Naringenin Protects against 1-Methyl-4-Phenylpyridinium- Induced Neuroinflammation and Resulting Reactive Oxygen Species Production in SH-SY5Y Cell Line: An *In vitro* Model of Parkinson's Disease

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ABSTRACT

Background: Parkinson's disease is a progressive neurodegenerative disorder which affects 1% of the population worldwide. It is well known that 1-methyl-4-phenylpyridinium (MPP^+) selectively and potentially inhibit Complex I of the mitochondrial electron transport chain. This inactivation leads to the generation of reactive oxygen species (ROS), which in turn damage neurons. In addition, neuroinflammation plays a major role in neurodegeneration processes. **Objective:** In the present study, the effect of naringenin (NGN), on MPP^+-induced neuroinflammation and ROS generation in SH-SY5Y cells were investigated. **Materials and Methods:** Cells were pretreated with (0.1% dimethyl sulfoxide) or NGN (25, 50, and 100 µm/mL) for 24 h, and then induced with 1 mM MPP^+ for 15 min. Following overnight incubation, cells were harvested for ROS staining, gene expression of apoptotic markers such as B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax), inflammatory markers such as tumor necrosis factor-alpha (TNF-α), and nuclear factor-κB (NF-κB) and protein expression of neuronal markers such as dopamine transporter (DAT), tyrosine hydroxylase (TH), and α-synuclein (SYN). **Results:** In this study, NGN exhibited oxidative protection by decreasing ROS generation which is evidenced through significant regulation of oxidative stress markers. Likewise, NGN decreased TNF-α, Bax, and NF-κB and increased Bcl-2 gene expressions in MPP^+-induced SH-SY5Y cells compared to normal SH-SY5Y cells. Further, NGN significantly and dose-dependently decreased SYN and increased DAT and TH levels in MPP^+-induced SH-SY5Y cells in comparison to the normal cells. **Conclusion:** The results obtained from the present study revealed that NGN has the potential to encounter MPP^+-induced dopaminergic degeneration through regulating ROS generation and neuroinflammation.

Key words: 1-methyl-4-phenylpyridinium, naringenin, neuroinflammation, Parkinson's disease, reactive oxygen species, SH-SY5Y

SUMMARY

• The data obtained from the present study showed that NGN possess neuroprotection by ameliorating neuroinflammation and oxidative stress in MPP^+ induced neurotoxicity in SH-SY5Y cells. Moreover, NGN significantly regulated inflammatory marker (TNF-α) and oxidative stress markers (SOD, LPO, NO and % ROS). Furthermore, NGN improved neuronal markers like DAT, TH and SYN which clearly reveals its protective effect. The results showed that NGN may be the better therapeutic choice in the treatment of PD and further investigations with detailed mechanistic interventions may pave the way to undertake the drug from bench to bedside.

Neuroprotective Effect of Naringenin against MPP^+-Induced Neuroinflammation
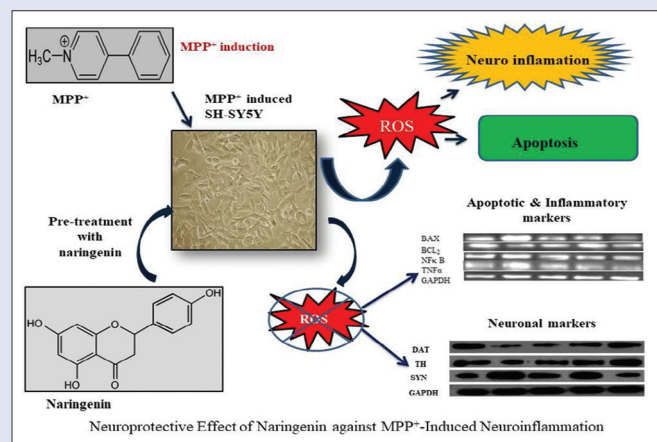
Abbreviations used: TNF-α: Tumor Necrosis Factor alpha; NF-κB: Nuclear factor; SYN-α: synuclein; NGN: Naringenin; MPP^+: 1-methyl-4-phenylpyridinium; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD-Parkinson's disease; DCFH-DA: 2',7' - dichlorofluorescin diacetate; SOD: Superoxide dismutase; NO: Nitric oxide; MDA: Malondialdehyde; DAT: Dopamine transporter; TH: Tyrosine hydroxylase; ANOVA: Analysis of variance; SEM: Standard error of the mean; RT-PCR: Reverse transcriptase-polymerase chain reaction; BSA: Bovine serum albumin; PBS: Phosphate buffered saline; TBST: Tris-Buffered saline with Tween; RNA: Ribonucleic acid.

