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Research Article

Morinda Citrifolia Modulate Bax/Bcl-2 Ratio to Inhibition of Apoptosis Induced By 6 OHDA – in SY-5Y Cells Model

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Abstract

Objectives: This study was conducted to research the protective effects of *Morinda citrifolia* (Noni) against the neurotoxicity, of 6-Hydroxydopamine (6-OHDA)-induced in the neuroblastoma (SH-SY5Y) cells model.

Methods and Results: 200 µM 6-OHDA were applied on SH-SY5Y cells under in vitro conditions to model Parkinson's Disease. Before the 6-OHDA application, it was treated with different doses of Noni extract (5, 10, 25, and 50 µg/mL). Cell viability was measured with routinely used 3-(4,5-Dimethyl Thiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and cytotoxic activity was determined by lactate dehydrogenase (LDH) assays. Besides cell viability, oxidative stress was evaluated by total antioxidant capacity (TAC), total oxidant status, superoxide dismutase assay, malondialdehyde, and glutathione reductase. The effect of Noni on *Bax*/Bcl2 gene regulation in SH-SY5Y cells was investigated by real-time PCR. 6-OHDA 200 µM decreased cell viability by approximately 60% in SH-SY5Y cells and increased LDH level. With 50 µg/mL Noni treatment, cell viability increased to 88.32%, while the LDH level decreased. Similarly, Noni re-regulated *Bax* and *Bcl-2* level, modified 6-OHDA, and decreased oxidative stress levels ($p<0.05$ and $p<0.001$).

Conclusion: These findings prove that noni exhibits a neuroprotective effect against 6-OHDA induction, through *Bax*/Bc-l2 gene regulation and decreasing oxidative stress.

Keywords: Bax, LDH, 6-OHDG

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The formation of Lewy bodies and loss of dopaminergic neurons in the substantia nigra (SN) are among the most characteristic features of Parkinson's disease (PD). PD clinically presents with symptoms such as muscle stiffness, postural instability bradykinesia, and tremors in the distal extremities.[1,2] Although the molecular mechanisms are unclearly understood, the decreased in mitochondrial complex-I activity and increased in stress due to ROS production are common mechanisms relate to dopaminergic neurons' death in PD patients.[3,4] ROS generation in PD not only includes damage to cellular organelles, loss of bioenergetic function, and disruption of mitochondrial-dependent redox signaling but also activates the apoptotic cascade, ultimately leading to neuronal loss.[5,6] *Bax* and *Bcl-2* (Apoptosis) genes are key biomarkers that are involved in PD. *Bax* and *Bcl-2* have roles in mitochondrial cytochrome-c (Cyc 1) release, and caspase-9 activation.[7] Apoptosis-related changes have been reported in patients with PD in the SN.[8,9] Current PD drugs can treat the symptoms of the disease but have a slight effect on stopping or delaying the degeneration of dopaminergic neurons.[10,11] *Bax/Bcl-2* plays a substantial role in the pathophysiology of apoptosis, thus targeting these molecular pathways may lead to reshaping new therapeutic strategies for the PD.[12]

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Newly, it was shown that strong antioxidant capacity can prevent oxidative stress damage and become a potential therapeutic strategy in neurodegenerative diseases like PD. One of the major sources of antioxidants is a plant like *Morinda citrifolia* (Noni).[13] It is reported that noni juice obtained from *M. citrifolia* fruit has many bioactive phytochemical components such as glycosides, iridoids, anthraquinones, flavonoids, phenolic acid, and coumarins and is widely used.[13,14] Noni juice has been proven to exhibit many pharmacological properties, inclusive anti-inflammatory, antioxidant, and antitumor effects.[15-17] The main components in noni are compounds in the class of flavonoids, coumarins, anthraquinones, iridoids, and polysaccharides.[16] In the light of the findings in the literature, Scopoletin is accepted as the main component of noni (*M. citrifolia* L), which contributes to its antioxidant and other therapeutic effects.[16,17] However, studies examining the neuroprotective effect of Noni on Parkinson's models and its effects on *Bax/Bcl-2* gene regulation have not been conducted yet. Therefore, in our study, it was planned to evaluate neuroprotection effect of noni against the neuro damage caused by 6-OHDA on SH-SY5Y cells. Noni's protect effect was investigate by various oxidative stress and apoptotic (*Bax/Bcl-2*) parameters.

