

# Combined effects of EGCG and retinol on viability and apoptosis-related gene expression in Caco-2 and HCT-116 colon cancer cells

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## ABSTRACT

**Objective(s):** Colorectal cancer remains a leading cause of cancer-related deaths worldwide, emphasizing the need for novel therapeutic strategies. Epigallocatechin-3-gallate (EGCG), a major green tea polyphenol, and retinol, a vitamin A derivative with antioxidant properties, have shown anti-cancer activity individually. This study investigates the combined effects of EGCG and retinol on human colorectal cancer cell lines, Caco-2 and HCT-116.

**Materials and Methods:** Cytotoxic effects of EGCG and retinol, alone and in combination, were evaluated using resazurin-based cell viability assays. Intracellular reactive oxygen species (ROS) levels were measured by DCFDA assay to assess antioxidant activity. Changes in expression of apoptosis- and survival-related genes (p53, p21, AKT, and NF-κB) were analyzed using quantitative real-time PCR (RT-qPCR).

**Results:** Combined treatment with EGCG and retinol reduced cell viability and intracellular ROS levels in both Caco-2 and HCT-116 cells. This treatment also modulated gene expression by increasing pro-apoptotic markers (p53 and p21) and decreasing anti-apoptotic markers (AKT and NF-κB), suggesting enhanced regulation of apoptosis-related pathways and inhibition of survival signaling.

**Conclusion:** The combined effects of EGCG and retinol result in enhanced cytotoxicity, antioxidant activity, and modulation of apoptosis- and survival-related genes in colorectal cancer cells, supporting their potential as a combined therapeutic approach. Further studies are required to confirm these findings and explore clinical relevance.

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## Introduction

Colorectal cancer (CRC) is one of the most common and deadly malignancies worldwide, ranking as the third most frequently diagnosed cancer and the second leading cause of cancer-related mortality (1). Despite advances in diagnostic tools and therapeutic strategies, patients with advanced CRC still experience poor outcomes, largely because of resistance to conventional treatments and the high propensity of the disease to metastasize (2). These challenges underscore the urgent need for alternative or complementary therapeutic approaches, including those based on natural bioactive compounds that may offer efficacy with fewer adverse effects.

Apoptosis, or programmed cell death, is a fundamental mechanism for maintaining tissue homeostasis by eliminating damaged or abnormal cells. Inducing apoptosis in cancer cells remains a major therapeutic objective, and natural compounds capable of promoting apoptotic pathways have gained growing attention as potential adjuncts to chemotherapy or as standalone agents to

mitigate treatment-related complications (3).

Epigallocatechin gallate (EGCG), the predominant polyphenol in green tea, exhibits diverse biological activities, including anti-inflammatory, antioxidant, and anticancer effects. It modulates key regulatory pathways such as PI3K/Akt, MAPK, and NF-κB, thereby suppressing cancer cell proliferation and promoting apoptosis in several tumor models (4,5). More recent findings indicate that EGCG can enhance the chemosensitivity of CRC cells to irinotecan by inducing GRP78-mediated endoplasmic reticulum stress, suggesting potential value in combination-based therapeutic strategies (6).

Retinol, a fat-soluble form of vitamin A, plays essential roles in cell differentiation, growth, and immune regulation. Retinoids—including retinol and its derivatives—exert anticancer effects primarily through activation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which regulate genes involved in cell-cycle arrest and apoptosis (7, 8). Recent studies further highlight that retinoic acid signaling can promote differentiation toward

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the absorptive lineage in CRC cells, underscoring its relevance to tumor biology (9).

Although both EGCG and retinol demonstrate anticancer properties individually, their combined influence—particularly in CRC—remains insufficiently understood. Interactions between bioactive compounds with distinct mechanisms may produce additive or complementary effects on cell survival pathways. A recent review also emphasized the potential therapeutic value of natural compounds used alongside conventional treatments in suppressing CRC progression and metastasis (10).

In this study, we examined the effects of EGCG and retinol, individually and in combination, on apoptosis in two human CRC cell lines: Caco-2 and HCT-116. Caco-2 cells, derived from human intestinal epithelium, are widely used as a model of enterocyte-like differentiation, whereas HCT-116 cells represent a more aggressive CRC phenotype frequently studied in the context of metastasis and therapy resistance (11, 12). Investigating these two cell lines allows for a broader evaluation of how these compounds influence CRC cell behavior.

By assessing apoptosis following treatment with EGCG and retinol—either alone or in combination—we aimed to characterize whether their co-administration modifies apoptotic responses. A better understanding of these interactions may contribute to developing more effective and potentially less toxic therapeutic strategies for CRC.

## Materials and Methods

### Cell culture and reagents

Human CRC cell lines, Caco-2 (NCBI Code: C139) and HCT-116 (NCBI Code: C570), were obtained from the Pasteur Institute of Iran. Caco-2 cells were characterized as described by Pinto *et al.* (11), while HCT-116 cells were detailed by Crowley-Weber *et al.* (12). The cells were cultured in RPMI 1640 medium (Bio-idea) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin, under standard conditions at 37 °C, 5% CO<sub>2</sub>, and 95% humidity. Cells were maintained in a sub-confluent state and passaged every three days to ensure healthy growth and viability.

