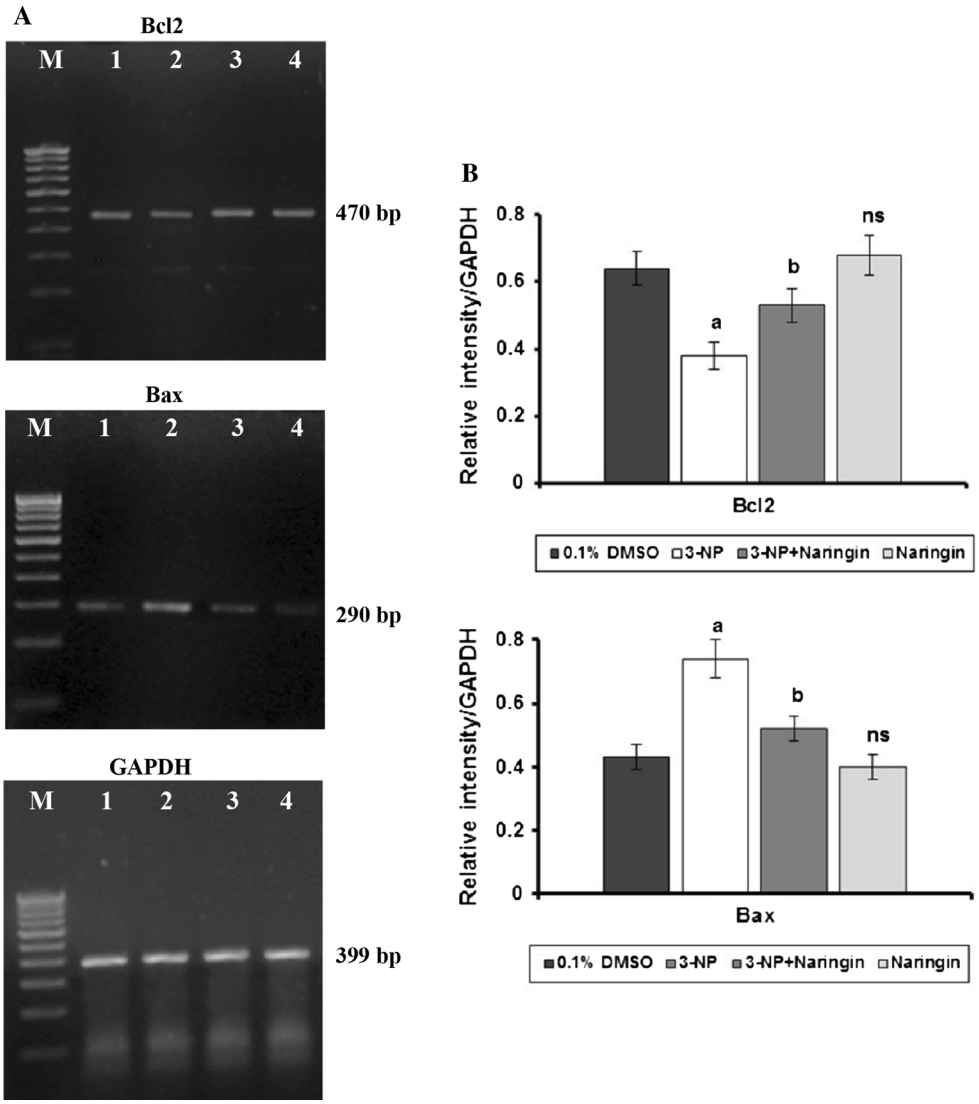

Fig. 7 Reverse transcriptase polymerase chain reaction analyses of Bcl-2 and Bax in PC12 cells. **A** 1.4 % agarose gel electrophoresis of RT-PCR products. *M* 100 bp molecular weight marker, *Lane-1* control, *Lane-2* 3-NP-induced, *Lane-3*: Naringin-treated, *Lane-4* Naringin alone. **B** Quantitative data expressing the ratio of Bcl-2 or Bax/GAPDH mRNA levels. Values are represented as the mean ± SD of three independent experiments. Values are given statistically significant at $p < 0.05$, [a]3-NP versus control, [b]3-NP + Naringin versus 3-NP, *ns* non-significant as compared to control

3-NP-induced neuronal death. Considering the important role of ROS in 3-NP-induced oxidative stress, the effect of naringin on ROS in PC12 cells was investigated. Administration of PC12 cells with 3-NP resulted in an elevated ROS production. Increase in the level of ROS has been documented in cells treated with 3-NP [55]. In this study, naringin protected the PC12 cells by decreasing the 3NP-induced ROS accumulation by its ability to scavenge free radical. Flavonoids have been shown to be effective scavengers of ROS [56]. This data suggest that increase in ROS generation by 3-NP causes cytotoxicity; however, treatment with naringin inhibits the ROS generation, thus rendering protection.