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder associated with symptoms such as resting tremor, rigidity, bradykinesia, and postural instability.[1] On a molecular and neurochemical level, PD is characterized by selective loss of dopaminergic (DA) neurons[2] in substantia nigra pars compacta (SNpc) and depletion of dopamine in striatum. The reasons behind the loss of DA neurons could be attributed to reactive oxygen

species (ROS),[3] mitochondrial dysfunction,[4] excitotoxicity,[5] and inflammation.[6] The mitochondrial defect in PD is linked with inhibition of electron respiratory chain Complex I activity. This inactivation leads to the generation of ROS which in turn damage neurons in association with neuroinflammation.[7]

The regulation of intracellular ROS is an important physiological aspect in the normal functioning of a system and disruption of this leads to deleterious effect in the body both at cellular and molecular level. Lipid peroxidation (LPO) is the primary result of oxidative stress and affects the level of malondialdehyde (MDA), a metabolite of membrane LPO, resulting in cell death.[8] Nitric oxide (NO) is also considered to be the key mediator in oxidative stress-induced cell death. Previous studies have shown that an increasing superoxide dismutase (SOD) activity protects the DA neurons against the toxin-induced neurodegeneration.[9]

1-methyl-4-phenylpyridinium (MPP+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has been proven for its selective complex I inhibition of the mitochondrial electron transport chain.[10] MPP+ and MPTP are well-proven models to study the efficacy of various drugs in PD.[11] Generations of intracellular ROS and neuroinflammation have been demonstrated in MPP+-induced neurotoxicity.[12]

Naringenin (4, 5, 7-trihydroxy flavonone; NGN) is a naturally-occurring bioflavonoid that is found in citrus fruits and vegetables.[13] NGN possess pharmacological properties such as neuroprotective,[14] anticancer,[15] antiatherogenic,[16] hepatoprotective,[17] and nephroprotective[18] activities. In the present study, the effect of NGN on neuroinflammation and intracellular ROS generation in MPP+-induced neurotoxicity in human neuroblastoma SH-SY5Y cells were investigated.

MATERIALS AND METHODS
Chemicals
NGN, MPP+, 2', 7'-dichlorofluorescin diacetate (H$_2$DCFDA), dimethyl sulfoxide (DMSO), MDA, thiobarbituric acid, and TRIzol reagent were purchased from Sigma Aldrich, USA. Mouse anti- α-synuclein (SYN), rat anti-dopamine transporter (DAT), rabbit anti-tyrosine hydroxylase (TH), and their respective secondary antibodies were procured from Santa Cruz, USA. Polymerase chain reaction (PCR) master cycler gradient was purchased from Genet Bio, Korea. DMEM/Ham's F-12, heat-inactivated fetal calf serum (FCS), L-glutamine, penicillin, streptomycin, and nonessential amino acids were procured from Gibco, USA. Primers were procured from Eurofins India. SH-SY5Y cell lines were procured from the National Centre for Cell Science, Pune. Unless mentioned, all other chemicals and reagents were of analytical grade.

Maintenance of cell-line
Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle Medium/Ham's F-12 medium, supplemented with 10% (v/v) FCS, 2 mmol/L L-glutamine, 50 U/mL penicillin, 100 mg/L streptomycin and nonessential amino acids, and maintained at 37°C in a humidified 5% CO$_2$ and 95% O$_2$ atmosphere. The medium was changed every 3 days and the cells were passaged every 4–5 days.

Cell viability assay
SH-SY5Y cells were seeded on 96-well plates at a density of 0.5×10^5 cells/well. The cultures were grown for 24 h followed by the addition of fresh medium containing different doses of NGN (1, 10, 100, 500, 1000 μM/mL) for 24 h duration before they were subjected to MPP+ (1 mM) exposure. Cell viability was determined by MTT assay.[19] After incubation for 24 h with the desired drug, 50 μl of MTT reagent (5 mg/

mL MTT in phosphate-buffered saline (PBS) containing 10 mM HEPES; filter with 0.22 μm) was added to each well and incubated in a CO$_2$ incubator for 4 h. The medium was aspirated from each well and dissolved the remaining MTT-formazan crystals by adding 200 μL of DMSO to all the wells in column, and the absorbance was measured in a microplate reader at 570 and 630 nm. Based on the MTT assay, the doses (25, 50, 100 μM/mL) were selected for further studies.

