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Neuroprotective effect of olive leaf extract in the mouse neuroblastoma cell line (NB2a): A good model to evaluate the neuroprotective effects of natural products

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Abstract

Chronic neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, whose prevalence increases with aging, cause serious health problems with inadequate treatment options. Long-term exposure to low doses of pesticides like chlorpyrifos has been linked to an increased risk of developing neurodegenerative diseases. This study assessed the effects of olive leaf extract (OLE) on cell viability and neurite outgrowth inhibition induced by chlorpyrifos (25 µM) using the mouse NB2a neuroblastoma cell line. High concentrations of OLE as 100 to 3000 µg/ml reduced cell viability, likely due to its anticancer properties. On the other hand, lower concentrations of OLE as 3, 10, 30 and 100 µg/ml significantly reversed the inhibition of neurite outgrowth caused by chlorpyrifos exposure, demonstrating a neuroprotective effect. In conclusion, the regular consumption of OLE may effectively slow or prevent the advancement of neurodegenerative diseases linked to long-term exposure to low-dose organophosphate pesticides. Furthermore, the chlorpyrifos-induced moderate neurotoxicity model can be employed as a valuable screening method for evaluating the neuroprotective potential of natural products, including various plant extracts.

Keywords: Neurodegenerative diseases, olive leaf extract, chlorpyrifos, neurite outgrowth

Introduction

Chronic neurodegenerative disorders like Alzheimer's disease (AD) and Parkinson's disease (PD), along with acute neurodegenerative conditions such as stroke and spinal cord injury, pose major health challenges, particularly for the elderly. Unfortunately, effective treatment options for these conditions remain limited [1-3]. Additionally, acute brain injuries were reported as a risk factor associated with chronic neurodegenerative diseases [4-7].

The efficacy of natural plant components in foods against chronic diseases has been demonstrated in many studies [8-10]. In this context, substantial evidence suggests that the Mediterranean diet (MD) may help prevent neurodegeneration, likely because of its high phenol content. The MD typically includes the daily consumption of 25 to 50 grams of extra virgin olive oil, and the phenols present in olive oil are believed to contribute significantly to its beneficial effects [11-14].

The olive tree (Olea europaea L.) produces various phenols, primarily located in its leaves and fruits. These phenols are effective against fungi and microorganisms, and also deter insects from feeding on the leaves due to their unpleasant taste [15]. The potential neuroprotective effects of olive leaf and its phenols such as oleuropein have been shown in some in vivo and limited number of in vitro studies [16-20].

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On the other hand, exposure to specific types of pesticides has been reported 2-3-fold increase in the risk of developing PD, especially in patients with additional risk, such as traumatic brain injury or genetic polymorphism that increases susceptibility to disease [5,21,22]. Chlorpyrifos (CPS: 0-0-diethyl 0-3,5,6-trichloro-2-pyridyl phosphorothioate) is one of the most widely used organophosphate (OP) pesticides. CPS is metabolically converted into its oxon or oxygen analogue, wherein the sulfur in its P=S group is replaced by oxygen. CPS-oxon in the body results in neurodegenerative changes in central and peripheral nerves as moderate acute toxicity in susceptible species [23].

Neurite outgrowth, observed during neuronal differentiation in culture, is a physiological process that serves as an indicator of cellular well-being. Neurite outgrowth, a process specific to the nervous system, relies on crucial cellular functions like axonal transport. As a result, it serves as a valuable in vitro model for assessing neurotoxicity and has been effectively used to evaluate the neurotoxic or neuroprotective effects of various agents [24,25].

NB2a, a mouse neuroblastoma cell line, is frequently used to study the neurotoxic effects of different agents on neuronal cells, as it is a sensitive predictor of neurotoxicity, relatively easy and reproducible. When the damage is moderate, the nerve cell retracts its neurite outgrowth [24,26].The moderate neurotoxic effect of CPS and neuroprotective effect of pioglitazone, an antidiabetic drug, by reversing CPS-induced neurite outgrowth inhibition have been demonstrated in this in vitro cell culture model [26].

