The Influence of *Cichorium Endivia* and *Urtica Dioica* Extracts Against H2O2-Induced Stress in SH-SY5Y Cells[*]

Hatice Feyzan AY[**], Serap YEŞİLKIR-BAYDAR[***], Rabia ÇAKIR[****]

Abstract

Aim: In neurodegenerative diseases, increased production of hydrogen peroxide (H_2O_2) causes oxidative stress in neurons, and when the damage is severe, it is irreversible and apoptosis is induced. In recent years, phytochemicals that exhibit neuroprotective and antioxidant properties to protect neurons from oxidative stress have gained importance. Therefore, herbal extracts with high antioxidant capacity and anti-apoptotic properties may provide a protective strategy against H_2O_2-induction. This study aims to examine the influence of *Cichorium endivia* liquid extract (CEE) and *Urtica dioica* liquid extract (UDE) against H_2O_2-induction on SH-SY5Y cells *in vitro*.

Method: For this purpose, cells were treated with the extracts and then exposed to H_2O_2. The neuroprotective effect and cell viability of these extracts were evaluated by XTT method. Antioxidant activity was determined by the CUPRAC method. Finally, the apoptotic cell death and reactive oxygen species (ROS) levels were examined with DAPI and DCFH-DA, respectively.

Results: The results obtained showed that CEE and UDE have an influence on neuroprotection, anti-apoptotic, and antioxidant effects by reducing H_2O_2-induced toxicity, apoptotic cell death, and ROS levels.

Conclusion: H_2O_2 causes neurotoxicity in neurons through oxidative damage. To prevent this, traditional plant extracts with high antioxidant activity can provide a neuroprotective strategy. As a consequence, it was shown in an *in vitro* model that these herbal extracts could be a neuroprotective effect.

Keywords: *Cichorium endivia*, *Urtica dioica*, neuroprotective effect, neurotoxicity, oxidative damage, hydrogen peroxide (H_2O_2).

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[**] Corresponding Author, PhD Student, Yildiz Technical University, Faculty of Chemistry and Metallurgical, Department of Bioengineering, Istanbul, Türkiye. E-mail: h.feyzanay@gmail.com ORCID https://orcid.org/0000-0001-7874-8885
[***] Asst. Prof., Istanbul Gelisim University, Faculty of Engineering and Architecture, Department of Biomedical Engineering, Istanbul, Türkiye ; Istanbul Gelisim University, Life Sciences and Biomedical Engineering Application and Research Centre, Istanbul, Türkiye. E-mail: syesilkir@gelisim.edu.tr ORCID https://orcid.org/0000-0001-6311-4302
[****] Prof., Yildiz Technical University, Faculty of Chemical and Metallurgical Engineering, Department of Bioengineering, Istanbul, Türkiye ; Health Institutes of Türkiye (TUSEB), Biotechnology Institute, Istanbul, Türkiye.
E-mail: rabiacakir@gmail.com ORCID https://orcid.org/0000-0002-8545-9878.

Cichorium Endivia ve _Urtica Dioica_ Ekstraktlarının SH-SY5Y Hücrelerinde H_2O_2 Kaynaklı Strese Karşı Etkisi

Öz

Amaç: Nörodejeneratif hastalıklarda artan hidrojen peroksit (H_2O_2) üretimi nöronlarda oksidatif strese neden olur ve hasar şiddetli olduğunda geri dönüşümsüz olur ve apoptoz tetiklenir. Son yıllarda nöronları oksidatif stresten korumak için nöroprotektif ve antioksidan özellikler sergileyen fitokimyasallar önem kazanmıştır. Bu nedenle yüksek antioksidan kapasitesine ve anti-apoptotik özelliklere sahip bitkisel ekstraktlar H_2O_2 indüksiyonuna karşı koruyucu bir strateji sağlayabilmektedir. Bu çalışma, *Cichorium endivia* sıvı ekstraktının (CEE) ve *Urtica dioica* sıvı ekstraktının (UDE) SH-SY5Y hücreleri üzerindeki H_2O_2 indüksiyonuna karşı *in vitro* etkisini incelemeyi amaçlamaktadır.

