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Investigation of the Protective Effects of Chrysin Against Paclitaxel-Induced Oxidative Stress and Apoptosis in Human Neuronal SH-SY5Y Cells

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Abstract: In this study, the potential protective effects of chrysin, an important flavonoid, against paclitaxel-induced cell toxicity in the SH-SY5Y nerve cell line as an *in vitro* model, were investigated by cell viability analysis, lipid peroxidation analysis and quantitative simultaneous PCR methods. In the study, firstly, paclitaxel and chrysin were applied to the SH-SY5Y cell line at different concentrations in the range of 0-30 µM, and the results showed that 15 and 30 µM paclitaxel reduced cell viability, and 500 and 1000 µM chrysin application reduced these effects. In addition, chrysin application has been shown to significantly reduce malondialdehyde levels in paclitaxel-induced cells. The study also examined the effects of paclitaxel and chrysin application on apoptotic and antiapoptotic genes, mostly located in the intrinsic pathway, and showed that chrysin significantly reduced the levels of c*aspase 10, caspase 8, caspase 6, p53* and *NFKB*, and increased the *Bcl-2* level compared to the paclitaxel-treated group. The results of this study suggest that chrysin's suppression of oxidative stress and apoptotic cell death may be an effective strategy for the treatment of paclitaxel-induced SH-SY5Y cytotoxicity.

Krisinin İnsan Nöronal SH-SY5Y Hücrelerinde Paklitaksel Kaynaklı Oksidatif Stres ve Apoptoza Karşı Koruyucu Etkilerinin Araştırılması

Anahtar Kelimeler
Paklitaksel,
Krisin,
Apoptoz,
Bcl-2,
Malondialdehit düzeyleri

Öz: Bu çalışmada, *in vitro* model olarak SH-SY5Y sinir hücre hattında paklitaksel kaynaklı hücre toksisitesine karşı önemli bir flavonoid olan krisinin potansiyel koruyucu etkileri hücre canlılık analizi, lipid peroksidasyon analizi ve kantitatif eş zamanlı PCR yöntemleri ile araştırılmıştır. Çalışmada ilk olarak SH-SY5Y hücre hattına 0-30 µM aralığında farklı konsantrasyonlarda paklitaksel ve krisin uygulanmış ve sonuçlar 15 ve 30 µM paklitakselin hücre canlılığını azalttığını ve 500 ve 1000 µM krisin uygulamasının da bu etkileri azalttığını göstermiştir. Buna ilaveten, krisin uygulamasının paklitaksel ile indüklenmiş hücrelerde malondialdehit düzeylerini anlamlı ölçüde azalttığı da gösterilmiştir. Araştırmada paklitaksel ve krisin uygulamasının çoğunlukla intrinsic yolakta yer alan apoptotik ve antiapoptotik genler üzerindeki etkileri de incelenmiş ve krisinin *kaspaz 10, kaspaz 8, kaspaz 6, p53* ve *NFKB* seviyelerini anlamlı şekilde azalttığını, *Bcl-2* seviyesini ise paklitaksel uygulanmış gruba göre arttırdığını göstermiştir. Bu çalışmanın sonuçları, krisinin oksidatif stres ve apoptotik hücre ölümünün baskılanmasının, paklitaksel ile indüklenen SH-SY5Y sitotoksisitesinin tedavisi için etkili bir strateji olabileceğini göstermektedir.

1. INTRODUCTION

A chemotherapy drug called paclitaxel is used to treat a number of cancers, including breast cancer, ovarian cancer, lung cancer, and others. Paclitaxel can have negative effects, just like many chemotherapy medications. Individuals may experience fewer adverse effects than others, and the intensity of these side effects can vary from person to person. Nausea and vomiting, hair loss, neutropenia, peripheral neuropathy, muscle and joint pain, exhaustion, changes in liver enzymes, and diarrhoea are some of the more frequent side effects of paclitaxel [1-3].

Nowadays, various treatment strategies have been practically applied to reduce side effects [4-8]. One of these strategies and the newest is antioxidants, which have a natural usage area. Natural antioxidants have a slowing and even stopping effect on the course of cancer and can be applied as a combined treatment with chemotherapy.