Methods

Chemicals and Reagents

6-OHDA, Phosphate buffer solution, Dulbecco Modified Eagles Medium F12 (DMEM-F12), fetal bovine serum (FBS), DMSO, MTT, Trypsin (with EDTA), and antibiotic were supplied from Sigma-Aldrich (St. Louis, MO, USA). Elisa kits were supplied from Elabscience (Houston, USA). Primers and regents for gene expression by real-time PCR were obtained from Roche (Darmstadt, Germany). Noni extracts were purchasing by Nature's Health Noni (Radiant Sentral Nutrindo, Indonesia).

Cell Cultures

SH-SY5Y Cell Culture

For our study, SH-SY5Y (CRL-2266™) was purchased ATCC. The cells were resuspended by fresh medium (high glucose DMEM), including antibiotic 1% (penicillin, amphotericin B, and streptomycin) and 10% FBS. The cells were seeded in different well plates (6 and 96-well plate; Corning, USA) and stored at an incubator (5% CO_2; 37°C).[18]

Drug Administration

After the cells reached 80–85% confluency, they were seeded into 96 well plates. Experimental groups were determined as Noni extract (5, 10, 25, and 50 µg/mL), 6-OHDA (positive control) and control group (healthy cells) were administered. It was incubated for 24 h at optimum conditions. Initially, different concentrations of Noni were applied to the cells to create a neuroprotective effect. After half an hour, all wells were treated with 6-OHDA (except for the healthy control) and incubated 24 h at the optimum condition.

MTT Assay

After 24 h of treatment, MTT (10 µL) is added to each well of the 96-well plate and the plate was incubated for 4 h in at CO2 incubator. After 4 h, DMSO (100 µL) solution was added (to liquify Formazan crystals). The optic density was read by the Spectrophotometer reader at 570 nm.[19]

Lactate Dehydrogenase (LDH)

LDH level was examined into the LDH detection kit. All procedure conducted by the manufacturer's instructions. Optic density was measured at 490 nm.[20] LDH level was calculated according to kit protocol and the results were given as %.

Total Oxidant Status (TOS) and Total Antioxidant Capacity (TAC)

TOS and TAC levels were measured spectrophotometrically (Multiskan ™ GO Microplate Spectrophotometer reader). The density of the color is relevant to the number of oxidants and antioxidants in the sample, according to commercial kit instructions.[21]

Catalase (CAT), Glutathione (GSH), Malondialdehyde (MDA), and Superoxide Dismutase Assay (SOD) Analysis

CAT, GSH, MDA, and SOD were detected by ELISA kits (Elabscience, United States). The oxidative damage analyses were performed based on the manufacturer's instructions. The absorbance was determined by spectrophotometer at 450 nm.[20]

Real-Time Polymerase Chain Reaction (PCR)

Gene Expression

Total RNA was extracted from the cells with The High Pure RNA Isolation Kit (Roche Mannheim Germany). The cells were removed with absolute methanol. We performed a real-time PCR using a High-Capacity First Strand cDNA Synthesis Kit for RT-PCR (AMW) by Roche (Darmstadt, Germany) and specific primers, their sequences are listed Table 1. (Roche; Darmstadt, Germany Results were expressed as relative expression changes (fold changes) between Noni extract (5 µg/mL, 10 µg/mL, 25 µg/mL and

Table 1. Primers and their sequences

Gene	Forward	Reverse
β-actin	5'-CCAACCGCGAGAAGATGA-3'	5'-CCAGAGGCGTACAGGGATAG-3'
Bax	5'-ATG GAC GGG TCC GGG GAG CA-3'	5'-CCC AGT TGA AGT TGC CGT CA-3'
Bcl2	5'-GTG AAC TGG GGG AGG ATT GT-3'	5'-GGA GAA ATC AAA CAG AGG CC-3'

50 µg/mL), 6OHDA (positive control) and control group (healthy cells). We normalized the expression of target genes to beta-actin (reference control gene) using the ΔΔCt method.[22, 23]

Statistical Analysis

Statistical difference between groups was calculated using Tukey HSD method and one-way ANOVA. All calculations were conducted using SPSS 26 software for statistical analysis, $p<0.05$ and $p<0.001$ were considered a statistically significant difference in all tests. Results are presented as mean and standard deviation (mean±SD).