Yöntem: Bu amaçla hücreler ekstraktlarla muamele edildikten sonra H_2O_2'ye maruz bırakıldı. Bu ekstraktların nöroprotektif etkisi ve hücre canlılığı XTT yöntemi ile değerlendirildi. Antioksidan aktivite CUPRAC yöntemiyle belirlendi. Son olarak ise apoptotik hücre ölümü ve reaktif oksijen türleri (ROS) düzeyi sırasıyla DAPI ve DCFH-DA ile incelendi.

Bulgular: Elde edilen sonuçlar, CEE ve UDE'nin H_2O_2 kaynaklı toksisiteyi, apoptotik hücre ölümünü ve ROS düzeylerini azaltarak nöroproteksiyon, anti-apoptotik ve antioksidan etkiler üzerinde etkiye sahip olduğunu gösterdi.

Sonuç: H_2O_2 oksidatif hasar yoluyla nöronlarda nörotoksisiteye neden olmaktadır. Bunu önlemek amacıyla yüksek antioksidan aktiviteye sahip geleneksel bitki ekstraktları nöroprotektif bir strateji sağlayabilmektedir. Sonuç olarak *in vitro* modelde bu bitkisel ekstraktların nöroprotektif etki sergileyebileceği gösterilmiştir.

Anahtar Sözcükler: *Cichorium endivia*, *Urtica dioica*, nöroprotektif etki, nörotoksisite, oksidatif hasar, hidrojen peroksit (H_2O_2).

Introduction

Neurodegenerative diseases such as Alzheimer's Disease (AD), Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis are progressive disorders that are characterized by neurons loss in specific regions of the brain[1]. The loss of neuronal activity causes neurodegeneration and neurological symptoms such as impairments of cognitive, movement, and behaviours as seen in AD[2,3]. Aging mainly causes neurodegeneration and today with the development of medical technology, the world is currently facing an aging population. Due to the fact that people can benefit more from health services, worldwide cognitive decline and dementia have become a growing load on patients and national healthcare systems[4,5]. World Health Organization declared that approximately 55 million people have dementia, worldwide. Ongoing studies indicate

that there is an increment increase in the AD ratio in nearly every country, unfortunately, the affected population is expected to rise to 78 million in 2030, and 139 million in 2050[6].

Although the main factor associated with AD is not known with certainty, many responsible factors are known and oxidative stress is thought to play an essential role[7]. The increased reactive oxygen species (ROS) accumulation, which eventually results in oxidative stress, leads to the depletion of the body's natural antioxidant system by overstimulating the natural antioxidant defence system[8]. The development and application of antioxidants are critical for the prevention and treatment of several diseases. Plants have been also a source of drugs in medicine in addition to food, cosmetics, and dyes. The therapeutic potential of medicinal plants as molecular resources has been proven long ago[9]. They have numerous biological actions that are advantageous to human health. The natural substances with the antioxidant ability to scavenge ROS can prevent and treat neurodegeneration[10]. In such cases, herbal polyphenolic compounds that suppress the increase in ROS levels are subjected. These herbal phenolic compounds show intracellular antioxidant effects and they can be used as neuroprotective agents, thereby reducing the risk of neurodegenerative diseases[11,12].

Also, hydrogen peroxide (H_2O_2), a member of the ROS group, is produced as a result of amyloid beta, and plays an essential role in the pathogenesis of AD. Therefore, in the treatment and prevention of H_2O_2-induced damage, the presence of antioxidant compounds is critical. We have previously reported that Silybum marianum extract gives a neuroprotective effect through its high antioxidant capacity[13]. Alternative neuroprotective herbal agents for AD are mentioned according to their anti-apoptotic, anti-inflammatory, and antioxidant effects such as chicory leaf (*Cichorium endivia*), nettle (*Urtica dioica*), and their active ingredients[14,15]. Various extracts of the chicory leaf plant have been found to contain phenolic acids and flavonoids such as cichoric acid, sesquiterpene lactones, coumaric acid, caffeic acid, chlorogenic acid, caffeoyl acid, kaempferol, quercetin, and coumarins[16,17]. In the studies in nettle extracts, it was determined that 2-O-caffeoyl malic acid, chlorogenic acid, and rutin are mostly found. It has also been shown to contain carvacrol, quercetin, kaempferol, campesterol, β-carotene, lutein, myricetin, carotenoids, and gallic, ferulic, salicylic, malic, and caffeic acids[18,19]. Especially since carvacrol has a neuroprotective effect, it suggests that nettle extracts will also provide neuroprotective properties[20].

