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ORIGINAL PAPER

Betanin Attenuates Oxidative Stress Induced by 6-OHDA in PC12 Cells via SAPK/JNK and PI3 K Pathways

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Abstract

Parkinson's disease is a neurodegenerative disorder which accompanied with cognitive decline, chorei form moves and behavioral difficulties. Oxidative stress which promote the apoptotic cell death are responsible for neurodegeneration in Parkinson. The purpose of this study is to evaluate the protective effects of betanin against toxicity and oxidative damage induced by 6-hydroxydopamine (6-OHDA) and hydrogen peroxide (H_2O_2) in PC12 cells as an appropriate model of Parkinson's cell damage. PC12 cells pretreated with betanin (1–200 µM) for 24 h, and exposed to either 6-OHDA (100 µM) or H_2O_2 (150 µM) for 24 h. Cell survival and intracellular reactive oxygen species (ROS) production analyzed by resazurin and DCF-DA assay. The anti-apoptotic effects of betanin in PC12 cells were studied using flow cytometry of PI stained cells. Also, western blot analysis of survivin, Cyt c, Phospho SAPK/JNK, SAPK/JNK, Phospho-PI3 kinase P85, PI3 kinase P85 was performed for detection of apoptosis. Betanin (1–200 µM) significantly decreased the 6-OHDA and H_2O_2 cytotoxicity also attenuated the ROS level. Cell apoptosis significantly increased after 6-OHDA (100 µM) treatment, compared to the control. However, pretreatment with betanin (20 and 50 µM), protected against apoptosis. Western blot analysis of PC12 cells showed that 100 µM 6-OHDA could increase the proteins involved in apoptosis signaling and betanin (20 and 50 µM), could decrease the apoptosis. The results show that betanin has antioxidant and anti-apoptotic effects and may have the ability to prevent or delay the progress of neural death in Parkinson's disease.

Keywords Parkinson's disease · 6-OHDA · H_2O_2 · Apoptosis · Betanin

Introduction

Parkinson's is a progressive disease of the nervous system that affect body movement. Vibration in rest, bradykinesia, rigidity and difficulty at the start of the move are the main symptoms [1]. Signs of Parkinson's disease at the cellular level include selective deprivation of dopaminergic neurons in substantia nigra pars compacta and the presence of Lewy bodies containing α-synuclein in neuronal cytoplasm [2]. The death cause of dopaminergic neurons in Parkinson's disease has not been clearly clarified. In the last two decades, in addition to underlying genetic factors, oxidative stress and mitochondrial function impairment have been identified as the main contributors to the neuronal degeneration of Parkinson's disease [3]. 6-hydroxydopamine (6-OHDA) leads to a syndrome similar to Parkinson's in humans and rodents. Impair in the function of mitochondria through inhibition of mitochondrial complex I has been shown with 6-OHDA [4]. Oxidation of dopamine increases the amount of hydrogen peroxide (H_2O_2) and changes the mitochondrial function [5, 6]. Antioxidants preserve the redox/oxidation balance in central nervous system and protect against oxidative damage [7]. Over the past few years, the use of natural compounds has been noticed in protection against neurological diseases. Anthocyanin pigments are among the natural products which have attracted much attention as antioxidant [8]. Betalains which present in red beetroot (*Beta vulgaris* L.) are considered as hydrophilic nitrogen-based pigments [9, 10]. Betanin is one of the abundant compounds among betalains [11] with excellent antioxidant and anti-inflammatory effects [12–14].

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Phenolic and cyclic groups within the betanin, trigger the free-radical scavenging function [15]. Interestingly, as a potent NO generator with high content of betanin, beetroot help in improvement of the cognitive responses and importantly cerebrovascular blood flow [16, 17].

Since there is not a report about the putative mechanism of betanin against neurodegeneration, in the present study, we have evaluated the protective effect of betanin on toxicity and oxidative damage induced by 6-OHDA and H_2O_2 in PC12 cells as an appropriate model of Parkinson's cell damage.

Materials and Methods

Materials

Betanin, Resazurin, the fluorescent probe propidium iodide (PI), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), H_2O_2 33%, 6-OHDA and 1640 RPMI medium and Quanti-Pro™ BCA Assay Kit from Sigma (Germany); rabbit anti-serum against survivin, rabbit polyclonal anti-serum against Cyt c, rabbit polyclonal Phospho SAPK/JNK, rabbit polyclonal SAPK/JNK, rabbit polyclonal Phospho-PI3 Kinase P85, rabbit polyclonal PI3 Kinase P85, β-Actin (13E5), anti-rabbit IgG HRP-linked antibody from Cell Signaling Technology (USA); fetal bovine serum (FBS) and penicillin-streptomycin (PS) from Gibco (USA); dimethyl sulfoxide (DMSO) from Merk (Germany); differentiated rat pheochromocytoma PC12 cells purchased from Pasteur Institute (Iran).

