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RESEARCH ARTICLE

Does Umbelliferone Protect Primary Cortical Neuron Cells Against Glutamate Excitotoxicity? [1]

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Abstract

Glutamate is the major excitatory neurotransmitter in the central nervous system. Excessive glutamate is known to cause excitotoxicity. Umbelliferone is a coumarin derivative compound and has antioxidant, anti-inflammatory, and neuroprotective effects. Also, umbelliferone can show neuroprotective effect by crossing the blood-brain barrier. In our study, it was aimed to investigate the neuroprotective effect of umbelliferone on primary cortical neuron (PCN) culture. Umbelliferone was isolated from the roots of *Ferulago cassia* dichloromethane sub-extract. The cerebral cortex of newborn Sprague Dawley rats was used to obtain PCNs. To stimulate glutamate excitotoxicity, cells were exposed to $6x10^{-5}$M glutamate. Then different concentrations (10-1000 µM) of umbelliferone were added into the medium and allowed to incubate for 24 and 72 h. MTT assay was used to measure cell viability. Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) analyzes were used to evaluate reactive oxygen species. MTT results showed that cell viability was decreased with glutamate application. 25-250 µM umbelliferone had a significant protective effect against glutamate excitotoxicity at 72 h ($P<0.05$). Consistent with MTT results, TAS analysis results showed 50-250 µM umbelliferone increase the level of antioxidants in cells, which can help protect neurons against glutamate-induced excitotoxicity. In this study, umbelliferone showed a neuroprotective effect in PCN against glutamate excitotoxicity. These results suggest that umbelliferone may be used as therapeutic agent against glutamate excitotoxicity.

Keywords: *Glutamate, Ferulago, Primary cortical neuron culture, Umbelliferone*

Umbelliferon, Glutamat Eksitotoksisitesine Karşı Primer Kortikal Nöron Hücrelerini Korur mu?

Öz

Glutamat merkezi sinir sisteminin ana uyarıcı nörotransmitteridir. Aşırı glutamatın, glutamat reseptörlerinin uzun süreli aktivasyonu nedeniyle eksitotoksisiteye neden olduğu bilinmektedir. Umbelliferon, antioksidan, antiinflamatuar ve nöroprotektif etkilere sahip kumarin türevi bir bileşiktir. Ayrıca, umbelliferon kan beyin bariyerini geçerek nöronal hücreleri ölümden koruyabilmektedir. Bu çalışmada glutamat eksitotoksisitesine maruz bırakılan primer kortikal nöron (PCN) kültürlerinde umbelliferonun nöroprotektif etkisinin araştırılması amaçlanmaktadır. Umbelliferon, *Ferulago cassia* diklorometan alt ekstraktının köklerinden izole edildi. PCN'ler yenidoğan Sprague Dawley cinsi sıçanların serebral korteklerinden elde edilmiştir. Glutamat eksitotoksistesi oluşturmak için hücreler $6x10^{-5}$ M glutamata maruz bırakıldı. Daha sonra hücrelere farklı konsantrasyonlarda (10-1000 µM) umbelliferon uygulanarak 24 ve 72 saat inkübasyona bırakıldı. Hücre canlılık oranı MTT yöntemi ile belirlendi. Hücrelerde oluşan reaktif oksijen türleri Total oxidant status (TOS) ve Total antioxidant status (TAS) yöntemleri ile değerlendirildi. MTT analiz sonuçlarına göre, glutamat uygulaması ile hücre canlılığının azaldığı görüldü. 72. saatte 25-250 µM umbelliferonun glutamat eksitotoksisitesine karşı nöronlarda anlamlı düzeyde koruyucu etkiye sahip olduğu tespit edildi ($P<0.05$). MTT sonuçlarıyla tutarlı olarak TAS analizi sonuçları, 50-250 umbelliferonun hücrelerdeki antioksidan düzeyini artırdığını ve bu da nöronların glutamata bağlı eksitotoksisiteye karşı korunmasına yardımcı olabileceğini gösterdi. Bu çalışmada umbelliferon, PCN hücrelerinde glutamat eksitotoksisitesine karşı nöroprotektif bir etki göstermiştir. Bu sonuçlar ile umbelliferonun glutamat eksitotoksisitesine karşı terapötik bir ajan olarak kullanılabileceği sonucuna varıldı.