Results

MTT Assay Results

Cytotoxicity results of all groups after 24 h of application were revealed by the MTT test and the cell viability graph is given in Figure 1. Cell viability was considered as 100 in the control group (negative control). In the 6-OHDA 200 µM group, this rate was found to be 44.14% at the end of 24 h. Cell viability significantly was reduced in the 6-OHDA 200 µM group ($p<0.001$). Almost 60% of decrease in this viability proves the cytotoxic effect of 6-OHDA. The viability rate of Noni 5 and 10µg/ml was found to be 48.41% and 59.21%, respectively ($p>0.05$). The highest cell viability ratio was found to be 88.32 in the Noni 50 µg/mL group after 24 h ($p<0.001$).

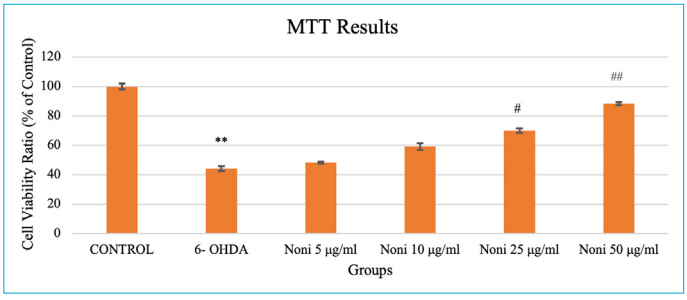

Figure 1. MTT Results of Application Group. Cells were cultured in 96-well plates treated with Noni extract (5, 10, 25, and 50 µg/mL) and 6-OHDA 200 µM for 24 h. The results represent the average of three separate experiments. Data are specified as the means±SD. **p<0.01 values are very significant for control group#: p<0.05;##: p<0.001 for 6-OHDA.

LDH Results

Since LDH is released by necrotic cells, it is an excellent metabolic marker of cell viability. The effect of treatment with Noni on SH-SY5Y cells LDH activity was determined using an LDH kit. The measured LDH activity of treated cells, expressed as % of the standard (designated as 100%,) is presented in Figure 2. As a result of exposure of cells with only 6-OHDA 200 µM, LDH activity increased in correlation with the decrease in cell viability. LDH level was observed as 38.79% in the 6-OHDA 200 µM group, and it was figured out to be significant compared to the control group ($p<0.01$). For neuroprotective activity, LDH level gradually decreased in a dose-dependent manner in the groups that were administered Noni before 6-OHDA administration. The most significant result was that at 50 µg/ml Noni LDH level decreased, approaching the control group (16.37) compared to the 6-OHDA 200 µM group ($p<0.05$ and $p<0.01$). These data show us, Noni significantly reduces the cytotoxic effect of 6-OHDA.

Redox State in SH-SY5Y Cells Treated with Noni TAC and TOS results

The TAC value of SH-SY5Y cells determined spectrophotometrically in the control group was 15.96 mmol Trolox equiv./L, and the TAC value in the 6-OHDA 200 µM group was 3.70 mmol Trolox equiv./L (Fig. 3). Compared with the control group, 6-OHDA reduced cellular antioxidant activ-

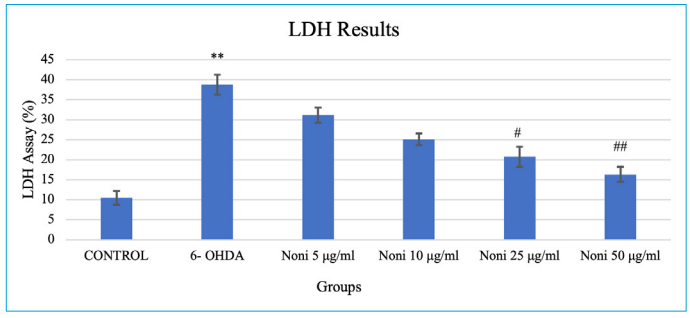

Figure 2. LDH activity Results of Application Group. Cells were cultured in 96-well plates treated with Noni extract (5, 10, 25, and 50 µg/mL) and 6-OHDA 200 µM for 24 h. The results represent the average of three separate experiments. Data are specified as the means±SD. **p<0.01 values are very significant for control group#: p<0.05; ##: p<0.001 for 6-OHDA.