Trypan blue exclusion assay
SH-SY5Y cells were incubated with different concentrations of NGN for 24 h, then added 1 mM MPP+ and incubated for 2 h. Following overnight incubation of fresh media with NGN, the number of viable cells was determined using trypan blue exclusion assay. Cells were collected and rinsed with PBS. After which they were immediately stained with 0.4% trypan blue solution, and the number of viable cells were counted using a hemocytometer under a light microscope.[20]

Measurement of intracellular reactive oxygen species generation
SH-SY5Y cells were treated with three different concentrations of NGN (25, 50, 100 μM/mL) and/or MPP+ and incubated at 37° C as mentioned above. Following incubation, the media was removed, and the cells were washed 3 times with 1X PBS (pH 7.2). 10 μM of 2',7' - dichlorofluorescin diacetate was added to all the wells, and they were incubated at 37° C for 30 min and fluorescence intensity of DCF was measured in a microplate-reader (Thermo Scientific Multiskan spectrophotometer, USA) at excitation wavelength 485 nm and emission wavelength 535 nm.[21]

Antioxidant potential of naringenin against 1-methyl-4-phenylpyridinium-induced neurotoxicity in SH-SY5Y cells
Superoxide dismutase
SH-SY5Y cells were mechanically harvested and then collected by centrifugation at 2,000 g for 10 min at 4° C. The pellets were homogenized in 1 ml of cold 20 mM HEPES buffer, pH 7.2 (1 mM Ethylene Glycol Tetra acetic Acid, 210 mM mannitol and 70 mM sucrose) and sonicated (3 times, 5 s). The samples were then centrifuged at 1,500 g for 5 min at 4° C. To 0.1 ml of the supernatant, 0.25 ml of sodium pyrophosphate buffer (0.025 M), 0.025 ml of phenazonium methosulphate (186 μM), and 0.075 ml of nitro blue tetrazolium (300 μM) were added. The reaction was triggered by the addition of 0.075 ml of NADH (780 μM). After incubation at 30° C for 90 s, the reaction was terminated by addition of 0.25 ml glacial acetic acid. The mixture was stirred vigorously and shaken with 2 ml of n-butanol and allowed to stand for 10 min and centrifuged. N-butanol alone served as blank. The color intensity of the chromogen was read at 560 nm in Thermo Scientific Multiskan spectrophotometer, USA.[22]

Nitric oxide level
NO has been identified as a mediator of cytokine action in various cell types. NO is an intra- and intercellular messenger that mediates a variety of important physiological processes.[23] Changes in NO production were measured indirectly as the accumulation of nitrites (the end-product of NO metabolism) in the medium using Griess assay.[24] Neuroblastoma SH-SY5Y cells were incubated with NGN (25, 50, 100 μM/mL) and/or MPP+ (1 mM) as mentioned above. After incubation period, 1 ml of the culture supernatant was mixed with 1.0 ml of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2%–5% H$_3$PO$_4$) for 10 min at room temperature. The concentration of nitrite was measured by microplate reader (Thermo Fisher Scientific,

USA) at 540 nm, and the nitrite concentration was calculated using a standard curve of sodium nitrite.

Lipid peroxidase

SH-SY5Y cells were seeded in a six-well plate. After cells achieved the 65% confluence, LPO was determined by measuring the amount of thiobarbituric acid reactive substances formed during the decomposition of lipid hydroperoxides, mostly formed from MDA. Cells were collected in 2 ml of culture medium and sonicated for 3 s × 5 s intervals at 40 V over ice. Sodium dodecyl sulfate (SDS) lysis solution (1.0 ml) was added to the sample solution in a microcentrifuge tube and mixed thoroughly. Then, 2.5 ml of TBA reagent were added to each sample and standard to be tested, incubated at 95° C for 60 min. Each sample were loaded (in duplicate) into a clear 96-well plate and the absorbance at 532 nm was recorded using a microplate reader (Thermo Fisher Scientific, USA).[25]

