Research Article

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Potential effects of parietin on apoptosis and cell cycle related genes in SH-SY5Y neuroblastoma cells

Parietinin SH-SY5Y nöroblastom hücrelerinde apoptoz ve hücre döngüsü ile ilgili genler üzerindeki potansiyel etkileri

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Abstract

Purpose: Ingredients obtained from natural products have been used in cancer treatments for years. High diversity and non-toxicity compared to chemotherapeutic agents are the main reasons for their preference. Lichens having potential for treatment of cancer consist of fungus and 1-2 species of algae. Under the name of lichen substances, many of them have also been synthesized as specific substances. The secondary metabolites in lichens are generally insoluble in water, and have many biological activities such as antiviral, antitumor, antibacterial, and antioxidant; they store in the fungal cell or on the surface of the hyphae and can only be extracted with organic solvents. Parietin extracted from lichen species such as xanthoria parietina is an anthraquinone pigment and a secondary metabolite. In our study, the effects of parietin on cytotoxicity, gene expression, migration, invasion, and colony formation in neuroblastoma cells treated with parietin were investigated. SH-SY5Y cell line without parietin was used as the control group.

Materials and methods: The IC_{50} value of the parietin was determined using XTT assay. The total RNA extractions were performed from the cells using the Tri-Reagent kit. The expressions of BAX, CASPASE3, CASPASE8, CASPASE9, P53, PUMA, NOXA, TIMP1, TIMP2, BCL2, BCL-XL, CASPASE10, BID, CYCLIND1, CDK6, P21, MMP2, MMP9, TRADD and FADD genes were investigated by Lightcycler 480 (Roche) using SYBR Green dye. Migration analysis of the control and the dose group cells were performed in accordance with the Wound-healing assay protocol. Invasion activities were determined using the “Invasion Chamber” (BD Biosciences) protocol. Colonies were treated with crystal violet and observed under the light microscope.

Results: The IC_{50} value of the parietin used for 48-hour treatment on the cells was determined as 35 μM. It was found that the expression levels of BCL-XL, BCL-2, MMP2, MMP9, P21, and CYCLIN D1 mRNA were downregulated, and it was also shown to be upregulated the expression levels of CASPASE3, CASPASE9, BAX, P53, PUMA, and NOXA to be upregulated. It was determined that parietin suppressed both cell invasion and migration, and colony formation in the neuroblastoma cells.

Conclusions: Thus, it can be possible parietin to be used as an alternative, complementary, and supportive agent together with the other drugs in the treatment of neuroblastoma. However, more comprehensive studies supporting these significant effects of parietin will increase its potential in the application.

Keywords: Parietin, neuroblastoma, cell culture, gene expression, migration.

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Öz

Amaç: Doğal ürünlerden elde edilen bileşenler yıllardır kanser tedavisinde kullanılmaktadır. Kemoterapötik ajanlara göre çeşitliliğinin fazla olması ve toksik olmaması tercih edilmelerinin başlıca nedenleridir. Kanser tedavisi potansiyeli olan likenler mantar ve 1-2 tür algden oluşmaktadır. Liken maddeleri adı altında birçoğu spesifik maddeler olarak da sentezlenmiştir. Likenlerdeki sekonder metabolitler genellikle suda çözünmez ve antiviral, antitümör, antibakteriyel ve antioksidan gibi birçok biyolojik aktiviteye sahiptir; mantar hücresinde veya hifanın yüzeyinde depolanırlar ve yalnızca organik çözücülerle ekstrakte edilebilirler. Xanthoria parietina gibi liken türlerinden ekstrakte edilen parietin, bir antrakinon pigmenti ve ikincil bir metabolittir. Çalışmamızda parietin ile tedavi edilen nöroblastoma hücrelerinde parietin'in sitotoksisite, gen ekspresyonu, migrasyon, invazyon ve koloni oluşumu üzerine etkileri araştırılmıştır. Parietin tedavisi uygulanmayan SH-SY5Y nöroblastom (NB) kontrol grubu olarak uygulanmıştır.