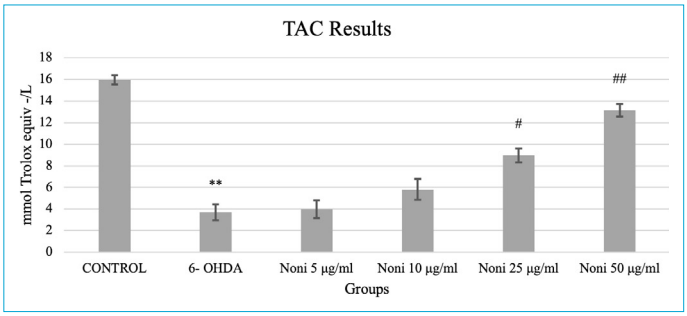

Figure 3. TOS Results of Application Group. Cells were cultured in 96-well plates treated with Noni extract (5, 10, 25, and 50 µg/mL) and 6-OHDA 200 µM for 24 h. The results represent the average of three separate experiments. Data are specified as the means±SD. **p<0.01 values are very significant for control group#: p<0.05; ##: p<0.001 for 6-OHDA.

ity and led the cells to cytotoxicity (p<0.01). Antioxidant activity of cells started to increase with Noni, but significant results were not obtained at low doses. The TAC value was found to be 8.97 mmol Trolox equiv./L in the Noni 25 µg/mL group, and 13.14 mmol Trolox equiv./L in the Noni 50 µg/mL group (Fig. 3). The TAC value approached the control group at the highest.

Well in correlation with the TAC results, the Noni' treatment (Fig. 4) was found to decrease SH-SY5Y cell TOS levels, dependent on concentration. The most prominent effects were obtained after of treatment with Noni 50 µg/m (p<0.01).

CAT, GSH, MDA, and SOD Analysis Results

As shown in Figure 4, CAT, GSH, and SOD levels decreased meaningfully in the 6-OHDA 200 µM group compared to the control group, while MDA levels increased significantly (p<0.001). CAT, GSH, and SOD activities increased in the Noni group due to the increased concentration compared to the 6-OHDA 200 µM group, while MDA levels decreased

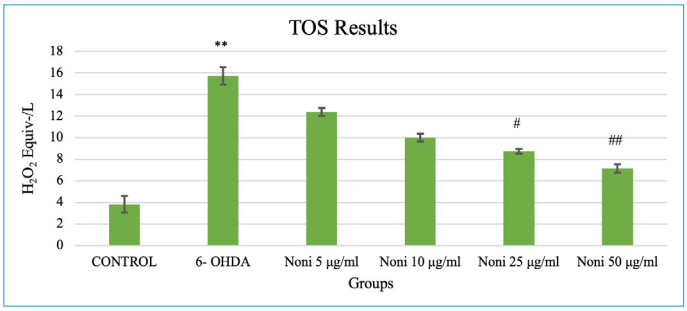

Figure 4. TOS Results of Application Group. Cells were cultured in 96-well plates treated with Noni extract (5, 10, 25, and 50 µg/mL) and 6-OHDA 200 µM for 24 h. The results represent the average of three separate experiments. Data are specified as the means±SD. **p<0.01 values are very significant for control group#: p<0.05; ##: p<0.001 for 6-OHDA.

meaningfully (p<0.001). The findings support both TAC and TOS data as well as MTT and LDH data (Fig. 5).

The Effect 6-OHDA and Noni on Bax and Bcl-2 Gene Expressions Level

Gene expression of *Bax* and *Bcl-2* gene was measured by Real-Time-PCR analysis at the 24-h point of various treatments. This approach showed that 200 µM of 6-OHDA significantly up-regulated *Bax* expression (p<0.01). Specifically, a 1.98-fold increase indicates 6-OHDA induced apoptotic factors. Nano was not effective at low doses and could not stop apoptosis. On the contrary, high concentrations of 25 and 50 µg/mL Nano increased the *Bax* level to the level of control cells and protected the cells from the apoptotic effect of 6-OHDA (Fig. 6).