Gene expression analysis of apoptotic and inflammatory markers

Reverse transcriptase (RT)-PCR was performed to determine the level of mRNA expression of Bcl-2-associated X protein (Bax), B-cell lymphoma-2 (Bcl-2), tumor necrosis factor-alpha (TNF-α), and nuclear factor κB (NF-κB). The housekeeping gene GAPDH was used in this study as a reference for relative gene expression. Total ribonucleic acid (RNA) was extracted from cultured SH-SY5Y cells using TRIzol Reagent (Sigma, USA). After retrieving the cells, the tubes were incubated for 10 min and centrifuged at 1000 rpm for 5 min. To the supernatant, 100 µl of chloroform was added, incubated for 5 min at room temperature and centrifuged at 12,000 rcf for 20 min. Then, 250 µl of isopropyl alcohol was added to the supernatant to precipitate the total RNA and centrifuged at 12,000 rcf for 15 min following the

incubation period of 10 min. The supernatant was decanted carefully, and the pellet was washed three times with 75% ethanol and centrifuged at 12,000 rcf for 15 min and the pellet was dried. The dried pellet was resuspended in 20 µl of RNase-free water and stored at −80°C until use. The isolated RNA was allowed to undergo reverse transcription and polymerization reaction to get cDNA using PCR master cycler gradient (Genet Bio, Korea). The gene expression was analyzed using the bands formed in agarose gel electrophoresis, captured using Gel documentation unit (VilberLaumar, Germany) and quantified by ImageJ software. The primer sequences used in the study are presented in Table 1.

Effect of naringenin on apoptosis and inflammatory related proteins

Cells were lysed with 0.1 mL of lysis buffer (1% Nonidet P-40; 50 mM Tris HCl, pH 7.6; 5 mM EDTA), followed by 30 min incubation on ice. Lysate was centrifuged at 15,000 g for 10 min at 4°C. The supernatant (total lysate) was collected and protein levels were determined. Samples containing 40 µg protein were separated in SDS-PAGE (100V) and transferred to polyvinylidene fluoride polyvinylidene difluoride membrane (230 mA for 90 min). Membranes were blocked with 5% milk for 1 h and washed three times with tris-buffered saline (TBS) for 5 min each. Primary rabbit polyclonal TH (1:100), mouse monoclonal SYN (1:100), rat monoclonal DAT (1:50) in 1% bovine serum albumin (BSA), 0.1% Tween-20, and 0.02% sodium azide in TBS. After washing with BSA in TBS, the membrane was incubated in anti-rabbit IgG (1:300), anti-mouse IgG (1:100), anti-rat IgG (1:100) secondary antibody in BSA for 1 h, and washed with TBS with Tween. The bands were visualized with enhanced chemiluminescence solution.

Statistical analysis

Data were expressed in mean ± standard error of mean. Mean difference between the cells were analyzed using one-way ANOVA followed by
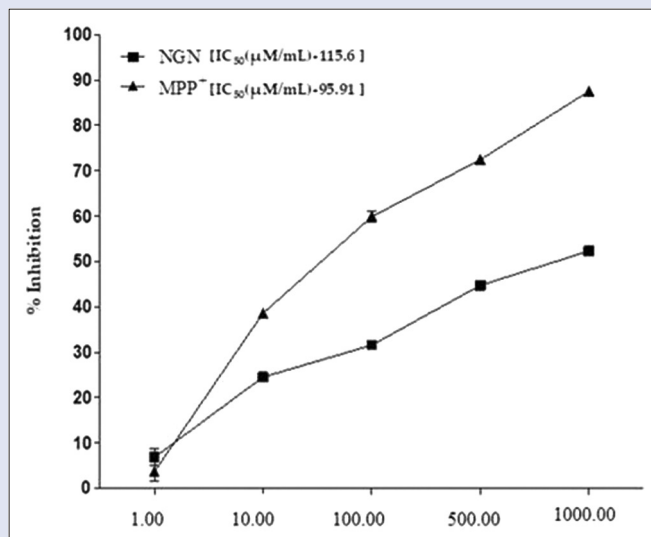

Figure 1: Cell viability assay for Naringenin and 1-methyl-4-phenylpyridinium. Values are expressed in mean ± standard error of mean
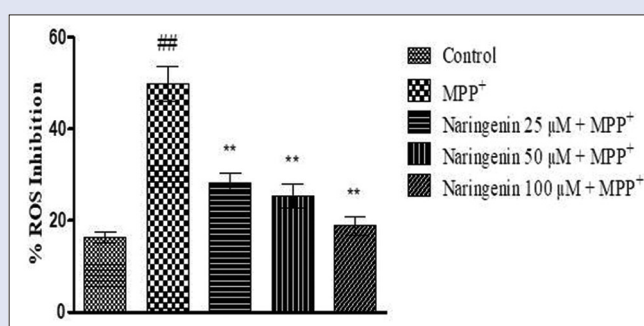