Flavonoids are an important class of compounds found naturally in plants. They serve as colour pigments in plants and are widely found in various plant materials. Flavonoids play a protective role for plants against environmental stresses and have potential biological effects that may provide beneficial effects to humans. Flavonoids have various effects on health. These effects can be summarized as antioxidant properties, anti-inflammatory effects, cardiovascular effects, anticancer effects, anti-diabetic and antimicrobial effects [9-11]. Chrysin is a bioactive compound naturally found in some plants. It is a type of plant pigments called flavonoids. Chrysin is found primarily in flowering plants and can also be isolated from propolis, honey and some fruits. One of the scientific highlights of chrysin is that it has antioxidant properties. There has also been some research on the anti-inflammatory, antimicrobial and anti-cancer potential of chrysin [12-15].

The aim of this study was to evaluate the possible protective effects of chrysin against paclitaxel-induced toxicity in SH-SY5Y neuron cells as an *in vitro* model through oxidative damage and apoptosis. Thus, possible damage and cell losses that may occur in neuronal cells induced by paclitaxel can be ameliorated. In this study, human neuroblastoma SH-SY5Y cells was used as a model to study neuronal damage caused by paclitaxel-induced oxidative damage and apoptosis in neurodegenerative diseases.

2. MATERIAL AND METHODS

2.1. Materials

SH-SY5Y (ATCC® CRL-2266™) cells were graciously donated by Ataturk University, Turkey. Chrysin was attained from Sigma-Aldrich (St Louis, MO, USA) company with 97% purity. Malondialdehyde bis (PubChem CID:67147) was procured from Merck (Germany). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were acquired from GIBCO (Gibco, USA).

2.2. Cell Culture

SH-SY5Y cells were grown at 37 °C in a humidified 5% CO_2 environment with 10% FBS and 1% penicillin-streptomycin in DMEM. For 24 hours, the cells were sown in 75 cm^2 cell culture flasks.

2.3. Cell Proliferation Assay

After the SH-SY5Y cells cultivated in a flask reached sufficient growth, they were removed with Trypsin-EDTA, stained with trypan-blue on the thoma slide, and then counted under an inverted microscope. The calculated cells were seeded in 96-well plates with $7x10^3$ cells in each well. Then, different concentrations of paclitaxel (3.75 µM, 7.5 µM, 15 µM, 30 µM) were applied to the cells to determine the most effective cytotoxic paclitaxel concentration on the cells. After determining the most effective paclitaxel concentration, chrysin was applied to the cells at different concentrations (125 µM-1000 µM) together with paclitaxel in order to investigate the potential of chrysin to prevent paclitaxel-induced cytotoxicity. After 24 hours of incubation, 3 µl of the WST-1 cell viability kit was added to each well, incubated for 4 hours, and cell viability analyzes were performed by measuring at 595 nm on the ELISA micro plate reader device.

2.4. Measurement of Malondialdehyde Levels

By lipid peroxidation analysis, the extent to which 30 µM Paclitaxel and 500-1000 µM chrysin applied to the SH-SY5Y cell line had a scavenging effect on lipid peroxidation was determined by MDA measurement. The night before, MDA solution (500 µl from the MDA stock was taken and transferred to the tube containing 450 µl of pure water, and 50 µl of the mixture was taken from each tube (except MDA stock) and transferred to the next tube, diluted and kept at +4 °C. In the next stage, SH- SY5Y cells were removed with Trypsin-EDTA solution and then counted on the thoma slide and planted in 6-well plates with 10^6 cells in each well. After the cells were incubated in a CO_2 incubator for 24 hours, determined amounts of paclitaxel and chrysin were applied to the cells. The next day, the cells were centrifuged and the above cells were incubated. The medium was removed and 1 ml of 0.8% TBA (thiobarbutic acid) solution and 250 µl of TCA (trichloroacetic acid) solution were added and mixed to ensure sufficient homogenization. Afterwards, the cells were boiled in boiling water at 95 °C for 30 minutes. The water bath process was shocked. For this purpose, the cells were placed in ice and treated for 5 minutes. After the quenching process, the cells were centrifuged at 1000 rpm and the supernatant on the cells was removed and transferred to suitable plates for measurement. The absorbance of the cells placed in the ELISA reader device was calculated at 532 nm.

