Research Article

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Antitumor Activity of Etoposide, Puerarin, Galangin and Their Combinations in Neuroblastoma Cells

Cagatay Oltulu[1]*, Melek Akıncı[2], Elvan Bakar[3]

ABSTRACT

In this study we aimed that the evaluation of the mRNA expression levels of apoptosis-related genes by treatment with Etoposide, a topoisomerase 2α inhibitor, galangin and puerarin, plant-based antioxidants, in neuroblastoma *in vitro*. The effects of etoposide, galangin, puerarin and combinations of etoposide+galangin and etoposide+puerarin on neuroblastoma and astrocyte cells' apoptotic process were examined. IC_{50} dose was determined by MTT test in neuroblastoma and healthy astrocyte lines from *Mus musculus*. Expressions of apoptosis-related gene topoisomerase 1 and 2α, caspase 3, caspase 9, BAX, BCL-2, IL-1β, TNFα, p53 were observed in astrocyte and neuroblastoma cells at the dose of neuroblastoma IC_{50}. The neuroblastoma IC_{50} dose was lower than the healthy astrocyte cell IC_{50} dose in all groups. mRNA expression of apoptosis-related genes increased in the neuroblastoma cancer line. The mRNA expression changes in the astrocyte cell line did not cause apoptosis. Antiproliferative effect of etoposide+galangin and etoposide+puerarin combinations were decreased relative to etoposide group. We concluded that single therapy of galangin and puerarin may be promising in the treatment of neuroblastoma.

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Introduction

Neuroblastoma is a solid tumor originating from the peripheral sympathetic system mostly seen in children less than 5 years of age [1, 2]. It is the most common extracranial tumors in childhood [3]. In the treatment of neuroblastoma, such methods like chemotherapy, radiation therapy, surgery, immunotherapy, and stem cell transplantation are used. While better treatment results are obtained for those in the low and medium risk groups, the long-term survival rate in the high-risk group neuroblastoma is 50%. Therefore, there is a need to develop new treatment methods [4].

Topoisomerase 1 and 2 are essential for cell survival. It is involved in DNA metabolism and regulation of its topological structure and inhibition of these enzymes creates adverse

[1] Trakya University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology Edirne, Turkey.
[2] Trakya University, Faculty of Pharmacy, Department of Pharmacology, Edirne, Turkey.
[3] Trakya University, Faculty of Pharmacy, Department of Basic Phamaceutical Sciences, Edirne, Turkey.
*Corresponding Author: Cagatay Oltulu, e-mail: cagatayo@trakya.edu.tr

effects on cell viability [5]. Topoisomerase 1 disrupts the structure of the DNA by destructing single strand and topoisomerase 2 disrupts the structure of the DNA by destructing double strands to regulate the topological structure of DNA [5]. Etoposide, known to be a topoisomerase 2 inhibitor, belongs to the class of epipodophyllotoxin [6]. Etoposide acts on topoisomerase 2, forming a covalent complex between topoisomerase 2 and DNA [1]. Etoposide inhibits the catalytic activity of topoisomerase 2, creates DNA damage and by impairing DNA metabolism, disrupts transcription and replication. Since etoposide forms a DNA complex with a high amount of topoisomerase 2, it converts topoisomerase 2, an essential enzyme, into a toxic compound. Cells that express topoisomerase 2 at a higher level are more sensitive. The cell may develop resistance against topoisomerase 2 inhibitor drugs by reducing topoisomerase expression [6].

Apoptosis is a form of programmed cell death of damaged and unwanted cells to prevent the formation of substances that will damage the surrounding tissue. Induction of apoptosis is one of the ways used in cancer treatment. The damaged cell identifies the DNA damage caused by topoisomerase 2-DNA complex created by etoposide and it is eliminated via apoptosis [7]. p53 protein can contribute to DNA repair, cell cycle arrest, or apoptosis development [8]. In case of DNA damage, the tumor suppressor gene p53 tries to repair the damage by stopping the cell cycle in G1 phase, and apoptosis occurs if the repair is not successful [9]. Apoptosis can be triggered in two ways. The first one is through an intrinsic pathway. The B-cell lymphoma 2 (BCL-2) protein is antiapoptotic and keeps the cell alive by regulating the potential of the mitochondria outer membrane. Proapoptotic proteins such as BCL-2 associated X (BAX) are normally inactive. Due to various signals, antiapoptotic proteins are suppressed by proapoptotic proteins. BAX protein, which is kept inactive by BCL-2, gets activated with other proapoptotic proteins, leads changes in membrane potential. This leads to activation of initiator caspases such as caspase 9 and effector caspases such as caspase 3, causing to the breakdown of cellular proteins and ultimately apoptosis. The second way is the extrinsic pathway. Apoptosis cascade from the extrinsic pathway is activated by the activation of death receptors such as Tumor Necrosis Factor (TNF) located on the cell surface.

Galangin is reported as plant-based antioxidants induce apoptosis in cancer cells [10, 11]. Galangin is a flavonol, a type of flavonoid, which is antiproliferative, antimutagenic, anticlastogenic, antigenotoxic, antioxidant, neuroprotective and has free radical capturing

quality [10-13]. It is stated that it induces apoptosis of hepatocellular carcinoma cells (HEPG2) in the melanoma cell line [12, 14]. It is also reported that galangin inhibits cell progression in retinoblastoma and induces apoptosis by activating protein of human phosphatase and tensin homolog (PTEN) and caspase 3 pathways [15]. The puerarin found in Pueraria is an isoflavonoid. It is reported that it has antioxidant and estrogenic activity, anti-inflammatory, antidiabetic, and anticancer effects [16, 17].

