FABAD J. Pharm. Sci., 48, 3, 373-384, 2023
Doi: 10.55262/fabadeczacilik.1301973
RESEARCH ARTICLE

Evaluation of The Effect of Topotecan, Carvacrol, Epigallocatechin Gallate and Their Combination on Apoptotic Process in Neuroblastoma and Astrocyte Cells

Çağatay OLTULU[*°], Melek AKINCI[**], Elvan BAKAR[***]

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SUMMARY

Neuroblastoma is a sympathetic nervous system cancer. Neuroblastoma most commonly affects children under five years of age and is the most common solid tumor in childhood. Topotecan is a topoisomerase 1 inhibitor. Carvacrol and epigallocatechin gallate are naturally derived substances with anticancer, antioxidant, and apoptotic properties. Our study aimed to evaluate the effects of topotecan, carvacrol, epigallocatechin gallate, topotecan+carvacrol, and topotecan+ epigallocatechin gallate combinations on the apoptotic signaling pathway. IC50 values were determined in neuroblastoma and healthy astrocyte cells using the MTT assay. Apoptotic mRNA expressions (topoisomerase 1 and 2, p53, BCL2, BAX, caspase 9, caspase 3, IL1, TNFα) in astrocytes and neuroblastoma cells at the neuroblastoma IC50 dose were analyzed using quantitative real-time PCR. Topotecan and carvacrol did not exhibit selective cytotoxic effects between healthy astrocytes and neuroblastoma cells. However, we found that the combination of topotecan+ epigallocatechin gallate and topotecan+carvacrol with epigallocatechin gallate showed selective cytotoxic effects on the neuroblastoma cell line compared to astrocyte cells. The obtained mRNA results can be interpreted as the initiation of apoptosis in neuroblastoma cells in the topotecan, carvacrol, epigallocatechin gallate, and topotecan+epigallocatechin gallate groups. Further studies are needed to investigate this matter in more detail.

Key Words: *Topotecan, carvacrol, epigallocatechin gallate, apoptosis, cytotoxicity*

Topotekan, Karvakrol, Epigallokateşin Gallat ve Kombinasyonlarının Nöroblastom ve Astrosit Hücrelerinde Apoptotik Süreç Üzerine Etkisinin Değerlendirilmesi

ÖZ

Nöroblastom, sempatik sinir sistemi kanseridir. Genellikle 5 yaş altındaki çocukları etkiler ve çocukluk çağının en yaygın solid tümörüdür. Topotekan, topoizomeraz 1 inhibitörüdür. Karvakrol ve epigallokateşin gallat ise doğal kaynaklı maddeler olup antikanser, antioksidan ve apoptotik özelliklere sahiptir. Bu çalışmanın amacı, topotekan, karvakrol, epigallokateşin gallat, topotekan+karvakrol ve topotekan+epigallokateşin gallat kombinasyonlarının apoptotik sinyal yolakları üzerindeki etkilerini değerlendirmektir. MTT testi kullanılarak nöroblastom ve sağlıklı astrosit hücrelerinde IC50 değerleri belirlenmiştir. Nöroblastom IC50 dozunda, astrosit ve nöroblastom hücrelerinde apoptotik mRNA ifadeleri (topoizomeraz 1 ve 2, p53, BCL2, BAX, kaspaz 9, kaspaz 3, IL1, TNFα) kantitatif gerçek zamanlı PCR yöntemiyle analiz edilmiştir. Topotekan ve karvakrolün, sağlıklı astrositlerle ve nöroblastom hücreleriyle arasında seçici sitotoksik etkiler sergilemediği bulunmuştur. Ancak, epigallokateşin gallat ile birlikte topotekan+karvakrol ve topotekan+epigallokateşin gallat kombinasyonlarının, astrosit hücrelerine göre nöroblastom hücre hattında seçici sitotoksik etkiler gösterdiği bulunmuştur. Elde edilen mRNA sonuçları topotekan, karvakrol, epigallokateşin gallat, topotekan+epigallo kateşin gallat gruplarında apoptozun nöroblastom hücrelerinde başlatıldığı şeklinde yorumlanabilir. Bu konuyu daha detaylı araştırmak için ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: *Topotekan, karvakrol, epigallokateşin gallat, apoptoz, sitotoksisite*

Received: 24.05.2023
Revised: 10.08.2023
Accepted: 11.08.2023

[*] ORCID: 0000-0002-6051-3479, Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Trakya University, Edirne, Turkey.
[**] ORCID: 0000-0003-3879-4232, Department of Pharmacology, Faculty of Pharmacy, Trakya University, Edirne, Turkey.
[***] ORCID: 0000-0001-5703-3469, Department of Basic Pharmaceutical Sciences, Faculty of Pharmacy, Trakya University, Edirne, Turkey.