Recent studies have implicated the potential role of mitochondria in cell death mechanisms, since mitochondrial dysfunction results in the release of factors that initiate and amplify numerous signals resulting in apoptosis [57]. Further, mitochondrial dysfunction and concomitant bio-energetic failure can lead to abnormal cellular ionic homeostasis, eventually leading to neuronal death [58]. Several studies have reported the occurrence of mitochondrial dysfunction in brain of HD [59, 60]. In this study, PC12 cells induced with 3-NP exhibited mitochondrial depolarization and decrease in mitochondrial respiratory chain enzymes, succinate dehydrogenase and cytochrome *c* oxidase activities, and these cells might be undergoing the apoptotic processes as similar to previous report [61]. The naringin treatment protected PC12 cells from mitochondrial damage by ameliorating 3-NP-mediated reduction in mitochondrial transmembrane potential. Further, naringin offered considerable degree of protection against the decrease in mitochondrial complex enzyme activities, suggesting its potential to preserve the integrity of mitochondrial respiratory chain through its antioxidant effect.

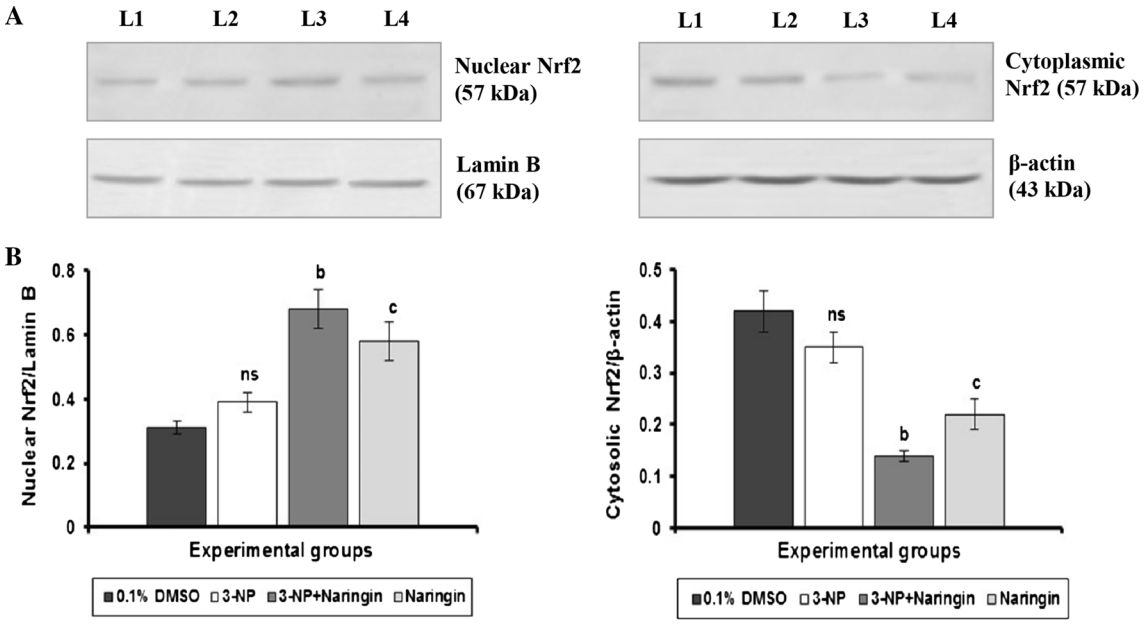

Fig. 8 Immunoblot analysis of Nrf2 in nucleus and cytosolic fractions of PC12 cells. **A** Immunoblot analysis was performed with the antibody against Nrf2. *Lane-1* control, *Lane-2* 3-NP-induced, *Lane-3* Naringin-treated, *Lane-4* Naringin alone. **B** Quantitative data expressing the corresponding protein levels were assessed using densitometry and are expressed as the ratio of Nrf2 with lamin B/β-actin. Values are represented as the mean ± SD of three independent experiments. Values are given statistically significant at $p < 0.05$, [b]3-NP + Naringin versus 3-NP, [c]Naringin alone versus control, *ns* non-significant as compared to control