On the other hand, 6-OHDA 200 µM significantly reduced the level of *Bcl-2*. After 6-OHDA treatment, *Bcl-2*, which was 0.42-fold down-regulated, increased to 0.78 with Noni 25 µg/ml and to 0.864 with 50 µg/mL concentration of Noni, approaching the control level. The reason why cell viability remained at approximately 88% could be related to the variation of Noni on the *Bax*/*Bcl-2* ratio (p<0.05) (Fig. 6).

Discussion

6-OHDA has been widely using to understand of the physiological and pharmacological mechanisms underlying PD symptoms.[24, 25] Therefore, in the present study, it was planned to ameliorate the neurodegenerative damage in 6-OHDA-applied SH-SY5Y cells with Noni to examine the characteristics of PD pathology and to explain the mechanism.

Various pathological factors have been recommended as important mechanisms of PD, including overproduction of ROS, dysfunction of iron metabolism, oxidative stress, apoptosis activation, and neuroinflammation.[26-30] The toxic effect of 6-OHDA on the dopaminergic system in the nigrostriatal pathway is due to its auto-oxidation caused by ROS production. As a result, superoxide radical intermediates are formed.[31] SOD is a potent endogenous antioxidant enzyme for Superoxide radicals. It was found to be significantly reduced in PD models created with 6-OHDA both in vivo and in vitro.[32] In this study, Noni was proven to be able to reverse the oxidative stress properties induced by 6-OHDA, including increased in ROS and MDA, and decreased in CAT, GSH and SOD. By causing an increase in 6-OHDA oxidative stress level, it decreased SOD activity and had a cytotoxic effect on cells. Its inhibitory effect on SOD activity suggests that the cytotoxicity induced by 6-OHDA will be mediated by oxidative stress. It also showed that Noni could rescue 6-OHDA-induced cell death in SH-SY5Y cells through its

Figure 5. CAT, GSH, MDA, and SOD Results of Application Group. Cells were cultured in 96-well plates treated with Noni extract (5, 10, 25, and 50 µg/mL) and 6-OHDA 200 µM for 24 h. The results represent the average of three separate experiments. Data are specified as the means±SD. **p<0.01 values are very significant for control group #: p<0.05; ##: p<0.001 for 6-OHDA.