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Gereç ve yöntem: Parietin'in IC_{50} değeri XTT testi kullanılarak belirlendi. Total RNA ekstraksiyonları, Tri-Reaktif kiti kullanılarak hücrelerden gerçekleştirilmiştir. BCL-XL, BCL-2, MMP2, MMP9, P21, CYCLIN D1, CA3, CASAPASE-9, BAX, P53, PUMA ve NOXA genlerinin ekspresyonları Lightcycler 480 (Roche) ile SYBR Green boyası kullanılarak araştırılmıştır. Kontrol ve doz grubu hücrelerinin invazyon, yara iyileşmesi testi protokolüne uygun olarak gerçekleştirilmiştir. İnvazyon, "İnvazyon odacıkları" (BD Biosciences) protokolü kullanılarak belirlenmiştir. Koloni testi için hücreler kristal viyole ile muamele edilmiş ve ışık mikroskobu altında gözlenmiştir.
Bulgular: Hücrelerde 48 saatlik tedavide kullanılan parietin'in IC_{50} değeri 35 μM olarak belirlenmiştir. BCL-XL, BCL-2, MMP2, MMP9, P21 ve CYCLIN D1 mRNA'nın ekspresyon seviyelerinin downregüle edildiği; CASPASE3, CASPASE9, BAX, P53, PUMA ve NOXA'nın upregüle edildiği tespit edilmiştir. Parietin'in nöroblastoma hücrelerinde hem hücre invazyonu ve migrasyonu hem de koloni oluşumunu baskıladığı belirlenmiştir.
Sonuç: Böylece parietin'in nöroblastom tedavisinde diğer ilaçlarla birlikte alternatif, tamamlayıcı ve destekleyici bir ajan olarak kullanılması mümkün olabilir. Ancak parietin'in bu önemli etkilerini destekleyen daha kapsamlı çalışmalar uygulamadaki potansiyelini artıracaktır.

Anahtar kelimeler: Parietin, nöroblastoma, hücre kültürü, gen ekspresyonu, migrasyon.

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Introduction

Neuroblastomas (NB), malignant solid tumors that arise from out-of-control primitive cells of the sympathetic nervous system, are often seen in early childhood; It is believed these tumors to stem from primitive neural crest cells. Some of the primitive cells undergo changes and form tumors, and the reason for the changes is genetic or environmental factors. The disease usually occurs at an early age. The reason for this is that the cells are of embryonal origin and the faulty development in nerve cells starts before the baby is born. Chromosomal changes and/or faulty gene regulation cause this faulty development in nerve cells [1]. Interestingly, the occurrence of neuroblastoma does not vary across different racial groups.

Neuroblastoma develops mainly in the adrenal medulla or paravertebral sympathetic ganglia at any level from the neck to the pelvis [2]. It can occur anywhere along the sympathetic nervous system and the most common primary site is an adrenal mass [3]. Compared with other tumors; The primary tumor may show different clinical manifestations of metastatic disease and paraneoplastic syndromes. Spontaneous regressions and differentiation into benign disease can be given as examples of different behaviors of neuroblastoma. It may be more aggressive in older children [4]. The SH-SY5Y NB cells are a triple cloned sub-colony of the SK-N-SH cell line (SK-N-SH-> SH-SY-> SH-SY5-> SH-SY5Y-CRL-2266). SH-SY5Y, showing dopamine-β-hydroxylase activity, can convert glutamate to the neurotransmitter Gamma-Aminobutyric acid (GABA). GABA, or Gamma-Aminobutyric Acid, is widely recognized as a key presynaptic inhibitor in both the central nervous system and the retina. This neurotransmitter holds significant importance in regulating brain metabolism and functioning. For these reasons, the SH-SY5Y cell line was chosen.

In our study, the expression levels of apoptosis, cell cycle and tumor suppressor genes were investigated. Many genes are involved in the orientation of the cell to apoptosis. The first of these is the Bcl2 family. The Bcl2 family is divided into two groups as proapoptotic and antiapoptotic members, and the excess of proapoptotic members increases the apoptosis tendency of the cell. Bad, Bax, Noxa, and Puma genes are proapoptotic genes and cause programmed death of cells by inducing apoptosis. Bcl-XL and Bcl2 are antiapoptotic genes. These genes suppress apoptosis and cancer cells affected by suppression continue to proliferate uncontrollably [5].

Caspases are essential proteins required for apoptosis. It consists of two pathways, intrinsic and extrinsic. While the intrinsic pathway starts with caspase 9 activation, the extrinsic pathway starts with caspase 8 activation and both pathways converge in caspase 3 [6].

The studies carried out in recent years shows that natural compounds have been used in the treatment of cancer as well as chemotherapeutic agents due to their non-toxicity.

Since time immemorial, plants and lichens have been used as drugs for medicinal

purposes in many countries within the scope of traditional treatment. There are studies that lichens are used as therapeutic agents in some diseases such as cancer, arthritis, diabetes, eczema, respiratory and circulatory tract and have a therapeutic effect thanks to secondary metabolites such as cystic acid, gyrophoric acid, and norstic acid from lichen species. Anthraquinones (AQs) are the predominant type of quinones found in nature and are abundant in various sources [7]. Parietin, an anthraquinone pigment, is located in the upper part of the upper cortex of lichens. They are localized as small extracellular crystals and play a protective role against sunlight, giving them a strong orange-brown color.

The antifungal and antibacterial activities of parietin were investigated in various studies. It has been reported that parietin exhibits significant antiangiogenic activity in breast cancer by inhibiting subcytotoxic concentrations, migration of endothelial cells and tube formation [8].