The compounds used in this study included Epigallocatechin-3-gallate (EGCG, Sigma-Aldrich, #989-51-5), all-trans retinol (Sigma-Aldrich, #68-26-8), dimethyl sulfoxide (DMSO, Sigma-Aldrich, #67-68-5), and resazurin (Sigma-Aldrich, #62758-13-8). Additionally, 2',7'-Dichlorofluorescein diacetate (DCFDA) and tert-Butyl hydroperoxide (TBHP) were purchased from Abcam (#ab113851). Phosphate-buffered saline (PBS) and trypsin were supplied by Bio-idea Co.

### Cell viability assay

Caco-2 and HCT-116 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells between passages 5 and 15 were used, and initial viability before treatment was confirmed to be >95% by trypan blue exclusion. For cytotoxicity assessment, cells were seeded at a density of 8,000 cells per well in 96-well plates and allowed to attach for 24 hr. Four treatment setups were prepared: (1) varying concentrations of EGCG (0, 5, 25, 50, and 100 μM); (2) different doses of retinol (0, 0.5, 1, 5, and 10 μM); (3) several retinol concentrations combined with a fixed EGCG dose; and (4) several EGCG concentrations combined with a fixed retinol dose (13). All treatments were applied for 24 hr. Following treatment, 0.1 mg/ml resazurin solution was added to each well, and plates were incubated

for an additional 3.5 hr at 37 °C. Fluorescence was measured using a PerkinElmer plate reader at 560 nm excitation and 590 nm emission wavelengths.

### Isobologram and combination index analysis

Cytotoxic interactions between EGCG and retinol were further evaluated using isobologram analysis. IC<sub>50</sub> values for each compound were determined from the resazurin assay. Combination index (CI) values were calculated according to the Chou-Talalay method (14).

CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively. Isobolograms were plotted with the doses of EGCG on the x-axis and retinol on the y-axis. Data are presented as mean ± SD from three independent experiments.

### Reactive oxygen species (ROS) assay

Intracellular ROS levels were measured using the DCFDA cellular ROS detection assay, following the protocol of Wang *et al.* (15). Caco-2 and HCT-116 cells were plated at a density of 25,000 cells per well in complete RPMI medium. After 24-hour treatment with EGCG and retinol, cells were incubated with 20 μM DCFDA for 30 min at 37 °C in the dark. Fluorescence was measured using a PerkinElmer fluorescence spectrophotometer at 485 nm excitation and 535 nm emission. 50 μM tert-Butyl hydroperoxide was used as a positive control.

### RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from treated cells using the Favorgen RNA extraction kit (#FABRK001) according to the manufacturer's instructions. RNA purity and concentration were assessed by 2% agarose gel electrophoresis and using a NanoDrop 1000 spectrophotometer.

cDNA synthesis was performed using 1 μg of total RNA and a commercially available cDNA synthesis kit (Parstous, #A101161). RT-qPCR was conducted on a Roche LightCycler 96 system using SYBR Green chemistry (Ampliqon, #A325402), following the standard protocols described by Bustin *et al.* (16).

Gene-specific primers targeting p53, p21, AKT, NF-κB, and the housekeeping gene GAPDH (internal control) were obtained from [www.pga.mgh.harvard.edu](http://www.pga.mgh.harvard.edu) and verified using BLAST. Primer sequences are listed in Table 1.

The PCR reaction mixture (12 μl) consisted of master mix (containing SYBR Green, dNTPs, MgCl<sub>2</sub>, and Taq DNA polymerase), 2 μl of cDNA template, and 0.3 μM of each primer. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 15 min; 40 cycles of denaturation at 95 °C for 30 sec, and annealing/extension at 58 °C for 60 sec.

Specificity of amplification was confirmed by melting curve analysis performed immediately after PCR, where a single sharp peak at the expected melting temperature (T<sub>m</sub>) indicated the absence of non-specific amplification and primer-dimer formation.

Standard curves were generated by serial 10-fold dilutions of PCR products for each gene to calculate amplification efficiency using the formula:

$$\text{Efficiency} = [10^{(-1/\text{Slope})} - 1] \times 100$$

Relative quantification of gene expression was calculated using the comparative Ct method ( $2^{-\Delta\Delta C_t}$ ), normalized to

**Table 1.** Sequences of primers used for RT-PCR analysis to investigate the mRNA expression of p53, p21, AKT, NF-κB and GAPDH

Gene	Primer sequences (5'-3')	Primer
p53	5'-ACCCCTGCTTGAATAGGTG-3'	Forward
	5'-AACAAAACACCGATGCAGGC-3'	Reverse
p21	5'-TGTCGGTCAGAACCCTATGC-3'	Forward
	5'-AAAGTCGAAGTTCATCGCTC-3'	Reverse
AKT	5'-CTACCCACACAGCAGTACGC-3'	Forward
	5'-AAGTCGCTGGTGTAAAGCCG-3'	Reverse
NF-κB	5'-AACAGAGAGGATTTCGTTCCG-3'	Forward
	5'-TTTGACCTGAGGGTAAGACTTCT-3'	Reverse
GAPDH	5'-ACAACTTTGGTATCGTGAAGG-3'	Forward
	5'-GCCATCACGCCACAGTTTC-3'	Reverse

GAPDH expression (17).