[°] Corresponding Author: Çağatay OLTULU
Phone: +90 (284) 235 01 80, E-mail: cagatayo@trakya.edu.tr

INTRODUCTION

Neuroblastoma is a malignant tumor that develops in the sympathetic nervous system and primarily affects pediatric patients. Neuroblastoma is a tumor that originates from neural crest cells of the autonomic nervous system and is typically found in children under the age of five, although it can also occur in older individuals. Neuroblastoma is responsible for approximately 15% of pediatric cancer-related mortalities (Maris, 2010). The tumor is most commonly located in the adrenal medulla (Maris, 2010). Neuroblastoma is classified according to prognostic factors such as stage, age, and molecular changes (Ponzoni et al., 2022). Among neuroblastoma patients with high-risk metastases, 60% are diagnosed, and only 40% survive (Chaturvedi et al., 2016). Effective treatments need to be developed to treat high-risk neuroblastoma (Ponzoni et al., 2022; Qiu & Matthay, 2022).

Astrocytes are the most abundant type of glial cell in the brain, serving essential roles in supporting neuronal function, such as providing nutrients, structural support, waste clearance, and immune regulation (Salles et al., 2022). Cells of neuroblastoma originate derive from neural crest cells, which serve as precursors to neurons and glia (Oltulu, Akinci, & Bakar, 2022). We utilized the C8-D1A (Astrocyte type I clone) astrocyte cell line to evaluate the effects of topotecan, carvacrol, and EGCG on the apoptotic signaling pathway. The C8-D1A astrocyte cell line was selected for its well-characterized nature, ease of cultivation, and manipulability. Astrocyte cell lines are also commonly used as controls in studies examining neuronal cell death (Messeha et al., 2016; Mojić et al., 2014; Oltulu et al., 2022).

DNA topoisomerases regulate DNA topology for DNA replication, repair, and transcription. Topoisomerase 1 functions in the regulation of DNA topology by transiently generating a single-strand break in the absence of ATP, allowing the relaxation and passage of the DNA supercoil by the other strand (Ormrod & Spencer, 1999). In rapidly dividing cells such as neuroblastoma, topoisomerase 1 creates a single-strand break in the DNA supercoil, temporarily relaxes it by passing the other strand through the break, repairs the transient single-strand break, and dissociates from the DNA. At the same time, transcription and replication processes continue (Ormrod & Spencer, 1999). Topotecan, a camptothecin derivative, is a topoisomerase 1 inhibitor (Ormrod & Spencer, 1999). It induces a permanent single-strand break by preventing the dissociation of the DNA-topoisomerase 1 complex (Ormrod & Spencer, 1999). Topoisomerase 2, on the other hand, regulates DNA topology by creating double-strand breaks. Accumulation of this damage leads to cell death. In addition, it has been suggested that topotecan induces cell death by generating free radicals (Sinha, Tokar, & Bushel, 2020). p53 is involved in cell cycle arrest, DNA repair, or apoptosis induction. If repair is unsuccessful, the cell undergoes apoptosis. Anti-apoptotic proteins like B-cell lymphoma 2 (BCL2) promote cell survival.

Researchers have been studying naturally sourced substances for their potential antioxidant and anticancer properties. Carvacrol is a compound found in the essential oils of aromatic plants such as oregano and thyme (Baser, 2008). Carvacrol is a phenolic monoterpenoid that can be used as a food additive and has antibacterial, antifungal, antioxidant, neuroprotective, and anticancer effects (Magi, Marini, & Facinelli, 2015; Suntres, Coccimiglio, & Alipour, 2015; Undeger, Basaran, Degen, & Basaran, 2009; Yu et al., 2012). 25 μM and 50 μM carvacrol administration to KELLY and SH-SY5Y human neuroblastoma cell lines have been reported to cause antiproliferative effects in both cell lines (Calibasi Kocal & Pakdemirli, 2020). Carvacrol has been shown to induce apoptosis in colon cancer cells through the influx of calcium ions into the mitochondria by disrupting the mitochondrial membrane potential (Fan et al., 2015). There are not enough studies on the cytotoxic mechanism of action of carvacrol on neuroblastoma and astrocyte cells.

Epigallocatechin gallate (EGCG) is a polyphenolic compound with anticancer, apoptotic, chemopreventive, and antioxidant properties found in green

tea (Berger, Gupta, Belfi, Gosky, & Mukhtar, 2001; Castellano-González et al., 2016; Chakrabarti & Ray, 2016). It has been reported that EGCG modulates cytochrome oxidase activity and increases ATP production in neurons and astrocytes (Castellano-González et al., 2016). It has been shown that low-dose application of EGCG at 10-50 μM induces apoptosis in MCF-7 cells, while high-dose application leads to necrosis (Hsuuw & Chan, 2007).

Apoptosis is a natural process that occurs in all living cells. It is a tightly regulated process that ensures that cells die in a controlled manner. Apoptosis is essential for maintaining tissue homeostasis and preventing the development of cancer. Two main pathways can trigger apoptosis: the intrinsic pathway and the extrinsic pathway. In the intrinsic pathway, apoptosis is initiated through mitochondrial involvement due to reasons such as increased intracellular stress. The anti-apoptotic protein BCL2 is responsible for maintaining mitochondrial membrane potential. Pro-apoptotic proteins, including BCL2-associated X (BAX), induce disruptions in mitochondrial membrane potential and permeability. This disruption triggers the release of cytochrome c from the mitochondria into the cytoplasm, resulting from various cellular stressors and factors. As a result, apoptotic changes occur in the cell through the activation of initiator caspase, caspase 9 (CAS9), and subsequent activation of executioner caspase, caspase 3 (CAS3). The extrinsic apoptosis pathway leads to cell death through the initiation of the caspase cascade by death receptors, such as the tumor necrosis factor-alpha (TNFα) receptor located outside the cell membrane.