The objective of this study is to investigate the anticancer effects of parietin on SH-SY5Y cells. In addition, it was also aimed to reveal the molecular mechanisms such as therapeutic activity, cell proliferation, invasion, colony formation, cell cycle and apoptosis.

Material and methods

Cell Culture-Cytotoxicity Test (XTT)

SH-SY5Y cells were obtained from ATCC (American Type Culture Collection, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 2mM L-Glutamine, Penicillin-streptomycin (20 units/mL-20 µg/mL), and 10% Fetal Calf Serum under 5% CO_2 and 37°C conditions. The cytotoxicity assays were performed when the cells multiplied with DMEM reached a sufficient number. The cytotoxicity assays were made using 1×10^4 cells. Cytotoxicity experiments and the determination of IC_{50} of Parietin were done by XTT method in accordance with the protocol. The cells were seeded into 96-well plates using the DMEM being prepared.

In order for the cells to adhere to the bottom of the plate, they were incubated in the oven for 24 hours under appropriate conditions. At the end of the incubation, certain doses (10-250 µM) of parietin were added to the wells. 24 and 48 hours after the addition of parietin, 10 µL of XTT Reagent was added per well in accordance with the XTT protocol and incubated for 2 hours in an oven with 5% CO_2 and 37°C conditions.

At the end of the incubation period, absorbance detections at a wavelength of 450 nm were performed by a microplate reader (Reference wavelength=630 nm). The percentage of the cell viability was determined using the equation below:

The measured mean optical densities (MOD) of the sample was used in the equation below to determine the percentage of viable cells: % viability = [(MOD of sample) / (MOD of control)] x100

Total RNA isolation and PCR

Total RNA was extracted from the SH-SY5Y cells and exposed to the IC_{50} dose of parietin from the 5×10^6 cell flasks, so as to be 1000 µl per flask, the cells were removed from the base using Trizol, and cell scrapers and transferred to eppendorf tubes (1 mL).

After adding 200 µL of chloroform to each eppendorf tube, the tubes were incubated for 15 min at 25°C. Centrifugation was performed at 15,000 rpm and 4°C for 20 minutes with a refrigerated centrifuge; after that, the collected supernatants were placed to new eppendorf tubes.

After adding 500 µL of isopropanol to the collected supernatant, this mixture was incubated at 25°C for 10 minutes. After that, the resultant mixture was centrifuged at 15,000 rpm, 4°C for 30 minutes. After adding 70% ethanol to the pellet, centrifugation was performed at 12,000 rpm and 4°C for 10 minutes. The supernatants were discarded, and the pellet was dissolved with 40 µL of RNase-DNase free water. The concentration and purity of total RNAs were measured using the Thermo Nanodrop device.

The process of cDNA synthesis was carried out utilizing the First-Strand cDNA Synthesis Kit. Gene expressions of BAX, BCL-2, BCL-XL, CASPASE3, -8, -9, -10, BID, TRADD, CYCLIND1, CDK6, P53, P21, FADD, PUMA, NOXA, MMP-2, MMP-9, TIMP-1, and TIMP2 were analyzed using specific primers and the

Real-Time RT-PCR Applied Step One Plus instrument following the SYBR Green qPCR Master Mix protocol. The obtained expression results were compared with the expression levels of the GAPDH gene, which served as the housekeeping gene in the study.

Cell migration, invasion, colony analysis

a. Migration

Migration analysis of the control and the experiment group cells were performed in accordance with the wound-healing assay protocol. Its working principle is based on the Culture-Insert 2 Well placed on the surface of the petri dish, which provide two cell culture reservoirs separated by a 500 µm wall, and thus the migration was observed [9, 10].

Adding the cell suspension to the reservoirs allowed the cells to grow only in the designated growth areas. $3x10^5$ cells/mL per reservoir were seeded and 24 hours later after seeding, the inserts were first removed, and then the determined IC_{50} dose of Parietin was applied to the cells.

Only medium with serum was given to the control group. A gap of approximately 500 µm formed after the insert was removed. An inverted microscope was used to evaluate the migration process. The images at different times (0h and 24h) were compared during the evaluation.

b. Invasion

Percent invasion ratio of the control and the experiment group cells were determined using 2.5 $x10^5$ cells/mL on 24-well culture plates in accordance with the "Invasion Chamber" (BD Biosciences) protocol. According to the kit protocol, the filters were established with methanol for 10 minutes and dyed with crystal violet dye. The cells reaching the lower surface of the filter were counted under a light microscope. Migration analysis was confirmed using analysis of the wound healing assay. Percent invasion formula was calculated using the following equation:

Invasion (%) = (Number of cells in matrigel matrix basement membrane) / (Number of cells in control membrane) x 100.

c. Colony analysis

The cells were seeded in 6-well culture dishes for colony formation analysis. The parietin-treated cell group and the control group cells were incubated in incubators, and in a humid environment containing 5% CO_2 at 37°C. The cells were kept under constant observation and checked with a light microscope. The medium was removed by an aspirator and washed with 1X PBS at the end of the 48th hour and this process was repeated 3 times. The cells were detached with trypsin and centrifugation was used at 500 g for 5 min to collect the cells and the parietin - treated cell group and the control group cells were inoculated into 24-well plates at $2x10^5$ cells/mL per well and incubated in a 37°C incubator, and a humid environment containing 5% CO_2 for 1-2 weeks.