We hypothesized that combination of etoposide, galangin, puerarin and galangin+puerarin combination with etoposide may have apoptotic and antiproliferative effect on neuroblastoma cell line while do not have apoptotic effect at same dose on healthy astrocyte cell line. The aim of our study was to investigate the effects of etoposide, galangin, puerarin and their combinations with etoposide for 24 hours on apoptotic process in neuroblastoma line and in healthy astrocyte cell lines at the dose of neuroblastoma IC_{50}.

Material and Methods

Groups

The study was planned in six groups as control, etoposide, galangin, puerarin, etoposide+galangin combination and etoposide+puerarin combination.

Chemicals

HAMS F 12 (318-010-CL), Eagle's Minimum Essential Medium (EMEM) (320-026-CL) and trypsin/EDTA (325-542-EL) from Multicell (Wisent Bioproducts, St-Bruno, QC, Canada); Dulbecco's modified Eagle's medium (DMEM) (320-026-CL), L-glutamine (Gibco 25030081), fetal bovine serum (FBS) (Gibco 26140079) and penicillin-streptomycin (Gibco 15070063) from Gibco (Thermo Fisher Scientific, Waltham, MA, USA); Thiazolyl Blue Tetrazolium Bromide (MTT) were obtained from Biocompare (New York, USA). Etoposide (Sigma E1383), galangin (Sigma 282200) and puerarin (Sigma 82435), was obtained from Sigma (St. Louis, MO, USA). Phosphate buffered saline (PBS) (Merck 524650) and dimethyl sulfoxide (DMSO) (Merck 67-68-5) were taken from Merck-Millipore (Darmstadt, Germany). The PureLink RNA Mini Kit (121-830-18A) was supplied from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA); SYBR Select Master Mix and High Capacity cDNA Reverse Transcription Kit (8368814) from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture

Mus musculus brain neuroblastoma cell line (N1E-115; ATCC® CRL-2263 ™) and healthy *Mus musculus* brain astrocyte cells (C8-D1A; ATCC® CRL-2541 ™) 5% heat-inactivated fetal bovine serum (FBS); nutrient medium contains 100 IU/ml penicillin, 10 mg/ml streptomycin and 1% L-glutamine, 1:1 ratio of Eagle's Minimum Essential Medium (EMEM), DMEM, HAMS F12 are seeded in flasks and they are placed in the incubator that contains 95% moisture and 5% CO_2 at 37 °C. Our study started in the 5th passage and ended in the 12th passage.

Determination of substance concentrations to be administered to cell lines by MTT method

In order to determine IC_{50} values of all groups to be used in the study, 180 µL cells were seeded in 96 well plates to have $1x10^6$ cells in each well, and they were left to incubate for 24 hours to enable the cells to adhere onto the plate wells. Etoposide, galangin, puerarin were prepared as an aqueous solution containing 0.01% DMSO. Combinations of etoposide+galangin and etoposide+puerarin were prepared in a 1:1 ratio (v/v). All substances were administered to all groups except for the control group, in a volume of 20 µl as 1.25 µM, 2.5 µM, 5 µM, 10 µM, 20 µM and were left in the incubator (37 °C, 5% CO_2) for 24 hours. An aqueous solution containing 0.01% DMSO was applied to the control group. After 3 hours, 200 µl of 0.01% DMSO was added to dissolve the formazan crystals and the absorbance value was determined at 492 nm with the microplate reader (Thermo Scientific Multiskan Go). The control group was considered 100% alive and the IC_{50} dose was calculated by probit analysis. MTT test was run in four replicates in all groups.

RNA isolation and cDNA synthesis

Neuroblastoma and astrocyte cells were seeded 3 times in culture plates to have $3x10^6$ cells in each well. After 24 hours, the chemical applications of the experimental groups were administered at the dose of neuroblastoma IC_{50} for 24 hours. RNA isolation from the obtained cells was done with PureLink RNA Mini Kit according to the manufacturer's instructions. Concentrations and purity values of the obtained RNA samples were determined with nanodrop (NaNoQ OPTIZEN). cDNA synthesis was carried out from RNA samples, according to the manufacturer's instructions with the High Capacity cDNA Reverse Transcription Kit.

qRT-PCR analysis

qRT-PCR analysis of gene expressions of the cells associating with topoisomerase 1, topoisomerase 2α, caspase 3, caspase 9, BCL-2, BAX, Interleukin 1β (IL-1β), Tumor Necrosis Factor Alpha (TNFα) and p53 was performed with Quant Studio 6 Flex device of SYBR Select Master Mix. PCR conditions are determined as; 1 cycle is 2 minutes at 50 °C, 10 minutes at 95 °C, afterwards 50 cycles for denaturation are 15 seconds at 95 °C, and 1 second at 60 °C for annealing and extension. mRNA expression levels were analyzed by comparative cycle threshold (2-ΔΔCt) method (User Bulletin 2, Applied Biosystems). To obtain a copy of the Topoisomerase 1 and Topoisomerase 2α gene sequences: was selected "Nucleotide" from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/guide/). After, the relevant organism/gene name was entered in the search box, and FASTA was determined and the relevant genes were designed. Gene expressions were determined as relative fold-change compared to the control group and normalized by β-actin mRNA expression (Table 1).