This study aims to investigate the effects of protectivity, antioxidant, anti-apoptotic and suppressing of ROS formation of *C. endivia* liquid extract (CEE) and *U. dioica* liquid

extract (UDE) against induced oxidative stress and neurotoxicity in the SH-SY5Y cell line treated with H_2O_2.

Material and Methods

Material

SH-SY5Y cell line were obtained from American Type Culture Collection (ATCC). Foetal bovine serum (FBS) and Trypsin-EDTA were purchased from Gibco®. Dulbecco's Modified Eagle's Medium/F-12 (DMEM-F12) and penicillin-streptomycin antibiotic solution were purchased from PAN Biotech. Phosphate buffered saline (PBS), phenazine methosulfate (PMS), hydrogen peroxide (H_2O_2), and 2',7'-dichlorofluorescin diacetate (DCFH-DA) were purchased from Merck (USA), while 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) salt and 4′,6-diamidino-2-phenylindole (DAPI) from Santa Cruz Biotechnology® (Texas, USA). *C. endivia* liquid extract (CEE), and *U. dioica* liquid extract (UDE) were obtained from Immunat Herbal Pharmaceuticals (Mugla, Türkiye).

Cell Culture

SH-SY5Y cell line is a human neuroblastoma cell that is frequently used as an *in vitro* model of neurological diseases[21]. The SH-SY5Y cells were cultured in DMEM/F12 supplemented with 10% FBS and 0.5% penicillin/streptomycin and incubated in CO_2 incubator at 37°C.

In Vitro Cytotoxicity of the Extracts

The extracts were evaluated for toxic effects on the SH-SY5Y cell line using the XTT assay. SH-SY5Y cells were seeded into 96 well plates at a 1×10^4 cells/well and incubated for 24 hours. Then, the cells were treated with CEE and UDE at the 5 concentrations between 1.25 and 20 µg/mL (1.25, 2.5, 5, 10, and 20 µg/mL) for 24 hours. XTT was carried out by multiplate reader to evaluate cell viability.

Neuroprotective Effect of the Extracts on H_2O_2-Induced Cell Damage

The neuroprotective effect of extract concentrations that were shown to have no toxic effect, against H_2O_2-induced cell damage was investigated. SH-SY5Y cells were seeded into 96-well plates and incubated for 24 hours. After the cells were treated with extracts at concentrations of 1.25, 2.5, 5, 10, 15, and 20 µg/mL for CEE and 1.25, 2.5, 5, and 10 µg/mL for UDE for 24 and 48 hours, were exposed to 1 mM H_2O_2 for 30 minutes and cell viability was determined by XTT assay[22].

Measurement of Antioxidant Activity

The antioxidant activities of the CEE and UDE were examined by using the Cupric Reducing Antioxidant Capacity (CUPRAC) method. Briefly, 1 mL of each copper (II) chloride ($CuCl_2$) solution, ammonium acetate (NH_4Ac) solution, and neocuproin (Nc) solution were mixed. Trolox compound, which is known to have antioxidant properties, as a standard, and the same concentrations of CEE and UDE were added to this mixing. The solutions were incubated for 30 minutes in the dark and at room temperature and were measured in a microplate reader at a wavelength of 450 nm. The calibration curve of trolox was obtained from the concentration-absorbance plot of trolox. The locations of the absorbance values of the extracts corresponding to the trolox calibration curve were determined.

Determination of Apoptotic Cell Death

The effect of the extracts on H_2O_2-induced apoptotic cell death was determined using the DAPI staining method. SH-SY5Y cells were seeded in 48 well plates and incubated for 24 hours in CO_2 incubator. After being treated with extracts (at concentrations determined in prior tests) for 24 hours, the cells were exposed to 1 mM H_2O_2 for 30 minutes. Then the medium was replaced with DAPI solution, and incubated in CO_2 incubator at 15 minutes in the dark. Cells were examined with a fluorescence microscope (Leica, DM3000). Cells in bright blue colour represent the apoptotic cells.