When the incubation period concluded, the cells fixed by 100% methanol for 20 min were treated with crystal violet for 5 min. After staining, the cells washed with 1X PBS were allowed to dry overnight. After all these procedures, the cells were observed under the light microscope.

Statistical analysis

Analysis of the results was performed by the ΔΔCT calculation method. The comparison of the two groups, and the evaluation of the expression changes were performed by the "Volcano Plot" analysis from "RT^2ProfilesTMPCR Array Data Analysis". The student t-test was used to analyze the results, and to determine statistical significance; the p-value that meets the condition of $p<0.05$ was considered to be statistically significant.

Since our study is an *in vitro* study, ethics committee approval is not required.

Results

Cytotoxicity analysis with XTT method

Parietin was implanted to the SH-SY5Y neuroblastoma cells at the doses of 10-20-35-50-75-100-200-250 µM. The cells without parietin were considered as the controls. The IC_{50} dose of the parietin in the 48th hours were determined as 35 µM by the XTT assay. The dose and the time graph of percentage cell viability are shown in Figure 1.

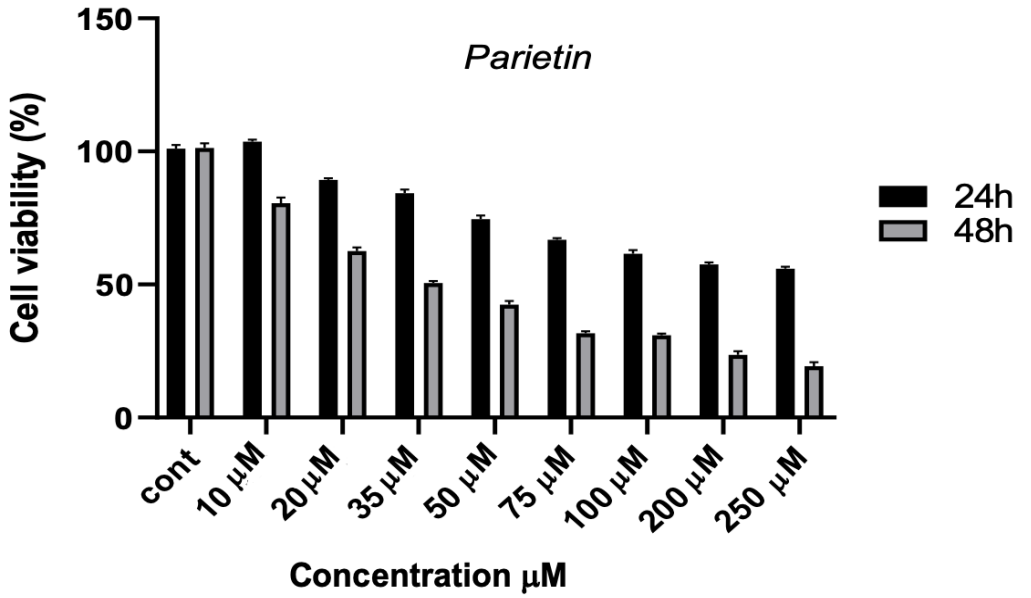

Figure 1. The effect of parietin on the viability of SH-SY5Y neuroblastoma cells was studied. The cells were exposed to various concentrations of parietin over different time intervals, and their growth was evaluated using the XTT assay. The presented data represents the averaged outcomes from three separate experiments

Invasion

In the SH-SY5Y cells treated with the IC_{50} dose of parietin, the invasion rate was reduced three times compared to the control group; the % invasion rates are shown in Figure 2. The percentage invasion rate of 70% in the control group decreased to 40% in the cells in the parietin-treated group. 10 representative fields were used to count the cells passing through the membrane.

Migration

Migration analysis was performed on the SH-SY5Y cells treated with 35 μM Parietin. The results obtained were compared with that of the control group. Inverted microscope images, taken at the 0 and the 24 hours, are shown in Figure 3. It was found that the parietin-treated SH-SY5Y cells suppressed migration compared to the control group.

Colony formation

Parietin decreased colony- forming ability of neuroblastoma cells. The effect of parietin on neuroblastoma cells is shown in Figure 4. While an average of 82 colonies were counted in the control group, this number decreased to an average of 40 in the parietin group (Figure 5).