Topotecan, carvacrol, EGCG, and their combinations are all-natural compounds with potential anticancer activity by inducing apoptosis. However, these agents can also harm normal cells, such as astrocytes. Astrocytes are a type of glial cell that support neurons in the brain. They play an essential role in many brain functions, including neuroprotection and inflammation. The potential presence of these compounds in astrocyte cell lines raises concerns about their impact on brain astrocytes, which could result in side effects like neurotoxicity and inflammation.

Our hypothesis is that topotecan, carvacrol, EGCG, and their combinations will exhibit selective targeting effects, showing no cytotoxic results on the healthy astrocyte cell line but inducing cytotoxic impact on the neuroblastoma cell line. In our study, we aimed to evaluate the apoptotic effects of topotecan combinations with carvacrol and EGCG on neuroblastoma cancer cells and healthy astrocytes.

MATERIALS AND METHODS

Groups

Our study included the following groups: control, topotecan, carvacrol, EGCG, topotecan+carvacrol combination, and topotecan+EGCG combination.

Cell Culture

In our investigation, we employed two cell lines: the N1E-115 (ATCC® CRL-2263™) neuroblastoma cancer cell line and the C8-D1A (ATCC® CRL-2541™) healthy astrocyte cell line, both obtained from Mus musculus. The cells were cultured in a nutrient medium consisting of a 1:1 ratio of Eagle's Minimum Essential Medium (EMEM) (MultiCell, Quebec, Canada), DMEM (MultiCell, Woonsocket, RI, USA), and HAMS F12 (MultiCell, Quebec, Canada), supplemented with 10 mg/ml streptomycin (Multicell, Wisent, USA), 5% heat-inactivated fetal bovine serum (FBS) (Multicell, Toronto, Canada), 100 IU/ml penicillin (Multicell, Quebec, Canada), and 1% L-glutamine (Multicell, Toronto, Canada). The cells were seeded in flasks and incubated in a humidified atmosphere with 95% moisture and 5% CO_2 at 37 °C. The study was initiated using cells from the 5th passage and continued until the 12th passage.

Determination of IC_{50} doses by MTT (3-(4,5-dimethyldiazol-2-yl) -2,5 diphenyl tetrazolium bromide) Method

To determine the IC_{50} values of all groups used in the study, cells were seeded in 96-well plates at a density of $1x10^6$ cells per well, using 180 μL of cell suspension. The cells were incubated for 24 hours to allow them to adhere to the plate wells. All substances ex-

cept the control group were administered to all groups in a volume of 20 µL at the concentrations shown in Figure 1. The cells were incubated (37 °C, 5% CO_2) for 24 hours. An aqueous solution containing 0.01% DMSO (Merck, Darmstadt, Germany) was applied to the control group. Topotecan (Sigma t2705 St. Louis, Missouri, United States), carvacrol (Sigma 282197, Steinheim, Germany), and EGCG (Sigma 1236700, St. Louis, Missouri, United States) solutions were prepared in 0.01% DMSO. Drug combinations were prepared in 0.01% DMSO at a 1:1 ratio. For example, 20 µM topotecan+carvacrol was prepared by combining 40 µM topotecan and 40 µM carvacrol. After 3 hours, 200 µL of 0.01% DMSO solution was introduced, and the absorbance at 492 nm was quantified using a microplate reader (Thermo Scientific Multiskan Go, USA). The control group was considered 100% viable, and the IC_{50} dose was calculated using probit analysis. The MTT assay was performed in four replicates.

Isolation of RNA and synthesis of cDNA

Neuroblastoma and astrocyte cells were seeded three times in culture plates to achieve a density of $3x10^6$ cells per well. After 24 hours, the experimental groups were treated with chemicals at the dose of neuroblastoma IC_{50} for 24 hours. RNA was isolated from cells using the PureLink RNA Mini Kit. The concentration and purity of RNA samples were determined with NanoDrop (NaNoQ OPTIZEN, Korea). Complementary DNA (cDNA) was synthesized from RNA samples with a High Capacity cDNA Reverse Transcription Kit.

Analysis of quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to analyze gene expression levels of cells associated with topoisomerase 1, topoisomerase 2, p53, BCL2, BAX, CAS3, CAS9, interleukin 1 (IL1), and TNFα. The qPCR was performed using a Quant Studio 6 Flex instrument with SYBR Select Master Mix. The PCR conditions were as follows: 1 cycle at 50 °C for 2 minutes, 10 minutes at 95 °C, 50 cycles at 95 °C for 15 seconds, 60 °C for 1 second. mRNA expression levels were analyzed using the comparative cycle threshold (2-ΔΔCt) method (Table 1). Gene expression was normalized to β-actin mRNA expression and then compared to the control group to determine relative fold-change.

Table 1. Sequences of primers used in qRT-PCR.

