The SH-SY5Y Human Cell Line: *Hawthorne Berry (Crataegus spp.)* Protects against 6-OHDA Induced Neurotoxicity In Vitro Model of Parkinson's Disease

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Abstract

Aim: We purposed to study the neuroprotective effects of *Hawthorn berry* (*crataegus spp.*) extract, which is familiar to have antioxidant and anti-inflammatory features, opposite the neurotoxicity led to by 6-OHDA in SH-SY5Y cells.

Method: SH-SY5Y cells were treated with *Hawthorn berry* (25-50-75 and 100 µg/mL) for two hours ago 6-OHDA administration. Cells were exposed to 200 µM 6-OHDA for 24 hours to mimic the in vitro Parkinson's disease model. After one day, cell viability was measured by lactate dehydrogenase and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis. Oxidative stress was evaluated with tumor necrosis factor-α, interleukin-1β, superoxide dismutase, catalase, glutathione, glutathione peroxidase, myeloperoxidase, and malondialdehyde assays.

Results: It was found that the viability rate of *Hawthorn berry* increased depending on the concentration and the cell viability was 94% at the highest concentration ($p<0.001$). Also, 6-OHDA raised lactate dehydrogenase leakage in SH-SY5Y cells ($p<0.001$). While 6-OHDA exacerbated oxidative stress by enhancing tumor necrosis factor-α, interleukin-1β, myeloperoxidase, and malondialdehyde ($p<0.001$), pretreatment with *Hawthorn berry* alleviated these toxic effects of 6-OHDA through antioxidant capacity by increasing glutathione peroxidase, superoxide dismutase, catalase and glutathione ($p<0.05$), ($p<0.001$). In line with all findings, *Hawthorn berry* attenuated neuronal cell demise in a dose-dependent manner.

Conclusion: Considering its neuroprotective role as well as its effects on oxidative stress, *Hawthorn berry* could be a potential natural bio-medicine to prevent the development of Parkinson's disease.

Keywords: Antioxidant, *Hawthorn berry*, 6-OHDA, Parkinson's disease, SH-SY5Y cell line

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SH-SY5Y İnsan Hücre Hattı: *Hawthorne Berry* (*Crataegus spp.*) Parkinson Hastalığının İn Vitro Modelinde Oluşturulan 6-OHDA Kaynaklı Nörotoksisiteye Karşı Korur

Öz

Amaç: Çalışmada antioksidan ve antiinflamatuar özelliklere sahip olduğu bilinen *Hawthorn berry* (*crataegus spp.*) ekstraktın, SH-SY5Y hücrelerinde 6-OHDA ile meydana gelen nörotoksisiteye karşı nöroprotektif etkilerini araştırmayı amaçlanmıştır.

Yöntem: SH-SY5Y hücreleri, 6-OHDA uygulamasından önce iki saat boyunca *Hawthorn berry* (25, 50, 75 ve 100 µg/mL) ile muamele edildi. Hücreler, in vitro Parkinson hastalığı modelini taklit etmek için 24 saat boyunca 200 uM 6-OHDA'ya maruz bırakıldı. Bir gün sonra, 3-(4,5 Dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromür ve laktat dehidrogenaz tahlilleri ile hücre canlılığı belirlendi. Oksidatif stres tümör nekroz faktör-α, interlökin-1β, süperoksit dismutaz, katalaz, glutatyon, glutatyon peroksidaz, miyeloperoksidaz ve malondialdehit analizleri ile değerlendirildi.

Bulgular: Canlılık oranında *Hawthorn berry*'nin konsantrasyona bağlı olarak bir artış gösterdiği ve en yüksek konsantrasyonda hücre canlılığı % 94 oranında bulundu (*p<0,001*). Ayrıca, 6-OHDA SH-SY5Y hücrelerinde laktat dehidrogenaz sızıntısını arttırdı (*p<0,001*). 6-OHDA tümör nekroz faktör-α, interlökin-1β, miyeloperoksidaz ve malondialdehit artırarak oksidatif stresi şiddetlendirirken (*p<0,001*), *Hawthorn berry* ile ön tedavi süperoksit dismutaz, katalaz, glutatyon ve glutatyon peroksidazı artırarak antioksidan kapasite yoluyla 6-OHDA'nın bu toksik etkilerini hafifletti (*p<0,05), (p<0,001*). Tüm bulgular doğrultusunda *Hawthorn berry* nöronal hücre ölümünü hafifleterek doza bağlı bir şekilde önledi.