It was shown parietin to reduce colony formation in the SH-SY5Y neuroblastoma cells.

Real-Time PCR

Based on the Real-Time PCR results, it was revealed that the mRNA expression levels of BCL-XL, BCL-2, MMP2, MMP9, P21 and CYCLIN D1 were downregulated in the parietin-treated groups of the cells. Conversely, it was revealed the expression levels of CASPASE3, CASPASE9, BAX, P53, PUMA and NOXA to upregulate in the same group.

The mRNA expression levels of PUMA and NOXA were doubled compared to the control group; BAX, CASPASE9 triple; p53 about four times; CASPASE3 increased fivefold. The mRNA expression level of BCL-2 was observed to decrease twofold when compared to the control group.

The effect of parietin on fold changes in mRNA level of genes in neuroblastoma cells is shown in Figure 6.

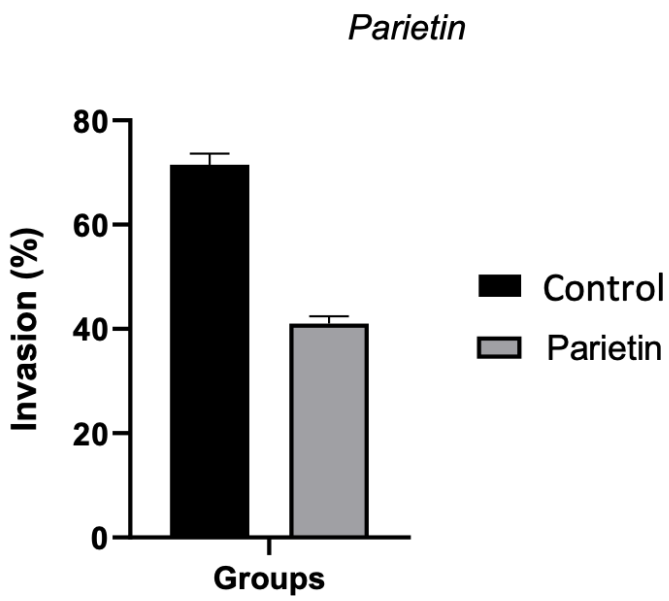

Figure 2. The invasion ratio of SH-SY5Y cells in the control group and the dose group was calculated as the percentage of cells that successfully passed through the membrane. The counting was conducted in 10 specific areas chosen as representatives

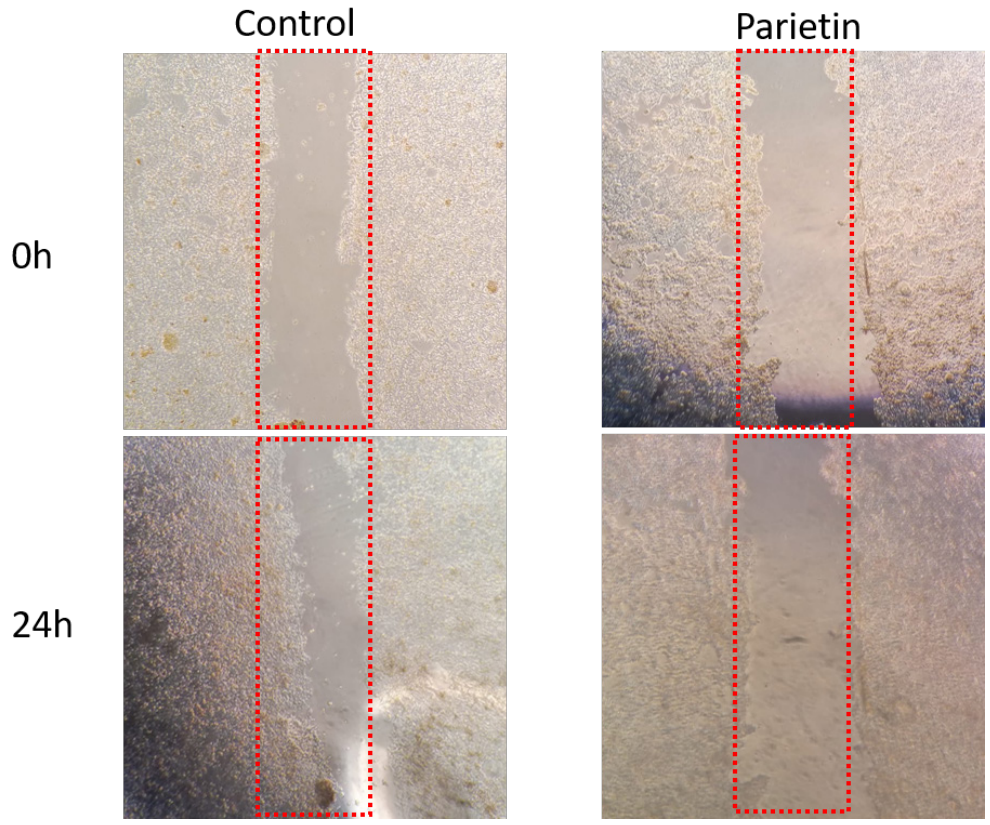

Figure 3. The findings from the wound-healing assay indicated that parietin effectively decreased cell migration. Images from both the control and dose groups were captured at 0 hours and 24 hours to observe the progression of the wound closure over time