Cell Culture and Treatment

Differentiated rat pheochromocytoma PC12 cells maintained in RPMI-1640 medium with 1% penicillin and streptomycin, 10% (v/v) FBS. Cells incubated at temperature of 37 °C, a relative humidity of about 95% and 5% CO_2 concentration. 6-OHDA and betanin were dissolved in DMSO to obtain a 40 mM stock solution. PC12 cells pretreated with betanin (1–200 µM) for 24 h. Then exposed to either 6-OHDA (100 µM) or H_2O_2 (150 µM) for the next 24 h. The optimum time and concentration points were used according to the pre-test evaluation with 6-OHDA (12.5, 25, 50, 100, 200 and 50) for 24 h (Data not shown) and previous studies [18].

Analysis of Cell Viability

Resazurin reduces to resorufin and the change in the color is proportional to metabolic activity of the cell which is measured by colorimetric or fluorometric methods [19]. 1×10^4 PC12 cells seeded in each well of 96-well culture plates. Betanin was added 24 h before 6-OHDA and H_2O_2 exposure. After 24 h, 20 µl of resazurin was added to each well and incubated at 37 °C for 4–6 h. Cellular viability was determined by Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA) and the absorbance intensity measured in 570 and 600 nm.

ROS Generation

To determine the amount of reactive oxygen production, DCFH-DA is added to the cells. Lipophilic and non-fluorescent DCFH-DA passes through the cell membrane, de acetylated with intracellular esterase and finally converted to the fluorescent DCF via interaction with intracellular ROS [20]. 1×10^4 PC12 cells per well were seeded in 96-well culture plates. Betanin (1–200 µM) was added to the cultures 24 h before 6-OHDA (100 µM) and H_2O_2 (150 µM) exposure. After 24 h, DCFH-DA was added to the cultured cells. Then, the cells were exited at 485 nm and the emission at 538 nm was plotted with a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA). Data expressed as proportional to the amount of active species of mitochondrial oxygen.

Flowcytometric Apoptosis Assay

To sub G1 peak in the flow cytometry histogram of PI stained cells determine the amount of apoptosis in cells [21, 22]. 10^5 PC12 were seeded in each well of a 12-well plates and treated with betanin (20 and 50 µM) for 24 h before 6-OHDA (100 µM). In the following, cells were harvested and 400 µl of a hypotonic buffer containing 50 µg/mL PI in 0.1% sodium citrate plus 0.1% triton X-100 was added to each sample. Then, analyzed by FACS Scan flow cytometer (BD Biosciences, CA, USA).

Western Blotting

About 10^6 PC12 cells were treated with betanin (20 and 50 µM) for 24 h before 6-OHDA (100 µM). The cells were harvested and washed with cold PBS, then the western blot test performed according to the instructions previously published [23]. The membrane exposed to rabbit monoclonal survivin, polyclonal Cyt c, rabbit polyclonal Phospho SAPK/JNK, rabbit polyclonal SAPK/JNK, polyclonal Phospho-PI3 kinase P85, polyclonal PI3 kinase P85 and β-actin (13E5) as primary antibodies and anti-rabbit IgG, a HRP-linked antibody as secondary antibody. The values obtained from each sample were divided into its respective β-actin content. In this way, we calculated how much bandwidth has changed over control (intensity of the related β-Actin band) using Gel-pro Analyzer V.6.0 Gel Analysis Software. (Media Cybernetics, InG, Bethesda, MD).

Statistical Analysis

All data were expressed as Mean ± SD compared with the respective control using one-way ANOVA, followed by Dunnett's *post hoc* test in Graph Pad Prism 5 software. For all the findings differences in levels $p < 0.05$ are considered as significant levels.

Results

Effects of Betanin on Cell Viability

To determine the optimal protective concentration of betanin, the cell cytotoxicity of the betanin was measured. Betanin did not show cytotoxic effects in any concentration compared to the control group (Fig. 1a).

