Neuroprotective Effects of Different *Lavandula stoechas* L. Extracts Against Hydrogen Peroxide Toxicity *in vitro*

Mümin Alper Erdoğan ¹, Ceylin Bayar ², Ekim Özkaya ², Ayşegül Metin ², Derviş Birim ³, Güliz Armağan ³, Serdar Demir ⁴, Gözde Elgin Cebe ⁴*

¹ Izmir Katip Celebi University, Faculty of Medicine, Department of Physiology, Izmir, Turkey
² Aydın Science High School, Aydın, Turkey
³ Ege University, Faculty of Pharmacy, Department of Biochemistry, Izmir, Turkey
⁴ Ege University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Izmir, Turkey

*Corresponding author

Gözde Elgin Cebe, Associate Professor, PhD
Ege University
Faculty of Pharmacy
Department of Pharmaceutical Botany
Izmir, TURKEY
Email: gozde.elgin.cebe@ege.edu.tr, gozde.elgin@gmail.com
Phone: +90532 292 55 40

https://orcid.org/0000-0002-7253-3864
LiveDNA: 90.37947

**Running title:** Neuroprotective Effects of *Lavandula stoechas* L. Extracts

**Conflict of Interest:** The authors declare no conflict of interest.

**Contribution of Author:** Detail of the each author with his/her contribution in this paper is as under:

<table>
<thead>
<tr>
<th>Name of the author</th>
<th>Types of contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mümin Alper Erdoğan</td>
<td>Concept and Design, Control, Sources and Materials, Data Collection and Processing, Analysis, Literature review, Manuscript writing, Critical review</td>
</tr>
<tr>
<td>Ceylin Bayar</td>
<td>Data Collection and Processing, Literature review</td>
</tr>
<tr>
<td>Ekim Özkaya</td>
<td>Data Collection and Processing, Literature review</td>
</tr>
<tr>
<td>Ayşegül Metin</td>
<td>Data Collection and Processing, Literature review</td>
</tr>
</tbody>
</table>
ABSTRACT

Background and Objective: Cell damage caused by oxidative stress is one of the mechanisms that has been implicated for a long time in various neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. *L. stoechas* has been used in European-Iranian traditional medicine for the treatment of various diseases, primarily cardiovascular and neurodegenerative disorders. In this study, the neuroprotective effects of *Lavandula stoechas* L. extracts against H$_2$O$_2$ toxicity in SH-SY5Y cells were investigated in vitro. Materials and Methods: Several extracts were prepared from the flowering branches of the *L. stoechas*. The neuroprotective activity of the extracts against H$_2$O$_2$ damage in SH-SY5Y cells and their effects on apoptotic processes were evaluated based on cell viability against H$_2$O$_2$ toxicity, colony formation capacity and effect on apoptotic protein levels. Results: It was determined that pretreatment with 1-10 ppm doses of *L. stoechas* extracts (2-24 hours) had a significant protective effect against 250 μM H$_2$O$_2$ toxicity. It was shown for the first time that *L. stoechas* extracts exhibited a significant protective effect for colony formation at 1-10 ppm doses and protected the colony numbers by 50% against H$_2$O$_2$ toxicity. In addition, it was determined that they were able to exert anti-apoptotic effects by decreasing Bax levels and increasing Bcl-2, thereby decreasing the Bax/Bcl-2 protein ratio. Conclusion: In this study, neuroprotective activities of different *L. stoechas* extracts were compared for the first time. Total methanol, ethyl acetate and water (infusion) extracts have shown potent neuroprotective effects and the cellular processes underlying its effects have been proven by these preliminary data.

Keywords: *Lavandula stoechas*, neuroprotection, SH-SY5Y cells, oxidative stress, viability, colony formation, apoptosis
INTRODUCTION

Oxidative stress has been linked for a long time to both the physiological mechanism of ageing and a variety of neurodegenerative diseases, including Alzheimer’s and Parkinson’s. Reactive oxygen species created by cell disruption, oxidative respiration, or the excessive accumulation of free transition metals may damage DNA, proteins and lipid membranes, compromising cell function and viability. One of the primary reactive oxygen species, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), is formed all through the oxidation/redox reaction and is regarded as a transmitter in intracellular signalling pathways. Furthermore, it is widely understood that H\textsubscript{2}O\textsubscript{2} may induce DNA damage and lipid peroxidation, leading to apoptosis in a variety of cell subtypes. Therefore, therapeutic strategies to prevent or delay apoptosis induced by reactive oxygen species are considered suitable approaches for the treatment of these diseases. Among the different treatment methods, increasing or enhancing the endogenous defence system towards oxidative stress by nutritional or pharmaceutical antioxidant consumption is a potential option. Numerous synthetic substances, including phenolic compounds, have been demonstrated to be powerful radical scavengers, although they frequently have severe adverse effects. In this context, in recent years, the focus has been on the research of natural substances with neuroprotective potential.