The objective of this study was to demonstrate the possible neuroprotective effect of olive leaf extract (OLE) on the CPS toxicity by using mouse NB2a neuroblastoma cell line.

Material and Methods

Materials

Mouse NB2a neuroblastoma cells were obtained from European Collection of Cell Cultures (ECACC) (cell line: 89121404). Chemicals and culture flasks and plates were provided from Sigma (St. Louis, MO, USA) and Falcon/Fred Baker (Runcorn, Cheshire, UK), respectively. Gentamicin was supplied from from I. Ethem (GentaR 20 mg ampul, I. Ethem, İstanbul, Türkiye).

OLE, prepared as previously described [27] and standardized to contain 15% oleuropein, was generously provided by Kale Naturel (Edremit-Balıkesir, Türkiye). Briefly, olive leaves were harvested from the Balikesir-Edremit region of Türkiye in February 2018. After the leaves were washed, they were dried under suitable conditions at room temperature and homogenized with a blender. Extraction was carried out by continuously shaking the powder in 80% ethanol at room temperature. Stability and purity of extract were also confirmed. The OLE preparation was dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted with medium to achieve a maximum final DMSO concentration of 0.2% for cell culture experiments. This stock solution was prepared immediately before use and filtered through microbial filters for sterilization. Culture medium was used for subsequent OLE dilutions.

Antioxidant Activity of OLE

The antioxidant activity of OLE (1 mg/mL) was assessed by measuring its radical scavenging activities against 2,2′-Azino-bis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals. The percentage scavenging activity values were calculated using the formula: Inhibition% = [(Ab – As) / Ab] x 100, Where Ab is the absorbance of the control reaction and As is the absorbance of the test sample. Each analysis was performed in triplicate and final results were obtained from the average values.

ABTS Assay

ABTS solution was mixed with potassium persulfate (K2S2O8) and after allowing to react at room temperature for 16 h. The solution was diluted with acetic acid (20 mM, pH 4.5) to achieve an absorbance of 0.70±0.02 at 734 nm. Then, 2 mL of this solution was mixed with 200 µL of the diluted sample (1 mg/mL). Absorbance was measured after 15 minutes at 734 nm [28]. Trolox served as the standard solution.

DPPH Radical Scavenging Assay

The DPPH scavenging activity of the extracts was measured using the method described earlier [29]. Specifically, 4 mL of 0.004% DPPH in ethanol was mixed with 1 mL of ethanol extracts (1 mg/mL). The absorbance was assessed at 517 nm after 30 minutes. Ethanol was used as blank. α-Tocopherol was used as standard.

Cell Culture

Neuroblastoma cells were proliferated in a 37°C incubator at 5% CO_2 atmosphere in culture flasks. The culture medium consisted of high glucose Dulbecco's Modified Eagle Medium (DMEM) with Glutamax-1, containing 5% horse serum, 5% fetal calf serum, 25 µg/mL gentamicin and 1% penicillin/streptomycin solutions (10000 U/10 mg).

Cell Viability

In order to evaluate the cytotoxicity of OLE, the CellTiter-Glo® luminescent cell viability assay (Promega, Madison, WI) was employed for the experiments. NB2a neuroblastoma cells were seeded into 96-well plates at a density of 10,000 cells per well and allowed to adhere to the bottom of the plates for 24 hours. Subsequently, the cells were treated with

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a serial dilution of OLE ranging from 10 to 3000 µg/mL for an additional 24 hours before the assay. Medium (100 µL) without any cells and OLE was used as negative control, and medium without OLE was used as positive control. This method quantifies ATP levels to assess the number of viable cells. Briefly, 100 µL of CellTiter-Glo® reagent mixture was added directly to the cells without removing the medium and mixed for 2 minutes to facilitate cell lysis. The luminescent signal was measured using a GloMax® 96 microplate luminometer (Promega, Madison, WI) following a 10-minute incubation of the plate. Ultra-Glo-luciferase, an enzyme that catalyzes the oxidation of luciferin in the presence of ATP, which is released by metabolically active cells, served as an indicator of cell viability. The dose-response curve was plotted based on OLE concentration and the corresponding luminescence signal [30]. All treatments were performed three times and the average values were used as the final results.