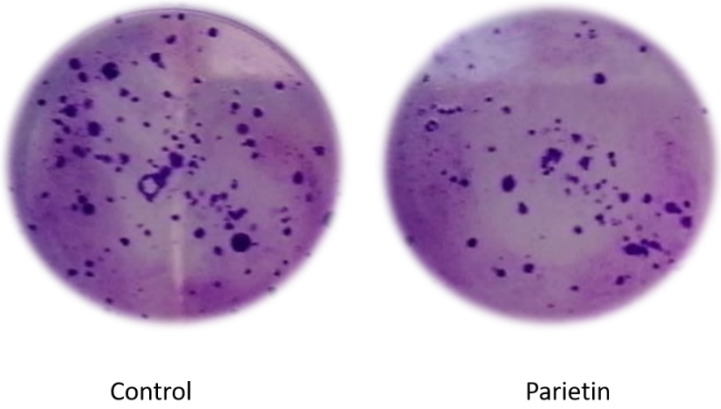

Figure 4. Parietin decreases colony formation. The colonies were stained with crystal violet

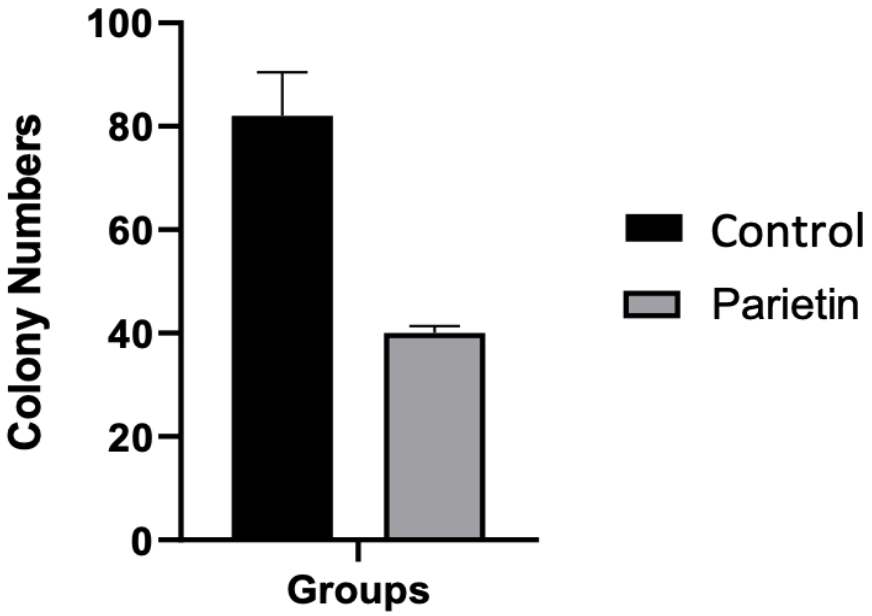

Figure 5. The number of colonies were significantly decreased in the cells treated with ferulic acid compared with the control cells