Anahtar sözcükler: *Glutamat, Ferulago, Primer kortikal nöron kültürü, Umbelliferon*

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Introduction

Glutamate, is a major excitatory neurotransmitter in the central nervous system. It is important in various physiological processes such as learning, memory, synaptic plasticity, and other cognitive functions [1,2]. Most excitatory neurons in the central nervous system are glutamatergic, more than 50% use glutamate as a neurotransmitter [3,4]. Glutamate shows its effect on the postsynaptic cell surface by stimulating its ionotropic and metabotropic receptors. Thus, it is resulted with action potential by depolarizing the cell membrane [5]. Glutamate is present in millimolar concentrations in the mammalian central nervous system [6]. Glutamate can not cross the blood-brain barrier, as it is all the glutamate present in the central nervous system (CNS) are produced here. Glutamate is synthesized from glutamine by glutaminase in presynaptic neurons or α-ketoglutarate by glutamate dehydrogenase enzyme. The synthesized glutamate is taken into vesicles by the vesicular transporter. After then, they are lead to an increase in glutamate concentration by excreting their contents into the synapse cavity by exocytosis in response to presynaptic depolarization [7,8]. Glutamate concentration in the synaptic cleft is increased by synaptic activity, but extracellular glutamate concentration is protected by glutamate uptake by glutamate transporter [9,10]. Hence, unused glutamate during synaptic transmission must be cleared from the extracellular space, rapidly. Glutamate clearance is achieved by astrocytes and is mediated by glutamate uptake transporters [11]. Although glutamate plays an important role in brain functions, its high concentration in CNS causes the neurotoxic effect [12]. The excessive increase of glutamate leads to prolonged activation of glutamate receptors and leads to excito-toxicity due to intracellular overload of Ca^{2+}. This state plays an important role in neurodegeneration, protease activation, mitochondrial dysfunction. Moreover, the reactive oxygen species (ROS) are increased with excessive intracellular Ca^{2+} concentration and, neuronal cell death occurs [13]. Glutamate-induced toxicity plays an important role in the pathogenesis of various neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's Disease (AD), Huntington's Disease, Parkinson's Disease [14,15]. One of the important factors that cause neuronal cell death in neuropathological processes is oxidative stress [12,16]. AD arises from the accumulation of α and β plaques and tau protein hyperphosphorylation in neurons plays role in pathogenesis. The most important reason is thought of as the development of oxidative stress due to the generation of ROS [17]. The excessive glutamate concentration causes oxidative stress by inhibiting glutathione synthesis and leading to increased ROS production [12,16]. Therefore, therapeutic approach for neurodegenerative diseases may be protection of neuronal cells against glutamate-induced excitotoxicity [12]. Treatment with plants is a traditional process for the improvement of modern medicine from ancient times [18,19]. The use of herbal medicines is increasing worldwide, and although they have some negative effects, consumers believe that traditional herbal medicines are safe [20]. Since it is thought that plant origin compounds are effective in the treatment of many diseases and in reducing the prognosis, effective compounds obtained from plants by various methods have gained importance today. However, these compounds can be used as active substances in the preparation of drugs, and preclinical studies should be done initially.