Table 1. Primer sequences of analyzed genes for qRT-PCR analysis

Gene	**Primer sequences (Forward/Reverse)**	
Topoisomerase 1	5’-TCATACTGAACCCCAGCTCC-3’ 5’-GTCCTGCAAGTGCTTGTTCA-3’	
Topoisomerase 2α	5’-CTTCTCTGATATGGACAAACATAAGATTCC-3’ 5’-GGACTGTGGGACAACAGGACAATAC-3’	
p53	5’-CACGAGCGCTGCTCAGATAGC-3’ 5’- ACAGGCACAAACACGCACAAA-3’	[18]
Caspase 3	5’-GGTATTGAGACAGACAGTGG-3’ 5’-CATGGGATCTGTTTCTTTGC-3’	[19]
Caspase 9	5'-GAGTCAGGCTCTTCCTTTG-3’ 5’-CCTCAAACTCTCAAGAGCAC-3’	[20]
BAX	5’-TTCATCCAGGATCGAGCAGA-3’ 5’-GCAAAGTAGAAGGCAACG-3’	[21]
BCL-2	5’-ATGTGTGTGGAGAGCGTCAA-3’ 5’-ACAGTTCCACAAAGGCATCC-3’	[22]
TNFα	5’-TCAGCCTCTTCTCC-3’ 5’-TCAGCTTGAGGGTT-3’	[23]
IL-1β	5’-GCACGATGCACCTGTACGAT-3’ 5’-CACCAAGCTTTTTTGCTGTGAGT-3’	[24]
β-actin	5’-AGAGCTACGAGCTGCCTGAC-3’ 5’- AGCACTGTGTTGGGCGTACAG-3’	[25]

Immunofluorescence assay

Cell Viability Imaging Kit Caspase 3/7 staining was utilized to determine the morphological changes induced by etoposide, galangin, puerarin and combinations to neuroblastoma (N1E-115) cells. To evaluate the apoptotic activity, cancer cells which were plated at 1×10^5 cells/well into a 24-well chamber plate, and cells were treated with substances and incubated for 24 h. After 24 h of incubation, cells were washed with PBS and stained by Cell Viability Imaging Kit Caspase 3/7 according to manufacturer instruction. To decide the cell death, the cells were seeded in 24-well plates and treated IC_{50} of the concentration of formulations. The morphological change was investigated from stained dead, live and apoptotic cells under a fluorescent microscope (Carl Zeiss, Axio Observer, Germany).

Statistical analysis

IC_{50} value was calculated by applying probit analysis to percent viability data obtained by MTT test. After application of the neuroblastoma line at IC_{50} doses for 24 hours to the astrocyte and neuroblastoma cells, one-way ANOVA test, post hoc Tukey were administered to the relative fold-change values of gene expressions. Values of $p<0.05$ was considered significant. Probit analysis and ANOVA test were done with SPSS 20 software (IBM).

Results

IC_{50} doses were obtained by applying probit analysis to the results obtained after 24 hours exposure to the N1E-115 neuroblastoma cell line (Fig. 1). IC_{50} doses were determined as 3.75 μM in etoposide, 3.75 μM in galangin, 5.26 μM in puerarin, 5.81 μM in etoposide+galangin, 17.54 μM in etoposide+puerarin. In the MTT test conducted to examine the effects of the dose range applied in neuroblastoma cells on the healthy cell, IC_{50} doses for astrocyte were determined as 14.16 μM in the galangin group and 17 μM in the etoposide+galangin group, while IC_{50} dose for astrocyte was higher than our tested dose scale in the other groups.