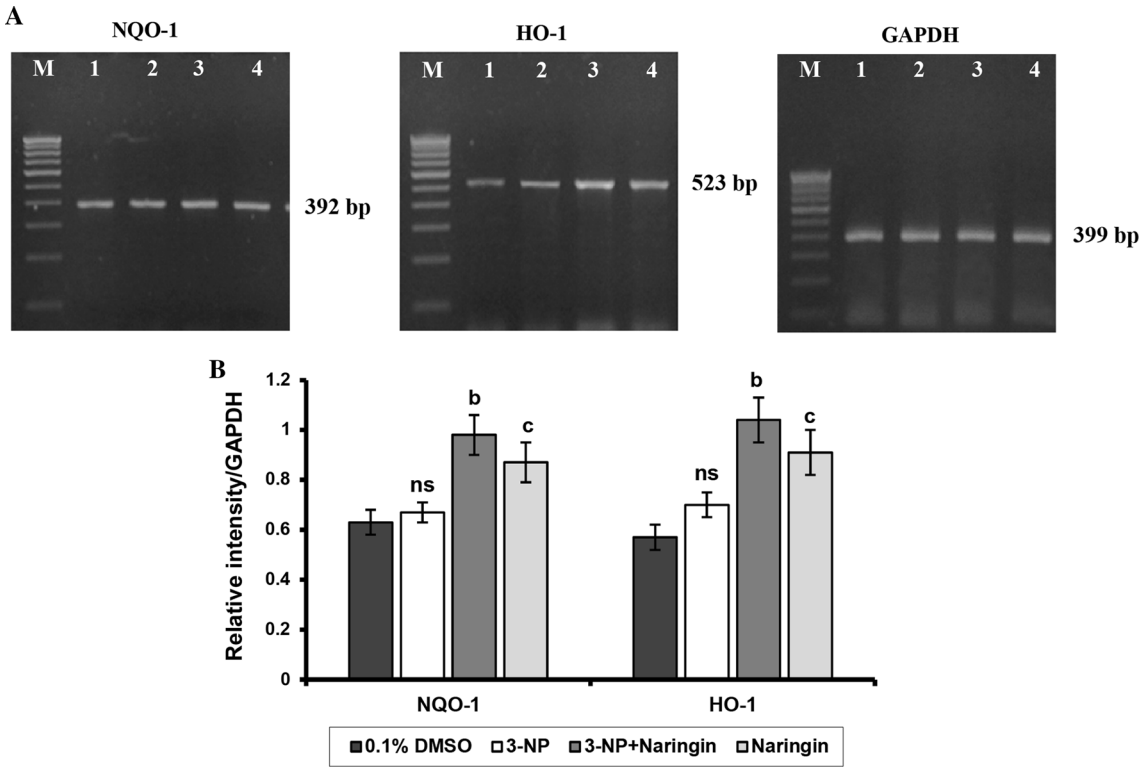

Fig. 9 Reverse transcriptase polymerase chain reaction analyses of NQO-1 and HO-1 in PC12 cells. **A** 1.4 % agarose gel electrophoresis of RT-PCR products. *M* 100 bp molecular weight marker, *Lane-1* control, *Lane-2* 3-NP-induced, *Lane-3* Naringin-treated, *Lane-4* Naringin alone. **B** Quantitative data expressing the ratio of NQO-1 or HO-1/GAPDH mRNA levels. Values are represented as the mean ± SD of three independent experiments. Values are given statistically significant at $p < 0.05$, [b]3-NP + Naringin versus 3-NP, [c]Naringin alone versus control, *ns* non-significant as compared to control