Umbelliferone, a member of coumarin derivatives, is found in fruits, vegetables, and herbs such as citrus fruits and golden apples. It is a compound with antioxidant and free radical scavenging properties [21]. Umbelliferone is widely consumed by humans as a medicine and dietary supplement [22]. It has been shown that it can be used safely and effectively in the diet and is not toxic at low doses [23]. It has also been shown to have a neuroprotective effect in the study conducted with the experimental Parkinson's Disease model [24]. Also, an important property of umbelliferone is that it can cross the blood-brain barrier [25]. In recent years, because coumarins have an anti-neurodegenerative disease potential, the search for medicinal plants that can be used in the isolation of coumarins has become the focus of researchers as they are natural sources of coumarins [26]. Therefore, it can be considered that umbelliferone has a protective potential in neurodegenerative diseases. The various studies showed that umbelliferone, is a plant-derived coumarin derivative, has anticancer, antitumoral, anti-inflammatory, neuroprotective, and antioxidant effects [27-29]. However, the neuroprotective effect of umbelliferone against glutamate excitotoxicity is unknown. Therefore, in our study, we aimed to investigate the neuroprotective effect of umbelliferone isolated from *Ferulago cassia Boiss* (Apiaceae) dichloromethane sub-extract in PCN cultures exposed to glutamate excitotoxicity.

Material and Methods

Ethical Statement

This study was approved by Atatürk University Local Animal Experiments Ethics Committee with the work permit dated 28.03.2019 and numbered (93722986-000-E.1900094667) and was carried out in the cell culture laboratory of the Medical Pharmacology Department in the Atatürk University Faculty of Medicine.

Reagents

In this study, Neurobasal medium (NBM), fetal bovine serum (FBS) (Gibco, USA), B-27, Penicillin/Streptomycin-Amphotericin B (Thermo Fisher, Germany), dimethylsulfoxide (DMSO) (Roche) (Santa Cruz), sterile culture dishes (Petri), 96-well cell culture plate (Greiner), MTT kits (Cayman

Chemical, Ann Arbor, MI, USA) ve Hanks'Balanced Salt solution (HBSS), Trypsin-Ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich®, ABD) were used. Umbelliferone was isolated from the roots of *Ferulago cassia* dichloromethane sub-extract.

Plant Material, Extraction, and Purification of Umbelliferone

Plant material of *Ferulago cassia* extraction and purification of umbelliferone were obtained using the method described via Karakaya et al.[30] 200 g dried powdered root was used and 12.32 g dichloromethane sub-extract was obtained. Eluting with Hexane: Ethyl acetate over silica gel column gave known compound umbelliferone (215 mg).

Preparation of Primary Cortical Neuron Culture

In this study, the cortex neurons, which were obtained from a newborn Sprague-Dawley rat that did not complete 24 h, were used. Twenty rat cubs were quickly decapitated and, their brain cortexes were removed. The extracted brain cortexes were transferred to 5 mL of Hanks' Balanced Salt Solution (HBSS) and macro-fragments were performed in a petri dish with the help of a double scalpel. The crushed cortices were taken into DMEM solution. Then, ¼ ratio trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin-0.02% EDTA) was added and micro-fragmented. The cells were centrifuged 3 times at 1200 rpm for 5 min and the upper medium was changed each time. Culture medium containing 88% NBM, 10% FBS, 2% B-27 and 0.1% antibiotics (penicillin-streptomycin-amphotericin B) was added on the precipitated pure neuronal cells. And then, cells were added to 96-well polylysine coated plates at a concentration of 1×10^5 per well. The cells were incubated for 10 days (37°C and 5% CO_2) by changing the medium for 3 days intervals for branching.

Exposure of Glutamate Excitotoxicity and Treatment with Various Concentration of Umbelliferone

$6x10^{-5}$ M concentration of glutamate was applied to the culture medium to induce glutamate excitotoxicity. Then, various concentrations of umbelliferone (10-1000 µM) were applied to the medium and allowed to incubate for 24 and 72 h. After the incubation period, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, cell viability (cytotoxicity status) was evaluated by yellow tetrazole (MTT) assay.

MTT Analysis

The MTT analysis method is used for determination of cytotoxic and proliferative effects of substances, which is one of the enzymatic test methods commonly used in the determination of cytotoxicity. The proliferation-inducing effect on cell viability of umbelliferone on PCN cells with glutamate toxicity was determined by using MTT assay (Sigma, USA) concerning manufacturers' protocols (Cayman Chemical, Ann Arbor, MI, USA). Briefly, PCN cells were treated with various concentrations of umbelliferone as mentioned earlier and incubated for 24 and 72 h (37°C and 5% CO_2). The stock MTT solution prepared in sterile PBS was added to 96 well-plates at a concentration of 10%. After incubation for 4 h (37°C and 5% CO_2), it was provided that the formazan crystals were dissolved by adding 100 mL DMSO. Formazan crystal formation was evaluated by spectrophotometric method at 570 nm (reference wavelength 630 nm) using a microplate reader.