Sonuç: Oksidatif stres üzerindeki etkilerinin yanı sıra nöroprotektif rolü göz önüne alındığında *Hawthorn berry*, Parkinson hastalığının gelişimini önlemek için potansiyel doğal biyo-ilaç olabilir.

Anahtar Sözcükler: Antioksidan, *Hawthorn berry*, 6-OHDA, Parkinson hastalığı, SH-SY5Y hücre hattı.

Introduction

Parkinson's disease (PD) is a stage and age-related neurological illness characterized by the fastest-growing motor and non-motor signs in the world[1]. Intraneuronal accumulation of alpha-synuclein protein forming Lewy bodies and Lewy neurites and degeneration of dopaminergic neurons in the substantia nigra pars compacta are its main pathological features[2]. Recent works have reported a crucial role of neuroinflammation in neuronal cell apoptosis in PD[3,4]. Overexpression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are significant markers of the pathophysiology of PD and are familiar as one of its therapeutic goals[5]. Stimulation of oxidative stress is among the main mechanisms affecting the onset and development of PD[6]. 6-OHDA is a neurotoxin that induces oxidative stress and impairs mitochondrial function, leading to cell demise by activating the apoptotic path[7]. 6-OHDA has been used as a causative agent to investigate the molecular base of cytotoxicity in PD models and to study neuroinflammation, including neuronal demise activated by oxidative stress[8]. The SH-SY5Y cell line has a catecholaminergic phenotype and synthesizes both dopamine and noradrenaline

neurotransmitters. This cell line, which contains many features of dopaminergic neurons, has become a suitable in vitro model for PD studies[9].

A pharmacological approach may be one of the hopeful therapeutic strategies to reduce oxidative stress in neurodegenerative diseases due to increased oxidative damage in the brain and the weakening of antioxidant defense system activities[10]. It is known that most plant extracts show many pharmacological properties (cytotoxic, antiproliferative, anti-inflammatory and neuroprotective, etc.) up to natural polyphenolic antioxidants[11]. Today, there are many reports of herbal medicine that can help treat or delay neurodegenerative illnesses[12]. In this context, it is well known that Hawthorne berry (*crataegus spp.*) extract (HB) is used as herbal medicine[13]. The Hawthorn plant has powerful antioxidant properties that have the potential to protect the body from diseases caused by oxidative stress, and it is claimed that it may have the potential to prevent neurodegenerative diseases[14]. Although the antioxidant property of this plant extract is known, its neuroprotective effect opposite 6-OHDA stimulated neurotoxicity in SH-SY5Y cells in vitro has not been investigated Therefore, 6-OHDA was used to induce neurotoxicity in SH-SY5Y cells to research the potential protective effects of Hawthorn Berry extract. For this, cell viability and oxidative stress biomarkers were determined.

Material and Methods

Cell Culture and Neuroprotective Activities Assay

SH-SY5Y human neuroblastoma cells were taken from the Pharmacology Department at Ataturk University (Türkiye). This cell line was cultured with Dulbecco's modified eagle medium, antibiotic solution, and 10% fetal bovine serum (Sigma-Aldrich, USA) in a moisturized atmosphere of 5% CO_2 at 37 °C. Then, It was cultured in 96-well plates at an intensity of 1×10^4 cells/well in 100 µL for 24 hours. To establish the PD pattern in vitro, 200 µM 6-OHDA (Sigma-Aldrich, USA) was administered to the cells for 24 hours. Two hours ago the 6-OHDA application, cells were pre-treated with HB (25-50-75, and 100 µg/mL).

Neuroprotective activities of HB on 6-OHDA-stimulated cells were measured by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) analysis. The media were changed by a 20 µL medium including MTT solution (Sigma-Aldrich) and incubated for another 4 hours at the temperature of 37°C. Then the medium was cleaned and 100 µL of dimethyl sülfoksit was supplemented to cells to solve the formazan. The absorbance was read at 570 nm using a microplate reader (Bio-Tek, USA). The cell liveliness was expressed as the percentage, compared with the worth of the control group (100%). Moreover, cell demise was measured by using lactate dehydrogenase (LDH) (Elabscience, USA) released from cells with harmed membranes in agreement with the producer's instructions. The optical intensity was measured at 450 nm with a spectrophotometer plate reader.