Effects of Betanin on 6-OHDA and H_2O_2 Induced PC12 on Cell Viability

6-OHDA (100 µM) significantly reduced the cell viability compared with the control group ($p < 0.001$) while pretreatment with betanin (5–200 µM) showed significantly higher cell survival rates compared with the 100 µM 6-OHDA ($p < 0.05$, $p < 0.01$ and $p < 0.001$) (Fig. 1b). H_2O_2 (150 µM) led to significant reduction in cell viability compared with the control group ($p < 0.001$) while pretreatment with betanin (5–200 µM) exhibited significantly higher cell survival rates compared with the 150 µM H_2O_2 ($p < 0.001$) (Fig. 1c). This finding show that betanin potentially can protect PC12 cells from 6-OHDA and H_2O_2 induced cell death.

Effects of Betanin on 6-OHDA and H_2O_2 Induced ROS Production

Treatment with 6-OHDA (100 µM) for 24 h induced a significant elevation in the cell fluorescence intensity compared with the control group ($p < 0.001$). After pretreatment with betanin (1–200 µM); however, the fluorescence intensity decreased significantly ($p < 0.001$) (Fig. 1d). H_2O_2 (150 µM) significantly increased cell fluorescence intensity compared with the control group ($p < 0.001$). After pretreatment with betanin (5–200 µM); however, the fluorescence intensity decreased significantly ($p < 0.001$) (Fig. 1e). This results can indicate that betanin reduces the production of 6-OHDA and H_2O_2 induced ROS.

Effects of Betanin on 6-OHDA Induced Apoptosis by Flow Cytometry

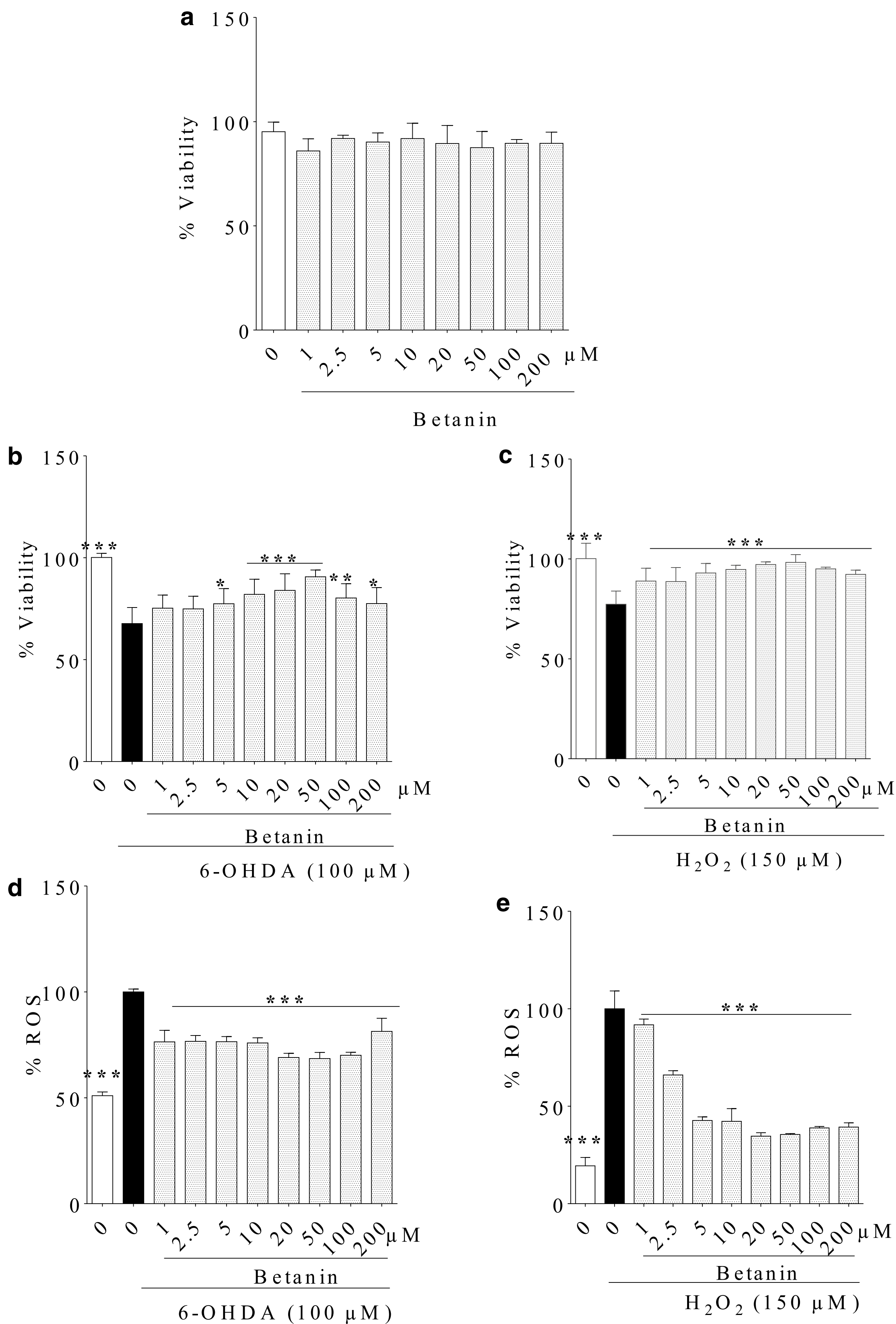
Effects of betanin on apoptosis induced by 6-OHDA in PC12 cells were examined using flow cytometry after PI staining. Cell apoptosis was significantly increased to 75.9% after treatment with 6-OHDA (100 µM) compared to control (1.9%) ($p < 0.001$). After pretreatment with betanin (20 and 50 µM); however, apoptosis was significantly reduced to 15.3% and 31.3% ($p < 0.001$). The sub-G1 peak in flow cytometry histograms of PC12 cells showed that betanin could reduce the amount of apoptosis compared to 6-OHDA (100 µM) (Fig. 2a, b).