Measurements of Total Oxidant Status (TOS)

Total oxidant status of umbelliferone was determined with a method based on color change developed by Erel [31]. For this purpose, 10-1000 µM concentration range umbelliferone with PCN cells for 24 and 72 h were incubated and cell culture mediums were removed, and supernatants were analyzed to determine the TOS. Each group was repeated three times. If there are oxidants in the sample, it oxidizes the ferrous ion-o-dianisidine complex to the ferric ion. This reaction is increased with abundant glycerol molecules. In an acidic medium, the ferric ion formed a colored complex with xylenol orange. The intensity of the color formed, related to the total oxidant molecules amount in the sample, was measured by the spectrophotometric method. The method was calibrated with H_2O_2 and the obtained results were given as µmol H_2O_2 equiv./L. The precision of the method was lower than 2%.

Measurements of Total Antioxidant Status (TAS)

Total antioxidant status of umbelliferone was determined with a method based on color change developed by Erel [32]. For this purpose, 10-1000 µM concentration range umbelliferone with PCN cells for 24 and 72 h were incubated and cell culture mediums were removed, and supernatants were analyzed to determine the TAS. Each group was repeated three times. The novel automated method is based on the production of OH^- radicals by Fenton reaction and its reaction with the colorless substrate O-dianisidine to produce dianisyl radical, which has a bright yellowish-brown color. When adding a sample of cell culture medium, the oxidative reactions initiated by the OH^- radicals present in the reaction are suppressed by the antioxidants in samples, preventing the color change and thereby producing an effective way to determine the TAS levels. The obtained results were given as mmol Trolox Eq/L.

Statistical Analysis

All results were performed using SPSS 20 software for statistical analysis. The results were calculated as mean±standard error. Results were analyzed using the One-way ANOVA with Duncan's Posthoc test. P values <0.05 were taken in consideration to indicate statistical significance.

Results

MTT Analysis Results

The proliferative effect of umbelliferone on PCN was determined using MTT analysis. Various concentrations of umbelliferone (final concentration in each well; 10-1000 µM) were used to determine its proliferative effects on PCN culture with glutamate toxicity, with results shown in *Fig. 1* and *Fig. 2*. At the end of 24 and 72 h of incubations, it was determined that following the application of $6x10^{-5}$ M glutamate, cell viability decreased to 62.87% and 42.88%, respectively. It was shown that cell viability increase in PCN with glutamate toxicity following administration of low concentrations of umbelliferone. An increase in cell viability was observed in PCN with glutamate toxicity following administration of low concentrations of umbelliferone. Especially after 72 h of incubation, it was found that 25-250 µM concentration of umbelliferone have a statistically significant protective effect on cell viability compared to glutamate control ($P<0.05$) *(Fig. 1-A,B)*.

TAS and TOS Analysis Results

Following the application of umbelliferone at various concentrations (10-1000 µM) for 24 and 72 h in PCN culture against glutamate excitotoxicity, the cell culture medium was taken and total antioxidant and oxidant capacities were measured with a commercial kit. TAS results showed that cells treated with 50-250 µM umbelliferone statistically significantly increased the antioxidant capacity compared to glutamate control ($P<0.05$). Low antioxidant capacity was found in cells treated with a high dose (500-1000 µM) umbelliferone. It was also found that TAS results were in line with MTT results and that 50-250 µM umbelliferone had the highest effect on both cell viability and antioxidant capacity *(Fig. 2-A,B)*.