Measurement of Neurite Outgrowth

NB2a cells were plated in 24-well culture plates at a density of 20,000 cells per well and incubated for 24 hours in a proliferation medium in the experiments to assess neurite outgrowth. Subsequently, this medium was replaced with a serum-free medium that included 0.5 mM dibutyryl cyclic AMP to stimulate differentiation, leading to neurite outgrowth. The control group cells were treated solely with 0.2% DMSO, a concentration previously determined to have no impact on neurite growth. Meanwhile, negative control cells were maintained in a serum-containing medium, without any induction for differentiation. To explore the potential neurotoxic or neuroprotective effects of OLE, it was administered in concentrations ranging from 3 to 1,000 µg/mL, alone or combined with 25 µM CPS. This concentration of CPS was found to be the lowest and most effective concentration that inhibited the neurite outgrowth in previous studies [26]. Three wells were used for control and each treatment.

After an additional 24 hours, the cells were fixed in 4% (w/v) formaldehyde in phosphate-buffered saline (PBS) at 24°C for 10 minutes and then stained with Coomassie Blue dye (0.6% Coomassie Brilliant Blue G in 10% (v/v) methanol, 10% (v/v) acetic acid in PBS). The stained samples were washed twice with PBS and distilled water at 10-minute intervals. Three blinded observers photographed the samples using an Olympus BX-40 light microscope (Olympus, Tokyo, Japan) equipped with a JVC-TK-C 601 video camera (Tokyo, Japan) for digital imaging. Image analyses were performed with the Image-Pro Plus image analyzer (version 5.1.259, Bioscience Technology, Bethesda, MD, USA). Neurite lengths were measured in 100 cells from 10 different randomly selected sites under the light microscope from all three wells using the image analysis software. The measured neurite lengths were expressed as a percentage of the control values [26].

Statistical Analysis

The statistical analysis was conducted using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA), with data expressed as mean±S.E.M. Kolmogorov Smirnov test was used to determine whether the data set in question conforms to a normal distribution. One-way ANOVA followed by Tukey's multiple comparison test was employed to determine statistical significance. A p-value of less than 0.05 was considered to be statistically significant.

Results

Antioxidant Activity of OLE

ABTS and DPPH radical scavenging activities were calculated as 39.90±6.81 and 31.74±4.50% for 1 mg/mL OLE, respectively.

Effects of OLE on Cell Viability and Proliferation

In order to evaluate the cytotoxicity of OLE and its effect on cell proliferation, NB2a neuroblastoma cells were incubated with 10, 30, 100 and 300 µg/ml and 1 and 3 mg/ml concentrations of OLE, and compared with control and solvent (DMSO, 0.2%) groups. Measurements were made in 3 repetitions for each group. While OLE significantly reduced cell viability to 68.10±10.05% of the control ($p<0.05$) at the concentration of 100 µg/ml, it had a lethal effect on nearly all cells at concentrations of 1 and 3 mg/ml (Figure 1).