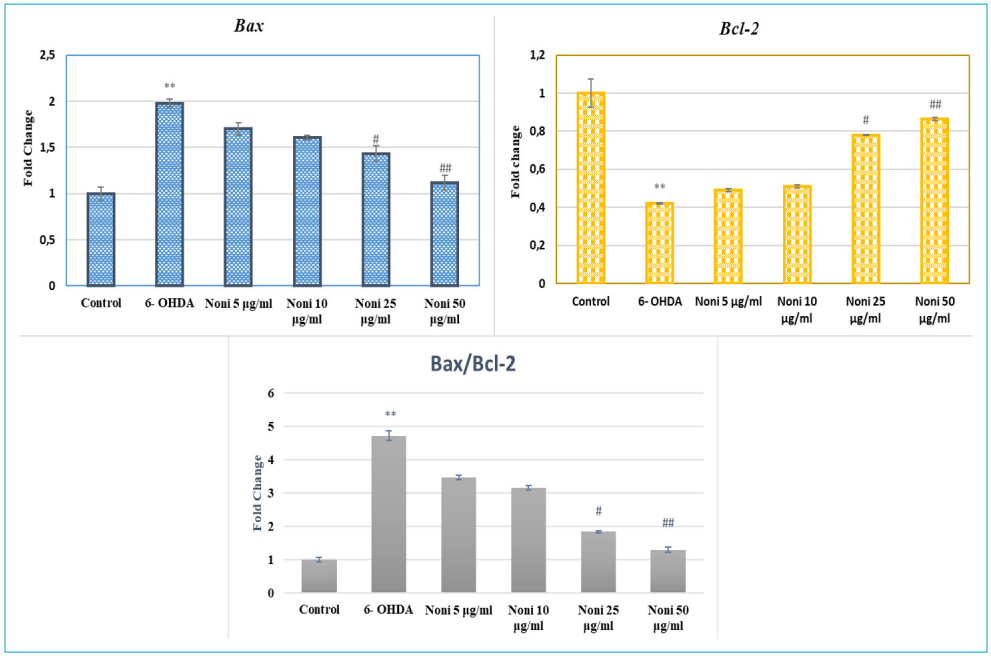

Figure 6. RNA expression levels of Bax and Bcl-2 in the SH-SY5Y of control and experimental groups. Cells were cultured in 96-well plates treated with Noni extract (5, 10, 25, and 50 µg/mL) and 6-OHDA 200 µM for 24 h. The results represent the average of three separate experiments. Data are specified as the means±SD. **p<0.01 values are very significant for control group #: p<0.05; ##: p<0.001 for 6-OHDA.

antioxidant activity and reduce ROS overproduction by inducing SOD, CAT, and GSH activity.

The increase in ROS production and mitochondrial dysfunction is thought to be an initial event mediating cell death after exposure to 6-OHDA. The most prominent features in PD are actually mitochondrial dysfunction and oxidative stress.[33-37] Mitochondrial dysfunction mediated by oxidative stress activates apoptosis pathways. Therefore, in this study, we examined the variation of *Bax* and *Bcl-2* levels. Cytotoxic drug-induced apoptosis, such as 6-OHDA, increases mitochondrial permeability and subsequent cytochrome-c release.[38] Thus, activation of caspase 9 and caspase 3 results from apoptosis.[39, 40] It has been clarified that the release of cytochrome-c is organized by *Bcl-2* family proteins. *Bcl-2* is in the outer mitochondrial membrane and stabilizes membrane permeability, maintaining mitochondrial integrity, and preventing cytochrome c release. Inducing cytochrome c release from mitochondria to the cytosol, *Bax*, on the other hand, is a pro-apoptotic protein that can affect membrane permeability.

The balance between *Bax* and *Bcl-2* plays a key role in sustaining cell integrity and controlling of cell viability,[41] and mutilation of this balance activates a signaling cascade that leads to the induction of cell death. ROS can induce apoptosis by regulating the phosphorylation and dissemination of *Bcl-2* family proteins, resulting in increased pro-apoptotic protein levels, and reduced anti-apoptotic protein expression level.[42]

The main components of noni are compounds in the class of anthraquinones, flavonoids, coumarins, iridoids, and polysaccharides. Mainly Scopoletin (6-methoxy-7-hydroxy-coumarine) is the most predominant among the ingredient compounds found in noni and is often used as the representative ingredient of noni. Scopoletin is one of the ingredients in noni that contributes to antioxidant, anti-inflammatory, immunomodulatory, and hepatoprotective properties. Therefore, the therapeutic properties of noni are associated with Scopoletin.[15-17] Our study shows that the protective effect of Noni against 6-OHDA is revealed by the regulation of *Bax* and *Bcl-2* gene regulation. 6-OHDA decreased *Bcl-2* gene regulation and increased *Bax* level. On the other hand, in the cells treated with Noni, the *Bax* level decreased while the *Bcl-2* level increased (Fig. 6). Apparently, Noni defends SH-SY5Y cells against 6-OHDAby inhibiting oxidative stress and modulating the mitochondrial apoptotic pathway.

Conclusion

6-OHDA application is caused to the increase of ROS and changes the *Bax*/*Bcl-2* ratioIn addition, activating the mitochondrial-dependent apoptotic pathway, it led to cytochrome-c release and reduced the viability of SH-SY5Y cells. Treatment with M. citrifolia, on the other hand, significantly blocked cytochrome-c release by preventing oxidative damage and potentiated the anti-apoptotic *Bcl-2*, thus increasing cell viability and attenuating 6-OHDA-induced apoptosis. This study shows that the anti-apoptotic activity of *M. citrifolia* can be used to alleviate nigral degeneration in PD.

Disclosures

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

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