Total oxidant status results showed the level of oxidant and free radical in the cell culture medium. The obtained results showed that the cells treated with 500-1000 µM umbelliferone had high oxidant capacity, which in turn induced toxicity and increased cell death by intracellular stress factor. Consistent with the TAS results, a statistically significant decrease in TOS levels was detected in the cells

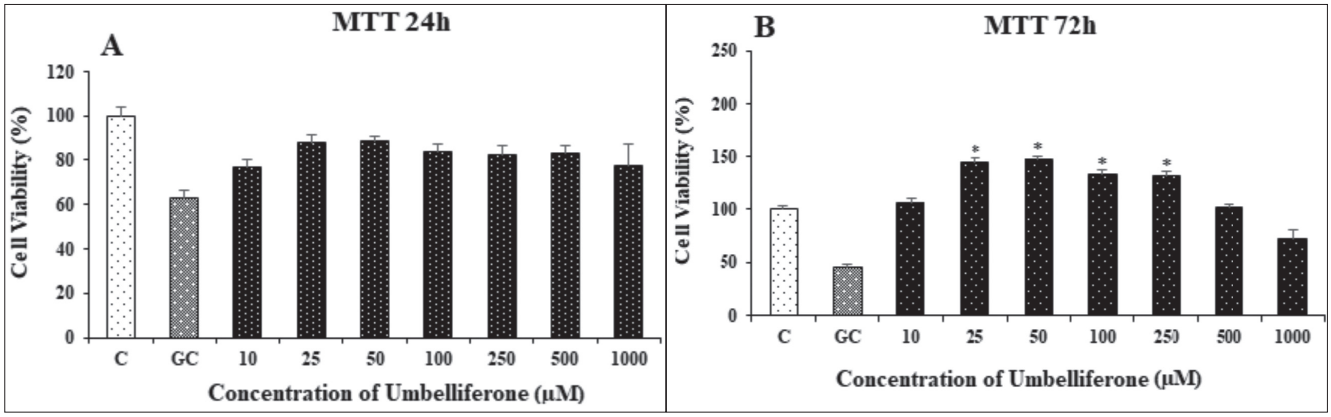

Fig 1. The proliferative effects of umbelliferone on the viability of primary cortical neuron cells with glutamate toxicity. **A:** in 24 h incubation, **B:** in 72 h incubation [results are given as mean ± SE, n = 8, * statistically significant in comparison with glutamate control, $P<0.05$, C: Control (untreated cells), GC: Glutamate control (Cells treated with $6x10^{-5}$ M glutamate)]

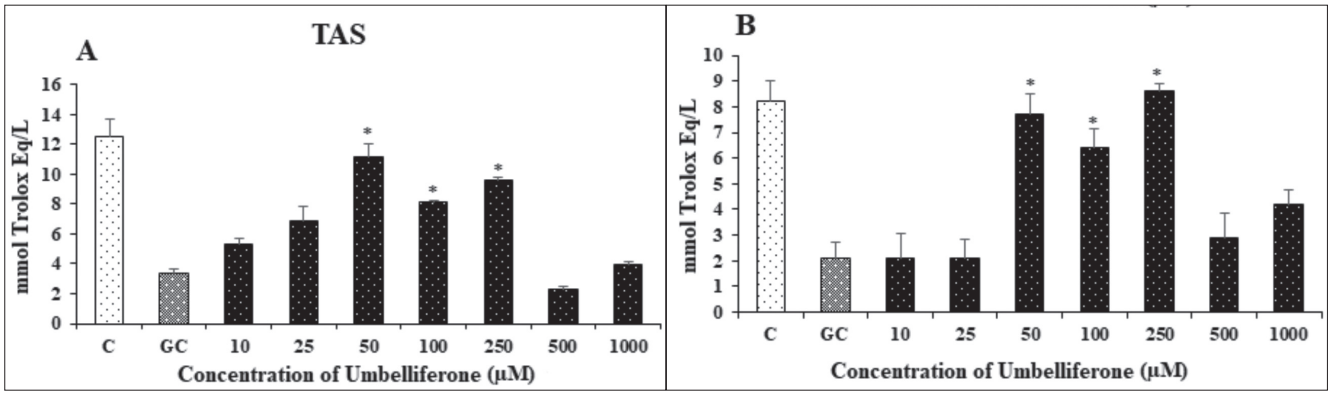

Fig 2. The determined TAS analysis for various concentrations of umbelliferone in primer cortical neuron culture against glutamate excitotoxicity. **A:** in 24 h incubation, **B:** in 72 h incubation [results are given as mean ± SE, * $P<0.05$ statistically significant compared to glutamate control; C: Control (nontreated cells), GC: Glutamate control ($6x10^{-5}$ M glutamate exposed cell)]