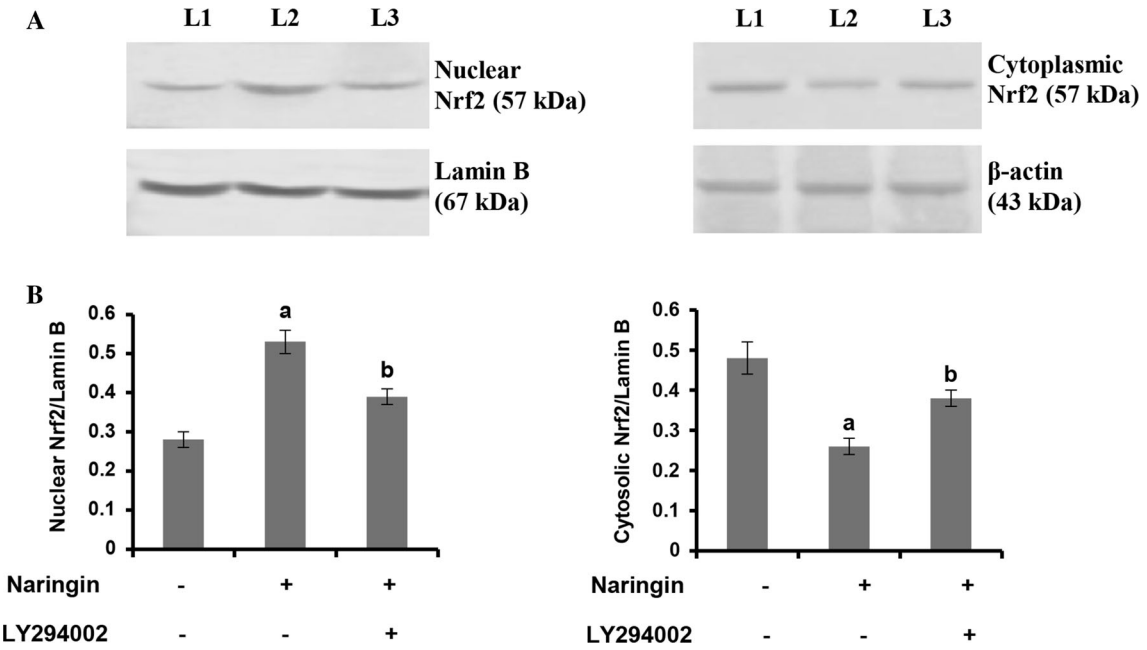

Fig. 10 Immunoblot analysis of Nrf2 in nucleus and cytosolic fractions of PC12 cells in the presence of PI-3K inhibitor LY294002. **A** Immunoblot blot analysis was performed with the antibody against Nrf2. *Lane-1* control, *Lane-2* Naringin alone, *Lane-3* Naringin + LY294002. **B** Quantitative data expressing the corresponding protein levels were assessed using densitometry and are expressed as the ratio of Nrf2 with lamin B/β-actin. Values are given statistically significant at $p < 0.05$, [a]Naringin alone versus control; [b]Naringin + LY294002 versus Naringin

