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Research Article

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

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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. *Lavandula 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 H₂O₂ 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 H₂O₂ damage in SH-SY5Y cells and their effects on apoptotic processes were evaluated based on cell viability against H₂O₂ 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 hrs) had a significant protective effect against 250 µM H₂O₂ 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 H₂O₂ 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.

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

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Data Availability: All relevant data are within the paper and its supporting information files.

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's¹⁻³. 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^{4,5}. One of the primary reactive oxygen species, hydrogen peroxide (H_2O_2), is formed all through the oxidation/redox reaction and is regarded as a transmitter in intracellular signalling pathways⁶. Furthermore, it is widely understood that H_2O_2 may induce DNA damage and lipid peroxidation, leading to apoptosis in a variety of cell subtypes^{7,8}. 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 effects⁹. 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 damage¹⁰. 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_2O_2 promotes apoptotic cell death in rat pheochromocytoma PC12 cells (a model for neurons that contain catecholamines)¹¹ and human neuroblastoma SH-SY5Y cells¹², according to previous experiments. However, the molecular mechanisms involved in oxidative stress-induced apoptotic neuronal cell death are complex and not yet fully elucidated^{13,14}.

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^{15,16}. This genus is native to West Africa, Europe, 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^{17,18}.

Lavandula 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 roadsides¹⁹.

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 infections¹⁸. 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 disease²⁰⁻²².

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 literature²⁰⁻²². In 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, H_2O_2 treatment of SH-SY5Y cells triggers cell apoptosis¹². The SH-SY5Y cells can be differentiated into neuron-like cells that acquire the characteristic morphological, neurochemical and electrophysiological features of neurons using retinoic acid²³⁻²⁵.

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 experiments²⁸.

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 H₂O₂ toxicity model. In this study, it was investigated whether *L. stoechas* extracts have neuroprotective effects against H₂O₂-induced apoptosis.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Physiology, Faculty of Medicine, Ege University and Departments of Pharmaceutical Botany and Biochemistry, Faculty of Pharmacy, Ege University, 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 hrs 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 min. 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 methods²⁹. 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 for 2 hrs (each 1.5L×3 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 hrs (300 mL×3 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.

The 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 U mL⁻¹).

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 passage²⁹.

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 5 × 10³ cells/well per 100 µL. After 24 hrs, 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⁻¹ 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 hrs and was applied. After a pretreatment period of 2 and 24 hrs with the extracts, the cells were exposed to 250 μ M hydrogen peroxide for 1 hr. At the end of 1 hr, 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).

About 20 μ L of the solution containing MTS and PMS (phenazine methosulfate) (20:1 v/v) was taken and added to each well containing 100 μ L of the medium. After 2-3 hrs 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 $\pm$ standard deviation with the help of the Microsoft Excel program²⁹.

Colony formation test: The SH-SY5Y cells were seeded in 6-well plates at an average of 2000-2500 cells/well per 2 mL. After 24 hrs 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 μ g mL⁻¹ 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 hrs and was applied. At the end of the 24 hrs pretreatment period with the extracts, the cells were exposed to 250 μ M H₂O₂ for 1 hr. At the end of 1 hr, 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 2nd 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. About 1 mL of this solution was added to the wells and waited for 5 min. 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 evaluated³⁰.

Protein analysis: The SH-SY5Y cells were seeded (25 $\times$ 10⁴) into 25 cm² T25 flasks and incubated for 24 hrs. After 24 hrs,

cells were pre-treated with 1 and 10 ppm doses of *L. stoechas* extracts for 24 hrs. After 24 hrs, the medium was aspirated and cells were washed twice with sterile PBS followed by exposure to 250 μ M hydrogen peroxide for 1 hr. Following H₂O₂ induction, cells were washed with cold 1X PBS solution and cell pellets were collected into individual Eppendorf tubes. The 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 method³⁰. Then, Western blot analyzes were performed using cell lysates²⁹.