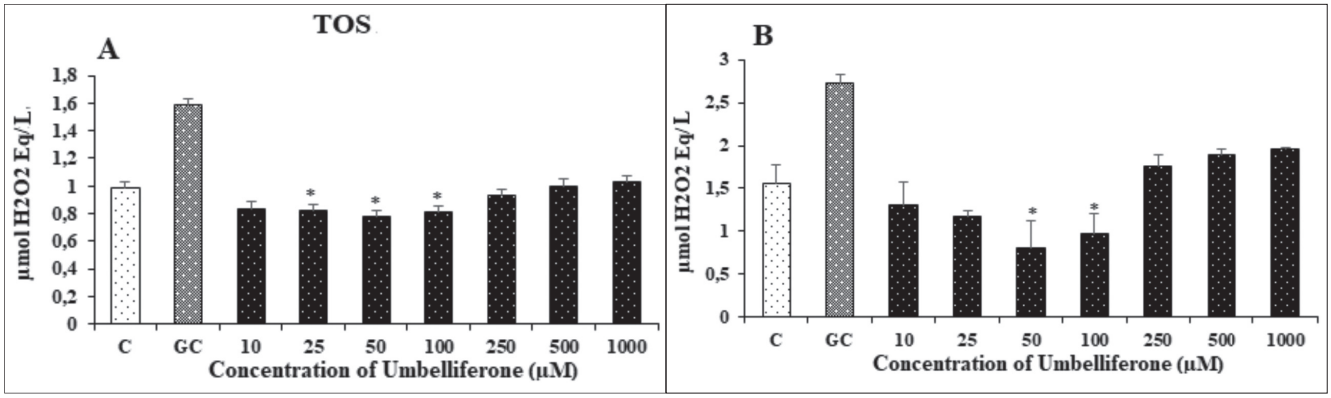

Fig 3. The determined TOS analysis for various concentrations of umbelliferone in primer cortical neuron culture against glutamate excitotoxicity. **A:** in 24 h incubation, **B:** in 72 h incubation [results are given as mean ± SE, * P<0.05 statistically significant compared to glutamate control; C: control (nontreated cells), GC: Glutamate control (6x10^{-5} M glutamate exposed cell)]

treated with 50-100 µM umbelliferone compared to the glutamate control (P<0.05). However, it was found that the oxidant level (stress factor level) was the lowest following the application of 50-100 µM umbelliferone, inconsistent with both our MTT and TAS results *(Fig. 3-A,B)*.

Discussion

The central nervous system has excitatory and inhibitory neurotransmitters that cause excitation and inhibition. One of the excitatory neurotransmitters is glutamate that can pass through the blood-brain barrier at presynaptic neuron terminals and is synthesized from glutamine [3,33]. Excessive glutamate, because of changes in glutamate metabolism trigger various pathological events, is known to cause excitotoxicity [34,35]. Glutamate excitotoxicity also accelerates neuronal cell death by causing oxidative stress [36,37]. Also, glutamate excitotoxicity has a role in the pathogenesis of various neurodegenerative diseases such as Alzheimer's and Parkinson's [38,39].

Umbelliferone is a 7-hydroxycoumarin derivative compound, which is a pharmacologically active agent. Studies have been shown that umbelliferone exhibit pharmacological activities in degenerative diseases caused by cancer cells, pro-oxidants, and ROS [40]. Given the role of oxidative stress in glutamate excitotoxicity, we assumed that the antioxidant activity of umbelliferone contributed significantly to its protective role against glutamate excitotoxicity. This study demonstrated the proliferative and antioxidant effects of umbelliferone against glutamate excitotoxicity in PCN cells. MTT, which is a colorimetric analysis, is a method that determines the amount of cell viability in proliferative and cytotoxic studies. Since MTT is a fast, useful, and low-cost technique, it has become a very popular method for determining the amount of cell viability in cell culture studies [41]. In the study, we used MTT assay and a significant decrease in cell viability was detected in PCN cells with glutamate toxicity. As a result of our study, following 50-250 µM concentration of umbelliferone application, an increase in cell viability on PCN cells against glutamate excitotoxicity was detected.