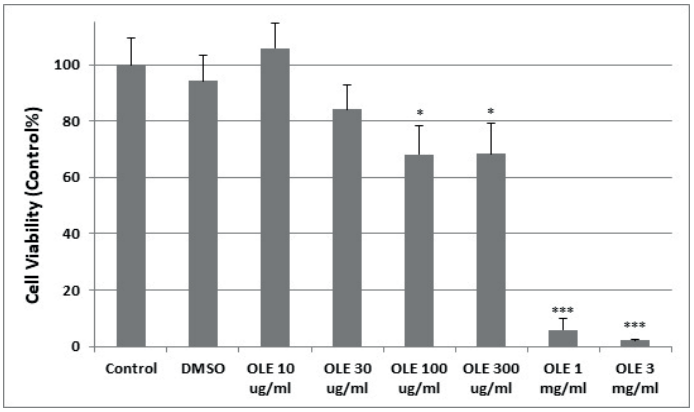

Figure 1. Effect of different concentrations of olive leaf extract (OLE) on cell viability in mouse neuroblastoma cell line (NB2a). DMSO: dimethyl sulfoxide as a solvent. Significant difference from control, *$p<0.05$, ***$p<0.001$

Effects of OLE on Neurite Outgrowth

Mouse neuroblastoma cells (NB2a) were grown in proliferation medium and replaced with differentiation medium in the presence of 1 mM dibutyryl cAMP, resulting in neurite outgrowth of the control group (Figure 2). Addition of solvent (DMSO, 0.2%) did not have any effect on neurite outgrowth.

In order to evaluate the possible neurotoxic (inhibition of neurite outgrowth) effect of OLE, 3, 10, 30, 100 and 300 µg/ml and 1 mg/ml concentrations of OLE were added into the differentiation medium, with a concentration in every three wells. 1 mg/ml OLE concentration showed severe neurotoxicity and completely blocked neurite outgrowth, consistent with the results of cell

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viability tests. Therefore, measurements for this concentration were not evaluated. A slight dose-dependent inhibition trend in neurite outgrowth was observed at 3, 10, 30, and 100 µg/ml concentrations of OLE, but there was no statistically significant difference compared to the control ($p>0.05$). It was determined that neurite outgrowth was inhibited significantly (52.51±9.74% of control, $p<0.05$) at 300 µg/ml concentration of OLE (Figure 3).