Effect of Betanin and 6-OHDA on Apoptosis Signaling Proteins

To determine the mechanism of protective effects of betanin versus 6-OHDA, the amount of apoptotic proteins (survivin, Cyt c, phospho SAPK/JNK46/54, SAPK/JNK46/54, PI3 Kinase P85, Phospho-PI3 Kinase P85) were compared in the PC12 cells treated with betanin and 6-OHDA. Our results showed that treatment with 6-OHDA (100 µM) for 24 h reduced survivin ($p < 0.05$) and significantly increased Cyt c ($p < 0.01$) whereas pretreatment with betanin (20 and 50 µM) markedly decreased Cyt c ($p < 0.01$). Also, 6-OHDA (100 µM) increased Phospho SAPK/JNK46/54 to SAPK/JNK46/54 compared with the control group ($p < 0.05$) ($p < 0.001$), and pretreatment with betanin (20 and 50 µM) inverted the 6-OHDA induced apoptosis ($p < 0.05$) ($p < 0.01$). In addition, 6-OHDA (100 µM) reduced the ratio of Phospho-PI3 kinase p85/p55 to PI3 kinase p85/p55 ($p < 0.01$) compared with the control group, and betanin (20 and 50 µM) protected the cells against apoptosis ($p < 0.01$) (Fig. 3).