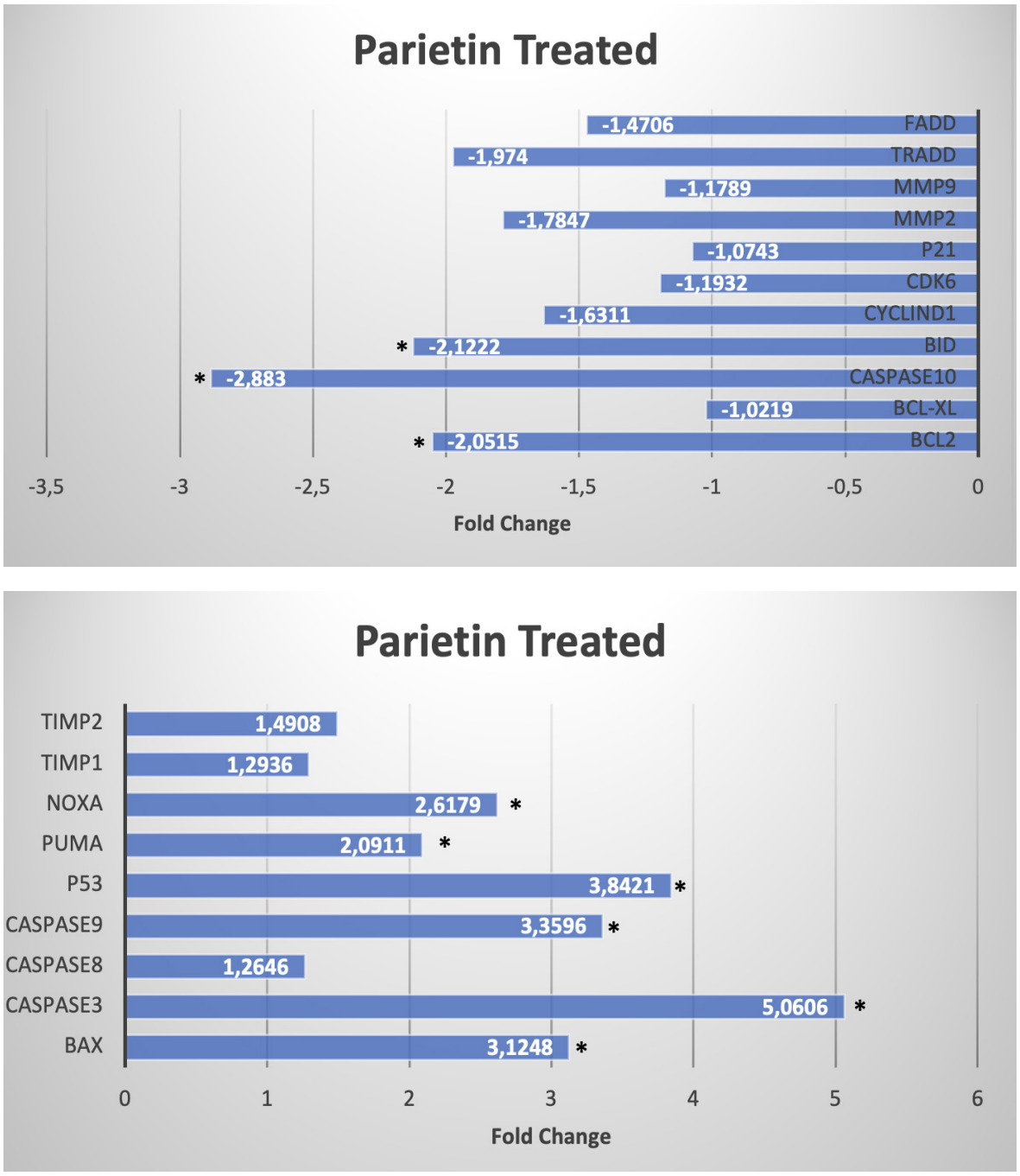

Figure 6. *mRNA* expression changes in parietin treated SH-SY5Y dose group
* $p<0.05$

Discussion

Cancer consists of cells that are unlimited and capable of dividing very rapidly. Cells have a certain number of divisions under normal conditions, but muscle and nerve cells do not have this feature.

Normal cells may turn into tumor cells over time due to genetic or environmental factors. When tumor cells come together, first a bulk and then cancer occurs. Neuroblastoma is malignant solid tumor originating from the sympathetic nervous system. It usually occurs in early childhood.

In a study, by isolating parietin from Rheum ribes L, it was obtained in different concentrations ranging from 1-100 µM and the cytotoxic effect of Parietin on Human Dermal Fibroblast cells was determined by MTT method. Concentrations in the range of 5-250 µM were chosen for parietin, and doses higher than 50 µM at 24 hours were found to have cytotoxic effects [11]. In our study, however, we found the IC_{50} value of parietin in the SH-SY5Y NB cells at a lower concentration to be 35 µM at the 48th hour.

In another study, the cytotoxic effect of parietin on cisplatin-resistant BRCA2-mutated human breast cancer (HCC1428), human breast ductal carcinoma (T-47D), and (HUVEC) cells were investigated and its effect on ROS accumulation and caspase 3 activation were determined.

Cell viability assays were performed by AlamarBlue method. The experiments, performed with parietin the calculated IC_{50} values of which for 48 hours treatment on the cells were higher than 600 µM, showed parietin to have less cytotoxic effects on healthy HUVEC cells than HCC1428 and T-47 D cells depending on concentration and time. Parietin showed significant anti-angiogenic and apoptotic activity at low concentrations, which are less or equal to 100 µM [12]. This result is in direct proportion to our study result.

Based on the results obtained from this study, it can be stated that it has been proven that parietin can be used in cancer treatment.