Measurement of Oxidative Parameters

The cell media obtained are interleukin-1β (IL-1β), glutathione (GSH), glutathione peroxidase (GPx), tumor necrosis factor-α (TNF-α), superoxide dismutase (SOD), myeloperoxidase (MPO), catalase (CAT) and malondialdehyde (MDA) (Elabscience, USA) was measured according to the kit instructions. The optical intensity was measured at 450 nm with the aid of a microplate reader.

Statistical Analysis

The quantitative data were stated as the mean ± standard deviation (SD). All assays were done by one-way assays of variance with post hoc Tukey's test (IBM SPSS 22.0) ($p<.05$).

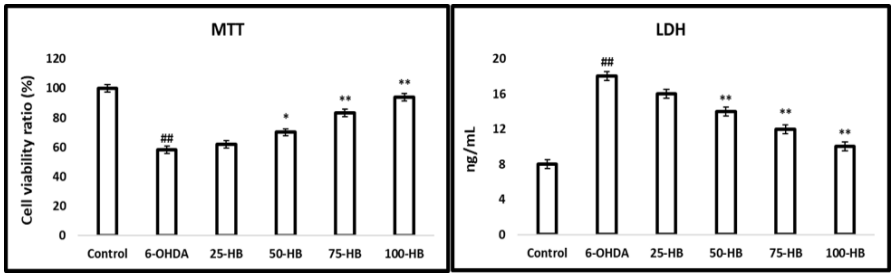
Results

Neuroprotective Effect of HB in 6-OHDA-Stimulated SH-SY5Y Cells

MTT analysis, which measures cellular metabolism, is widely used for examining the antiproliferative, cytotoxic, and anti-tumor effects of herbal extracts. After, the supplement of 200 μM 6-OHDA, cell liveliness declined significantly to 58% ($p<0.001$), when 75 and 100 μg/mL doses of HB extract were supplemented, cell liveliness was 83% and 94%, respectively ($p<0.001$). On the other hand, 25 μg/mL HB extract showed partial protection from the detrimental effects of 6-OHDA on cells; Again; this was found to be statistically meaningful ($p<0.05$) (Figure 1).

In addition, 6-OHDA encouraged significant LDH release from cells compared to the control group ($p<0.001$). When 50, 75, and 100 μg/mL doses of HB were supplemented, LDH release was significantly lower than the LDH release caused by exposure to 6-OHDA solo ($p<0.001$), demonstrating avoiding of the damage to membrane unity stimulated by 6-OHDA. In line with the MTT findings, LDH activities were not significantly different from those stimulated by treatment with 6-OHDA when an HB dose of 25 μg/mL was added.

Figure 1. Study of the cytotoxic effect of HB by MTT and LDH assay.