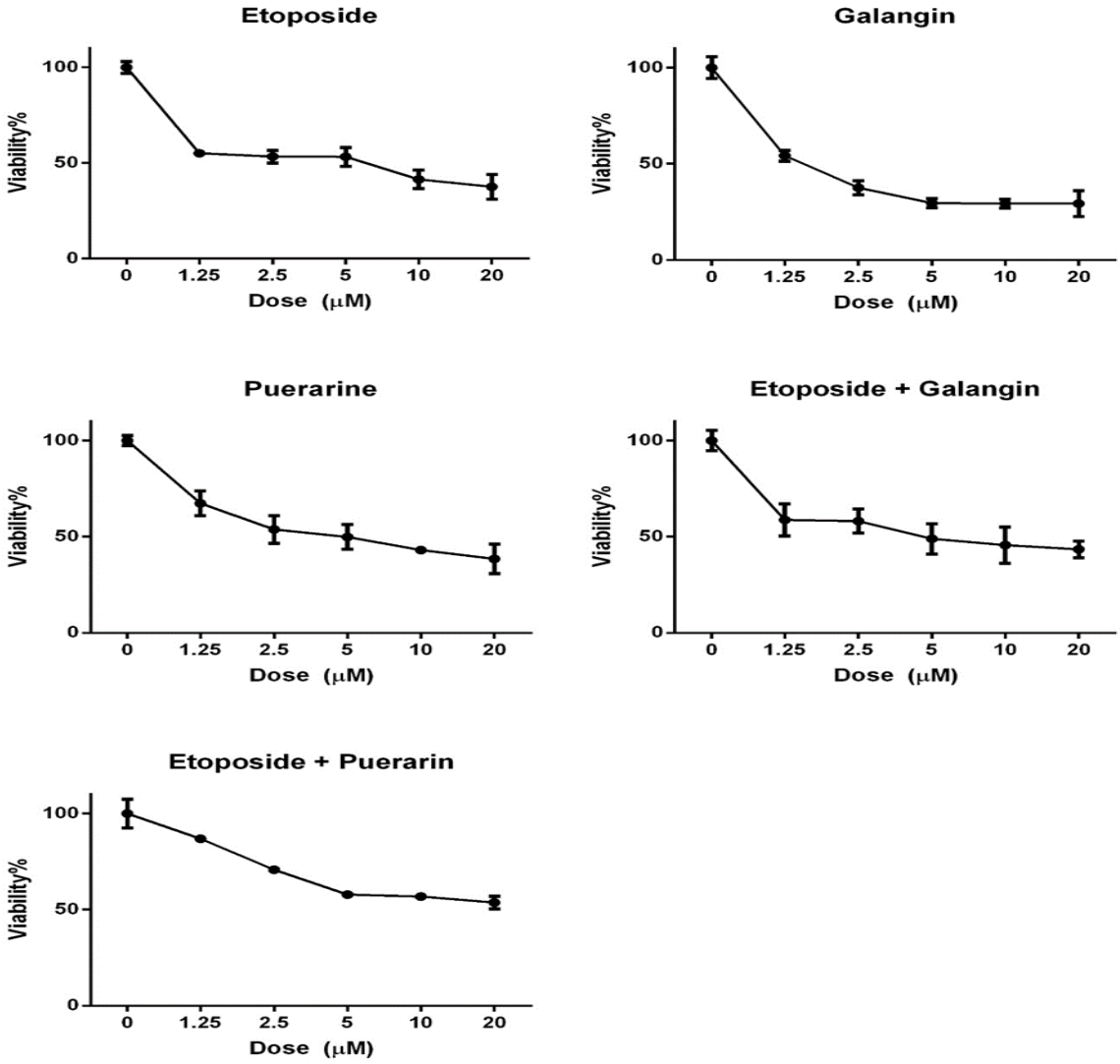

Fig 1. MTT assay results of each treatment group. Vertical bars represent standard deviation. (n=4, mean ±std dev.) (viability%=sample absorbance average/control absorbance average×100)

qRT-PCR

The statistical significance of fold-changes of mRNA expressions of topoisomerase 1, topoisomerase 2α, caspase 3, caspase 9, BAX, BCL-2, IL-1β, TNFα, p53 in neuroblastoma and astrocyte cell lines were evaluated in all groups by comparing with control group (Fig. 2). It was observed that expression of topoisomerase 1 in the astrocyte line increased in the puerarin group compared to both the control and the etoposide group (Fig. 2A). A decrease of the expression of topoisomerase 1 on the neuroblastoma line was detected in the etoposide, galangin and etoposide+galangin groups compared to the

control group. It was determined that enzyme expression in the neuroblastoma line was increased in the puerarin and etoposide+puerarin groups compared to the etoposide group.