Primer	Primer Sequence	Reference
Topoisomerase 1	F: TCATACTGAACCCCAGCTCC R: GTCCTGCAAGTGCTTGTTCA	(Oltulu et al., 2022)
Topoisomerase 2	F: CTTCTCTGATATGGACAAACATAAGATTCC R: GGACTGTGGGACAACAGGACAATAC	(Oltulu et al., 2022)
p53	F: CACGAGCGCTGCTCAGATAGC R: ACAGGCACAAACACGCACAAA	(Oltulu et al., 2022)
CAS3	F: GGTATTGAGACAGACAGTGG R: CATGGGATCTGTTTCTTTGC	(Oltulu et al., 2022)
CAS9	F: GAGTCAGGCTCTTCCTTTG R: CCTCAAACTCTCAAGAGCAC	(Oltulu et al., 2022)
BAX	F: TTCATCCAGGATCGAGCAGA R: GCAAAGTAGAAGGCAACG	(Oltulu et al., 2022)
IL1	F: GCACGATGCACCTGTACGAT R: CACCAAGCTTTTTTGCTGTGAGT	(Oltulu et al., 2022)
TNFα	F: TCAGCCTCTTCTCC R: TCAGCTTGAGGGTT	(Oltulu et al., 2022)
BCL2	F: ATGTGTGTGGAGAGCGTCAA R: ACAGTTCCACAAAGGCATCC	(Oltulu et al., 2022)
β-actin	F: AGAGCTACGAGCTGCCTGAC R: AGCACTGTGTTGGGCGTACAG	(Niiro et al., 2021)

Statistics

To determine the IC_{50} values, the percentage of viability data obtained from the MTT test were subjected to probit analysis. After administering the IC_{50} dose of the neuroblastoma cell line to astrocyte and neuroblastoma cell lines for 24 hours, we used a one-way ANOVA test to analyze the relative fold change values of gene expressions. Then, the difference between the groups was determined by post hoc Tukey test, as $p<0.05$ was accepted as statistically significant. All statistical analyses, including probit analysis and ANOVA test, were conducted using SPSS 25 software (IBM).

RESULTS AND DISCUSSION

Due to the high mortality rate in high-risk neuroblastoma, there is a need for the development of new treatments. In our study on neuroblastoma, we evaluated the cytotoxic and apoptotic effects of topotecan, carvacrol, EGCG, and their combinations on both neuroblastoma cell line and astrocyte cell line at the neuroblastoma IC_{50} dose for 24 hours. The similarity of IC_{50} values between topotecan and carvacrol for both healthy astrocyte cell lines and cancerous neuroblastoma cell lines suggests that these agents have a non-selective effect (Figure 1). Selective agents are needed in cancer treatment. When evaluating the IC_{50} values, it can be interpreted that EGCG and topotecan+carvacrol, topotecan+EGCG combinations may exhibit a selective effect between neuroblastoma cells and astrocyte cells.

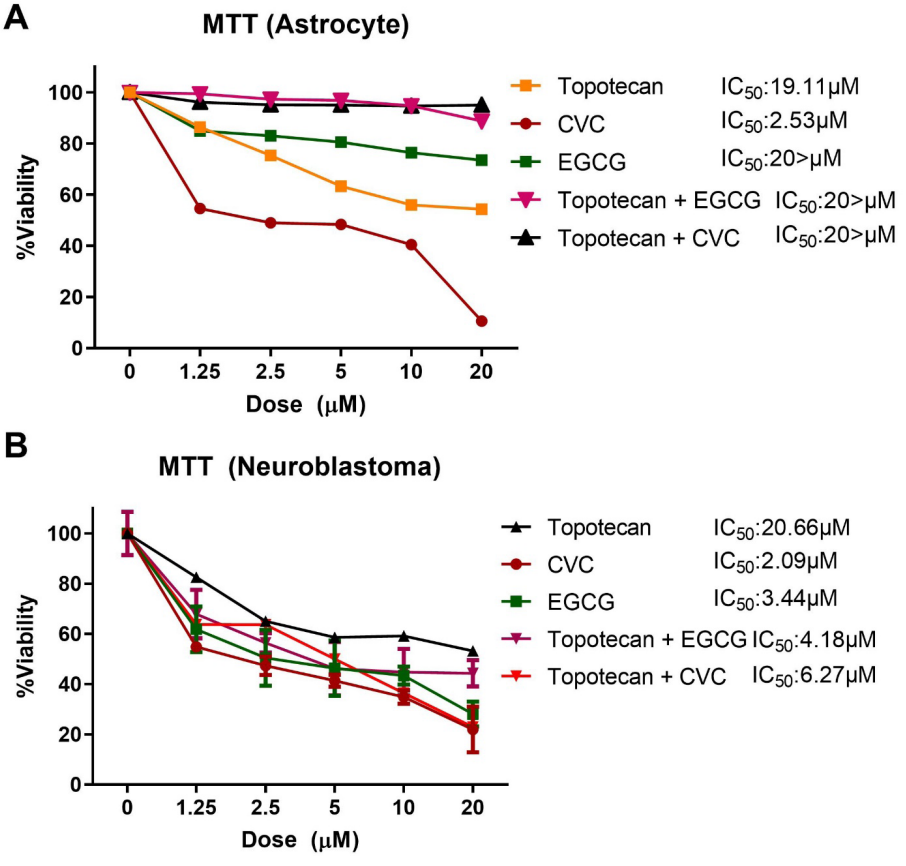

Figure 1. Graph showing the results of the 24-hour MTT test and IC_{50} values for (A) C8-D1A astrocyte cell line and (B) N1E-115 neuroblastoma cell line.