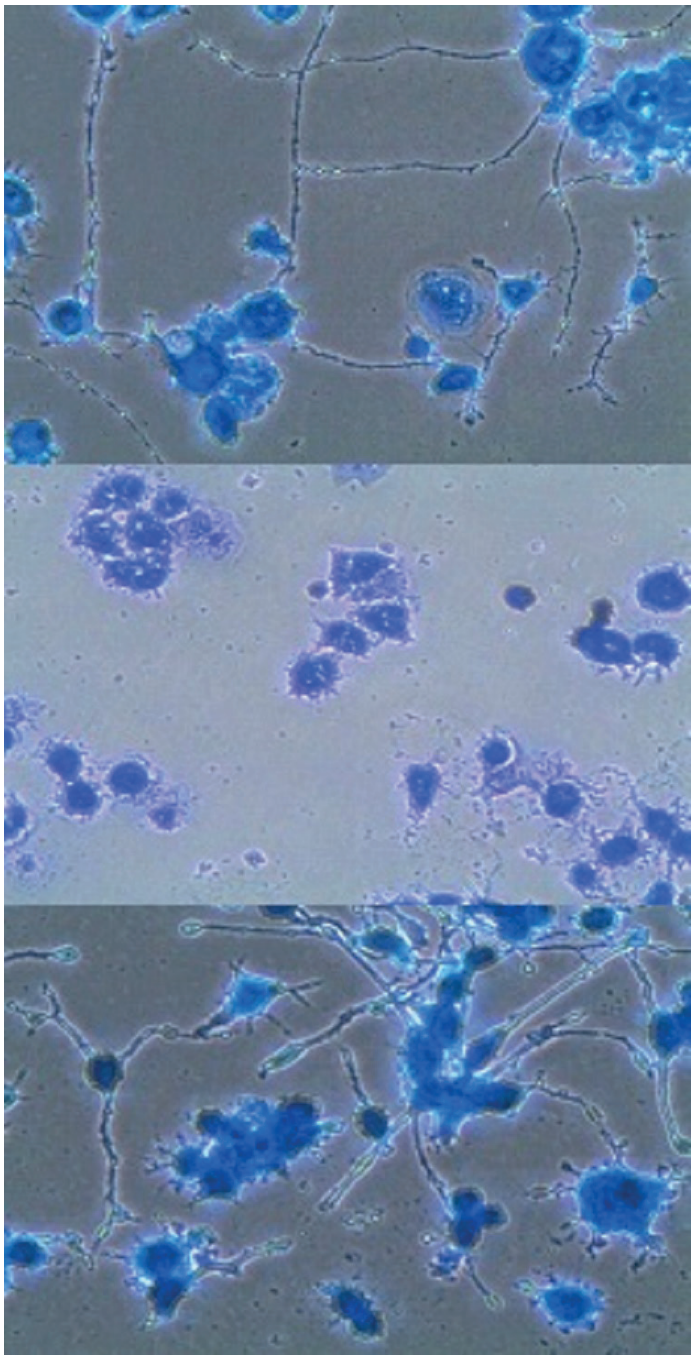

Figure 2. Neurite outgrowth of mouse neuroblastoma culture cells (NB2a) differentiated by d-cAMP (top), inhibition of neurite outgrowth in NB2a cells in the presence of chlorpyrifos (25 µM) (middle), reversal of chlorpyrifos induced neurite outgrowth inhibition by olive leaf extract at a concentration of 30 µg/ml (bottom). (Coomassie Blue staining, X400)

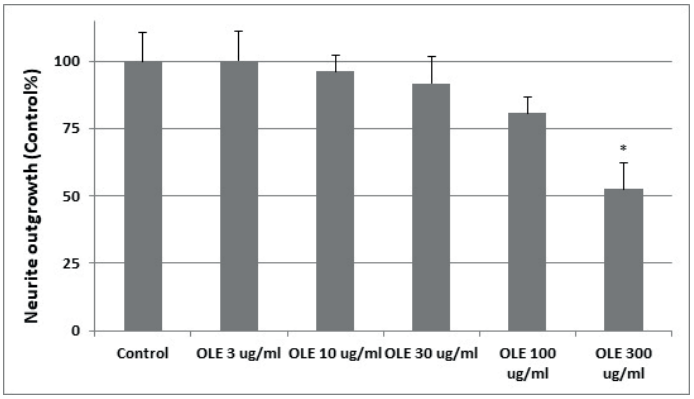

Figure 3. Effect of different concentrations of olive leaf extract (OLE) on neurite outgrowth in mouse neuroblastoma cell line (NB2a); significant difference from control, *$p<0.05$

Neuroprotective Effects of OLE

In studies to evaluate the possible neuroprotective effect of OLE, the addition of CPS (25 µM) into differentiation medium almost completely blocked neurite outgrowth in NB2a neuroblastoma cells (Figures 2 and 4). The effects of 3, 10, 30, 100 and 300 µg/ml concentrations of OLE on neurite outgrowth were evaluated in the presence of CPS. Although OLE at a concentration of 300 µg/ml partially reversed the inhibitory effect of CPS, no statistically significant difference was found between the CPS group ($p>0.05$). Other concentrations of OLE significantly decreased the inhibitory effect of CPS. While the protective effect on neurite outgrowth was observed most clearly at 10 and 30 µg/ml concentrations of OLE, no statistically significant difference was observed between the groups that received 30 µg/ml OLE (87.14±10.65%) and control group (Figures 2 and 4). Interestingly, a significant protective effect was found even at very low concentrations of 3 µg/ml of OLE (49.77±5.51% of control) (Figure 4).