Discussion

In this study, we examined the possible protective effects of betanin on the toxicity of 6-OHDA in PC12 cells. The findings of this study showed the protective effects of betanin as a potent antioxidant on PC12 cells against 6-OHDA and H_2O_2 toxicity. In addition, we examined the effects of betanin on apoptosis also related molecular mechanisms against 6-OHDA toxicity in PC12 cells. Previously it was shown that 6-OHDA produces hydrogen peroxide and hydroxyl radicals leading to the impairment of mitochondrial function [24]. Types of reactive oxygen are collectively referred to as reactive oxygen species (ROS) which many of them are free radicals. ROS is not always harmful. For example, they play a vital role in the killing of pathogenic microbes by phagocytes also they have other beneficial effects. Fortunately, ROS is naturally inhibited in the body by a variety of complex defense systems. These systems are perfect because they work on different ROSs or on different cellular constituents. Oxidative stress is a mechanism involved in the patogenesis of Parkinson's disease and may attribute to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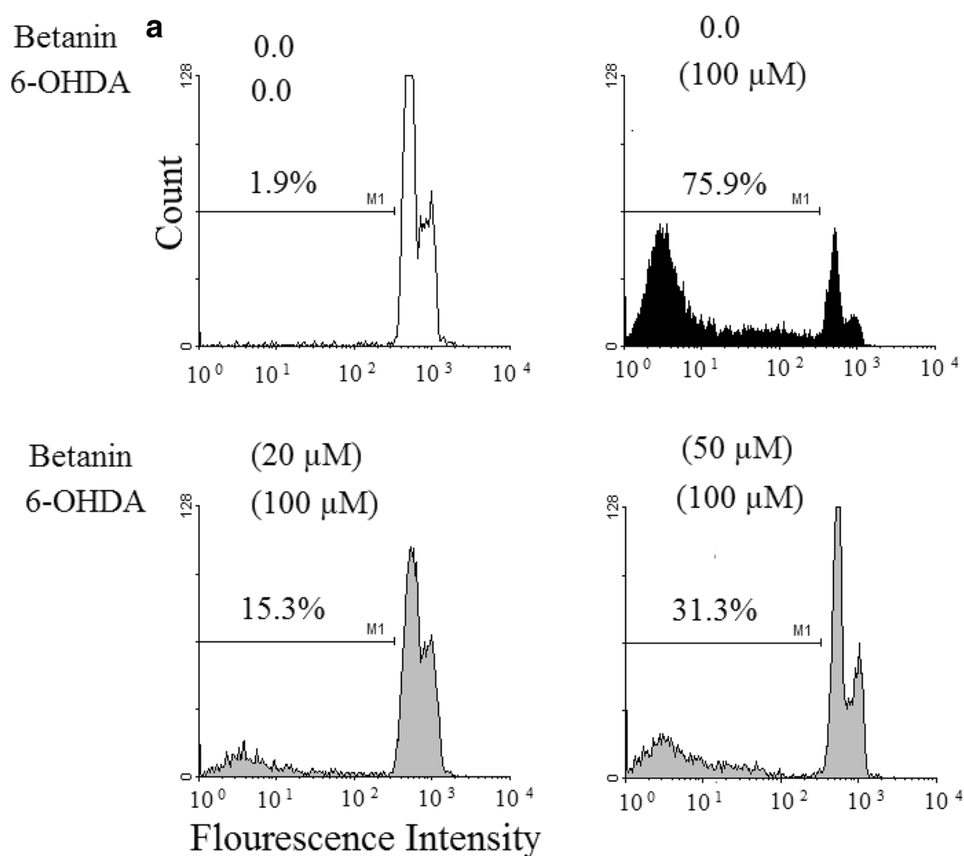
◂**Fig. 1** **a** Effects of betanin on cell viability. The viability of PC12 cells determined after treatment with betanin (1–200 µM) for 24 h. The data presented as the mean ± SD (n = 9) of three independent experiments. **b**, **c** Effects of betanin on 6-OHDA and H_2O_2 induced PC12 on cell viability. PC12 cells were pretreated with betanin (1–200 µM) 24 h before treatment with 100 µM 6-OHDA and 150 µM H_2O_2 for 24 h. The data presented as the mean ± SD (n = 9) of three independent experiments. $*p < 0.05$, $***p < 0.001$ and $**p < 0.01$ compared with 6-OHDA and H_2O_2 group (n = 3) in triplicate. **d**, **e** Effects of betanin on 6-OHDA and H_2O_2 induced ROS production. The PC12 cells pretreated with betanin (1–200 µM) for 24 h before treatment with 100 µM 6-OHDA and 150 µM H_2O_2 for 24. The data presented as the mean ± SD (n = 9) of three independent experiments. $***p < 0.001$ compared with 6-OHDA and H_2O_2 group

the apoptosis of dopaminergic neurons through mitochondrion defect. In the cellular model of Parkinson's disease with 6-hydroxy dopamine (6-OHDA) on PC12 cells, oxidative stress is increased which leads to inhibition of Sirtuin 1 function and cell death [25]. Investigations have focused on the highly complex and complementary relationships between oxidative stress and genes involved in Parkinson's disease. It is also shown that dopamine metabolism in the brain spontaneously leads to oxidative stress and this will cause changes in intracellular macromolecules which their performance is naturally essential for cell survival. In addition, activated microglia produc nitric oxide and superoxide during the inflammatory responses of neurons and this will be worse with the release of molecules such as α-synuclein, neuromelanin and matrix metalloproteinase-3 from the damaged dopaminergic neurons [26]. Polyphenolic compounds as unique antioxidants, regardless of their role to combat ROS, also may have synergistic effect with other natural compounds [27]. Red beetroot has high level of betalain pigments [28]. Recent studies show that betalain pigments in red beetroot extract has free radical-scavenging properties and as natural antioxidants, they have the ability to prevent and treat oxidative stress related diseases [9]. One of the reasons for the high antioxidant property of betanin is due to its high electron-donor properties [29]. Studies have shown betalains induce glutathione formation in human erythrocytes [30]. Also, betalain keeps the LDL (low density lipoprotein) particle safe from oxidation [29, 31] and reduce biomarkers of lipid oxidation [30]. Furthermore, in mice fed with red beet extract, betanin exert antioxidant activity [32] and has anti-inflammatory effects by inhibiting cyclooxygenase-2 [13]. There is a report implying that betanin may have protective effect on kidney of parquet treated rats by reducing oxidative stress and inflammatory reactions [33]. Another study has shown that betanin protects against parquet-induced acute lung injury and interstitial pneumonia in rats as a natural antioxidant through antioxidant and anti-inflammatory mechanisms [34]. Also, studies indicate that the presence of betanin in beetroot leads to reduction in ROS production, DNA damage and modify the neutrophil oxidative metabolism [14]. In human lymphocytes, betalains decrease the H_2O_2 induced damage of DNA [35]. Furthermore, in human hepatocytes, treatment with betanin activated the nuclear factor erythroid 2 related factor 2(Nrf2) dependent signaling pathway (NRF2-ARE) [36]. Treatment of Huh7 cells with betanin increased the expression of heme oxygenase 1(HO1) and paraoxonase 1 (PON1) genes [37]. In addition, treatment of rat liver cells with betanin in the red beetroot extract increased the quinone reductase [38].