Topoisomerase 1 is an enzyme that regulates DNA topology. Changes in the expression of topoisomerase 1 mRNA may be associated with DNA damage or genotoxicity in cells. Topotecan is a topoisomerase 1 inhibitor and exerts its cytotoxic effect by preventing the separation of the DNA-topoisomerase 1 complex, leading to the formation of toxic compounds. In the astrocyte cell line, a statistically significant increase in topoisomerase 1 mRNA expression has been observed in the carvacrol and topotecan+EGCG groups compared to the control and topotecan groups (Figure 2). In the neuroblastoma cell line, an increase in topoisomerase 1 mRNA expression has been found in response to topotecan, carvacrol, and topotecan+carvacrol combination compared to the control group. Among all groups, a statistically significant increase has been observed compared to the topotecan group.

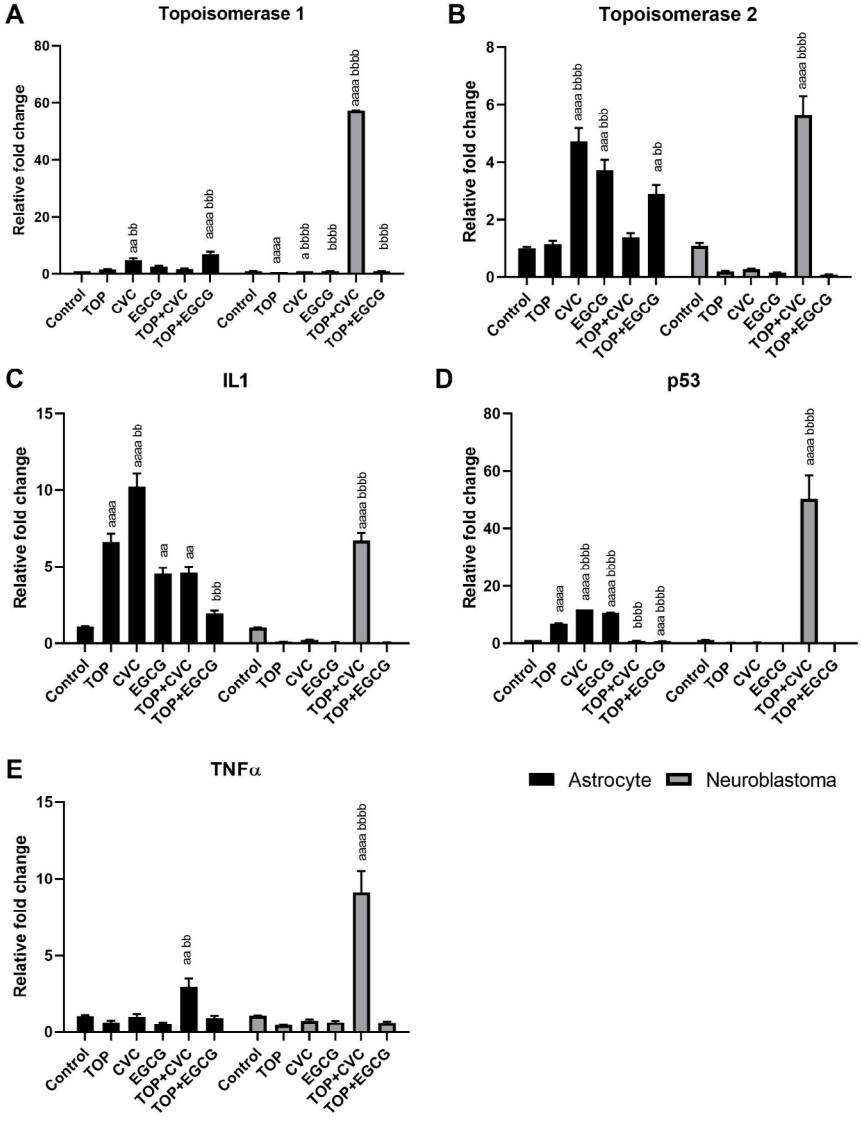

Figure 2. The mRNA expressions of topoisomerase 1 (A), topoisomerase 2 (B), IL1 (C), p53 (D), and TNFα (E) were shown in the qRT-PCR results graph. (a: $p < 0.05$, aa: $p < 0.01$, aaa: $p < 0.001$, aaaa: $p < 0.0001$ compared with the control group; b: $p < 0.05$, bb: $p < 0.01$, bbb: $p < 0.001$, bbbb: $p < 0.0001$ compared to the topotecan group; one-way ANOVA post hoc Tukey, $p < 0.05$)

The topoisomerase 2 enzyme is responsible for regulating DNA topology. In the astrocyte cell line, an increase in topoisomerase 2 mRNA expression has been observed in the carvacrol, EGCG, and topotecan+EGCG groups compared to both the control and topotecan groups. In the neuroblastoma cell line, an increase in Topoisomerase 2 mRNA expression has been found in the topotecan+carvacrol combination group compared to both the control and topotecan groups.