Oxidative stress-induced neuronal cell death includes necrosis and apoptosis with programmed cell death and the mode of death is dependent on the severity of oxidative damage. Apoptosis is a prolonged kind of cell death that results from the execution of genetic programming, as opposed to necrosis, which is an immediately developing type of cell death. H\textsubscript{2}O\textsubscript{2} promotes apoptotic cell death in rat pheochromocytoma PC12 cells (a model for neurons that contain catecholamines) and human neuroblastoma SH-SY5Y cells, according to previous experiments. However, the molecular mechanisms involved in oxidative stress-induced apoptotic neuronal cell death are complex and not yet fully elucidated.

The Lamiaceae family is one of the largest families in the world with 224 genera and approximately 5600 species. Lavandula (Lavender) genus is a member of the Lamiaceae family and is represented by approximately 49 species in the world. This genus is native to West Africa, Europe to North and East Africa, the Mediterranean, Southwest Asia and India. In Turkey, 3 Lavandula taxa have a natural distribution in Western and Southern Anatolia. It is known that some Lavandula species are cultivated in Turkey, Bulgaria, England, the USA and North African countries. Since cultural forms are widespread in the world, they also show natural spread outside their natural areas.
**L. stoechas**, is a shrub to 45 cm or more and tomentose. Verticillasters are 6-10-flowered and the corolla is blackish-purple. It spreads up to 700 m in open Pinus brutia forests, macchie, phrygana, rocky, calcareous and sandy places and roadsides\(^19\).

The plant is known locally as “Karabaş” in Turkey and it is recorded that it has been used traditionally as a pain reliever, sedative and expectorant. The plant is also used by the public for wounds, eczema and urinary tract infections\(^18\). It is known that the leaves and flowers of *L. stoechas* are used in European and Iranian Traditional Medicine for the treatment of central nervous system disorders such as epilepsy, dementia, migraine and Parkinson's disease\(^20\)-\(^22\).

*L. stoechas* is registered in the pharmacopoeias of many countries such as the German Pharmacopoeia, the French Pharmacopoeia, and the FFD (Pharmacognosy and Phytotherapy Association) Monographs. It has been revealed that the aerial parts of the plant contain alkaloids, carbohydrates, flavonoids, glycosides, phenols, proteins, saponins, steroids, tannins, terpenes and terpenoids\(^20\),\(^21\). The effects of *Lavandula* species on neurodegenerative disorders have been reported in the literature\(^20\)-\(^22\). This study, it was aimed to determine the neuroprotective activities of different extracts of *L. stoechas* flowers.

Human SH-SY5Y neuroblastoma cells are commonly utilized to examine neuronal cell apoptosis produced by the generation of reactive oxygen species. As shown by DNA fragmentation and the formation of apoptotic bodies, \(\text{H}_2\text{O}_2\) treatment of SH-SY5Y cells triggers cell apoptosis\(^12\). SH-SY5Y cells can be differentiated into neuron-like cells that acquire the characteristic morphological, neurochemical and electrophysiological features of neurons using retinoic acid\(^23\)-\(^25\).

Neurodegeneration is the progressive damage to neurons and consequently, death. Genetic factors are effective in the emergence of neurodegenerative diseases as well as environmental factors. Reactive oxygen species formed by intracellular and extracellular pathways and to which we are exposed to environmental factors can cause neurodegenerative diseases by damaging cellular proteins, lipids and nucleic acids\(^26\),\(^27\). Human neuroblastoma cells SH-SY5Y is a dopaminergic neuronal cell line that is often used as an *in vitro* model for neurotoxicity experiments\(^28\).

There were studies in the literature examining the powerful antioxidant effects of *L. stoechas* extracts and their effects on various diseases. However, there is no study evaluating the protective efficacy of different types of *L. stoechas* extracts against the \(\text{H}_2\text{O}_2\) toxicity model. In this study, it was investigated whether *L. stoechas* extracts have neuroprotective effects against \(\text{H}_2\text{O}_2\)-induced apoptosis.

**MATERIALS AND METHODS**
The study was carried out at Ege University Faculty of Medicine, Department of Physiology and Faculty of Pharmacy, Department of Pharmaceutical Botany, Izmir, Turkey from July 2021 to January 2022.

**Plant Material:** Aerial parts (inflorescences) of *L. stoechas* were collected from the wild populations at Koçarlı (Aydın-Turkey) (600-700 m) between April and May 2021. Crude plants were dried in shade and ground into coarse size powder. Plant specimens were identified by Dr. G. Elgin Cebe (Department of Pharmaceutical Botany). Voucher specimens of the plant are being kept for records in the IZEF Herbarium of Ege University, Faculty of Pharmacy.