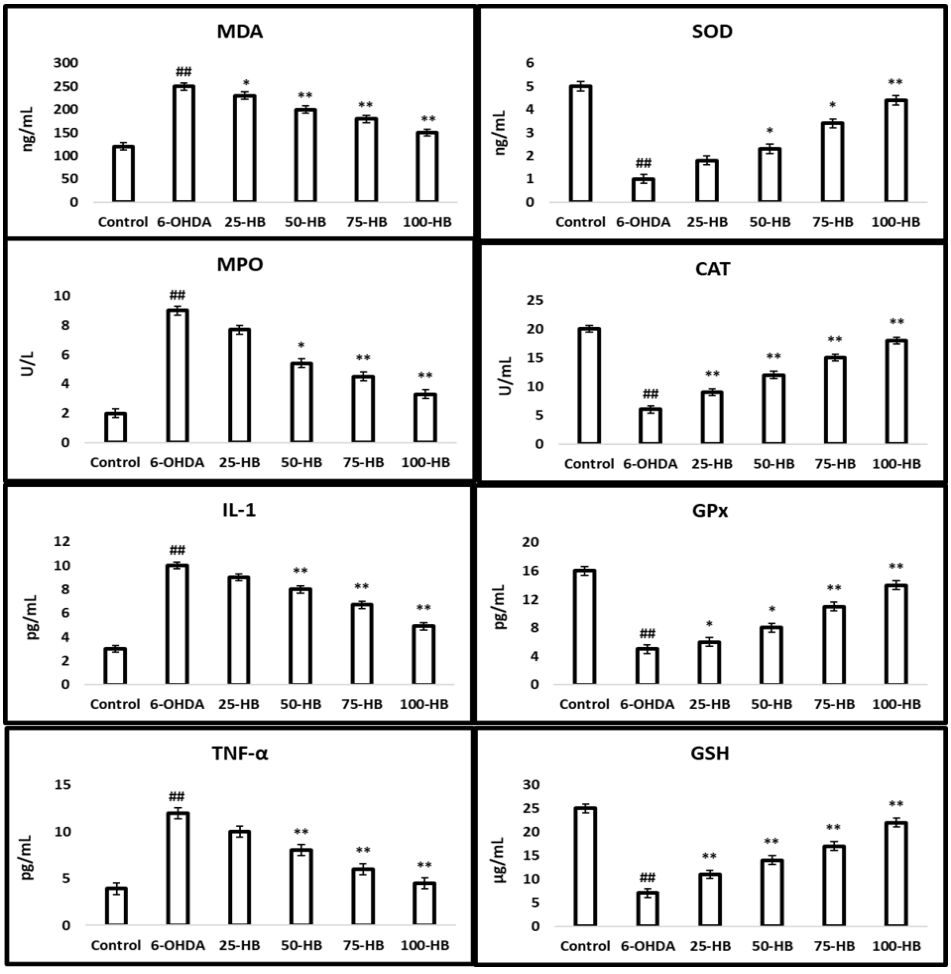
All values are stated as the mean ± SD. $^{\#\#}p<0.001$ vs. control group, $^{*}p<0.05$ vs. 6-OHDA group, $^{**}p<0.001$ vs. 6-OHDA group. HB, Hawthorne Berry; 6-OHDA, 6-hydroxydopamine.

HB Reduces 6-OHDA- Stimulated Oxidative Stress in SH-SY5Y Cells

To state whether HBs can suppress 6-OHDA-stimulated oxidative injury, we measured MDA, MPO, IL-1, TNF-α, SOD, CAT, GPx, and GSH levels. As demonstrated in Figure 2, After treatment

with 6-OHDA, MPO, MDA, IL-1, and TNF-α activities were notably raised, while SOD, CAT, GPx, and GSH activities were notably reduced compared to the control group ($p<0.001$), while means 6-OHDA' suggests that it may increase oxidative injury. When cells were pretreated with HBs (50, 75, and 100 µg/mL) followed by exposure to 6-OHDA, the activities of MDA, MPO, IL-1, and TNF-α were notably reduced by comparison to the 6-OHDA alone group ($p<0.001$). However, when 25 µg/mL HB concentration was applied, MPO, IL-1, and TNF-α activities were not different from cells treated with 6-OHDA alone. In addition, 50, 75, and 100 µg/mL HBs significantly increased SOD, CAT, GPx, and GSH activities in cells exposed to 6-OHDA ($p<0.05$, $p<0.001$).

Figure 2. Effects of HB on oxidative stress indications (MPO, MDA, IL-1, and TNF-α) and the antioxidant enzymes (CAT, SOD, GPx, and GSH).



All values are stated as the mean ± SD. $^{\#\#}p<.001$ vs. control group, $^{*}p<.05$ vs. 6-OHDA group, $^{**}p<.001$ vs. 6-OHDA group. HB, Hawthorne Berry; 6-OHDA, 6-hydroxydopamine.

Discussion

In this work, the neuroprotective effect of HB opposite an in vitro pattern of PD was investigated. SH-SY5Y cells, which have many features of dopaminergic neurons, are constantly used in the in

vitro PD pattern. It is widely familiar that 6-OHDA neurotoxin causes neuronal damage by stimulating oxidative injury of dopaminergic neurons leading to active apoptosis. We initially assessed cell liveliness using the MTT analysis in SH-SY5Y cells to investigate the effects of HBs on 6-OHDA-stimulated neurotoxicity. Pre-incubation of cells with 50, 75, and 100 μg/mL HBs two hours before exposure to 6-OHDA revealed the preventive features of this agent. Notably, 25 μg/mL HBs had no preventive effect opposite 6-OHDA-induced neurotoxicity. In addition, LDH is released into the cell supernatant when the neuronal membrane is injured by 6-OHDA[15]. We measured the LDH content in 6-OHDA-treated cells and found that pretreatment of 50, 75, and 100 μg/mL HBs greatly raised metabolic activity and decreased LDH release. For this, these findings suggest that HBs can suppress 6-OHDA-related oxidative injury and apoptosis.