Figure 2: Effect of naringenin on reactive oxygen species % inhibition in 1-methyl-4-phenylpyridinium-treated cells. Values were expressed in mean ± Standard error of the mean Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests, ## indicates $P < 0.01$ versus control cells, ** indicates $P < 0.01$ versus 1-methyl-4-phenylpyridinium

Table 1: Primer sequences

Product	Forward primer	Reverse primer
GAPDH	5' TTCACCACCATGGAGAAGGC 3'	5'TCATGACCACAGTCCATGCC3'
Bax	5'-TTTTGCTTCAGGGTTTCATC-3'	5'-GACACTCGCTCAGCTTCTTG-3'
Bcl-2	5'ATGTGTGTGGAGAGCGTCAACC-3'	5'TGAGCAGAGTCTTCAGAGACAGCC-3'
TNF-α	5'-ATGAGCACAGAAAGCATGAT-3'	5'-ACAGGCTTGTCACTCGAATT-3'
NF-κB	5'-ATGGCTTCTATGAGGCTGAG-3'	5'-GTTGTTGTTGGTCTGGATGC-3'

Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma-2; TNF-α: Tumor necrosis factor-alpha; NF-κB: Nuclear factor-κB

Tukey's multiple comparison test as *post hoc* using Graph Pad Prism 5.0. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Effect of naringenin on 1-methyl-4-phenylpyridinium-induced cytotoxicity

Cytotoxicity was performed to evaluate the cell survival as a function of mitochondrial viability. There was no significant difference between control and 0.1% DMSO-treated cells. A 24 h incubation period of MPP⁺ (1, 10, 100, 500, 1000 μM/mL) and NGN at increasing concentrations (1, 10, 100, 500, 1000 μM/mL) reduced the cell viability in a concentration dependent manner when compared with control [Table 2]. The LD_{50} was calculated using graph pad prism 5.0 the LD_{50} value for NGN and MPP⁺ was observed to be 115.6 and 95.91 μM [Figure 1].

Naringenin reduces the 1-methyl-4-phenylpyridinium-induced increase of reactive oxygen species in SH-SY5Y cells

ROS played a crucial role in cell apoptosis and mitochondrial transmembrane potential changes were considered to be in ROS production. ROS production was measured using fluorescent dye DCFDA. 1 mM MPP⁺ showed significant increase in ROS production (F [4, 10] = 27.86, $P < 0.01$) when compared to the normal cells. Whereas, NGN (25, 50, and 100 μM/mL) treatment reduced ROS production in MPP⁺-treated SH-SY5Y cells [Figure 2].

Antioxidant potential of naringenin against 1-methyl-4-phenylpyridinium-induced neurotoxicity in SH-SY5Y cells

To determine the suppression of MPP⁺-induced ROS production by NGN, we measured ROS production in SH-SY5Y under different conditions. Therefore, observed results suggest that NGN acts as an anti-oxidant agent to MPP⁺-mediated ROS generation. Moreover, the results showed that MPP⁺-induced cells significantly increased (F [4, 10] = 8.152, $P < 0.01$) MDA levels. On the other hand, NGN at 50 and 100 μM/mL concentrations significantly decreased ($P < 0.05$) MDA levels compared to 0.1% DMSO-treated MPP⁺-induced cells. MPP⁺-induced NO production in neuroblastoma SH-SY5Y cells were shown in 1 mM MPP⁺ with significant increase (F [4,10] = 6.656, $P < 0.01$). However, NGN at 100 μM/mL significantly decreased ($P < 0.05$) NO level in comparison to MPP⁺-treated cells. A significant decrease in SOD activity was also observed in MPP⁺-induced cells compared to control, whereas treatment with NGN at 100 μM/mL significantly increased ($P < 0.01$) SOD activity in comparison to MPP⁺ cells. Based on these findings, it is clear that NGN possess potent anti-oxidative properties against MPP⁺-induced oxidative stress in SH-SY5Y cells [Figure 3].