**Distillation Method:** To obtain essential oil from the plant, 100 g of the ground drug was subjected to hydrodistillation for 3 hours using a Clevenger apparatus. In summary; flowers were added to water and heated to boiling point. The essential oil, which was then entrained with the water vapour, was collected in a condenser. The distillate collected on top of the water was isolated and dried with the aid of anhydrous sodium sulfate.

**Infusion Preparation Method:** After adding 200 mL of boiling distilled water to 10 g of ground drug, the mixture was kept in a closed container for 30 minutes. Afterwards, the extract was filtered through filter paper and concentrated to dryness with an evaporator under low pressure at 40°C.

**Extraction Method:** Analytical grade *n*-hexane (Carlo Erba), ethyl acetate (Carlo Erba) and methanol (Carlo Erba) was used for the sequential extraction and total extraction methods\textsuperscript{29}. For sequential extraction; 100 g of the ground drug was added to *n*-hexane, ethyl acetate and methanol, respectively, and extracted using an ultrasonic water bath (Bandelin Sonorex RK 52 (240W, 35kHz) Bandelin Electronic, Berlin, Germany and NUVE MK 418 with heater (600W/1200rpm), NUVE, Ankara, Turkey) for 2 hours (each 1.5Lx3 replicates). Then the extracts were filtered through filter paper (Whatman filter, Whatman Clifton, NJ, USA) and combined separately.

For total extraction; only methanol was added to 20 g of ground drug and it was extracted in an ultrasonic water bath for 2 hours (300 mLx3 repetitions). Then the extracts were combined and filtered through filter paper. All filtered extracts were separately concentrated to dryness with an evaporator under low pressure at 40 °C and stored at -20°C until the experiments began.
Cell Culture Procedures: Cell culture processes were carried out in sterile laminar airflow (ThermoFisher Scientific, MA, USA) working cabinets. Cells stored at -80°C were thawed in a 37°C water bath and transferred to 75 cm² filtered cell culture flasks.

SH-SY5Y cell line was grown by adding 15% heat-inactivated fetal bovine serum to DMEM/F12 (Dulbecco's modified Eagle's minimal essential medium, High glucose, L-Glutamine, Sodium Pyruvate, Phenol Red) medium in an incubator at 37°C, containing 5% CO₂ and humidity. Penicillin and streptomycin were added to all media (100 units/mL).

Cell lines were monitored daily for viability, proliferation, and infection under an inverted microscope (Olympus CKX53, Olympus Corp, Tokyo, Japan). When a cell density of more than 80% was observed in the flasks, the cells were multiplied by passage.

MTS Cell Viability Test and L. stoechas Administration: The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] test, which is used to determine cell viability and proliferation, is based on the conversion of tetrazolium salt (water-soluble formazan product) into a coloured structure as a result of mitochondrial activities of cells living at 37°C and its measurement. The amount of formazan produced by dehydrogenase enzymes is directly proportional to the number of viable cells in culture and can be measured at 490 nm.

Reactions were set up for SH-SY5Y cells and each reaction was repeated 3 times. Cells were seeded in 96-well plates at an average of 5x10³ cells/well per 100 µL. After 24 hours, different doses of L. stoechas extracts were applied to the cells.

As L. stoechas extracts; infusion (IE), total methanol (TME), n-hexane (HE), ethyl acetate (EE), methanol (ME) extracts and essential oil (EO) were applied in cell studies. As applied doses, 10-100-1000 µg/mL for essential oil and 1, 10 and 100 ppm doses for other extracts were chosen.

The incubation period for these doses was determined as 2 and 24 hours and was applied. After a pretreatment period of 2 and 24 hours with the extracts, the cells were exposed to 250 µM hydrogen peroxide for 1 hour. At the end of 1 hour, H₂O₂ was withdrawn from the wells and fresh DMEM/FBS was added to their place and left for 1 day of incubation. The next day, the MTS assay was performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA).