We also investigated the effects of HB pretreatment on antioxidant enzymes and oxidative stress parameters in 6-OHDA-stimulated SH-SY5Y cells. 6-OHDA accumulates in the cytosol and undergoes rapid auto-oxidation, impairing mitochondrial function with the formation of high free radicals mostly composed of hydrogen peroxide. In addition, excess free radicals decrease the activities of antioxidant enzymes[16]. In this work, oxidative injury caused by 6-OHDA is manifested by high concentrations of MDA, MPO, IL-1, and TNF-α associated with an evident decrease in SOD, CAT, GPx, and GSH concentrations. This oxidative damage is associated with 6-OHDA in cells and is consistent with prior reports.

Oxidative injury is a marked pathogenic ingredient in neurodegenerative illnesses[17]. Since neurons are post-mitotic cells, they are very sensitive to free radicals. It leads to cell death in certain parts of the brain with the increase of free radicals that are active in neurons and glia[18]. Therefore, inhibiting free radical formation is a valued strategy to reduce oxidative stress-concern cell apoptosis[19]. GSH is a very important ingredient of cells and acts as a cofactor for signal transduction and antioxidant defense, especially in the brain[20]. GSH lack has been determined as having a causal role in the progression of PD. Many postmortem PD works have revealed that notable GSH consumption is associated with a rising proportion of oxidated GSH in the substantia nigra[21]. Ko et al[22]. showed that the application of 6-OHDA in neuroblastoma (SH-SY5Y) cells causes GSH consumption due to rising free radical formation, which finally leads to cell demise. In this work, oxidative stress caused by 6-OHDA was also proven by changing parameters indicating impaired antioxidant capacity, decreased SOD, CAT, GPx, and GSH, and increased MDA, MPO, IL-1, and TNF-α activities. This may be related to GSH consumption and lipid peroxidation, mainly due to 6-OHDA stimulated oxidative stress as previously defined[23]. Various studies have shown that varying oxidant and antioxidant levels are highly correlated with PD patients. It has been noticed that 6-OHDA increases the level of MDA in neuronal cells in the asset of oxidative stress[24]. It has been demonstrated that MDA levels are rising in the striatum of PD patients[25]. In the brain of PDs, the secretion of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α is closely related to the destruction of neurons[26]. Therefore, it is crucial to control the three

proinflammatory cytokines to prohibit PD advance[27]. In previous reports, it has been reported that high levels of antioxidant enzymes protect against mitochondrial membrane injury and cell demise[28]. The beneficial effects of HBs on neurodegenerative illness can be attributed to their potential free radical scavenging properties and increasing antioxidant capacity.

There are some limitations in the current study. Since 6-OHDA is insufficient to mimic idiopathic PD, the conclusions of in vitro and in vivo works may not be in line with clinical studies. Despite these restrictions, the 6-OHDA stimulated PD pattern is a crucial pattern for evaluating the neuroprotective effects of new agents opposite oxidative injury because 6-OHDA raises free radicals and induces changes similar to PD-stimulated neurodegeneration.

As mentioned above, the formation of 6-OHDA-induced free radicals in SH-SY5Y cells conclusions in mitochondrial membrane dysfunction and finally participates in neuronal cell demise associated with the pathogenesis of PD[29]. For the first time, we report the neuroprotective feature of HB opposite 6-OHDA-stimulated neurotoxicity in SH-SY5Y cells for PD.

Conclusion

Based on our findings, we think that HB protects against 6-OHDA-stimulated neuronal cell demise through the sustain of mitochondrial activity in cells in a dose-dependent manner. Taken together, our findings showed that HBs protected against 6-OHDA-stimulated neuronal cell demise by a decrease in oxidative injury. HB could be a potential natural biomedicine to prevent the development of PD through brain inflammation and the improvement of neurotransmitters.

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Informed Consent: Not applicable.

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