In current study, betanin (1–200 µM) does not show any toxicity compared to the control group (Fig. 1). While 6-OHDA (100 µM) and H_2O_2 (150 µM) decreased the cellular viability, betanin (5–200 µM) increased the cell survival. Pre-treatment of cells with betanin decreased the toxicity of 6-OHDA (Fig. 1). DCFH-DA fluorescence intensity reflect the ability of compounds to act as hydrogen atom donors. Treatment with 6-OHDA (100 µM) and H_2O_2 (150 µM) for 24 h induced a significant increase in the DCFH-DA fluorescence intensity in cells compared with the control group which inhibited with betanin (1–200 µM) (Fig. 1). It seems that betanin reduces the production of free radicals and protects the PC12 cells against 6-OHDA and H_2O_2 toxicity. So, one of proposed the mechanisms of betanin is to enhance the activity of intracellular antioxidant enzymes. Betanin (20 and 50 µM) decreased cell apoptosis induced by 6-OHDA (100 µM) which indicates the anti-apoptotic properties of betanin (Fig. 2).

To determine the mechanism of inhibition of apoptosis, expression of Cyt c, Phospho SAPK/JNK, SAPK/JNK, Phospho-PI3 kinase P85, PI3 kinase P85 protein was evaluated after 24 h pretreatment with 20 and 50 µM betanin and after treatment with 6-OHDA. Survivin (16 kD) is a member of the family of apoptosis inhibitors that inhibit the caspases, and negatively regulate the apoptosis [39]. Our results showed that betanin (20 and 50 µM) increased survivin rate relative to 6-OHDA (100 µM) and betanin have a protective effect against 6-OHDA induced-toxicity (Fig. 3). During apoptosis, Cyt c (14 kD) is released from mitochondria and activate caspase 9 [40]. The results of this study show that 6-OHDA (100 µM) increased the amount of Cyt c protein while betanin (20 and 50 µM) reduced the release of Cyt c (Fig. 3). MAPK (mitogen-activated protein kinase) is a type of protein kinase with amino acids serine and threonine that regulate cell activity including cell viability and apoptosis [41]. Studies have shown that blocking the transfer of SAPK/JNK (46/54 kD) to mitochondria prevents the toxic effect of 6-OHDA [42]. It seems when the cascade of 6-OHDA neural degeneration initiated, betanin reduces phosphorylation of SAPK/JNK and induction of apoptosis. Our results showed that 6-OHDA (100 µM) activated JNK, while betanin (20 and 50 µM) decreased the ratio of Phospho SAPK/JNK to SAPK/JNK and reduced the cell apoptosis (Fig. 3). The PI3K pathway regulates cell activity including survival.



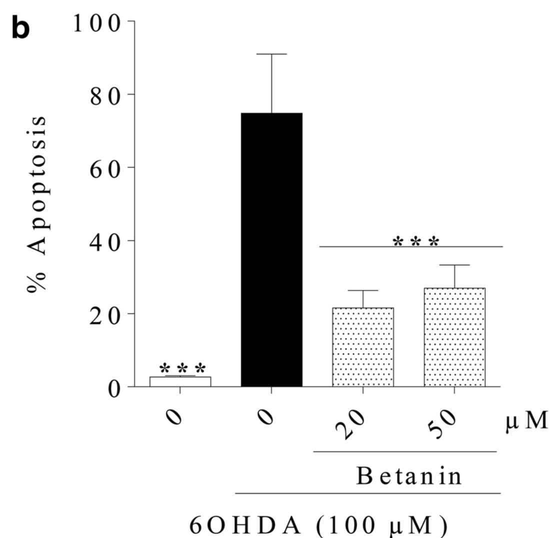


Fig. 2 Effects of betanin on 6-OHDA induced apoptosis by flow cytometry. The PC12 cells pretreated with betanin (1–200 µM) for 24 h before treatment with 100 µM 6-OHDA. The data presented as the mean ± SD (n = 9) of three independent experiments. ***$p < 0.001$ compared to 6-OHDA (n = 3)

When PI3K (85 kD) is activated, phosphorylation of the Akt (protein kinase B or PKB) protein occurs. The phosphorylated Akt, in turn, inhibit the pro-apoptotic family of protein including Bad, Bax, caspase-9, GSK-3, and FoxO1 [43]. Our results showed that 6-OHDA (100 µM) reduces the ratio of Phospho-PI3 kinase p85/p55 to PI3 kinase p85/p55 while betanin (20 and 50 µM) reduces the cell death (Fig. 3). So, probably betanin inhibits apoptosis by reducing phosphorylation of SAPK/JNK and PI3K pathways.