20 µL of the solution containing MTS and PMS (phenazine methosulfate) (20:1 v/v) was taken and added to each well containing 100 µL of the medium. After 2-3 hours of incubation at 37°C, a reading was taken at a wavelength of 490 nm in the spectrophotometer (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer, MA, USA). Means were calculated and graphs were drawn based on mean absorption ± standard deviation with the help of the Microsoft Excel program.
**Colony Formation Test:** SH-SY5Y cells were seeded in 6-well plates at an average of 2000-2500 cells/well per 2 mL. After 24 hours of incubation, pre-treatment applications of *L. stoechas* extracts were carried out after making sure that the cells adhered to the surface. This time, 1-10 μg/mL for essential oil, 1 and 10 ppm doses for other extracts were chosen as the doses applied. The incubation period for these doses was determined as 24 hours and was applied. At the end of the 24-hour pretreatment period with the extracts, the cells were exposed to 250 μM H₂O₂ for 1 hour. At the end of 1 hour, H₂O₂ was withdrawn from the wells and 2 mL of fresh DMEM medium was placed in their place and the cells were left for incubation. At the end of the second week, the experiment was terminated by considering the number and density of colonies in the untreated control well. After removing the medium, the wells were washed once with PBS (Phosphate Buffer Saline). The stock solution prepared by adding 0.2 g of crystal violet in 40 mL of methanol was diluted 1/10 with distilled water to obtain a working solution. 1 mL of this solution was added to the wells and waited for 5 minutes. In this way, cell colonies were stained with Crystal Violet. Afterwards, the wells were washed 3 times with distilled water for one minute. The plates were then left to dry with the lids open. Finally, pictures were taken from the plates and the colony numbers in the wells were compared and the results were evaluated.

**Protein analysis:** SH-SY5Y cells were seeded (25 x 10⁴) into 25 cm² T25 flasks and incubated for 24 hours. After 24 hours, cells were pre-treated with 1 and 10 ppm doses of *L. stoechas* extracts for 24 hours. After 24 hours, the medium was aspirated and cells were washed twice with sterile PBS followed by exposure to 250 μM hydrogen peroxide for 1 hour. Following H₂O₂ induction, cells were washed with cold 1X PBS solution and cell pellets were collected into individual Eppendorf tubes. 1X cell lysis buffer (added with phosphatase and protease inhibitors) was added to each sample and cells were lysed by vortexing on ice. Total protein amounts in these cell lysates were measured using the BCA (Bicinchoninic acid) protein determination method. Then, Western blot analyzes were performed using cell lysates.

**Western blotting:** Western blot analysis was performed by loading 30 μg protein samples into wells of 10% tris-glycine denaturing gels and then separating the proteins by electrophoresis and then transferring them to a PVDF (polyvinylidene difluoride) membrane. The membrane was then blocked with 5% non-structured milk powder. The membrane was then treated with anti-Bax rabbit monoclonal antibody (1:1000, Cell Signaling Technology) or anti-Bcl-2 rabbit monoclonal antibody (1:1000, Cell Signaling Technology) as primary antibodies, respectively, at +4°C overnight. After washing, the membrane was incubated for 1 hour at room temperature with HRP (horseradish peroxidase)-conjugated...
anti-rabbit secondary antibodies (1:2000, Cell Signaling Technology) that bind to these primary antibodies. The membrane was covered with ChemiGlow West Chemiluminescence Substrate Kit (Protein Simple, Cat. No:60-12596-00, San Jose, CA, USA) with the reagents mixed at a ratio of 1:1 and left for 1-2 minutes. Finally, the image of the membrane was recorded in tif format at optimal settings. The antibodies on the membrane were stripped by stripping buffer and then incubated with anti-β-actin mouse monoclonal antibody (1:1000, Cell Signaling Technology) for 1 hour at room temperature. Similarly, markings were made with secondary antibodies specific to this antibody and band images were taken. Visualization and densitometric analysis of protein bands were evaluated by scanning the membrane.

**Statistical analysis:** The analyses of the data obtained in the study were made using the SPSS 17.0 program. Obtained data are given as mean ± standard deviation (SD). The data were evaluated with a one-way analysis of variance (ANOVA), the posthoc Tukey HSD test was used to compare the groups, and the p<0.05 value was found to be statistically significant.

**RESULTS**

**Extraction:** From the aerial parts of the plant, 1.44 g water (infusion), 2.60 g n-hexane, 2.64 g ethyl acetate, 5.24 g methanol and 2.01 g total methanol extracts were obtained. As a result of the distillation process, 1.5 mL of essential oil was obtained from 100 g of dried plant material.