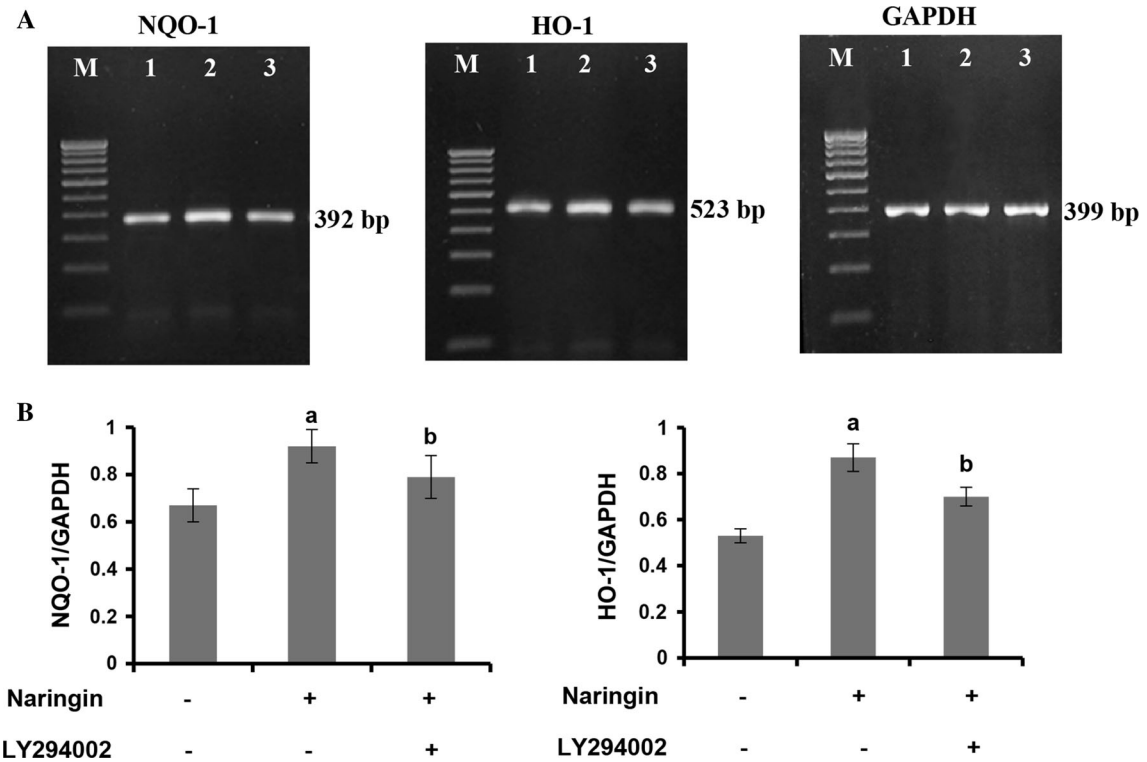

Fig. 11 Reverse transcriptase polymerase chain reaction analyses of NQO-1 and HO-1 of PC12 cells in the presence of PI-3K inhibitor LY294002. **A** 1.4 % agarose gel electrophoresis of RT-PCR products. *M* 100 bp molecular weight marker, *Lane-1* control, *Lane-2* Naringin alone, *Lane-3* Naringin + LY294002. **B** Quantitative data expressing the ratio of NQO-1 or HO-1/GAPDH mRNA levels. Values are given statistically significant at $p < 0.05$, [a]Naringin alone versus control, [b]Naringin + LY294002 versus Naringin

These results indicate that, 3-NP-induced cell death via mitochondrial dysfunction is decreased by the flavonoid naringin.

The mitochondrial death pathway is regulated by a fine balance between pro-apoptotic and pro-survival Bcl-2 family members [62]. In this study, 3-NP-administered PC12 cells exhibited decreased expression of Bcl-2 with elevated Bax. Naringin treatment decreased the apoptosis by ameliorating the expressions of Bcl-2 and Bax in cells exposed to 3-NP. Naringin has been previously reported to modulate the Bcl-2 genes and protect neurons from apoptosis [63]. Further, studies have shown that, Bcl-2 expression protects PC12 cells from 3-NP-induced mitochondrial dependent apoptosis [64]. This shows that naringin is effective enough to combat the 3-NP-induced cell death.

Activation of Nrf2 can confer significant protection to neurons, and drugs that activate this pathway have efficacy in blocking neuronal death [65, 66]. Certainly, a recent work has demonstrated that treatment with flavonoid could activate the Nrf2 pathway and confer protection against neurodegenerative diseases [67]. In the present study, increased Nrf2 nuclear accumulation was observed in naringin-treated cells. Thus, the assenting neuroprotective effect of naringin on 3-NP-induced neurotoxicity is through the modulation of Nrf2-signaling pathway. The induction of protective enzyme defense systems is important for defending cells from oxidative damage. The increased expressions of NQO-1 and HO-1 provide protection against ROS as a part of an adaptive response [68]. In this study, naringin-treated cells showed increased expressions of NQO-1 and HO-1 compared with 3-NP-induced cells, demonstrating that naringin reduces oxidative damage in PC12 cells induced by 3-NP with increased expressions of NQO-1 and HO-1 through Nrf2 activation.

The molecular mechanism underlying the Nrf2 activation by naringin has been the subject of extensive investigations. The PI-3K/Akt is a key survival-promoting signaling pathway that enhances cellular tolerance against multiple apoptotic insults [17, 69]. PI-3K/Akt-mediated Nrf2 activation determines the signals to govern the cellular defense system against inflammatory and oxidative insults [17]. Both P-I3K/Akt and Nrf2 pathways are implicated in the transcriptional regulation of NQO-1 and HO-1 [70]. Naringin was previously reported to activate PI-3K/Akt signaling [28]. In this study, inhibition of PI-3K by specific inhibitor LY294002 reduced the naringin-induced accumulation of Nrf2 in nucleus, and NQO-1 and HO-1 expression. These findings suggest that PI-3K/Akt signaling pathway is required for naringin-mediated Nrf2 activation, and subsequent expression of NQO-1 and HO-1 in PC12 cells.

Appending the results obtained from this study, a mechanism involved in the protective efficacy of naringin against the 3-NP-induced neurotoxicity is demonstrated. This study provides evidence that naringin exhibits neuroprotective effect on 3-NP-induced neurotoxicity through the PI-3K/Akt-dependent Nrf2 activation in PC12 cells.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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