Determination of Intracellular Reactive Oxygen Species (ROS)

The DCFH-DA assay was used to investigate the effect of extracts and H_2O_2 on ROS production. SH-SY5Y cells were seeded in 48 well plates and incubated for 24 hours in a CO_2 incubator. The cells were exposed to 1 mM H_2O_2 for 30 minutes after having been treated with extracts for 24 hours. Then the medium was replaced with DCFH-DA and incubated in CO_2 incubator for 30 minutes. SH-SY5Y cells were viewed by fluorescence microscopy in the dark.

Statistical Analysis

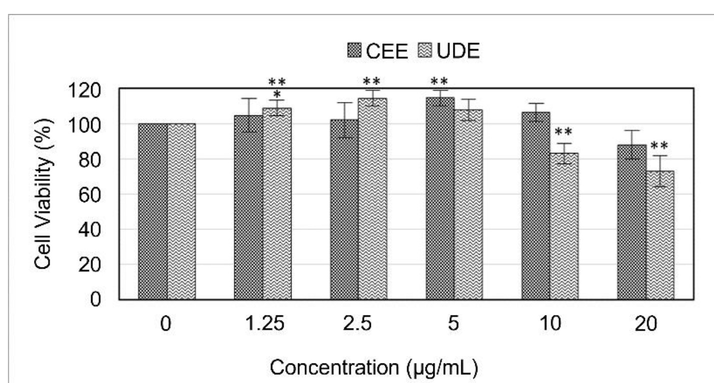
GraphPad software was used to perform statistical analysis on the data. In comparing the means of the sample and control groups, values were considered statistically significant at $p<0.05$. The data were then displayed as the mean±standard deviation.

Results

Determination of the Cytotoxic Effect of CEE and UDE.

To investigate the neuroproliferative role of CEE and UDE on SH-SY5Y cell line, we used several extract concentrations of up to 20 µg/mL were used. According to 24 hours of incubation results, it was seen that CEE showed a neuroproliferative effect at concentrations of 1.25 to 10 µg/mL and started to decrease cell viability at 20 µg/mL by approximately 10%. Also, 1.25 to 5 µg/mL concentrations of UDE were non-toxic while 10 and 20 µg/mL had reduced the viability for 15 to 25% (Figure 1).

Figure 1. Effect of different concentrations of CEE and UDE on the cell viability.

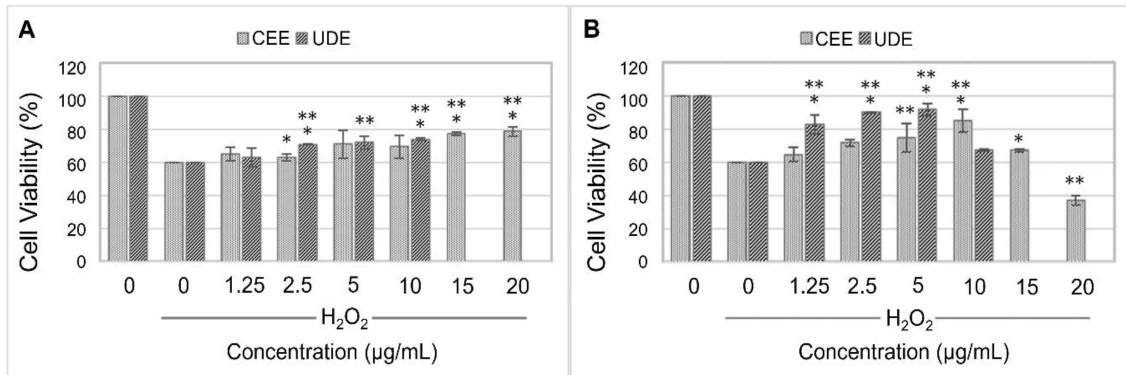


The results were indicated as *$p<0.001$, **$p<0.01$, and ***$p<0.05$ compared to the control.