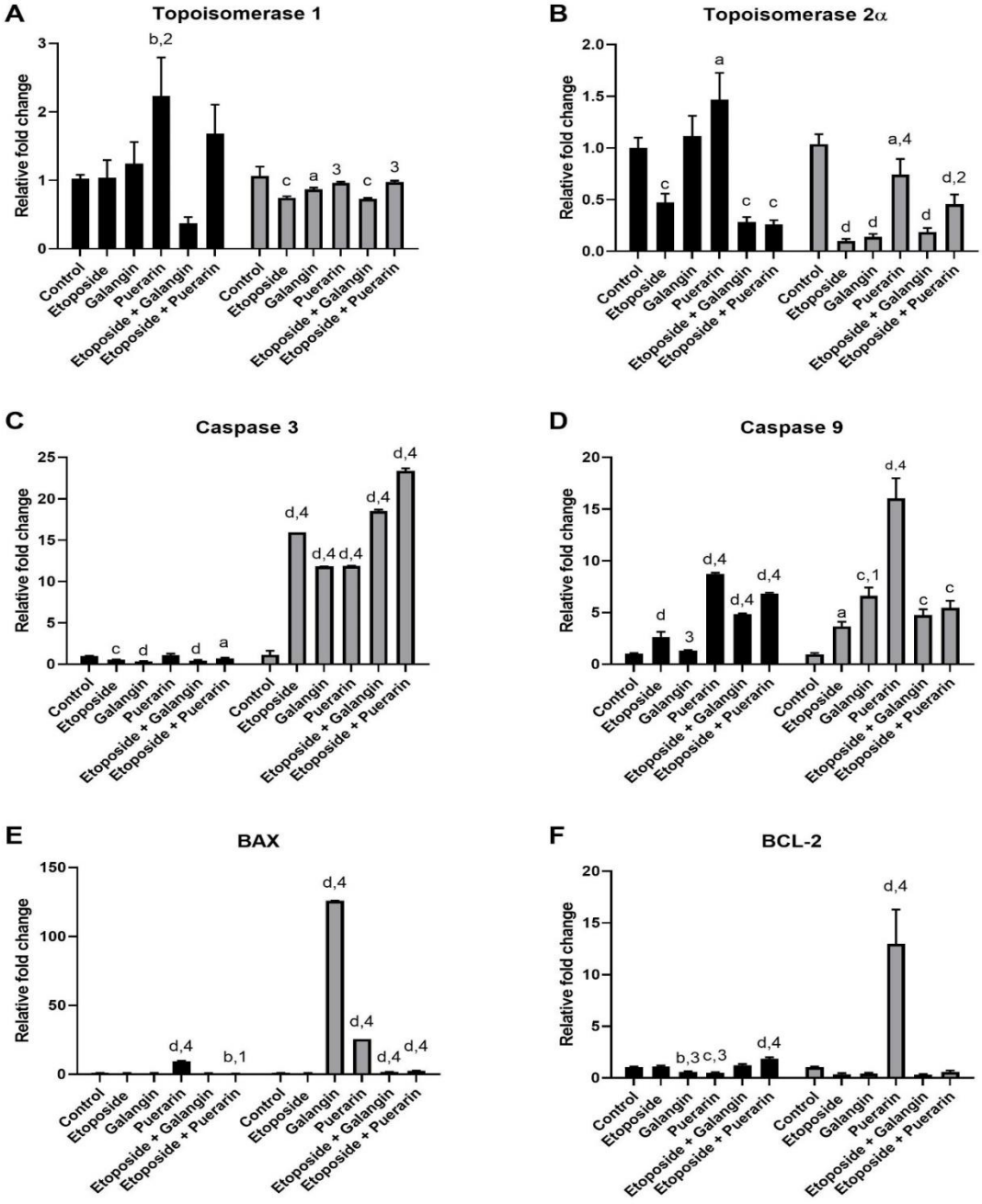

Fig 2. On N1E-115 Neuroblastoma and C8-D1A Astrocyte cell lines, the mRNA expression of topoisomerase 1 (A), topoisomerase 2α (B), caspase 3 (C), caspase 9 (D), BAX (E), BCL-2 (F) genes have been shown as relative fold-change. Black columns represent astrocyte cell line grey columns represent neuroblastoma cell line. (a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$, d: $p < 0.0001$ compared with the control group; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$, 4: $p < 0.0001$ compared to the etoposide group; vertical bars show standard deviation; one-way ANOVA post hoc Tukey, $p < 0.05$)

Expression of topoisomerase 1 was significantly increased in the astrocyte cell line Puerarin group compared to the control group. Expression of topoisomerase 1 in the neuroblastoma cell line was decreased in etoposide, galangin and etoposide+galangin groups compared to the control group. Expression of topoisomerase 1 in neuroblastoma cell line was increased in puerarin and etoposide+puerarin groups compared to etoposide group.

Expression of topoisomerase 2α on the astrocyte cell line decreased in etoposide, etoposide+galangin and etoposide+puerarin groups and increased in the puerarin group compared to the control group (Fig 2B). Topoisomerase 2α expression was decreased in all groups relative to the control group in neuroblastoma cell lines. On neuroblastoma cell lines, increase of Topoisomerase 2α expression was determined in the control, puerarin and etoposide+puerarin groups compared to the etoposide group.

The expression of caspase 3 decreased in all groups except puerarin group relative to the control group in the astrocyte cell line but increased in all groups relative to the control group in the neuroblastoma cell line (Fig. 2C). The expression of caspase 3 increased in the etoposide+puerarin and etoposide+galangin groups in the neuroblastoma line but decreased in the galangin and puerarin groups compared to the etoposide group.

Caspase 9 expression increased in etoposide+galangin and etoposide+puerarin groups relative to the control group and expression of caspase 9 increased in both groups compared to etoposide group on the astrocyte cell line (Fig. 2D). Expression of caspase 9 increased in all groups compared to control group and caspase 9 increased in etoposide+puerarin group compared to the etoposide group in the neuroblastoma cell line.

mRNA expression of BAX decreased in the etoposide+puerarin group compared to both the control group and the etoposide group, while it increased in the puerarin group on astrocyte cell line (Fig. 2E). An increase on the neuroblastoma line was observed in galangin, puerarin, etoposide+galangin and etoposide+puerarin groups compared to the control and etoposide groups.

BCL-2 gene expression increased in the etoposide+puerarin group compared to both the control group and the etoposide group while it decreased in the galangin and puerarin group in the astrocyte cell line (Fig. 2F). BCL-2 gene expression increased only in the puerarin group in neuroblastoma cell line when compared to the control and etoposide group.

IL-1β gene expression increased in all groups relative to the control group in the astrocyte cell line (Fig. 3A). IL-1β gene expression decreased in all groups relative to control group but increased when compared to the etoposide group and etoposide+puerarin group in the neuroblastoma cell line.