Effect of naringenin on 1-methyl-4-phenylpyridinium-induced alterations in Bcl-2-associated X protein and B-cell lymphoma-2

NGN protects cells from MPP⁺-induced apoptotic death which is clearly evidenced from the results obtained in gene expression studies. It was found that the expression of Bax and Bcl-2 genes were affected by MPP⁺ treatment. The intracellular Bax (F [4,10] = 4.731, $P < 0.05$) expression was increased significantly in 1 mM MPP⁺-treated cells compared with the control cells, whereas the intracellular Bcl-2 (F [4,10]

Table 2: Effect of naringenin on trypan blue dye exclusion assay

Concentration (μM/mL)	NGN (percentage of cell death)
1	13.94±1.90
10	16.52±0.97
100	31.58±0.80
500	44.73±0.22
1000	52.35±0.60

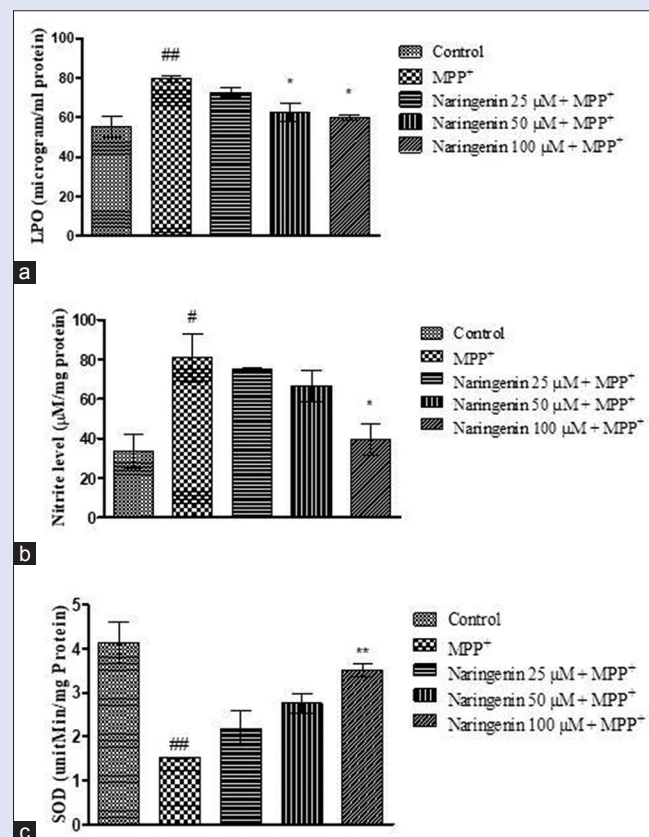Values were expressed in mean±SEM. SEM: Standard error of mean; NGN: Naringenin

Figure 3: (a) Lipid peroxidase (b) Nitrite nitrate (c) Superoxide dismutase. Effect of naringenin on superoxide dismutase, lipid peroxidation, and nitrite level in 1-methyl-4-phenylpyridinium-treated cells. Values were expressed in mean ± standard error of the mean Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests, #, ## indicates $P < 0.05$ and 0.01 versus control cells, *, ** indicates $P < 0.05$ and 0.01 versus 1-methyl-4-phenylpyridinium

= 6.756, $P < 0.01$) was down regulated in MPP⁺-treated cells. NGN treatment (100 μM) prevented MPP⁺-induced up regulation of Bax and down regulation of Bcl-2. These results suggest that MPP⁺ shifts the balance between pro-apoptotic and anti-apoptotic proteins whereas NGN prevented this type of alteration [Figure 4a and b].

Effect of naringenin on 1-methyl-4-phenylpyridinium-induced alterations in nuclear factor-κB and tumor necrosis factor-alpha

The main inflammatory factors, TNF-α increased during neurodegenerative diseases and induces apoptosis through a death receptor-dependent (extrinsic) or independent (intrinsic or

mitochondrial) pathway. Following 24 h treatment of SH-SY5Y cells with MPP⁺ (1 mM), we observed increase in TNF-α activity (F [4,10] = 7.326, $P < 0.01$). The addition of NGN at 50 and 100 µM significantly and dose dependently attenuated ($P < 0.05$ and 0.01, respectively) MPP⁺-induced TNF-α activation and reduced the active form of TNF-α in the NGN-treated cells. The NF-κB transcription factors have emerged as