Determination of the Neuroprotective Effect of CEE and UDE on H_2O_2-Induced Cell Damage

The effects of herbal extracts on cell viability against H_2O_2-induced oxidative damage in SH-SY5Y cells were evaluated. The results showed that CEE and UDE could reduce H_2O_2-induced toxicity (Figure 2). With 24-hour treatment of extracts (Figure 2A), 5 and 10 µg/mL CEE preserved cell viability up to approximately 70%, while 15 µg/mL protected up to 77% and 20 µg/mL up to 79%. Besides, 10 µg/mL UDE preserved up to 74%. According to the cell viability results at 48 hours (Figure 2B), 5 and 10 µg/mL CEE protected up to 75% and 85% against H_2O_2, respectively, while 1.25, 2.5, and 5 µg/mL UDE have preserved up to 82%, 89%, and 91%, respectively. However, 10 µg/mL UDE and 15 µg/mL CEE could not provide sufficient protection due to the high concentration and 20 µg/mL CEE increased the toxic effect. In subsequent experiments, 10, 15, and 20 µg/mL for CEE; 1.25, 2.5, and 5 µg/mL concentrations were used for UDE.

Figure 2. Neuroprotective effect of CEE and UDE against H_2O_2-damage. SH-SY5Y cells were treated with different concentrations of CEE and UDE for 24 hours (A) and 48 hours (B), then exposed to H_2O_2 for 30 minutes and cell viability was evaluated.



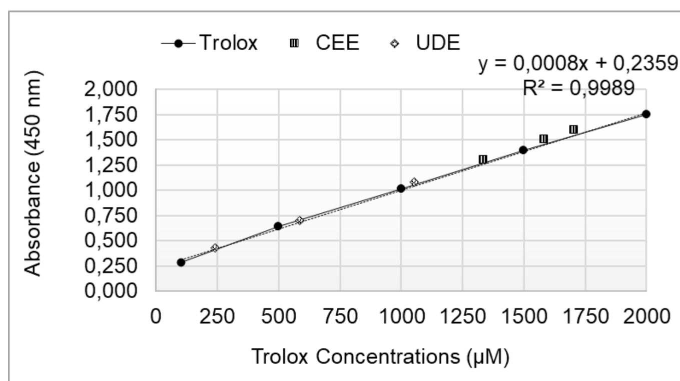
The results were indicated as *p<0.001, **p<0.01, and ***p<0.05 compared with the H_2O_2 group.

Determination of the Antioxidant Capacity

The calibration curve of trolox was obtained from the concentration-absorbance plot, and the line equation was $y=0.0008x + 0.2359$ and the R^2 value was found to be 0.9989. The absorbance values of the determined concentrations of the herbal extracts were substituted for the “y” value in the line equation, and the “x” values found were shown on the calibration curve. It was observed that the antioxidant activity also increased as the concentrations of herbal extracts increased (Figure 3).

It was observed that the antioxidant activity of 20 µg/mL concentration of CEE corresponded to the antioxidant efficiency of 1703 µM trolox compound, and it was concluded that 20 µg/mL CEE had the highest antioxidant activity. Following this, antioxidant activities were observed at 15 µg/mL and 10 µg/mL concentrations of CEE and then at 5 µg/mL, 2.5 µg/mL, and 1.25 µg/mL UDE, respectively.

Figure 3. Trolox calibration curve and antioxidant activity of CEE and UDE.

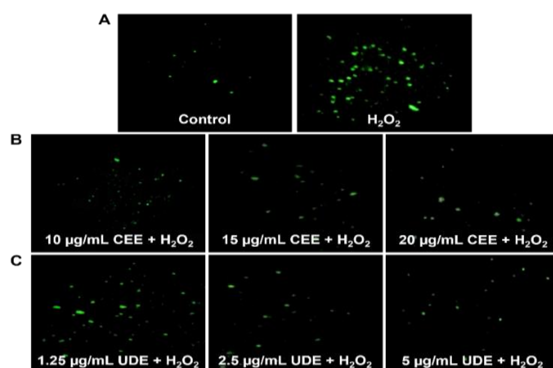


The antioxidant activity of determined concentrations of CEE and UDE was evaluated using Trolox as a standard.

Observation of the Effect of CEE and UDE on Intracellular ROS level

H2O2 was initially used to induce ROS formation in order to evaluate the inhibiting effect of herbal extracts on ROS formation (Figure 4). It was qualitatively established that cells exposed to 1 mM H2O2 produced higher levels of ROS compared to cells that were not exposed (Figure 4A). Then, after the cells were treated with determined concentrations of CEE and UDE, they were exposed to H_2O_2 and it was observed that as the concentrations were increased, the H_2O_2-induced ROS formation was suppressed (Figure 4B and C). It was observed that ROS levels decreased due to increasing CEE concentrations (Figure 4B), and UDE concentrations other than 1.25 μg/mL were effective on ROS (Figure 4C). Also, 20 μg/mL CEE inhibited ROS more than the other concentrations and UDE. Accordingly, when these were evaluated for their ability to scavenge ROS, it was established that CEE performed better than UDE.