Oxidative stress is an indicator of tissue damage due to increased ROS. The oxidant effect created by ROS is blocked by the antioxidant defense system. Disruption of the balance between free radicals and the antioxidant defense system causes oxidative stress and oxidative damage [42,43].

SOD activity and TAC are generally suppressed, redox balance cannot be maintained and oxidative stress occurs in the organism.

As a result of glutamate-induced excitotoxicity, an uncontrolled increase in ROS and intracellular Ca^{+2} concentration is observed due to excessive activation of glutamate receptors. At the same time, glutamate causes neuronal degeneration by causing partial depolarization in the mitochondrial membrane and triggering an increase in intracellular ROS concentration and oxidation [44]. Organisms protect the intracellular environment from the effects of ROS by activating their antioxidant systems to protect the cell from damage caused by free radicals [45].

Coumarins are known to have antioxidant and neuro-protective effects [46] Therefore, natural plants that can be used in the isolation of coumarins have become the focus of researchers [47]. Umbelliferone is one of the most widespread coumarin compounds found in the Apiaceae family and it has many biological effects like anti-inflammatory, anti-lipid peroxidation, antimicrobial, antidiabetic, anticancer, and antioxidant potential [48]. The antioxidant potential of umbelliferone has been previously reported in some studies. Umbelliferone has been shown to stop the cell cycle in the G0/G1 phase by increasing oxidative stress and induce apoptosis in human oral carcinoma cells through oxidative DNA damage [49]. Germoush et al.[50] showed umbelliferone modulates the glutamate-NO-cGMP pathway and prevents oxidative

damage in the brain of hyperammonemic rats. And also, in that study it was shown that umbelliferone suppressed oxidative stress, glutamine synthesis in the cerebrum and reduced the expression and activity of cerebral Na^+/K^+-ATPase. Hindam et al.[51] showed that umbelliferone or xanthotoxin treatments significantly mitigated the oxidative stress via decreased MDA levels in STZ-treated rats. Also, umbelliferone was reported to possess anti-oxidant properties in various tissues [50]. Karakaya et al.[52] showed that extracts of *Zosima absinthfolia* containing umbelliferone have a high anti-oxidant capacity.

In the current study, TAS-TOS analysis was used to evaluate its effect on oxidative damage. Umbelliferone was isolated from *Ferulago cassia*. The obtained results showed that the cells treated with 500-1000 µM umbelliferone had high oxidant and low antioxidant capacity, which in turn induced toxicity and increased cell death by intracellular stress factor. It was found that the antioxidant level was the highest following the application of 50-100 µM umbelliferone, consistent with both our MTT and TOS results. Similarly, in the literature, the antioxidant effect of umbelliferone was also shown on PCN against glutamate excitotoxicity.

In conclusion, umbelliferone, is a coumarin derivative compound, has a neuroprotective effect in PCN culture against glutamate toxicity. It is thought that umbelliferone shows this effect by increasing antioxidant properties in cells while decreasing oxidant capacity. In this study, we showed that umbelliferone can be a concentration-dependent agent that can be used as a protective and therapeutic agent against glutamate excitotoxicity. To better understand the effect of umbelliferone on glutamate toxicity, different comprehensive studies are needed both *in vitro* and *in vivo*.

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None.

Conflict of Interest

The authors' declares that they have no confict of interest.

Author Contributions

Concept: AKD, GG; Isolation of Umbelliferone: SK; Experimental of Cell Culture: GG, SYT, KAN, AH; Statistical analysis and Calculation: AKD, GG, SK, AH; All authors contributed on article writing and approved the final article.

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