Western blotting: Western blot analysis was performed by loading 30 μ g 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 hr 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 min. 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 hr 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 $\pm$ 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 hrs 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 hr. After one hour of H₂O₂ toxicity, fresh DMEM was added to the cells and incubated for another 24 hrs. 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 1, 64% at 10, 55% at 50, 53% at 100, 50% at 250, 10% at 800 and 9% at 1000 μ M (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 was 250 μ M ($p < 0.0001$). In the next tests, 1 hr application was preferred with this dose for the toxic model.

Effect of *L. stoechas* extracts on cell viability against H₂O₂

toxicity: The SH-SY5Y cells seeded in 96-well plates were pretreated with *L. stoechas* extracts for 2 hrs at the end of 24 hrs. At the end of the 2nd hrs, the cells were exposed to 250 μ M H₂O₂ to create a toxicity model. After 1 hr of H₂O₂ toxicity, a fresh DMEM medium was added to the cells and incubated for another 24 hrs. The next day, an MTS assay was performed to observe cell viability. In the results obtained, it was determined that the application of 250 μ M H₂O₂ 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 hrs 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 μ M H₂O₂ ($p < 0.0001$) (Fig. 2).

A similar procedure was applied to the same cells, this time with a 24 hrs pretreatment of *L. stoechas* extracts and then again with a 1 hr H₂O₂ toxicity model and close results were obtained. In the results obtained, it was determined that 250 μ M H₂O₂ 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 hrs pretreatment were