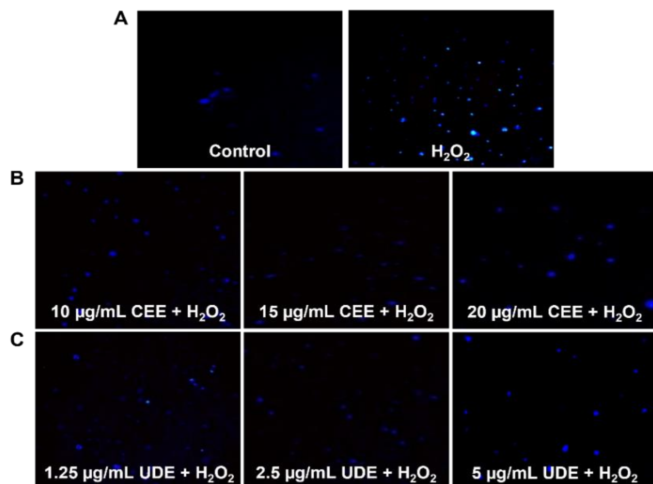
Figure 4. Effect of CEE and UDE on H_2O_2-oxidative stress. Effect of H_2O_2 compared to control (A) and concentration-dependent effect of CEE (B) and UDE (C) on the intracellular ROS level.



Observation of the Effect of CEE and UDE on Apoptosis

DAPI, a fluorescent dye, is used to visualize changes in the cell nucleus. DAPI staining shows cells with nuclear condensation and DNA fragmentation, which are considered apoptotic[23,24]. As DAPI dye is light sensitive and fluorescence rapidly fades under light, DAPI and DAPI-exposed cells must be kept in the dark. In order to evaluate the anti-apoptotic effect of herbal extracts, it was first determined that H_2O_2 caused apoptosis, and H_2O_2-exposed cells led to more apoptotic cell death than control cells (Figure 5A). Then, cells were treated with determined concentrations of CEE and UDE and following exposed to H_2O_2. In the images obtained, both CEE and UDE were found to have an anti-apoptotic effect. It was observed that as the concentrations of the extracts increased, the H_2O_2-induced apoptosis was more suppressed, and apoptotic cells were reduced (Figure 5B and C). The results showed that CEE was more effective than UDE and exhibited a better anti-apoptotic effect.

Figure 5. Effect of CEE and UDE on apoptotic cell. Effect of H_2O_2 compared to control (A) and concentration-dependent effect of CEE (B) and UDE (C) on the H_2O_2-induced apoptosis.



Discussion

Neurons have an antioxidant defence system to protect themselves from the damage of ROS formed during normal metabolic reactions, but an uncontrolled increase of ROS overstimulates this system, leading to the depletion of the body's antioxidant reserves[8,25]. This destroys cellular redox homeostasis, finally which leads to serious oxidative stress and damage to cell macromolecules[10,26,27]. This causes several neurodegenerative diseases. The cell may experience an increase in stress at different timelines and under different conditions, but it may not be predicted when this will occur or how much it will impact the cell. Therefore, taking preventative action in advance is quite beneficial.

Natural compounds offer neuroprotective potential because of their ability to regulate oxidative stress, which causes neurodegeneration. These compounds include flavonoids, alkaloids, polyphenols, coumarins, and quinones that can modulate the generation of reactive species and mitochondrial function. These natural compounds exhibit neuroprotective effects due to antioxidant, anti-apoptosis, and anti-inflammatory properties and can provide a therapeutic strategy against AD by decreasing ROS generation[10,28,29].