a major regulator of apoptosis or necrosis, which contributed a crucial anti-apoptotic effect during normal development. In this study, we observed that MPP⁺ alone could restrain the constitutive NF-κB activity significantly (F [4, 10] = 6.149, $P < 0.05$). On the other hand, NGN (50 and 100 µM) significantly reduced ($P < 0.05$) the nuclear factor expression in neuroblastoma SH-SY5Y cell line [Figure 4c and d].
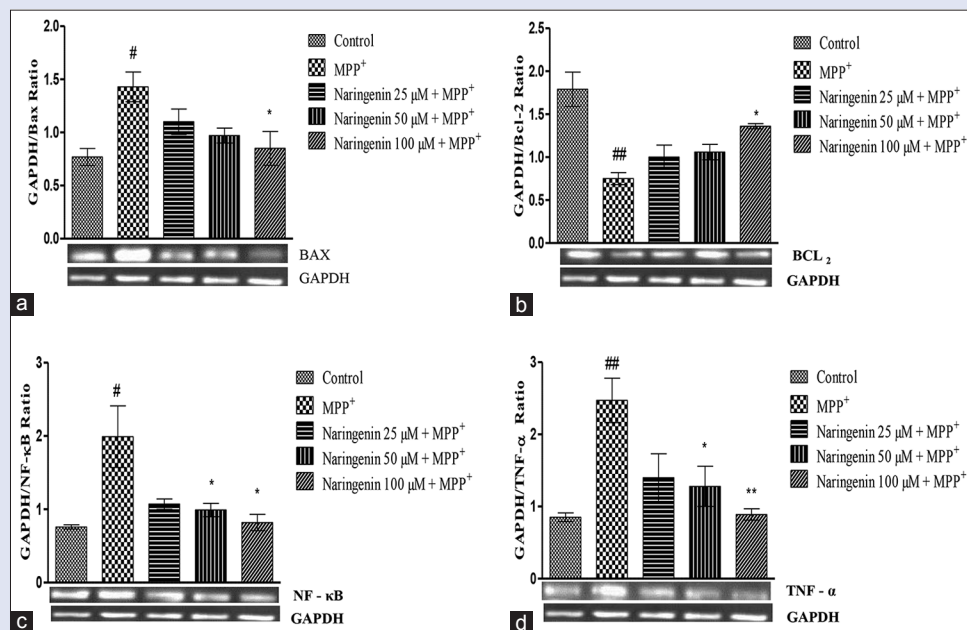

Figure 4: (a) BAX (b) BCL2 (c) NF-kB (d) TNF-α. Effect of naringenin on Bcl-2-associated X protein, B-cell lymphoma-2, NF-kB, tumor necrosis factor-alpha level in 1-methyl-4-phenylpyridinium- treated cells. Values were expressed in mean ± standard error of the mean statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests, #, ## indicates $P < 0.05$ and 0.01 versus control cells, *, ** indicates $P < 0.05$ and 0.01 versus 1-methyl-4-phenylpyridinium

Figure 5: (a) DAT (b) TH (c) SYN. Effect of naringenin on dopamine transporter, tyrosine hydroxylase and alpha α-synuclein level in 1-methyl-4-phenylpyridinium-treated cells. Values were expressed in mean ± standard error of the mean statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests, #, ## indicates $P < 0.05$ and 0.01 versus control cells, *, ** indicates $P < 0.05$ and 0.01 versus 1-methyl-4-phenylpyridinium
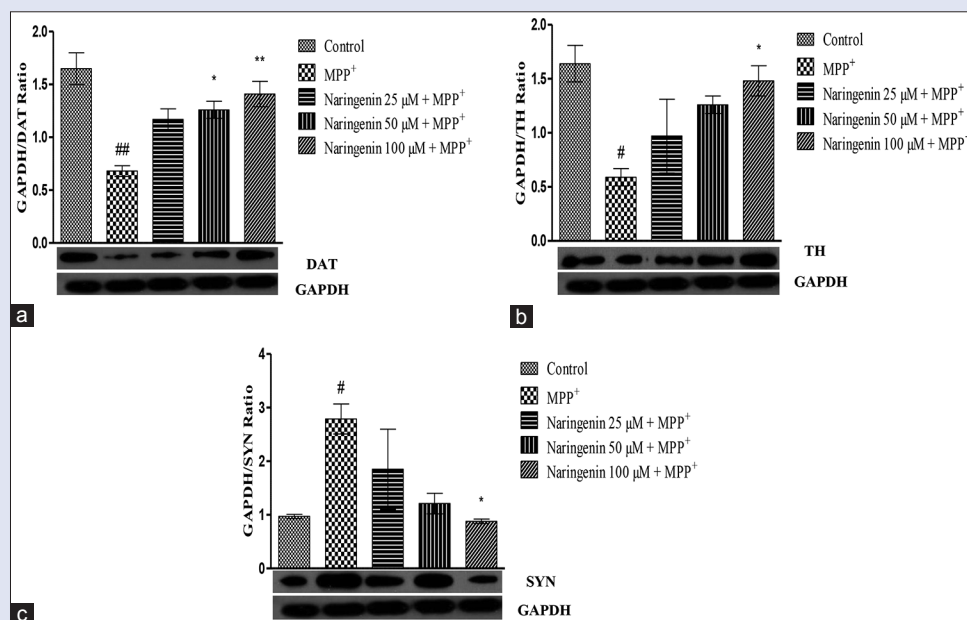
Effect of naringenin on 1-methyl-4-phenylpyridinium-induced alterations in dopamine transporter, tyrosine hydroxylase, and α-synuclein levels