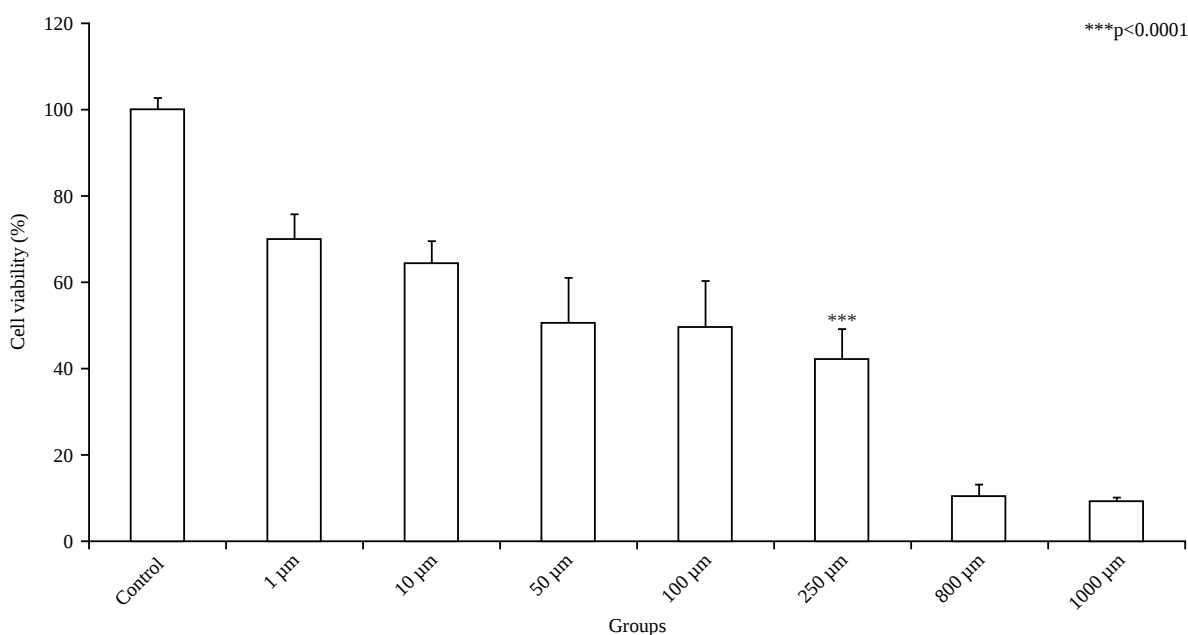


Fig. 1: H₂O₂ toxicity in SH-SY5Y cells

X-axis: Groups

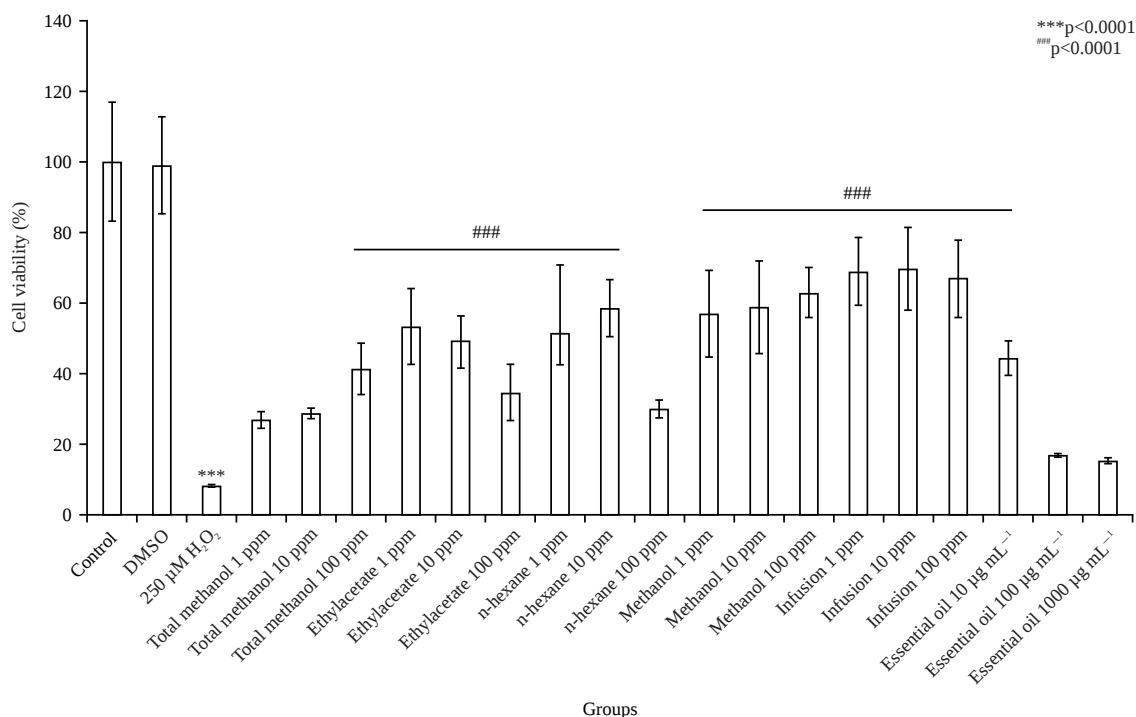


Fig. 2: Protective effect of *L. stoechas* extracts against H₂O₂ toxicity with 2 hrs of pretreatment

X-axis: Groups

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⁻¹). When all of these doses were compared to the group that received only 250 µM H₂O₂, 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 H₂O₂ toxicity group ($p < 0.0001$). It was observed that the percentage of colonies in the 250 µM H₂O₂ 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 H₂O₂ 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⁻¹, 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 H₂O₂ 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.

Figure 5a shows, there was a significant increase in Bax protein levels in H₂O₂-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 H₂O₂ 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 H₂O₂ group ($p < 0.0001$), 10 ppm IE and 1 and 10 ppm TME groups. It was found that it tended to increase (Fig. 5b).