AD is known to be characterized by the formation of senile plaques caused by accumulating amyloid beta (Aβ) peptides excessively, although the pathogenesis of AD

has not been completely explained until now[30]. It has been reported that the Aβ accumulation and the senile plaques lead to oxidative stress and consequent neurotoxicity, by inducing ROS production, and the oxidative stress can promote more Aβ formation[31,32]. Furthermore, it has been observed that the Aβ peptide can directly increase intracellular H_2O_2 levels by causing the formation of H_2O_2[33]. The obtained results showed that H_2O_2 both induces ROS formation and causes apoptosis and neurotoxicity in SH-SY5Y cells.

In our study, it is important to find out the non-toxic and neuroprotective concentrations of the extracts. For the first time in this study, it is shown that *Cichorium endivia* and *Urtica dioica* herbal extracts can be also effective against neurotoxicity caused by H_2O_2-induced oxidative stress.

Chicory is a plant that with antioxidant, anti-inflammatory, antibacterial, and many pharmacological and therapeutic effects[34]. The Cichorium genus has many species and usually, C. intybus was the researched one. As far as we know, the neuroprotective effect of *C. endivia* against H_2O_2 has not been previously reported, but several studies have shown that it possesses antioxidant activity and protective effects against different agents. It has been reported that *C. endivia* has the ability to free radical scavenging and protection against oxidative damage[35]. It has been reported that CEE has a hepatoprotective effect against oxidative stress-induced liver disease. In this study, CEE has been reported that displayed a neuroprotective effect by suppressing H_2O_2-induced oxidative damage and neurotoxicity. CEE, used in this study, has been proven to have a high antioxidant capacity as in the literature, and thus it has been found that inhibits the formation of ROS and has ROS scavenging effect. As a result of these effects, the CEE has been demonstrated to have neuroprotective and anti-apoptotic properties.

U. dioica extract has been demonstrated to have anti-inflammatory, antioxidant, and antiaging properties[36]. However, although their neuroprotective effects have been reported in the AD model created with different agents in many studies, their neuroprotective effect against H_2O_2-induced cell damage and oxidative stress has not been reported. Toldy et al. demonstrated that nettle supplementation can attenuate the loss in brain function and have a significant antioxidant effect by suppressing ROS generated by NMDA-induced brain lesions[37]. Ghasemi et al. investigated the effect of UDE on scopolamine-induced oxidative damage. Scopolamine led to an increase in malondialdehyde, a marker for oxidative stress, and *U. dioica* was found to suppress the induced MDA level. It has also been reported that scopolamine increases AChE activity

in the hippocampus and cortical tissues while *U. dioica* suppresses it[19]. Our results demonstrate that CEE and UDE have exhibited a neuroprotective effect by suppressing H_2O_2-induced oxidative damage and neurotoxicity. However, it was determined that the antioxidant capacity of the UDE we used in the study was lower than both the CEE and the literature. Nevertheless, it has been determined that UDE has a ROS scavenging effect by suppressing the formation of ROS and exhibits an anti-apoptotic effect by inhibiting apoptosis. These features also indicate that it has a neuroprotective effect. Identically, CEE, also, showed a neuroprotective effect by inhibiting both apoptosis and ROS. Although these effects of UDE are not as potent as CEE, it is identified to have a neuroprotective effect.

Each day, researchers pay more attention to disease-treating medicinal plant-derived natural compounds, and these natural products are becoming increasingly prominent. This study aims to highlight the possible antioxidant effects of UDE and CEE as promising alternatives for developing novel neuroprotective approaches. Considering these results, it is expected that more remarkable developments in neuroprotective natural compounds, which provide novel opportunities for the treatment and prevention of neurological diseases.

Conclusion

This study aimed to examine the anti-apoptotic and neuroprotective effects of CEE and UDE, as their efficacy against H2O2-induced apoptosis and ROS production in SH-SY5Y cells has not been previously studied. Oxidative stress, one of the pathogeneses of Alzheimer's Disease (AD), can be induced by H2O2 treatment. Therefore, we aimed to create an in vitro model of AD through H2O2 induction in SH-SY5Y cells. Cell viability studies showed that the extracts exhibited both neuroproliferative and neuroprotective effects against H2O2 toxicity. Moreover, both extracts were found to protect against apoptosis and ROS production by reducing the number of apoptotic cells and ROS levels. Further studies and in vivo investigations are required to better understand the neuroprotective impact of these two extracts in AD.

Conflicts of Interest (COI)

The authors declare no conflict of interests financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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