**Effect of H₂O₂ on viability in SH-SY5Y cells:** At the end of 24 hours for SH-SY5Y cells seeded in 96-well plates to adhere to the plate surface, a toxicity model was created by exposing the cells to different doses of H₂O₂ for 1 hour. After one hour of H₂O₂ toxicity, fresh DMEM was added to the cells and incubated for another 24 hours. Then MTS test was performed to observe cell viability. According to the results obtained, the cell viability decreased gradually in the groups in which toxicity was created with increasing doses of H₂O₂ compared to the control group. When the viability in the control group is accepted as 100%, the viability rates in the groups treated with H₂O₂ at different doses are respectively; it was measured as 70% at 1µM, 64% at 10µM, 55% at 50µM, 53% at 100µM, 50% at 250µM, 10% at 800µM, and 9% at 1000µM (Fig. 1). According to these results, the toxic concentration, that is, the IC50 dose, which caused a 50% decrease in viability, and the dose that showed a significant decrease compared to the control were 250µM (p<0.0001). In the next tests, 1-hour application was preferred with this dose for the toxic model.
Effect of *L. stoechas* extracts on cell viability against H$_2$O$_2$ toxicity: SH-SY5Y cells seeded in 96-well plates were pretreated with *L. stoechas* extracts for 2 hours at the end of 24 hours. At the end of the second hour, the cells were exposed to 250 μM H$_2$O$_2$ to create a toxicity model. After one hour of H$_2$O$_2$ toxicity, a fresh DMEM medium was added to the cells and incubated for another 24 hours. The next day, an MTS assay was performed to observe cell viability. In the results obtained, it was determined that the application of 250 μM H$_2$O$_2$ to SH-SY5Y cells showing dopaminergic neuronal cell line characteristics reduced cell viability to 8%. On the other hand, the most effective protective doses of the extracts applied as a 2-hour pretreatment were 100 ppm TME with 41%, 1 ppm EE with 53%, 10 ppm HE with 58%, and 1 and 10 ppm ME with 56% and 58% viability rates. Effective protective doses were determined for IE as 1 and 10 ppm with 68% and 69% viability rates and for EO it’s 10 ppm with 44% viability rate. All of these doses were shown to significantly preserve cell viability and exhibit neuroprotective effects when compared to the group that received only 250 μM H$_2$O$_2$ (p<0.0001) (Fig. 2).

A similar procedure was applied to the same cells, this time with a 24-hour pretreatment of *L. stoechas* extracts and then again with a 1-hour H$_2$O$_2$ toxicity model, and close results were obtained. In the results obtained, it was determined that 250 μM H$_2$O$_2$ application to SH-SY5Y cells reduced cell viability to 15%. On the other hand; the most effective protective doses of the extracts applied as a 24-hour pretreatment were 1 and 10 ppm TME with 86% and 71%, 1 and 10 ppm EE with 73% and 36%, 1 and 10 ppm HE with 44% and 45%, 1 and 10 ppm ME with 87% and 77%, 1 and 10 ppm IE with 82% and 85% viability rates. It was observed that the EO could not exert a sufficient protective effect this time (19% viability at 10 µg/mL). When all of these doses were compared to the group that received only 250 μM H$_2$O$_2$, it was determined that they could significantly preserve cell viability and exhibit neuroprotective effects (p<0.0001; p<0.001 for 1 and 10 ppm HE) (Fig. 3).

Effect of *L. stoechas* extracts on colony formation capacity: When the effects of *L. stoechas* extracts on colony formation capacities were examined, it was determined that all doses of the extracts significantly preserved the colony numbers compared to the H$_2$O$_2$ toxicity group (p<0.0001). It was observed that the percentage of colonies in the 250 μM H$_2$O$_2$ group decreased significantly to 3%. It was determined that 1 and 10 ppm doses of all extracts kept the colony numbers at an average of 50% and protected them from H$_2$O$_2$ toxicity (Fig. 4a). When compared to the other extracts, lower colony numbers were observed in the groups treated with EO at doses of 1 and 10 µg/mL, in parallel with the results of the MTS test (Fig. 4b).
Effect of *L. stoechas* extracts on apoptotic proteins: According to the results obtained by cell viability analysis, apoptotic pathways were examined to evaluate the mechanisms underlying the protective effects of *L. stoechas* extracts against H$_2$O$_2$ toxicity. The doses applied for the colony formation test were selected again (1 and 10 ppm). These samples were not included in the Western blot analysis, since effective results were not obtained in the MTS and colony formation tests performed with essential oil. The effects of the extracts on the expression levels of Bax (Bcl-2 Associated X-protein), a pro-apoptotic protein, and Bcl-2 (B-cell lymphoma-2), an anti-apoptotic protein, were evaluated using western blot analysis.

As shown in Fig. 5a, there was a significant increase in Bax protein levels in H$_2$O$_2$-treated cells compared to the control group (p<0.0001), while a concomitant decrease in Bcl-2 protein expression was observed (p<0.0001) (Fig. 5b).

On the other hand, pro-apoptotic Bax protein levels were significantly decreased at 1 and 10 ppm IE, 1 and 10 ppm TME, 1 ppm EE and 1 ppm ME doses versus H$_2$O$_2$ treatment (Fig. 5a). Accordingly, Bcl-2 protein levels were significantly higher for 1 and 10 ppm EE and 1 and 10 ppm ME doses compared to the H$_2$O$_2$ group (p<0.0001), 10 ppm IE and 1 and 10 ppm TME groups. It was found that it tended to increase (Fig. 5b).