2.5. Real Time PCR

The mRNA transcript level of the genes in the scope of current research were examined via qRT gene expression assay kit procured from Jena Bioscience Company using Rotor-Gene Q (Qiagen) device. For this study, mRNA was isolated utilizing the kit bought from Jena Bioscience. cDNA synthesis was accomplished in line with the supplied kit procedure (Jena Bioscience). The expression levels of apoptotic *caspase 10*, *caspase 8*, *caspase 6*, *p53* and *NFKB*, anti-apoptotic *Bcl-2* and *GAPDH* as a housekeeping gene were analyzed as explained in (Ref). Primer sequences (Molgen Biotechnology, Turkey) were shown in Table 1.

Table 1. Gene-specific primer sequences used in the qRT-PCR study (F: Forward, R: Reverse)

Genes	Primer sequences (5'-3')
Bcl-2 (F)	TTTAATTGTATTTAGTTATGGCCT
Bcl-2 (R)	AATAAACAATTCTGTTGACG
Caspase 8 (F)	CCTGTCCATCAGTGCCATAG
Caspase 8 (R)	CCTGTCCATCAGTGCCATAG
Caspase 6 (F)	GACCGACTAAATGCC
Caspase 6 (R)	AATTACTGTGCAAATGCC
Caspase 10 (F)	CCAGGCTATGTATCCTTTCGGC
Caspase 10 (R)	TCGTTGACAGCAGTGAGGATGG
NF-KB (F)	TGCAGCAGAACCAAGGACATG
NF-KB (R)	TGCATTGGGGGCTTTACTGCT
P53 (F)	CGACGGTGACACGCTTCC
P53 (R)	TTTCCTGACTCAGAGGGGGC
GAPDH (F)	GTATCGGACGCCTGGTTACC
GAPDH (R)	TTGAACTTGCCGTGGGTAGAG

2.6. Statistical Analysis

The data obtained from our experimental studies, which were performed in at least 3 repetitions, were statistically evaluated by comparing them with one-way ANOVA Tukey's Multiple Comparison test via GraphPad Prism 5.01 software, and p <0.001 was considered as significant.

3. RESULTS

3.1. Effects of Paclitaxel and Chrysin Application on Cell Viability

To examine the protective effects against paclitaxel-induced oxidative stress and apoptosis in the human neuronal SH-SY5Y cell line, cells were treated with chrysin and the extent to which the damage was minimized was evaluated by the following cell viability tests. To determine the damage status of SH-SY5Y cells, 4 different doses were applied: 3.75, 7.5, 15, 30 µM. It was observed that 15 to 300 µM paclitaxel caused a significant (p <0.001) decrease in cell proliferation, while 3.75 and 7.5 µM paclitaxel did not cause a significant change in cell proliferation (Figure 1).

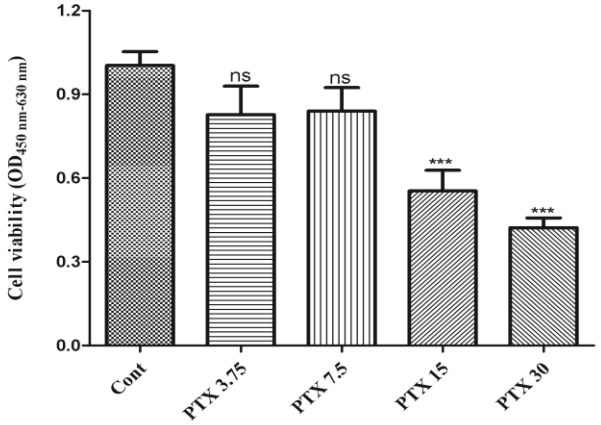

Figure 1. Effect of different doses of paclitaxel on cell proliferation of dopaminergic nerve line (SH-SY5Y) cells. ***p < 0.001: statistically significant differences between cont and other groups, ns: nonsignificant

After it was determined that 15 and 30 µM paclitaxel caused a significant (p <0.001) decrease in cell proliferation, 4 different concentrations of chrysin (1000, 500, 250, 125 µM) were injected into the cells together with paclitaxel in order to determine the protective effects of chrysin against paclitaxel-induced toxicity at the relevant dosages. When 1000 µM and 500 µM chrysin were applied to the cells together with 15 µM paclitaxel, it was determined that cell proliferation increased significantly compared to the cells applied paclitaxel, whereas the application of 250 µM and 125 µM chrysin did not show a protective effect (Figure 2). In the light of the findings, subsequent studies were carried out with 500 and 1000 µM chrysin compared to 30 µM paclitaxel.