SYN is a presynaptic neuronal protein that is linked with protein aggregation which leads to neuronal death. Results showed that SYN is significantly up-regulated in MPP+ (F [4, 10] = 4.646, $P < 0.05$) treated cells when compared to normal cells. TH is the rate limiting enzyme and DAT is the transporter for dopamine and found both the proteins were down-regulated (F [4, 10] = 4.945, $P < 0.05$ and F [4, 10] = 11.55, $P < 0.01$, respectively) in MPP+-treated cells when compared to control cells. On the other hand, NGN (100 μM) significantly increased DAT ($P < 0.01$) and TH ($P < 0.05$) levels and decreased SYN ($P < 0.05$) level in comparison to MPP+-treated SH-SY5Y cells [Figure 5].

DISCUSSION

The present study revealed that pretreatment with NGN significantly reduced the ROS production by decreasing oxidative stress markers such as LPO and NO and increasing SOD level. In addition, pretreatment with NGN decreased the inflammatory markers such as TNF-α and NF-$\kappa\beta$ in MPP+-treated SH-SY5Y cells. Further, NGN decreased the pro-apoptotic marker – Bax and increased the anti-apoptotic marker – Bcl-2 in MPP+-induced SH-SY5Y cells. These data clearly showed that pretreatment with NGN restores MPP+-induced neurotoxicity through regulating ROS production and neuroinflammation in SH-SY5Y cells.

Mechanism of MPP+-induced neurotoxicity is largely mediated through mitochondrial dysfunction. MPP+ enters DA cells through DAT and inhibits complex I enzyme in mitochondrial electron transport chain.[26] This decreases ATP production and triggers the generation of ROS and apoptosis which in turn leads to neuronal cell death.[27] TH, a rate-limiting enzyme was found to be significantly suppressed in MPP+-induced neuronal cells.[28] In the present study, pretreatment with NGN increased the DAT and TH levels in a dose-dependent manner which showed its protective effect against MPP+-induced neurotoxicity.

It is well known that MPP+ induces microglial activation through the activation of pro-inflammatory cytokines like TNF-α which then activates other cytotoxic molecules such as superoxide, NO, and ROS.[29] Further, NGN showed the involvement of NF-$\kappa\beta$ in both onset and determination of inflammatory process.[30] The observed decrease in inflammatory markers such as TNF-α and NF-$\kappa\beta$, and oxidative stress markers such as LPO, NO, and ROS in NGN-treated MPP+-induced SH-SY5Y cells clearly revealed its protective effect.

Overexpression of SYN is the key important marker and has been demonstrated in various experimental models of PD. Studies in numerous cell lines and primary neuronal culture demonstrated that overexpression of SYN induces apoptotic cell death. Evidence support the involvement of proapoptotic marker Bax in postmortem SNpc neurons in PD.[31] In the present study, pretreatment with NGN decreased pro-apoptotic marker-Bax and increased anti-apoptotic marker – Bcl-2 in MPP+-induced neurotoxicity in SH-SY5Y cells. These data clearly showed the unswerving involvement of NGN in the restoration of MPP+-induced alterations in an *in vitro* PD model.

SUMMARY AND CONCLUSION

In conclusion, NGN decreased neuroinflammation, ROS generation, and apoptosis and also SYN activation. In addition, pretreatment with NGN

increased DAT and TH levels in MPP+-induced SH-SY5Y cells. Further, NGN decreases NO, and MDA levels and increases SOD activity. Thus, it is clear that NGN has the potential to encounter MPP+-induced DA degeneration through regulation of ROS generation, neuroinflammation, and oxidative stress. Based on the results obtained, it is clear that NGN may be considered to be the better therapeutic choice in the treatment of PD. Further studies may warrant to clearly elucidate the molecular mechanism of NGN in protecting against neuronal cell death in PD.

Conflicts of interest

There are no conflicts of interest.

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