IL-1 is an inflammatory cytokine involved in tumor initiation and progression (Mantovani, Barajon, & Garlanda, 2018). Inhibiting IL-1 can lead to an anticancer effect. The expression of IL1 mRNA has increased in the astrocyte cell line in the topotecan, carvacrol, EGCG, and topotecan+carvacrol groups. IL1 expression has increased in the carvacrol group compared to the topotecan group, while it has decreased in the topotecan+EGCG group. In the neuroblastoma cell line, the topotecan+carvacrol group has shown an increase in IL1 expression compared to the control and topotecan groups.

p53 plays a crucial role in cell cycle arrest and can initiate apoptosis. It can also suppress tumor growth. The expression of p53 mRNA has increased in the astrocyte cell line in the topotecan, carvacrol, EGCG, and topotecan+EGCG groups. The expression of p53 has increased in the carvacrol and EGCG groups compared to the topotecan group, while it has decreased in the combination groups. In the neuroblastoma cell line, the expression of p53 mRNA has increased in the topotecan+carvacrol group compared to the control and topotecan groups.

TNFα is an inflammatory cytokine that may contribute to tumor development. It can also trigger apoptosis through the activation of the extrinsic pathway. In this study, we found that the expression of TNFα was significantly increased in both the astrocyte and neuroblastoma cell lines in the topotecan+carvacrol group compared to the control and topotecan groups.

BCL2 is a protein that inhibits apoptosis in cells. The expression of BCL2 mRNA has increased in the topotecan group compared to the control group in the astrocyte cell line. In the other groups, BCL2 expression was significantly decreased compared to both the control and topotecan groups. The decrease in BCL2 expression may be associated with the activation of apoptotic processes and increased cytotoxic activity in the cells. In the neuroblastoma cell line, BCL2 expression has decreased in all groups compared to the control group. Furthermore, in the neuroblastoma cells, BCL2 expression has also decreased in groups other than the control group compared to the topotecan group.

BAX is a proapoptotic protein. Increased expression of BAX is associated with the activation of apoptotic processes and the triggering of cell death in cancer cells. The expression of BAX mRNA has shown an increase in both the astrocyte and neuroblastoma cell lines in the carvacrol and topotecan+carvacrol groups compared to the control and topotecan groups.

CAS9 is involved in the mitochondrial pathway of apoptosis. The expression of CAS9 has increased in all groups compared to the control in the astrocyte cell line. The decreased expression has been observed in the carvacrol and topotecan+EGCG groups compared to the topotecan group, while increased expression has been observed in the topotecan+carvacrol group. In the neuroblastoma cell line, an increase in CAS9 expression has been observed in all groups except the topotecan group. The topotecan+carvacrol group has shown an increase compared to the topotecan group.

CAS3 is a protease that plays a role in apoptosis-induced cell death. In the astrocyte cell line, the expression of CAS3 has decreased in all groups except the carvacrol group compared to the control group. The carvacrol group showed a significantly higher expression of CAS3 than both the control group and the topotecan group. In the neuroblastoma cell line, CAS3 expression has decreased in the topotecan+carvacrol group compared to the control group, while it has increased in the other groups. A decrease has been observed in the topotecan group compared to the control group and the topotecan+carvacrol group, while an increase has been observed in the other groups.