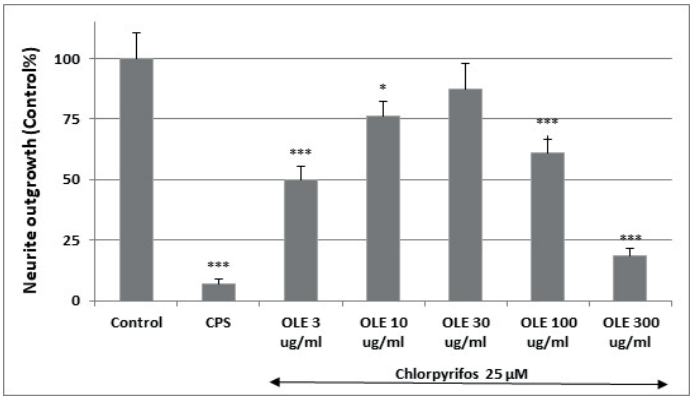

Figure 4. Effect of different concentrations of olive leaf extract (OLE) on the inhibition of neurite outgrowth by chlorpyrifos (CPS) in mouse neuroblastoma cell line (NB2a). Significant difference from control, *$p<0.05$, ***$p<0.001$

Discussion

The main finding of this study is that the acute neurotoxic effect observed in mouse-derived neuroblastoma cell (NB2a) culture, in the form of a nearly complete inhibition of neurite outgrowth

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by a widely used pesticide CPS, is significantly reversed even at very low concentrations of OLE (3 µg/ml). The most significant effect was observed at 10 and 30 µg/ml concentrations of OLE, and no statistically significant difference in neurite elongation was found between the 30 µg/ml concentration of OLE and the control group that did not receive CPS.

Chronic neurodegenerative diseases like AD and PD present significant health challenges, particularly in the elderly population. AD, the most prevalent form of dementia, affects over 27 million individuals globally. Its incidence rises from 1% in individuals aged 60-70 to 6-8% by age 85 [3]. PD, the second most common neurodegenerative disease, impacts 1-2% of people over the age of 65 [2].

The etiology of chronic neurodegenerative diseases is related to many factors; it includes interactions between environmental factors and genetic predisposition. OP compounds are the most widely used pesticides in agriculture, and there is evidence linking the etiologies of chronic neurodegenerative diseases such as PD and AD with low-dose/long-term pesticide exposure [5,21,22]. In a study of 357 idiopathic Parkinson's patients, the incidence of PD increased approximately 2-fold in patients with a history of head trauma, while the incidence increased 3-fold in those with concomitant paraquat exposure [5]. In addition, genetic mutations and polymorphisms of different variants of certain genes, such as paraoxonase and dopamine transporter, have been reported to increase susceptibility to both OP toxicity and neurological diseases [21,22]. In susceptible individuals with a polymorphism in the paraoxonase-1 gene, the increase in the incidence of PD under 60 years of age was greatest with one of the most widely used OP pesticides, CPS (approximately 2.6-fold increase) [22].

Furthermore, numerous clinical and epidemiological studies have shown that the MD, which prominently includes extra virgin olive oil, can lower the incidence of various chronic degenerative diseases, major cardiovascular events, type 2 diabetes mellitus, and certain cancers [13,14,31]. The impact of olive oil and its phenols on chronic neurodegeneration has primarily been explored in research focused on AD and PD. These phenols have been shown to reduce both Aβ plaques and neurofibrillary tangles by interacting to varying degrees with their production and clearance. In addition, antioxidant system strengthening and anti-inflammatory activities have also been shown concerning its protective effects in these diseases [12,16,32].

Since OLE contains olive oil phenols such as oleuropein and hydroxytyrosol in high amounts, which are known for their strong antioxidant properties, it is easy to obtain and the cost is cheap, it can be considered as a supplement or an alternative to olive oil consumption in terms of the beneficial effects mentioned. Neuroprotective effects of OLE have been demonstrated in many studies [16-20].

In the light of literature and the findings of our study, regular consumption of OLE may help prevent the development of chronic neurodegenerative diseases like AD and PD, potentially triggered by low levels of chronic pesticide exposure.