In addition to these, the ratio between Bax and Bcl-2 in the extract applied groups was also evaluated. It was shown that H$_2$O$_2$ administration induced apoptosis with an increase in Bax/Bcl-2 ratio (Fig. 5c). On the other hand, it was determined that Bax/Bcl-2 ratio decreased significantly compared to the group treated with infusion, total methanol, ethyl acetate and methanol 1 and 10 ppm H$_2$O$_2$ (Fig. 5c).

DISCUSSION

In the perspective of the present results, this research demonstrated for the first time that various extracts of *L. stoechas* may exhibit neuroprotective effects by preserving cell viability and colony formation capability against H$_2$O$_2$ neurotoxicity in SH-SY5Y cells. Furthermore, it was revealed that they might perform anti-apoptotic effects by lowering Bax levels, which play an active role in cell death (apoptosis) in response to H$_2$O$_2$, and boosting Bcl-2, hence decreasing the Bax/Bcl-2 protein ratio. In addition, as it was noted that methanol and ethanol extracts were employed in the experiments, it was also the first time that the activities of *L. stoechas* extracts produced by various extraction techniques were compared.

It has been demonstrated in several studies that oxidative stress is a significant driver of the cell damage seen in a wide range of human pathologies, including neurodegenerative diseases. Hydrogen peroxide, hydroxyl radical and the superoxide anion are all reactive oxygen species that may rapidly degrade biological materials, resulting in necrotic or apoptotic cell death$^4$. As a result, antioxidants may
be beneficial in avoiding oxidative cell death by removing excess reactive oxygen species or suppressing their production. Significant investigations on natural antioxidant compounds with neuroprotective properties have been done by scientists in recent years.  

*L. stoechas* has been used in European and Iranian traditional medicine for the treatment of various diseases, primarily cardiovascular and neurodegenerative disorders. Some studies are showing neuroprotective effects such as anti-inflammatory, anti-oxidant, anti-Alzheimer's and anti-epileptic properties. Among the many plant species used in complementary medicine today, *L. stoechas* has been used by the people in Anatolia for centuries for the treatment of some diseases. Today, it is seen that it is still used in the treatment of many diseases within the scope of what we can call folk medicine. The flowering branches of the *L. stoechas*, which are popularly called “karabas otu”, and “gargan otu” in Anatolia, are widely used as a tea for cough and bronchitis, common cold, headache, ulcer, stomachache, especially for heart ailments, as well as for diabetes. Leaves and stems within the scope of folk medicine; rheumatism, colds and digestive system diseases, and its extracts are used against wounds, eczema, urinary tract infections and heart diseases. It is known that the water extract in the form of tea obtained from its flowers is widely used in the Aegean region, especially for heart diseases. In this study, we investigated the protective effects of *L. stoechas* extracts on cell viability, colony formation capacity and cell death in a model of H\textsubscript{2}O\textsubscript{2}-induced neurotoxicity in SH-SY5Y cells.

In this investigation, the SH-SY5Y cell line was used to represent neuronal cells. This line is a subline of the SK-N-SH cell line that was cultured in 1970 from a bone marrow biopsy of metastatic neuroblastoma of a 4-year-old girl and has 3 different clonal selections. The SH-SY5Y cell strain has the modest activity of dopamine-β-hydroxylase and insignificant amounts of acetyl-cholinesterase, choline acetyltransferase and butyryl-cholinesterase, as well as baseline noradrenaline release and tyrosine hydroxylase expression when it was first characterized. The rate-limiting enzyme component in the catecholamine production cascade, tyrosine hydroxylase, transforms tyrosine into L-dopa, the progenitor of dopamine, which is then transformed to noradrenaline via dopamine-β-hydroxylase enzyme. Thus, since the SH-SY5Y cell line has the mechanism to synthesize both dopamine and noradrenaline, it may display a catecholaminergic phenotype. This cell line, which generates a model of the human neuronal system, benefits scientists in understanding brain function and the development of various neurological diseases. It is the most used cell line in AD studies. Both differentiated and undifferentiated SH-SY5Y cells can express dopaminergic neuronal markers, muscarinic, nicotinic and adrenergic receptors.

Although human SH-SY5Y neuroblastoma cells are widely used as a model cell system to study neuronal cell death induced by oxidative stress. In this sense, everything has not been sufficiently clarified. When cell studies with *L. stoechas* extracts were examined, no evaluation was found in the
H₂O₂ toxicity model in SH-SY5Y cells. In addition, it was realized that the extracts used in the studies are generally methanol and ethanol extracts. In this context, our study is a unique study in which the effects of different types of extracts such as sequential extracts, total methanol extract, infusion and essential oil in parallel with its use among the public are evaluated together.