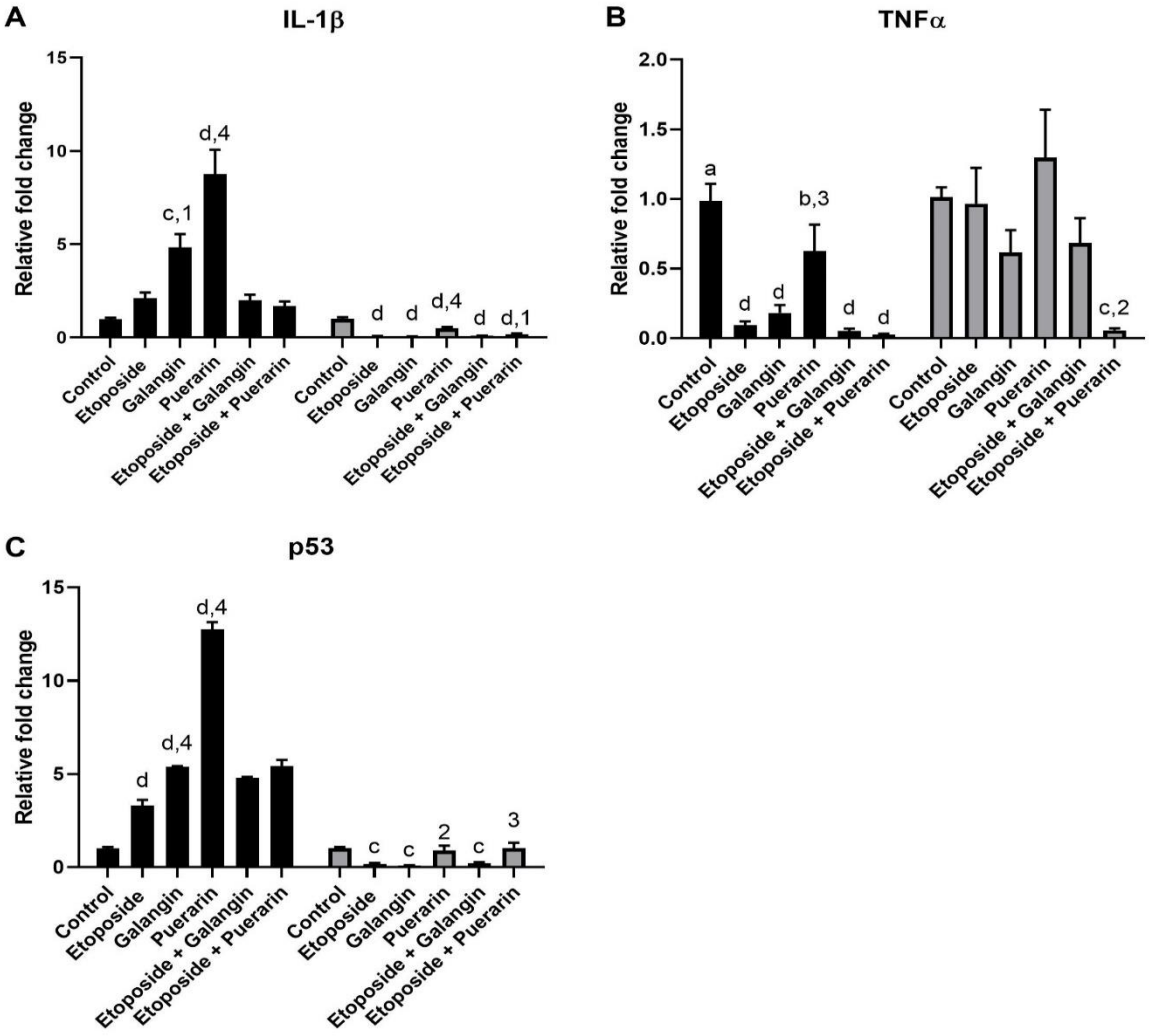

Fig 3. On N1E-115 Neuroblastoma and C8-D1A Astrocyte cell lines, the mRNA expression of IL-1β (A), TNFα (B), BAX (C), p53 (D) genes have been shown as relative fold-change. Black columns represent astrocyte cell line grey columns represent neuroblastoma cell line. (a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$, d: $p < 0.0001$ compared with the control group; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$, 4: $p < 0.0001$ compared to the etoposide group; vertical bars show standard deviation; one-way ANOVA post hoc Tukey, $p < 0.05$)

TNFα expression decreased in all groups relative to the control group in the astrocyte cell line (Fig. 3B). TNFα expression also decreased in the etoposide+puerarin group compared to the control group in the neuroblastoma cell line.

p53 expression increased in all groups relative to the control group (Fig. 3C) also in all groups except the control group when compared to the etoposide group in the astrocyte cell line. p53 gene expression decreased in the etoposide and etoposide+galangin groups compared to the control group in the neuroblastoma cell line.

Immunofluorescence assay

Since cell viability in MTT results is significantly inhibited by etoposide, galangin, puerarin, etoposide+galangin, and etoposide+puerarin, it is critical to classify which cell death is induced in N1E-115 cells. Therefore, an immunofluorescence staining experiment was conducted to determine the type of cell death induced by the administration groups. Apoptotic cells were determined in the viable part of the cell population with treatment groups for 24 hours (Fig. 4). In the immunofluorescence examination, it was observed that the cells in the N1E-115 cell line control group preserved their morphological integrity and were alive. It was observed that the cells in the N1E-115 cell line etoposide, puerarin, galangin, etoposide+galangin and etoposide+puerarin groups could not preserve their integrity, their cytoplasm stained caspase 3/7 positively and the cells were dead (Fig. 4).