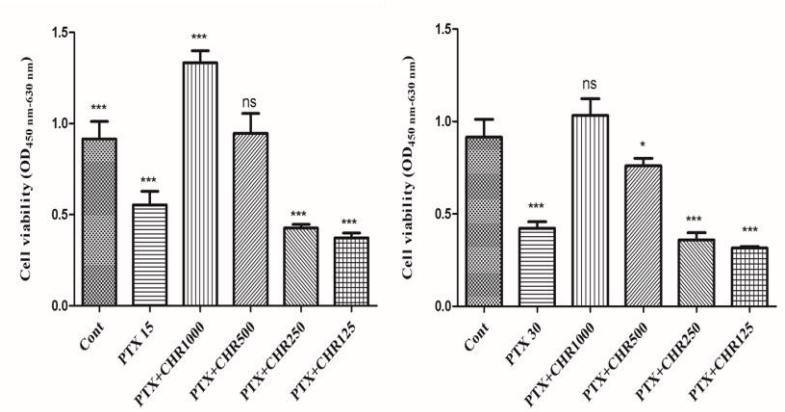

Figure 2. The effects of 500 and 1000 µM chrysin on cell proliferation on SH-SY5Y cells induced by paclitaxel, which was selected as the effective dose (15 and 30 µM). Cont: Control, PTX: Paclitaxel, CHR: Chrysin. ***p < 0.001 and *p < 0.1:statistically significant differences between cont and other groups, ns: nonsignificant

3.2. Lipid Peroxidation Assay

A lipid peroxidation experiment was conducted to observe to what extent the cells were damaged after the application of 30 µM paclitaxel, one of the paclitaxel-derived chemotherapeutics, to healthy human neuroblastoma cells and to what extent antioxidant treatment minimized this damage, and to determine the malondialdehyde level. To investigate whether chrysin contributes to suppressing nerve cell death from paclitaxel-induced toxicity and to determine the malondialdehyde level, LPO analysis was performed using paclitaxel+500 and 1000 µM chrysin vs 30 µM paclitaxel. While the MDA level in 30 µM paclitaxel-induced SH-SY5Y cells significantly increased ($p < 0.01$), chrysin pretreatment significantly reduced MDA levels compared to paclitaxel-induced SH-SY5Y cells ($p < 0.01$).

3.3. qRT-PCR Experiments

To test whether the mRNA transcript levels of anti-apoptotic and apoptotic genes were affected by paclitaxel and chrysin, the expression levels of Bcl-2, P53, Cas-6, Cas-8, Cas-10, and NFKB were measured by qRT-PCR. It was observed that the mRNA transcript levels of *p53*, *Cas-6*, *Cas-8*, *Cas-10* and *NFKB* were significantly increased after treatment with paclitaxel compared to the control, and the mRNA levels of *Bcl-2* were also significantly reduced (Figure 9). When chrysin and paclitaxel were administered together, it was observed that the mRNA transcript levels of *p53*, *Cas-6*, *Cas-8*, *Cas-10* and *NFKB* decreased compared to the paclitaxel-treated group, and the mRNA levels of *Bcl-2* increased significantly.

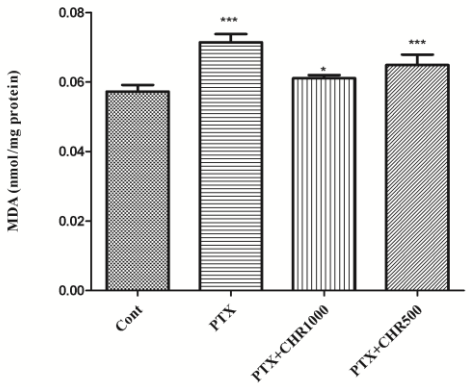

Figure 3. Measurement of MDA (malondialdehyde) released as a result of lipid peroxidation on the SH-SY5Y cell line induced by the simultaneous application of 30 µM paclitaxel, 30 µM paclitaxel + 500 and 1000 µM chrysin using the LPO Assay kit. ***$p < 0.001$ and *$p < 0.1$:statistically significant differences between cont and other groups, ns: nonsignificant