In the study, firstly, the IC₅₀ dose of H₂O₂ in SH-SY5Y cells was investigated and it was determined that this dose was 250µM. Then, in MTS cell viability analysis, it was determined that 1 and 10 ppm doses had a significant protective effect against H₂O₂ toxicity, which was formed after 2 and 24 hours of pretreatment with *L. stoechas* extracts. In this context, it was observed that the EO did not show sufficient protective effect compared to the others. The number of cell culture studies conducted with *L. stoechas* extracts in neuronal cells is very few in the literature. Consistent with our results, it was determined that pretreatment of ME at 2.5 and 5 µg/mL doses preserved cell viability against the 6-OHDA-induced apoptosis model in PC12 cells²². In another study with similar scope, it was reported that 100 µg/mL and 1 mg/mL water extracts of *Lavandula angustifolia* species significantly blocked glutamate-induced neurotoxicity in cerebellar granular cell culture¹⁸. As a result, its effects on cell viability against H₂O₂ toxicity in SH-SY5Y cells will be brought to the literature for the first time.

Examination of colony formation capacity is a valuable method for visualizing the effects on cell viability and proliferation. This method is generally used more frequently to evaluate the anticancer efficacy of various drugs. Therefore, there are not many studies performed with colony formation tests in cell culture in H₂O₂ toxicity models. In this study, when the colony formation capacities of SH-SY5Y cells were examined in the H₂O₂ toxicity model, it was shown for the first time that *L. stoechas* extracts showed a significant protective effect at 1 and 10 ppm doses and protected the colony numbers by 50% against H₂O₂ toxicity.

H₂O₂ is widely used as an inducer of oxidative stress in *in vitro* models⁴³. Hydroxyl and peroxy radicals have deleterious effects on proteins and lipids of the cell membrane when exposed to H₂O₂ in cultivated cells, causing instability in energy homeostasis. The current data revealed that H₂O₂ treatment caused a dose-dependent reduction of viability in cells (Fig. 1). Nevertheless, significant decreases in the viability of cells were detected after cells were pretreated using various doses of *L. stoechas* extracts (1 and 10 ppm) (Fig. 2 and 3).

Excessive reactive oxygen species ultimately lead to apoptotic or necrotic cell death. In this context, it was investigated whether *L. stoechas* extracts have a protective effect against neuronal cell apoptosis. Previous studies have shown that Bcl-2 protein family members are involved in both positive and negative regulation of cell apoptosis⁴⁴. Among them, Bcl-2 and Bcl-XL are anti-apoptotic, while Bax, Bel-Xs, Bad (Bcl-2 associated agonist of cell death), Bak (Bcl-2 antagonist killer) and Bik (Bcl-2 interacting killer) are pro-apoptotic. The balance of pro- and anti-apoptotic proteins is critical for the
survival of neurons. It has been shown that increased Bax and/or low Bcl-2 protein expression decreases the mitochondrial membrane potential and increases the production of reactive oxygen species in neurons, which are defined as early events in the apoptosis process\textsuperscript{45}. Consistent with previous studies\textsuperscript{22,46}, the current study showed a notable increase in protein levels, Bax, and a decrease in Bcl-2 after H\textsubscript{2}O\textsubscript{2} treatment (Fig. 5). It was determined that pretreatment with \textit{L. stoechas} extracts inhibited cell death by decreasing the H\textsubscript{2}O\textsubscript{2}-induced Bax/Bcl-2 ratio, decreasing Bax protein levels, and increasing Bcl-2 levels. Taken together, these results confirmed that \textit{L. stoechas} extracts could protect SH-SY5Y cells against H\textsubscript{2}O\textsubscript{2}-induced apoptosis and that modulation of apoptosis-related protein expression could contribute to the anti-apoptotic effect of \textit{L. stoechas} extracts.

In \textit{in vivo} studies, it was reported that \textit{L. stoechas} methanol extract alleviated the dementia process and findings by preventing oxidative damage in cholinergic neurons in mouse brain\textsuperscript{21}. In another similar study, it was stated that \textit{L. stoechas} methanol extract was able to ameliorate scopolamine-induced memory disorders and dementia in mice with antioxidant effects\textsuperscript{20}. In a very recent article from 2021, it is stated that active fractions of \textit{L. stoechas} have anticholinesterase activity in amnesic/dementia mice induced by scopolamine, and also reduce malondialdehyde levels, increase catalase, superoxide dismutase, glutathione levels, and improve behavioural deterioration with anti-oxidant effects and nootropic effects in mice\textsuperscript{47}. In addition, \textit{L. stoechas} essential oil reduces hyperglycemia and protects against oxidative stress in the alloxan-induced diabetic rat model\textsuperscript{48}. It has also been shown that \textit{L. stoechas} essential oil exerts hepatoprotective and nephroprotective effects against malathion-induced oxidative stress in young male mice\textsuperscript{49}.