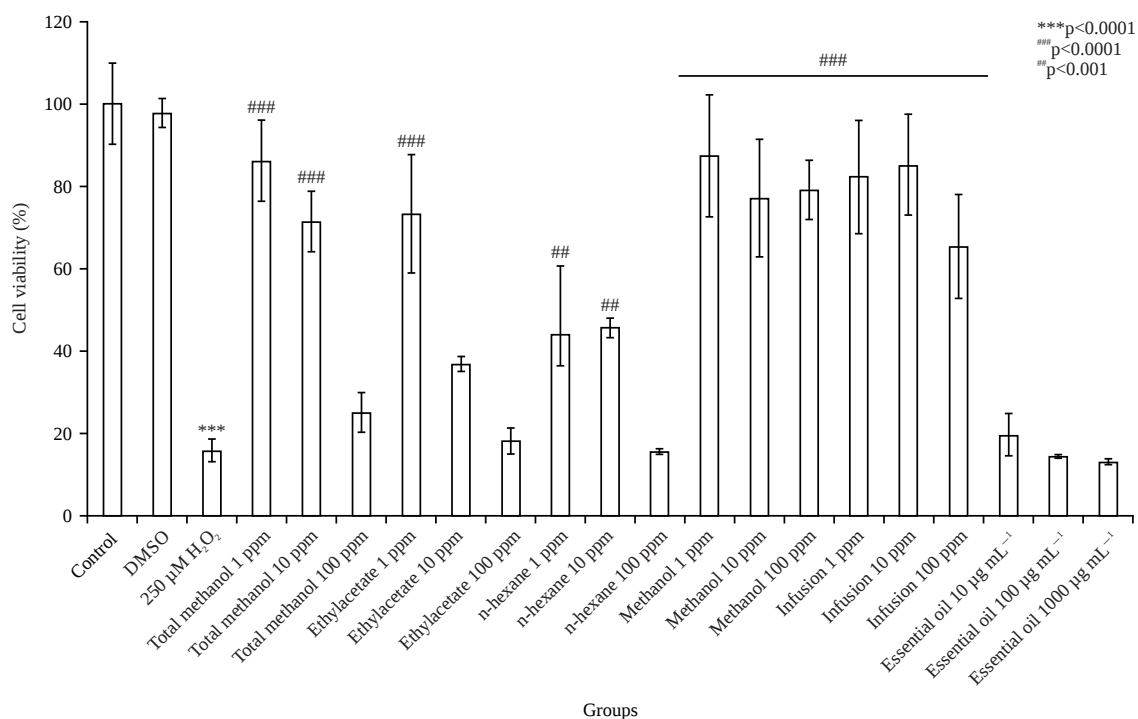


Fig. 3: Protective effect of *L. stoechas* extracts against H₂O₂ toxicity with 24 hrs pretreatment