In the one study, the cytotoxic effect of Parietin isolated from Rheum ribes L. plant on the HepG2 cell line was examined. HepG2 liver cancer cells were cultured under appropriate conditions and incubated for 24 to 48 hours by applying parietin at concentrations ranging from 25-1000 µM to the cells. The cell viability rate was determined as 25 µM at 48th hour by XTT method depending on dose and time [13]. The results are also in direct proportion to those obtained in the present study. We determined IC50 value of parietin in the SH-SY5Y cells as 35 µM using XTT viability assay. In another study, parietin was isolated from Xanthoria Parietina (L.). The effect of parietin ethanolic extract (XpE) on mouse skin fibroblast cells L929 was investigated. The cell viability test was performed using the XTT method and the IC50 value in the L929 cell lines was determined to be 768.01 mg/mL [14].

In another study, the anti-proliferative and cytotoxic effects of the lichen compounds (parietin, atranorin, usnic acid, and gyrophoric acid) on nine human cancer cell lines (A2780, HeLa, MCF-7, SK-BR-3, HT-29, HCT-116 p53(+/+), HCT-116 p53(-/-), HL-60 and Jurkat) were evaluated. Parietin was obtained from Xanthoria Parietina.

The cell viability was tested by MTT analysis. In the cytotoxicity analysis performed depending on the dose and time, the IC50 value of the parietin was determined as 50 µM. Other cell lines were found to be parietin-resistant [15]. Excessive glutamate release causes prolonged activation of glutamate receptors, leading to excitotoxicity. Excitotoxicity is a process by which nerve cells are damaged due to their overstimulation. In another study, the neuroprotective effects of parietin in primary cortical neuron cultures against glutamate toxicity were investigated. It was shown that parietin increases cell viability/ migration and speed up wound healing [16]. The most significant increase was observed at 5-10 µM parietin concentrations by 92% and 96.87%, while the least increase was observed at 500 µM parietin concentration by 67% [16].

In a study, the neurotoxicity and molecular mechanism of Difenoconozal in the SH-SY5Y cell line were evaluated in vitro. Difenoconozal is a triazole fungicide. The IC50 value of Difenoconozal in SH-SY5Y NB cells was found to be 55.41 µM at the 24 h exposure. Difenoconozal has been shown that it can cause apoptosis of SH-SY5Y cells [17].

The cytotoxic effects of vulpinic acid, one of the lichen metabolites, on CaCo2, HepG2, Hep2C, RD Wehi cancer lines and normal cells and the gene expression levels were investigated. It was shown that the IC50 value of vulpinic acid changed the mRNA levels of Bax, Bcl2, and P53 genes in the cancer cells being examined, and the increase in Bax gene expression was more than the Bcl-2 and P53 genes in all cell lines [18].

Usnic acid is a secondary metabolite of lichens like parietin, and it has been used in many studies. The effects of usnic acid on lung cancer cell lines A549 were investigated. The expression levels of the genes (1-10-50 µg/L) in usnic acid at different concentrations were evaluated by Reverse Transcriptase Polymerase Chain Reaction method.

ß-actin was used as a control while determining the expression levels of APOPT1, cytochrome C, APAF1, CASPASE3, CASPASE9, TNF, BCL2, BCL2L1, and AIFM1 genes.

It was obtained from the analysis that usnic acid at concentration of 1 µg/mL showed no significant effect compared to ß-actin, except for CASPASE3. APOPT1, cytochrome C, APAF1, CASPASE3. CASPASE9 genes were found to have higher gene expression results at 10 µg/mL and 50 µg/mL concentrations of usnic acid compared to ß-actin. It was observed that there were no significant differences in the RT-PCR analyzes of TNF, BCL2, BCL2L1 and AIFM1 [19].

In another study, it was found that olivetol at the doses of 50 and 100 µM decreased MMP2 and MMP9 gene expressions of SH-SY5Y NB cell line [20]. ß-actin has been used as a control to compare data from the experimental groups and gene expression analysis evaluated by RT-PCR method.

Considering the gene expression studies, it is understood that there is limited information about Parietin. Considering the results of the studies related to parietin-like lichen metabolites, significant differences have been observed in the results regarding BAX, BCL-2, P53, MMP2, MMP9, CASPASE3, and CASPASE9 genes in direct proportion to our study results.

Usnic Acid, atranorine, physodic acid, ramalin, and physciosporin are secondary metabolites obtained from lichens such as Parietin. The migration and invasion analyze of these metabolites were performed on various cell lines. The results from the experiments results, performed on lung, liver, colon, breast, and prostate cancer cell lines, have shown that the metabolites suppress migration and invasion in these cancer lines [21].

Parietin is also a secondary metabolite and we showed it to likewise suppress migration, invasion formation when compared with our results on the SH-SY5Y NB cells.

Conflict of interest: No conflict of interest was declared by the authors.

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Authors' contributions to the article

Y.D. have constructed the main idea and hypothesis of the study. M.S. and L.E. developed the theory and edited the material and method section. M.S. and G.G. have done the evaluation of the data in the results section. A.C. and N.S.G. have done the evaluation of the Discussion section of the article written by Y.D., A.C., N.S.G. and G.G. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.