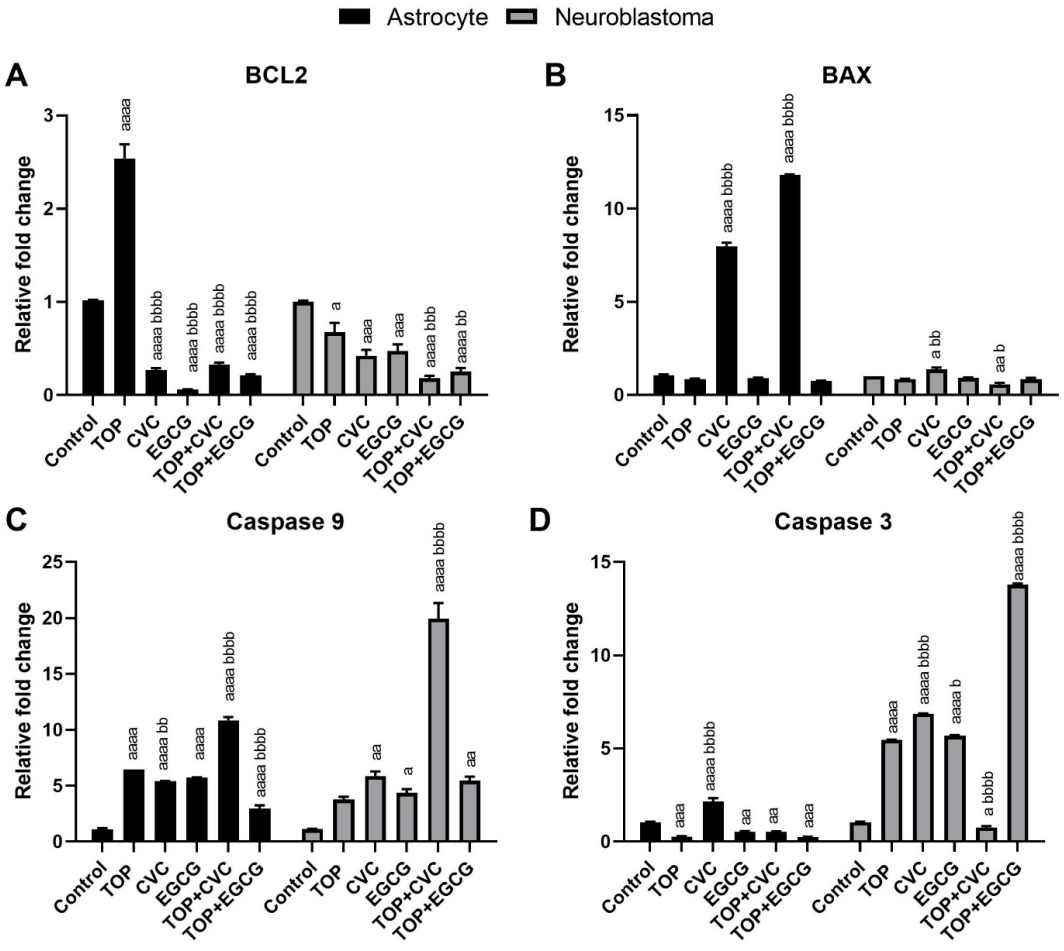

Figure 3. The mRNA expressions of BCL2 (A), BAX (B), caspase 9 (C), and caspase 3 (D) were shown in the qRT-PCR results graph. (a: p <0.05, aa: p <0.01, aaa: p <0.001, aaaa: p <0.0001 compared with the control group; b: p <0.05, bb: p <0.01, bbb: p <0.001, bbbb: p <0.0001 compared to the topotecan group; one-way ANOVA post hoc Tukey, p <0.05)

Astrocyte cells were treated with topotecan at the neuroblastoma cell line IC_{50} dose for 24 hours. The increase in p53 and IL1 mRNA expression in the cells may be attributed to cellular stress (Figure 2). An increase in CAS9 mRNA expression and a decrease in CAS3 expression can be interpreted as apoptotic signaling not triggered yet (Figure 3). BCL2 is a protein that protects cells from apoptosis by inhibiting the release of cytochrome c from the mitochondria, an essential step in the apoptotic pathway.

The application of topotecan at the neuroblastoma IC_{50} dose for 24 hours has resulted in an increase in CAS9 and CAS3 mRNA expression, indicating that the decrease in neuroblastoma cell viability may be due to apoptosis. Topotecan acts by disrupting the function of the DNA-topoisomerase 1 complex, preventing its separation. The decrease in topoisomerase 1 mRNA expression suggests the potential development of cell resistance. While previous studies have demonstrated genotoxic damage caused by topotecan in the SK-N-BE(2c) neuroblastoma cell line using the comet assay, our study did not show an increase in mRNA expression associated with DNA damage in the neuroblastoma cell line we used (McCluskey et al., 2008).

The increase in mRNA expression levels of topoisomerase 1, topoisomerase 2, and p53 in astrocyte cells after exposure to the neuroblastoma IC_{50} dose of carvacrol for 24 hours can be attributed to genetic damage and the activation of cellular repair mechanisms. Cellular stress and inflammation can cause the release of IL1, a cytokine that can lead to further inflammation and damage to cells. The increase in CAS3, CAS9, BAX, and p53 mRNA expression indicates a potential involvement of apoptosis. The lack of changes in BCL2 and TNF alpha mRNA expression suggests these genes may not play a significant role in apoptotic cell death mechanisms. These findings are consistent with our findings.

The similarity between the astrocyte IC_{50} value of carvacrol (2.53 μM) and the neuroblastoma IC_{50} value (2.09 μM) indicates that carvacrol exhibits a comparable cytotoxic effect on these cell lines, suggesting a lack of selectivity. The increase in BAX, CAS3, and CAS9 mRNA expression after 24 hours of treatment with carvacrol at the IC_{50} dose is consistent with apoptosis-mediated cell death in neuroblastoma cells. A study conducted on HELA cancer and CCD-1123Sk fibroblast human cell lines showed that the cytotoxic effect of carvacrol was non-specific, similar to our findings (Ranjitkar et al., 2021). It has been reported that carvacrol exhibits weak antioxidant activity and low anticancer efficacy in rat N2a neuroblastoma cells and primary rat neuron cell cultures, which is consistent with our study results (Aydın, Türkez, & Keleş, 2014).

The increase in p53 and Topoisomerase 2 mRNA expression in the astrocyte cell line after EGCG application indicates the presence of genetic damage. It suggests that the cell cycle has halted, with the cell initiating repair processes. Although there is an increase in CAS9 expression and a decrease in BCL2 expression, the absence of consistent changes in BAX and CAS3 implies that the apoptotic process has not been initiated yet. Additionally, the fact that the IC_{50} value for astrocytes is above 20 μM and the IC_{50} dose we applied corresponds to 3.44 μM in the neuroblastoma cell line is consistent with the mRNA expression patterns. The increase in IL1 mRNA expression can be attributed to cellular stress and inflammation.