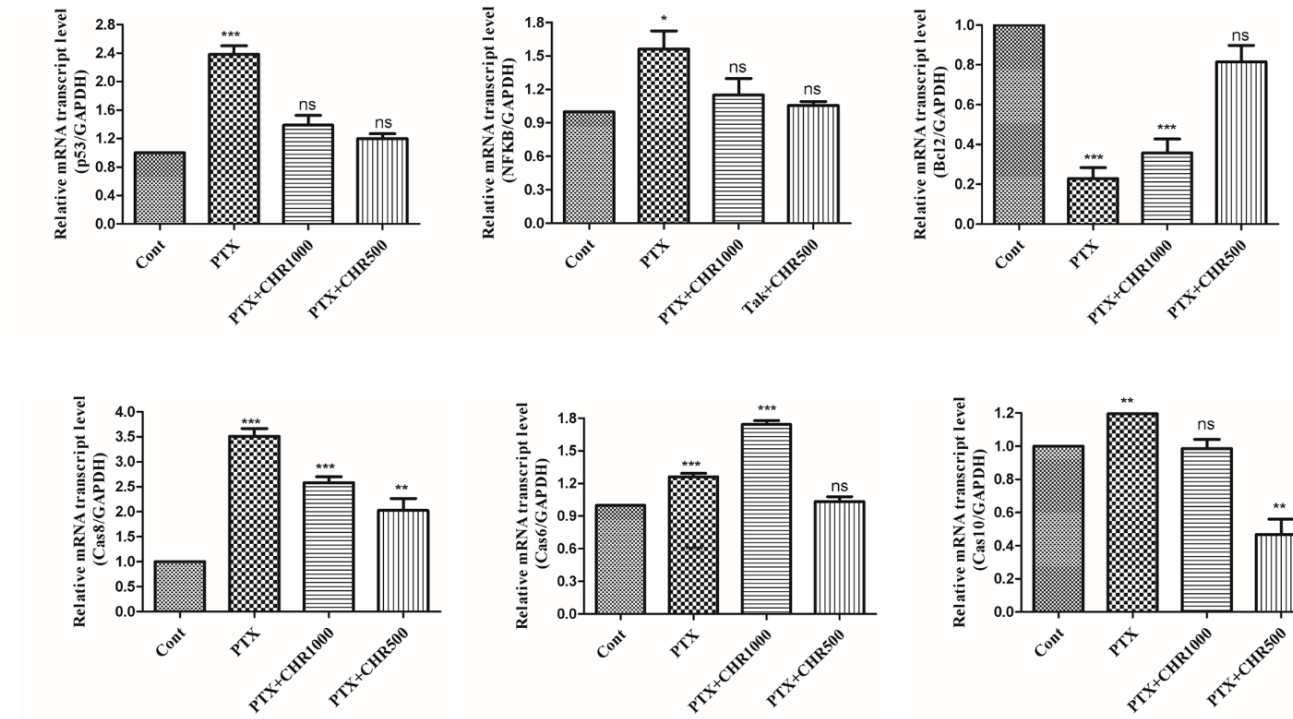

Figure 4. mRNA expression level of apoptotic and anti-apoptotic genes in SH-SY5Y cell line induced by 30 µM paclitaxel, 500 and 1000 µM chrysin. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.1$:statistically significant differences between cont and other groups, ns: nonsignificant

4. DISCUSSION

Paclitaxel is a chemotherapy drug widely used in the treatment of cancer. Chemotherapy drugs work by killing or preventing cancer cells from growing and multiplying [15]. However, cytotoxic drugs can act on healthy cells as well as cancer cells and cause some side effects. Many chemotherapeutic drugs can create oxidative stress in cells and lead to lipid peroxidation [16]. Paclitaxel may also be one of these mechanisms. When attacking cancer cells, chemotherapy drugs can cause peroxidation of lipids in cell membranes by increasing the formation of free radicals.