X-axis: Groups

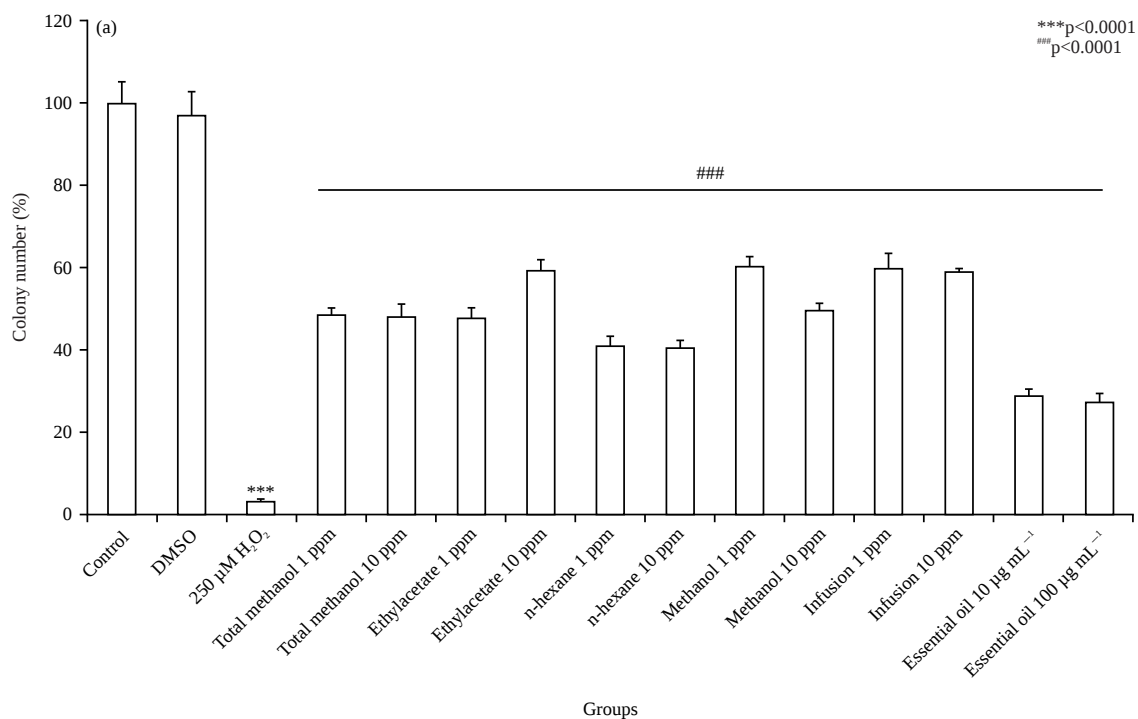


Fig. 4(a-b): Continue

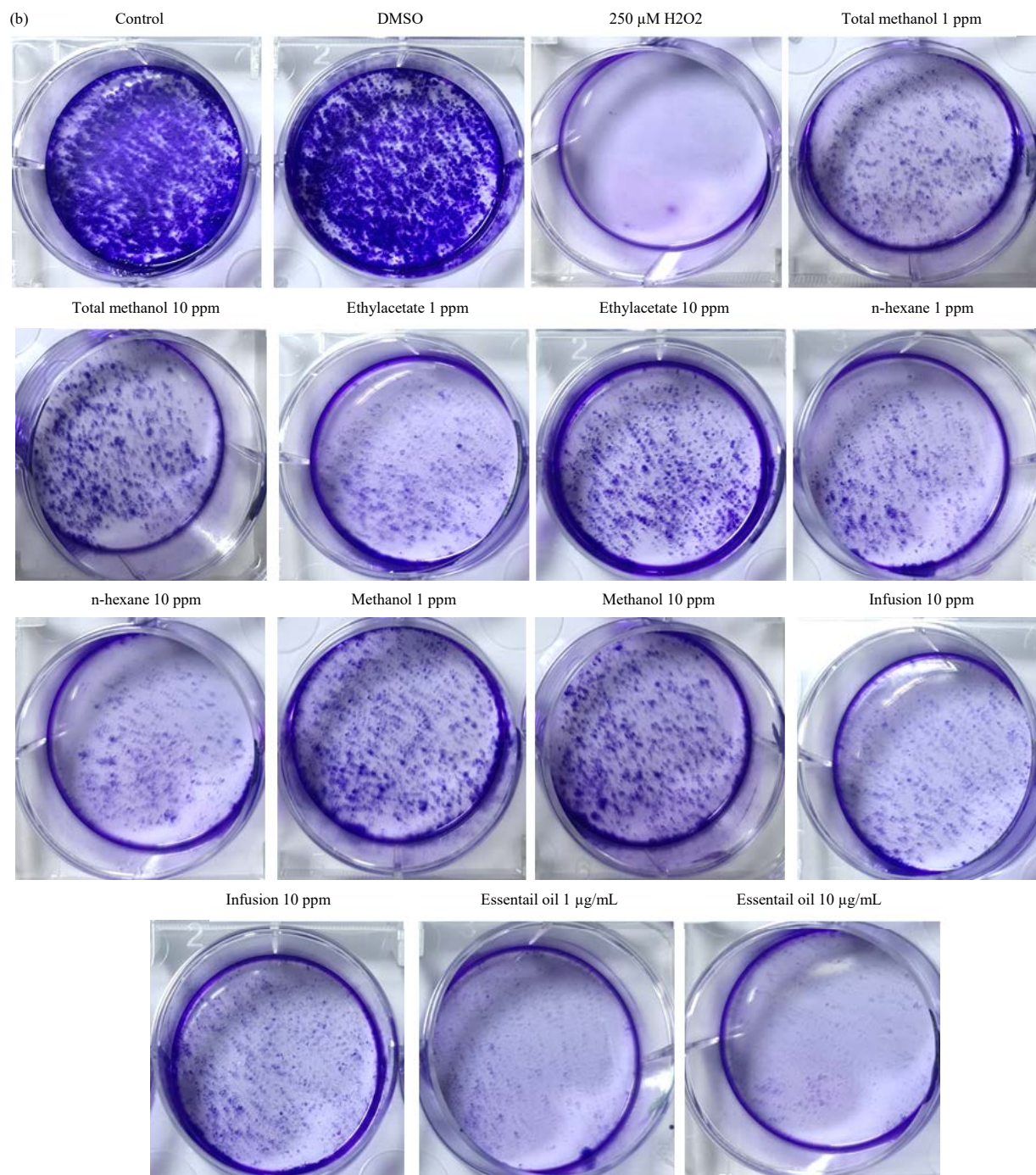


Fig. 4(a-b): Effect of *L. stoechas* extracts on colony formation capacity, (a) Graph of the colony numbers of each experimental group with percentage calculations and (b) Representative pictures showing the staining status of colonies belonging to each experimental group

X-axis: Groups

In addition to these, the ratio between Bax and Bcl-2 in the extract applied groups was also evaluated. It was shown that H₂O₂ 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 H₂O₂ (Fig. 5c).