The increase in CAS3 and CAS9 mRNA expression and the decrease in BCL2 observed in the neuroblastoma cell line after 24-hour treatment with the IC_{50} dose of 3.44 μM EGCG suggest that the reduction in cell viability is likely due to apoptosis. A study conducted on human undifferentiated hepatocellular carcinoma HLE cell line reported that in vivo and in vitro administration of EGCG induces apoptosis and dose-dependently activates caspases 3, 8, and 9, without affecting BAX (Nishikawa et al., 2006). Another study demonstrated that EGCG leads to a decrease in BCL-2 and an increase in p53, BAX, CAS3, and CAS9 in LM6 hepatocellular carcinoma cells, resulting in apoptosis. At the same time, it does not exhibit the same effect on healthy liver cells. This selective effect of EGCG is consistent with our findings (Zhang, Duan, Owusu, Wu, & Xin, 2015).

The increase in IL1 and TNF-alpha mRNA expression in the astrocyte cell line following the 24-hour treatment with the neuroblastoma IC_{50} dose of TOP+-CARV indicates their association with inflammatory-related proteins and can indicate cellular stress. The upregulation of CAS9 and BAX mRNA expression by carvacrol suggests that apoptotic signaling is being initiated. This is supported by the decrease in BCL2 expression, which is a protein that inhibits apoptosis. The decrease in CAS3 mRNA expression may be attributed to apoptosis not triggered yet. Despite these cellular signals, the combined application of topotecan and carvacrol in the astrocyte line has increased the IC_{50} dose compared to neuroblastoma and conferred a selective targeting feature.

The increase in P53, topoisomerase 1, and Topoisomerase 2 expression indicates DNA stress and the cell's effort to repair itself. The observed reduction in BAX and CAS3 expression, coupled with the

concurrent increase in CAS9 expression, implies that the caspase cascade has not yet been initiated. The increase in TNF-alpha expression is indicative of cellular stress in neuroblastoma.

The application of a combination of topotecan and EGCG at the neuroblastoma IC_{50} value for 24 hours on the astrocyte cell line, which is significantly below the astrocyte 24-hour IC_{50} value, may indicate an increase in the expression of topoisomerase 1 and topoisomerase 2, suggesting DNA damage. Despite the increase in CAS9 expression, the decrease in CAS3 and BCL2 expression can be explained by apoptosis not triggered.

The increase in CAS3 and CAS9 mRNA expression observed in neuroblastoma cells following the application of topotecan+EGCG at the neuroblastoma 24-hour IC_{50} dose suggests that the decrease in cell viability may be attributed to apoptosis.

We acknowledge that the differential effects observed on the astrocyte and neuroblastoma cell lines may arise from inherent cell type disparities beyond the distinction between cancerous and healthy cells. Nevertheless, employing an astrocyte cell line is a valuable starting point for further investigations.

One of the limitations of this study is that the assessment of apoptotic-related mRNA levels may not always be predictive of the synthesis of functional proteins or their proper activity. While mRNA expression provides valuable insights into cellular processes, it is essential to recognize that post-transcriptional and post-translational modifications can influence protein synthesis and activity. Additionally, protein degradation, localization, and interaction with other molecules may affect the ultimate functional outcome. Therefore, caution should be exercised when interpreting the results solely based on mRNA expression, as it does not necessarily reflect the actual protein levels or their functionality. Future studies incorporating protein-level analysis and functional assays will be essential to elucidate further the role of these apoptotic-related mRNA expressions in cellular processes.

Additionally, when evaluating the cytotoxic effects of a chemical substance on cancer cells, it is crucial to assess its cytotoxicity at a specific dose in healthy cells as well. The selected dose for our study was the IC_{50} value determined via the MTT assay, which represents the cytotoxic dose in the neuroblastoma cell line. The same dose needs to exhibit an acceptable level of cytotoxicity in healthy cells. In our study, topotecan was employed as an antineoplastic agent. If the administered carvacrol, EGCG, or combinations demonstrated significantly lower cytotoxic effects in healthy cells, even at higher doses than topotecan, they would possess a higher potential for clinical significance with fewer adverse effects. In this study, we will use neuroblastoma and astrocyte cells to investigate their selective effects in vitro, rather than solely focusing on their anticancer potential. Further in vivo studies are needed to develop and evaluate formulations that enhance the delivery of drugs to target tissues, as well as to assess the effects of systemic factors and metabolites on drug delivery in an in vivo setting.

CONCLUSION

Based on the data obtained in our study, it can be concluded that topotecan and carvacrol did not exhibit selective cytotoxic effects between the healthy astrocyte cell line and the neuroblastoma cell line. However, EGCG and the combinations of topotecan with carvacrol and topotecan with EGCG demonstrated specific cytotoxic effects on the neuroblastoma cell line.

Regarding evaluating apoptotic mRNA levels in our study, it is evident that the effects of topotecan, carvacrol, and EGCG on the apoptotic signaling pathway have been elucidated. Nonetheless, it is essential to note that mRNA expression levels may not directly reflect protein levels or functional activity. Further *in vitro* and *in vivo* studies are needed to confirm our findings and to gain a better understanding of the protein levels and functional aspects of apoptosis. These studies will provide a more comprehensive un-

derstanding of the mechanisms involved in apoptosis and shed light on the potential therapeutic implications.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis (ÇO, MA, EB), experimenting (ÇO, MA, EB), preparing the study text (ÇO, MA, EB), reviewing the text (ÇO, MA, EB), statistics (ÇO, MA, EB), analysis and interpretation of the data (ÇO, MA, EB), literature research (ÇO, MA, EB)

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