Lipid peroxidation is the process by which lipids (fats) in cells undergo oxidative damage. These reactions involve chain reactions initiated by highly reactive molecules called free radicals [17]. Free radicals affect cell membranes and intracellular components, causing a condition called oxidative stress. Lipid peroxidation is a process in which the lipids that make up the cell membrane, such as fatty acids, phospholipids and cholesterol, are targeted. Lipid hydroperoxides are formed by adding hydroxyl groups to these lipids under the influence of free radicals. Lipid hydroperoxides can disrupt the structural integrity of the cell membrane, deplete enzymatic and non-enzymatic antioxidant systems, and interact with other cellular components, leading to cell damage and cell death [18]. Lipid peroxidation is considered a marker of oxidative stress and plays an important role in many diseases. It may be especially effective in the development of cardiovascular diseases, diabetes, cancer, neurodegenerative diseases and other chronic diseases [19]. In response, the body has naturally developed antioxidant defense mechanisms against lipid peroxidation. Adopting a healthy lifestyle, a balanced diet and a diet enriched with antioxidants can play an important role in preventing lipid peroxidation. However, in some cases, especially in chronic diseases or cases of increased oxidative stress, supplements can be used to support a balanced antioxidant system. In a study conducted on experimental animals, it was reported that paclitaxel caused peripheral neuropathy by increasing lipid peroxidation [20]. Another study revealed that royal jelly, rich in antioxidant content, had a reducing effect on paclitaxel-induced lipid peroxidation in heart tissue [21]. In an experimental study conducted by Gür and his colleagues, it was reported that paclitaxel causes lipid peroxidation in liver cells and hesperidin minimizes this effect [22].

Paclitaxel has an effect that inhibits cell division and stops the growth of cancer cells. This effect prevents cancer cells from dividing and proliferating by interfering with the cell cycle [23]. However, paclitaxel also induces apoptosis [24]. Paclitaxel neutralizes structures called microtubules within the cell. Microtubules serve as the skeleton of the cell during cell division. By increasing the stability of these structures, paclitaxel prevents cell division from completing properly and leads to the death of cells [25]. It is known that cancer cells, in particular, divide and multiply faster than normal cells. Therefore, while paclitaxel works as an effective treatment against the rapid division of cancer cells, it interferes less with the division of normal cells and reduces damage to healthy cells [26]. Paclitaxel's triggering of apoptosis enables cancer cells to enter the process of programmed cell death. Apoptosis is important to keep abnormal growth of cells and potentially cancerous tumours under control. Therefore, drugs that induce apoptosis, such as paclitaxel, may be an effective tool in cancer treatment. However, these drugs can also affect healthy cells and cause some side effects during treatment. Healthy cells may respond in different ways than cancer cells, which can lead to side effects. Paclitaxel can damage healthy cells and cause cells to die by apoptosis. In parallel with the data obtained in the thesis, it has been reported in previous studies that paclitaxel causes apoptosis in the sciatic nerve by increasing *caspase-3* and *Bax* levels and suppressing Bcl-2 levels [27]. In another study, an increase in the mRNA transcript levels of *caspase-3*, *p53* and *Apaf-1* was observed in both the sciatic nerve and spinal cord after treatment with paclitaxel, while a decrease was observed in the mRNA transcript levels of *Bcl-2* and *Bcl-xL* [28]. Another study shows that chrysin application protects SH-SY5Y cells by reducing the expression of *Cas-3*, *Cyt c* and *Bax* in cyclophosphamide-induced SH-SY5Y cells and reducing the expression of the anti-apoptotic gene *Bcl-2* [6]. According to the results obtained in the study titled neuroprotective effects of chrysin on diclofenac-induced apoptosis in SH-SY5Y cells, chrysin reduces the expression of *Bax, cytochrome c, cas-3, cas-8* and *p53* and reduces the expression of the anti-apoptotic gene *Bcl-2*. It shows that it protects SH-SY5Y cells [29] further indicating that natural antioxidants have a slowing and even stopping effect on the course of cancer and can be applied as a combined treatment with chemotherapy [30,32]. The use of antioxidants as nutritional supplements, and chrysin in particular, reduces the apoptotic effects of paclitaxel in SH-SY5Y cells, which may add depth to therapeutic approaches to paclitaxel-induced cell injuries.

5. CONCLUSIONS

The purpose of this study was to examine the impact of different paclitaxel and chrysin concentrations on SH-SY5Y cell viability, MDA levels and mRNA transcript levels of some apoptotic and anti-apoptotic genes. In this study, the results showed that 15 and 30 µM paclitaxel reduced cell viability, and 500 and 1000 µM chrysin application reduced these effects. Chrysin treatment has been shown to considerably reduce MDA levels in paclitaxel-induced cells. The study also examined the effects of paclitaxel and chrysin application on apoptotic and antiapoptotic genes, and showed that chrysin significantly reduced the levels of *caspase 10, caspase 8, caspase 6, p53* and *NFKB*, and increased the *Bcl-2* level compared to the paclitaxel-treated group

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