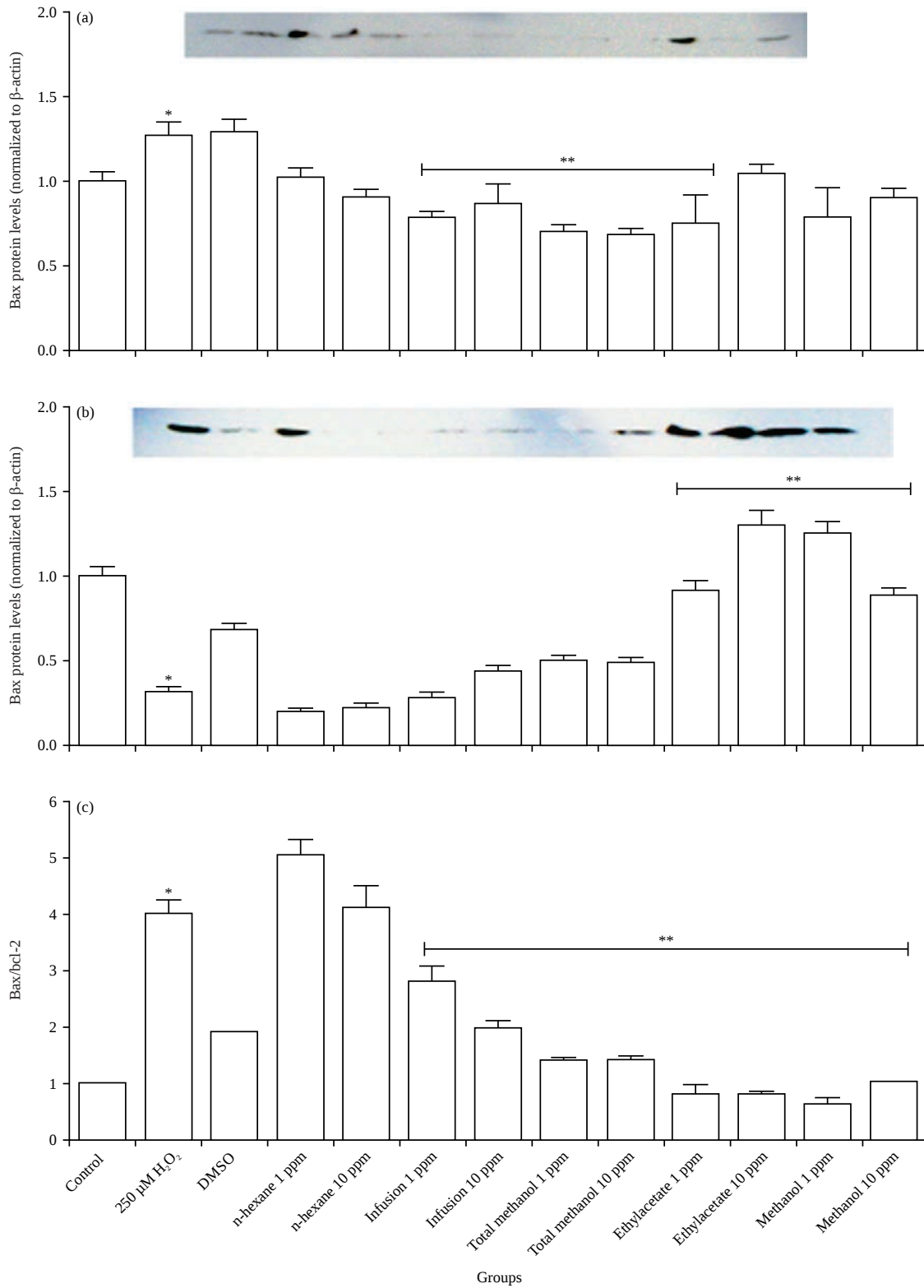


Fig. 5(a-c): Effect of *L. stoechas* extracts on apoptotic proteins, (a) Bar graph of Bax protein levels for each experimental group, (b) Bar graph of Bcl-2 protein levels for each experimental group and (c) Bar graph of Bax/Bcl-2 ratio for each experimental group

* $p < 0.0001$ different from Control, ** $p < 0.0001$ different from 250 μ M H_2O_2 and X-axis: Groups

DISCUSSION

In the perspective of the present results, this study demonstrated for the first time that various extracts of *L. stoechas* may exhibit neuroprotective effects by preserving cell viability and colony formation capability against H₂O₂ 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 H₂O₂ 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 death⁴. 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 years³¹.

L. stoechas has been used in European and Iranian traditional medicine for the treatment of various diseases, primarily cardiovascular and neurodegenerative disorders^{32,33}. Some studies are showing neuroprotective effects such as anti-inflammatory, anti-oxidant, anti-Alzheimer's and anti-epileptic properties^{18,34,35}. 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 diabetes³⁶. 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^{37,38}. 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 H₂O₂-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 years 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 phenotype³⁹. 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 receptors⁴⁰.

Although human SH-SY5Y neuroblastoma cells are widely used as a model cell system to study neuronal cell death induced by oxidative stress^{12,41,42}. 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, this 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 250 μ M. 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 hrs 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 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$ and 1 mg mL^{-1} 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 H_2O_2 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 H_2O_2 toxicity models. In this study, when the colony formation capacities of SH-SY5Y cells were examined in the H_2O_2 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 H_2O_2 toxicity.