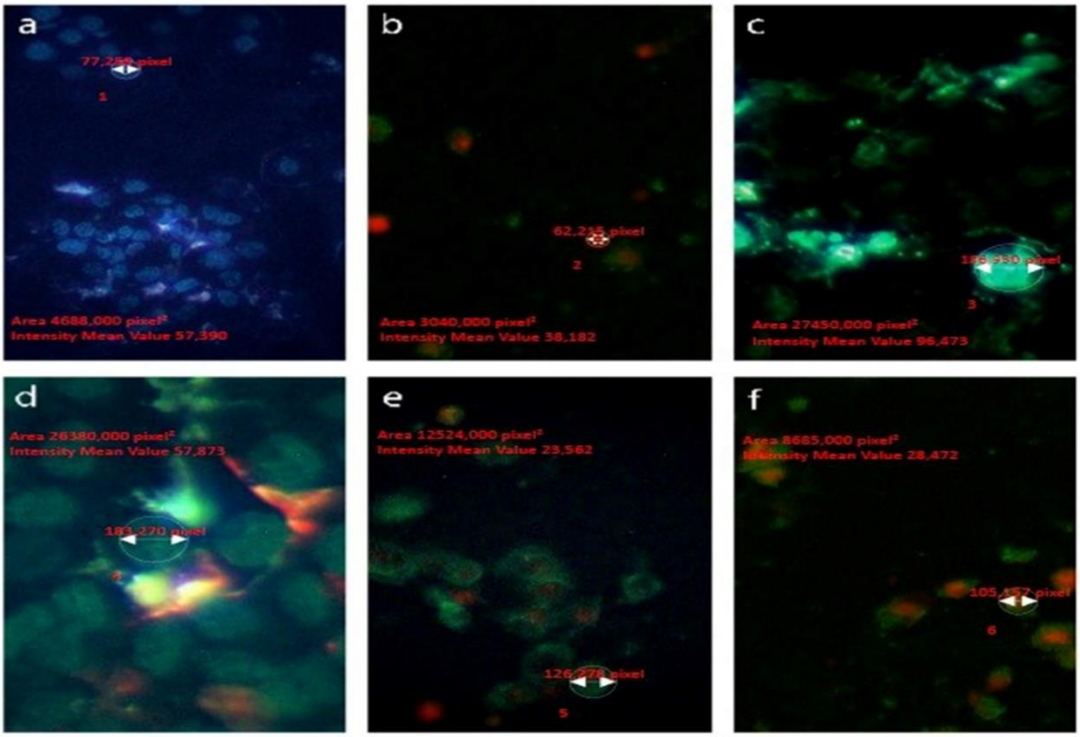

Fig 4. Immunofluorescent staining of control (a), etoposide (b), galangin (c), puerarin (d), etoposide+galangin (e), etoposide+puerarin (f) groups of neuroblastoma (N1E-115) cells by cell viability imaging kit caspase 3/7. (Blue: live cell; Red: dead cell; Green: caspase 3/7 positive)

Discussion

Etoposide is an antineoplastic drug as a topoisomerase 2 inhibitor. Galangin and puerarin are antiproliferative plant-based antioxidants that induce apoptosis in cancer cells [10, 11]. Accordingly, we hypothesized that etoposide and galangin/puerarin combinations with etoposide may have an antiproliferative and apoptotic effect on the neuroblastoma cell line when it is in a non-apoptotic level on a healthy astrocyte cell line in this study. Therefore, we investigated the effects of etoposide, galangin, puerarin and their combinations with etoposide on the mRNA expression of genes which are involved in apoptotic process on neuroblastoma and healthy astrocyte cell lines. The effects of drug administrations at the dose of neuroblastoma IC_{50} on apoptotic process of healthy astrocyte cell line were also examined in our study.

It was observed that topoisomerase 2α gene expression decreased in the etoposide (3.75 μM) group on the neuroblastoma and astrocyte cell line. The reduction in topoisomerase 2α gene expression can be explained by an improved resistance mechanism against anticancer agents acting on the mechanism of non-separation of the topoisomerase-DNA complex. This result obtained from our study is in line with other studies [6, 26]. It is a remarkable finding that the gene expression on the neuroblastoma line significantly decreases while overexpression of p53, the tumor suppressing gene, is observed on the astrocyte line. If astrocyte cell IC_{50} value of etoposide was not determined between administered dose range, this indicates that the IC_{50} value is higher than the administered dose and the decrease of the caspase 3 gene expression shows that etoposide has no effect on apoptotic death at the dose administered in healthy astrocyte cell. It was determined that it caused significant overexpression of caspase 3 in the neuroblastoma line. This result shows that the apoptosis signal pathway is activated. It was determined that p53 decreased significantly in this cell line. The significant increase in caspase 9 expression in the neuroblastoma line indicates that apoptosis occurs through the mitochondrial pathway. Depending on the damage to the mitochondrial membranes in the neuroblastoma line cells, the release of cytochrome c (cyc-c) may have initiated the caspase cascade and thereby leaded the cell into apoptosis through the mitochondrial pathway. However, it was determined that IL-1β expression did not change in the astrocyte line and decreased significantly in the neuroblastoma line. At the same time, TNFα gene expression decreased significantly in the astrocyte line, while no difference

was observed on the neuroblastoma line. Reduction of TNFα expression can be interpreted that apoptosis is not triggered through the Extrinsic pathway on the astrocyte cell line. The fact that the etoposide causes apoptosis in the neuroblastoma cell line, not in the astrocyte line, shows its selective effect.