The fact that the study was carried out only \textit{in vitro} and in this context, it was evaluated on a single cell line can be shown as the limitations of the study. As stated in the discussion section, this limitation has been tried to be overcome by using the most common cell line used in such models in the literature. Another limitation is the inability to use more advanced techniques such as flow cytometry to cell death. However, with the data found, it was tried to shed a light on the researchers in the field. In future studies, it is essential to examine these effects \textit{in vivo} in related animal models in detail and to determine the active substance and from which molecule or molecules the main effect originates.

**CONCLUSION**

As a result, in the light of current data, this study showed for the first time in the literature that different extracts of \textit{L. stoechas} can exert neuroprotective effects by protecting cell viability and colony formation capacity against H\textsubscript{2}O\textsubscript{2} neurotoxicity in SH-SY5Y cells. In addition, it was determined that they were able to exert anti-apoptotic effects by decreasing Bax levels, which plays an active role in cell
death (apoptosis) related to H$_2$O$_2$, and increasing Bcl-2, thereby decreasing the Bax/Bcl-2 protein ratio. In addition, since it was observed that methanol and ethanol extracts were used in the studies, it was also for the first time that activities of the extracts obtained from the _L. stoechas_ with different extraction methods were compared with each other. In this sense, the fact that TME, EE as well as IE extracts have shown very strong neuroprotective effects, and considering that this plant is consumed in the form of tea among the public, the cellular processes underlying its effects have been proven by preliminary data. Considering these potential effects, further basic and clinical studies should be continued by examining the ingredients of the extracts in detail and investigating them in various advanced cell cultures and animal models to determine their mechanism of action and safe doses.

**SIGNIFICANCE STATEMENT**

Considering its traditional use and antioxidant potential, we think that _L. stoechas_ extracts can be evaluated by the pharmaceutical and food industry to be used as medicinal tea, standardized extracts and aromatherapeutic oils. We believe that the plant, which has been enriched in terms of content and whose potential chemical composition has been standardized through breeding studies for its potential use, can be beneficial to the country's economy and the pharmaceutical industry by producing it from an ecological culture point of view.

**REFERENCES**


Figure 1. H$_2$O$_2$ toxicity in SH-SY5Y cells
Figure 2. Protective effect of *L. stoechas* extracts against H$_2$O$_2$ toxicity with 2 hours pretreatment
Figure 3. Protective effect of \textit{L. stoechas} extracts against $\text{H}_2\text{O}_2$ toxicity with 24 hours pretreatment.
A

**SH-SYSY L. stoechas Colony Formation**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Colony Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>120</td>
</tr>
<tr>
<td>DMSO</td>
<td>100</td>
</tr>
<tr>
<td>250 µM H2O2</td>
<td>80</td>
</tr>
<tr>
<td>Total Methanol 1 ppm</td>
<td>60</td>
</tr>
<tr>
<td>Ethylacetate 1 ppm</td>
<td>40</td>
</tr>
<tr>
<td>n-hexane 1 ppm</td>
<td>30</td>
</tr>
<tr>
<td>Methanol 10 ppm</td>
<td>30</td>
</tr>
<tr>
<td>Infusion 1 ppm</td>
<td>20</td>
</tr>
<tr>
<td>Essential oil 1 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>Essential oil 10 µg/ml</td>
<td>5</td>
</tr>
</tbody>
</table>

1 hour H2O2

B

**SH-SYSY L. stoechas Colony Formation**

- Control
- DMSO
- 250 µM H2O2
- Total Methanol 1 ppm
- Total Methanol 10 ppm
- Ethylacetate 1 ppm
- Ethylacetate 10 ppm
- n-hexane 1 ppm
- Essential oil 1 µg/ml
- Essential oil 10 µg/ml
Figure 4a-b. Effect of L. stoechas extracts on colony formation capacity

Footnote: A. The graph of the colony numbers of each experimental group with percentage calculations.
B. Representative pictures showing the staining status of colonies belonging to each experimental group.
Figure 5a-c. Effect of *L. stoechas* extracts on apoptotic proteins
Footnote: A. Bar graph of Bax protein levels for each experimental group. B. Bar graph of Bcl-2 protein levels for each experimental group. C. Bar graph of Bax/Bcl-2 ratio for each experimental group * p<0.0001 different from Control, ** p<0.0001 different from 250 µM H₂O₂