The possible mechanism of the protective effect of betanin to attenuate the oxidative stress induced by 6-OHDA in PC12 cells appear to be through reduction in phosphorylation of SAPK/JNK and increase in phosphorylation of PI3K. As mentioned, phosphorylation of PI3K let to inhibition

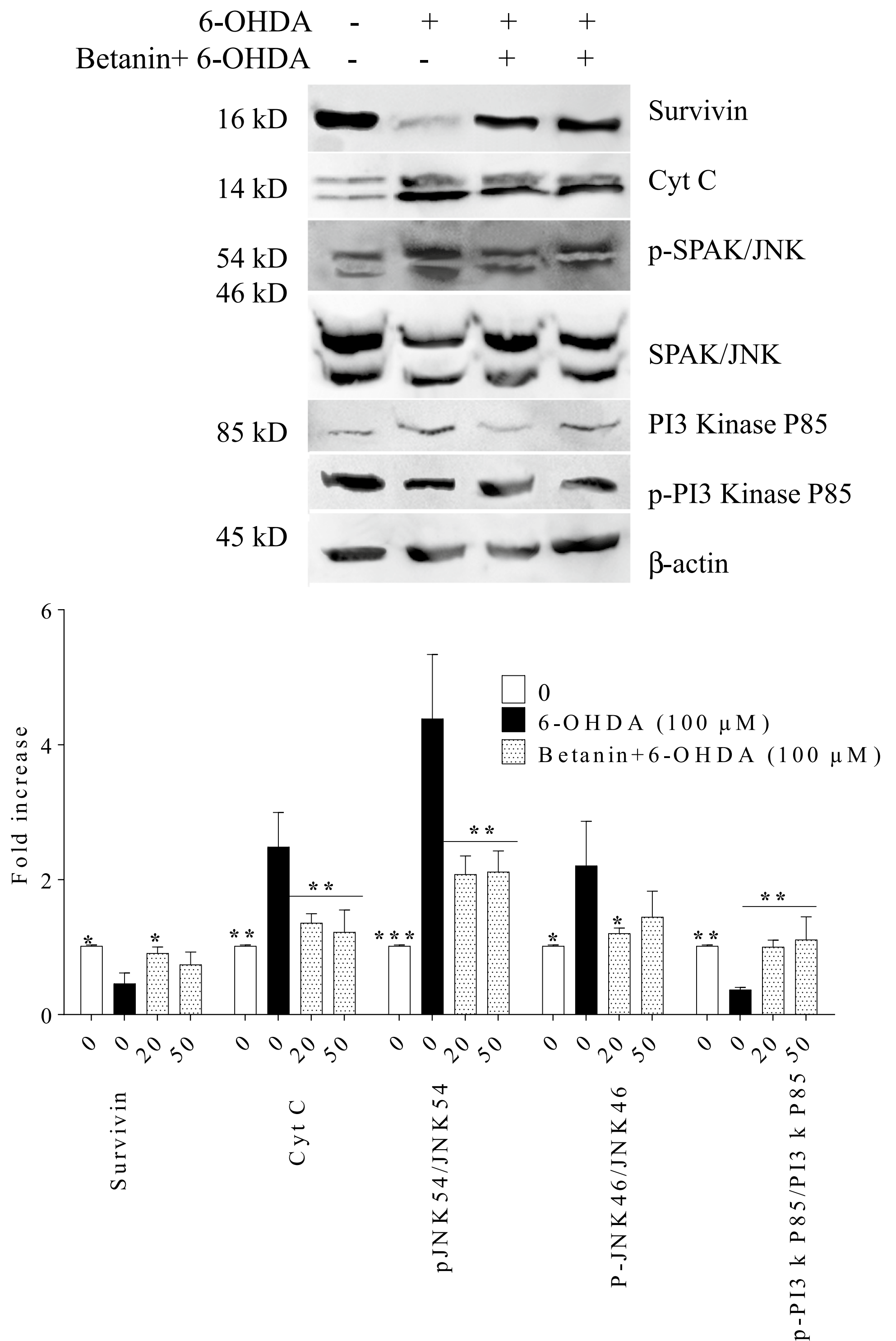


Fig. 3 Effect of betanin and 6-OHDA on apoptosis signaling proteins. About 106 PC12 cells were treated with betanin (20 and 50 μM) 24 h before 6-OHDA (100 μM) exposure. Images were quantified using Gel-pro Analyzer V.6.0 Gel Analysis Software. The data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the 6-OHDA