The H_2O_2 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 H_2O_2 in cultivated cells, causing instability in energy homeostasis. The current data revealed that H_2O_2 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, 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, Bcl-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 process⁴⁵. Consistent with previous studies^{22,46}, the current study showed a notable

increase in protein levels, Bax and a decrease in Bcl-2 after H_2O_2 treatment (Fig. 5). It was determined that pretreatment with *L. stoechas* extracts inhibited cell death by decreasing the H_2O_2 -induced Bax/Bcl-2 ratio, decreasing Bax protein levels and increasing Bcl-2 levels. Taken together, these results confirmed that *L. stoechas* extracts could protect SH-SY5Y cells against H_2O_2 -induced apoptosis and that modulation of apoptosis-related protein expression could contribute to the anti-apoptotic effect of *L. stoechas* extracts.

In *in vivo* studies, it was reported that *L. stoechas* methanol extract alleviated the dementia process and findings by preventing oxidative damage in cholinergic neurons in mouse brain²¹. In another similar study, it was stated that *L. stoechas* methanol extract was able to ameliorate scopolamine-induced memory disorders and dementia in mice with antioxidant effects²⁰. In a very recent article from 2021, it is stated that active fractions of *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 mice⁴⁷. In addition, *L. stoechas* essential oil reduces hyperglycemia and protects against oxidative stress in the alloxan-induced diabetic rat model⁴⁸. It has also been shown that *L. stoechas* essential oil exerts hepatoprotective and nephroprotective effects against malathion-induced oxidative stress in young male mice⁴⁹.

The fact that the study was carried out only *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 for 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 *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 *L. stoechas* can exert neuroprotective effects by protecting cell viability and colony formation capacity against H_2O_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_2O_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 a 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.

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