It was determined that IL-1β, p53 and caspase 9 gene expressions increased and caspase 3, BCL-2, TNFα expressions decreased as a result of the administration of galangin (3.75 μM) to the astrocyte cell line. The increase of caspase 9 on the astrocyte cell line depending on the administration of galangin can be considered as the cell's attempt to regulate balance with cytoprotective autophagy. Similarly, with the administration to the neuroblastoma cell line, it was determined that caspase 3, caspase 9, BAX expressions increased but IL-1β, p53, topoisomerase 1 and 2α expressions decreased. Increased tumor suppressor gene p53 mRNA expression in the astrocyte line and also decreased caspase 3 expression indicate that the apoptosis signal pathway does not cause apoptosis on the astrocyte line. The increase in caspase 3, caspase 9, BAX gene expressions in the neuroblastoma line and decrease in p53 indicate that apoptosis is stimulated. In a study conducted on the HEPG2 cell line, it was reported that cell death occurred via autophagy rather than apoptosis with the administration of galangin at 130 μmol/L, whereas when it was administered at 370 μmol/L dose, cell death occurred via apoptosis [27]. In a study on breast cancer, galangin was shown to increase BAX expression and decrease BCL-2 expression, and this result was found to be compatible with our findings [28]. It is demonstrated that the administration of galangin to hepatocellular carcinoma cells (HEPG2), by binding of the proapoptotic protein BAX to the mitochondria membrane, caused the apoptosis-inducing factor and cyc-c to be released into the cytosol [12], and caused apoptosis on B16F10 melanoma line but does not cause any changes on BAX [14]. In the present study, the observation of the increase in BAX gene expression was interpreted as the difference of cell lines. There is no study in the literature about the effect of galangin on neuroblastoma cell line.

In the etoposide+galangin (5.81 μM 1:1) combination group, mRNA expressions of tumor suppressor gene p53 and caspase 9 increased but topoisomerase 1, topoisomerase 2α, caspase 3, and TNFα decreased in the astrocyte cell line. However, caspase 3, caspase 9 and BAX gene expressions were significantly increased in the neuroblastoma line. The significant increase of caspase 3, caspase 9 and BAX gene expressions that took place

with the etoposide+galangin administration in the neuroblastoma cell line may be interpreted as apoptosis triggered via mitochondrial pathway. It can be evaluated that the increase in caspase 9 gene expression may depend on the stress of endoplasmic reticulum (ER). The significant decrease of p53 can be interpreted as apoptosis may have occurred independently from p53. Depending on the disruption in the mitochondrial membranes on the neuroblastoma cells, the cyc-c release may have initiated the caspase cascade and thus leaded the cell via the mitochondrial pathway into apoptosis.

While IL-1β expression did not change in the astrocyte line but a significant decrease was observed in the neuroblastoma line. It was observed that TNFα gene expression was significantly decreased in the astrocyte line but not changed in the neuroblastoma line. Reduction of TNFα expression on the astrocyte cell line can be interpreted as apoptosis is not triggered from the extrinsic pathway on the astrocyte cell line. We could not find any study investigating the effect of etoposide and galangin combination in neuroblastoma cell line. Therefore, we think that the findings of our study have a significant contribution to the future study.

Increased BCL-2 and p53 expressions and decreased BAX expressions in astrocyte cell line in consequence of etoposide+puerarin (17.54 μM 1:1) administration can be evaluated as the protective effect of puerarin. Similarly, it was reported that puerarin has a protective effect in astrocyte cells and reduces BAX expression depending on concentration [29]. In the literature, cytotoxicity studies in which the combination of etoposide and puerarin were administered were not found. The significant increase of caspase 3, caspase 9, BAX gene expression that took place with the etoposide+galangin administration in the neuroblastoma cell line may be interpreted as apoptosis triggered via mitochondrial pathway. Compatibly to our study, in another study conducted on human lung adenocarcinoma cell line, puerarin was found to induce apoptosis via mitochondrial pathway [30]. It was also considered that caspase 9 gene expression may have increased due to ER stress. In a study on human colorectal cancer line, the increase in BAX, decrease in BCL-2 and increase in caspase 3 activation are in line with our results [31]. In another study, it was stated that administration of puerarin inhibits proliferation in the bladder cancer T-24 cell line, and apoptosis occurs as a result of decrease in p53 and BCL-2 and increase in BAX protein expression [32]. Decrease in BCL protein expression and increase in BAX expression were consistent with our study. While IL-1β

expression did not change on the astrocyte line, it decreased significantly on the neuroblastoma line. TNFα gene expression was found to be significantly decreased on the astrocyte line and not changed on the neuroblastoma line. Constancy of IL-1β expression and reduction of TNFα expression on the astrocyte cell line can be interpreted as apoptosis is not triggered from the extrinsic pathway on the astrocyte cell line. In immunofluorescence examination, the apoptosis and cell death table observed in cells is consistent with the changes in mRNA expression. This result indicates that death in N1E-115 cells of administration groups is due to apoptotic cell death.

Conclusion

In all groups, IC_{50} dose was lower in neuroblastoma than astrocyte. This suggests that selective effect can occur. Moreover, it shows that the antiproliferative effect is decreased in the combination groups relative to the etoposide group. Available data indicate that apoptosis is triggered by the intrinsic pathway. Etoposide combinations have decreased antiproliferative effect compared to the etoposide group. Thus, the single treatment of galangin and puerarin in neuroblastoma treatment may be promising and proper attention should be paid to the dosage. We also consider that administrations of puerarin and galangin should be evaluated in terms of autophagy and ER stress.

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