of the pro-apoptotic family of protein including Bax and activation of the anti-apoptotic Bcl-2 protein. Subsequently the increase in the amount of survivin and reduction in the release of Cyt c from mitochondria, in turn inhabit the activation caspase 9 and caspase 3. Inhabiting caspase 3 activation reduces the cleavage of PARP and finally decrease apoptosis induced by 6-OHDA (Fig. 4). Of course, more

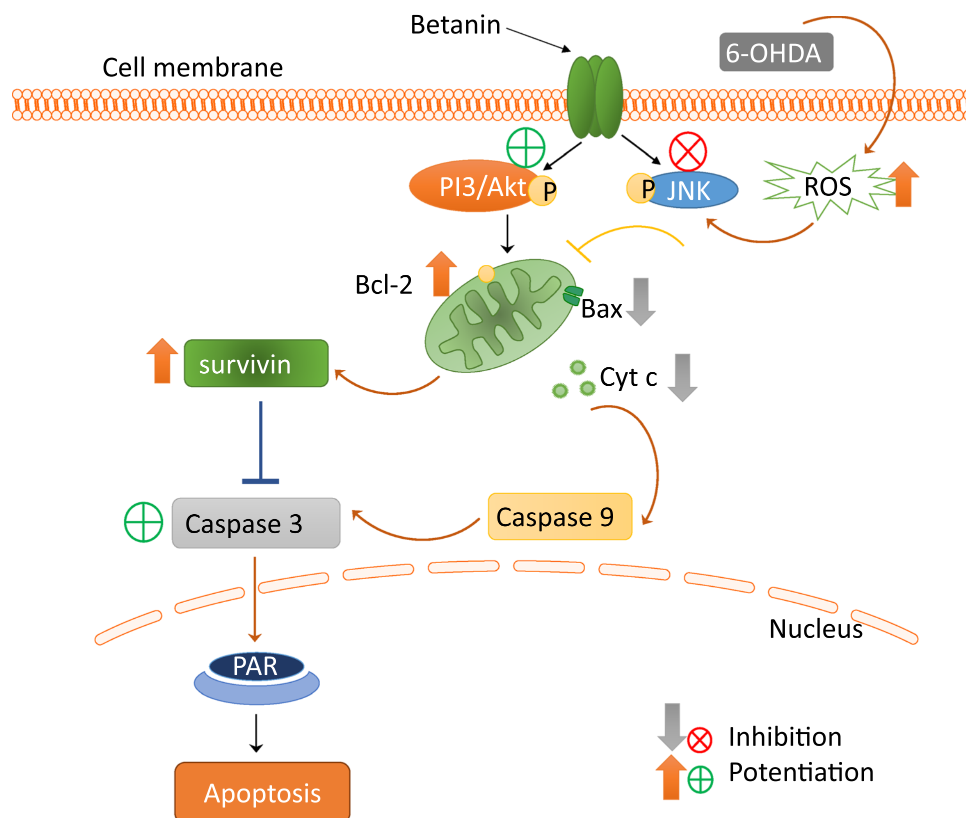


Fig. 4 Schematic representation of the protective role of betanin on 6-OHDA cytotoxicity. 6-OHDA induce apoptosis through SAPK/JNK and PI3 K pathways. Betanin decreased phosphorylation of SAPK/JNK and increased phosphorylation of PI3 K. Also, 6-OHDA activated the intrinsic pathway of apoptosis through reduction of Cyt c and increase survivin

research is needed to clarify the mechanism of the effect of betanin.

Conclusion

The results of our study show that 6-OHDA leads to apoptosis via PI3K/AKT and MAPK pathways. Also, it can activate the internal pathway of apoptosis. While betanin prevents 6-OHDA-induced apoptosis and may able to prevent the neural degeneration in Parkinson's disease as a potential treatment.

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Author Contributions EH performed the experiments and wrote the manuscript. MF and ZT-N conceived, designed, and supervised the project, wrote the manuscript nd provided financial support.

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest in this study.

Ethics Approval As this work is carried out in PC12 cells, there is no need for ethical clearance.

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