Although there are many in vivo and in vitro methods to examine the neurotoxic effects of different compounds, neuronal cell culture studies are highly preferred due to their relative ease and showing the toxic effects well at the cellular level. The neurotoxic effect of a compound can be observed in two ways, usually depending on dose or concentration. If the effect is severe, the cell exposed to the toxic effect dies. However, if the neuron is exposed to a mild or moderate toxic effect, it retracts its neurites and cannot complete its differentiation. Differentiation of neurons in cell culture is a physiological process and measurement of neurite outgrowth provides a valuable in vitro model for demonstrating the neurotoxic potential of a wide range of agents [24]. Severe neurotoxicity, on the other hand, is assessed using a variety of methods that demonstrate cell viability and proliferation [30].

CPS is a widely used OP insecticide employed against horticultural, agricultural, and forest pests, leading to frequent human exposure. In the body, CPS is converted into its oxygen or oxon analog by the liver's cytochrome P450-dependent monooxygenase system. This conversion to CPS-oxon is the main cause of its neurotoxic effects in susceptible species, leading to neurodegenerative changes in both the central and peripheral nervous systems [23]. CPS applied at a concentration of 25 µM has been reported to cause inhibition of neurite outgrowth, which is an indicator of moderate neurotoxic effect in neuroblastoma or other neural tissue cell line [23,26]. In a study by Vural & Seyrek [26], they showed that pioglitazone, an antidiabetic drug, significantly reversed neurite outgrowth inhibition induced by 25 µM CPS in a concentration-dependent manner. In the light of these findings, this model can be used as a screening test to reverse the moderate toxic effect (inhibition of neurite outgrowth) produced by CPS with various agents and to demonstrate the neuroprotective effects of these agents. In our study, we showed that OLE caused a neuroprotective effect by reversing the neurite outgrowth inhibition induced by CPS at certain concentrations, probably due to its strong antioxidant effect also shown in the study.

Another important finding of this study is that while OLE caused a statistically significant decrease in cell viability (68.10±10.05% of control) at 100 µg/ml concentration, it showed a lethal effect on almost all cells at 1 and 3 mg/ml concentrations. In addition, consistent with these findings, we demonstrated that neurite outgrowth was inhibited by approximately 50% at 300 µg/ml concentration of OLE, while completely blocked at 1 mg/ml concentration. In studies on the anticancer activity of OLE in T98G human Glioblastoma multiforme cell cultures, similar to our findings, OLE caused a significant decrease in cell viability at 1 and 2 mg/ml concentrations [27,33].

Despite the protection of cell viability against a neurotoxic agent to demonstrate neuroprotective effects of various plants or their extracts is a widely used method, since the cells used in cultures are mainly cancer cells and these plants generally have apoptotic

and anticancer properties, the results may be confusing. The results of our study also showed that the concentrations of OLE to decrease in cell viability as an indicator of anticancer activity and to reverse CPS-induced neurite outgrowth inhibition as an indicator of neuroprotective effect were found different in mouse-derived neuroblastoma cell culture. We demonstrated that the concentrations required for the anticancer effects were apparently higher than for the neuroprotective effects.

Conclusion

In this study, we demonstrated that OLE at 3-100 µg/ml concentrations shows a neuroprotective effect by significantly reversing the CPS-induced inhibition of neurite outgrowth in the mouse NB2a neuroblastoma cell line. That's why, the regular consumption of OLE may be useful to prevent neurodegenerative diseases linked to long-term exposure to low-dose OP.

On the other hand, higher concentrations of OLE reduced cell viability, probably due to its anticancer properties. Since cancer cells are mostly used in cell culture studies to evaluate neuroprotective effects of different agents, confusing results may be obtained in these studies assessing cell viability. Although further studies are needed, this moderate neurotoxicity model induced by CPS may be an important screening test for demonstrating the neuroprotective effects of natural products such as herbal extracts and may be useful for comparison with each other.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
Not applicable, as the study did not involve patients, volunteers or animals.

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