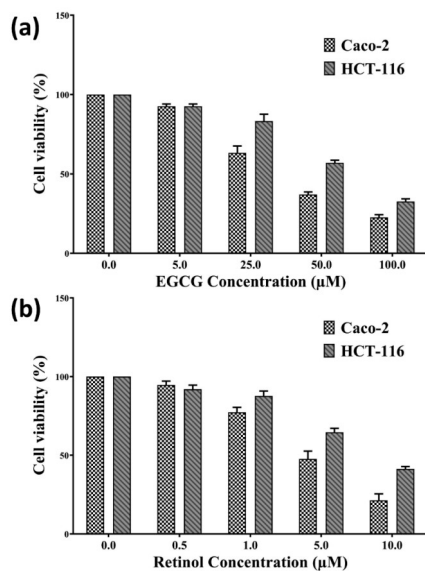
**Statistical analysis**

All experiments were performed in triplicate and repeated independently three times. Data are presented as mean ± standard deviation (SD). Statistical comparisons were performed using one-way ANOVA followed by Tukey's *post hoc* test for multiple group comparisons. For combination studies, CI values were analyzed according to the Chou-Talalay method. A *P*-value < 0.05 was considered statistically significant. GraphPad Prism (v.9.0) software was used for all analyses (18).

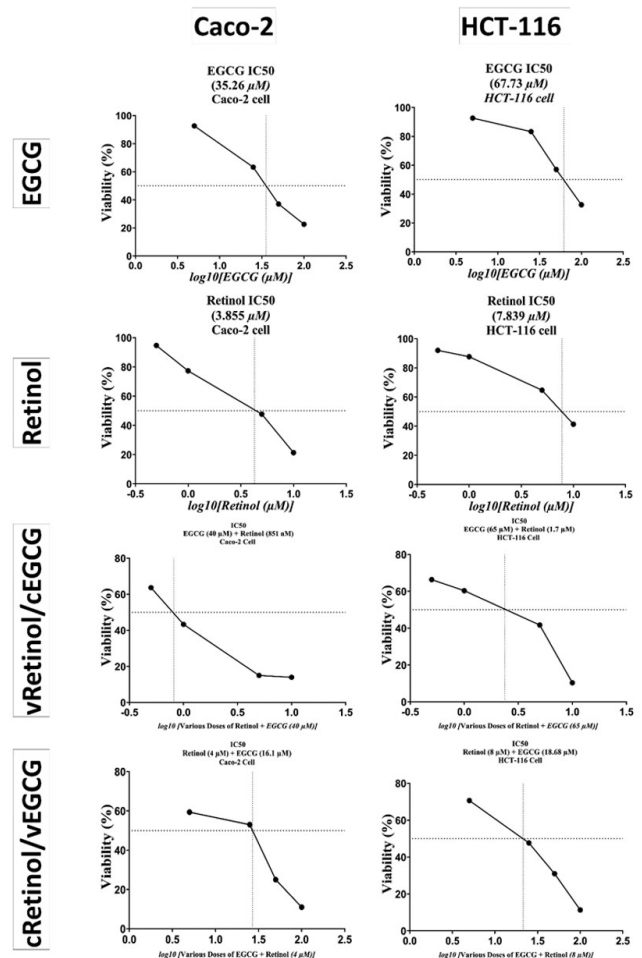
**Results**

**Effect of EGCG and retinol on cell viability**

The cytotoxic effects of EGCG and retinol, administered individually or in combination, were assessed in Caco-2 and HCT-116 cells. In single-agent treatments, Caco-2 cells exhibited greater sensitivity than HCT-116 cells, with IC<sub>50</sub>



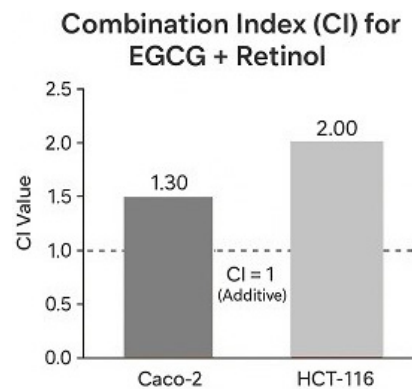
**Figure 1.** EGCG (a) and retinol (b) Cytotoxicity against Caco-2 and HCT-116 colon cancer cell by resazurin assay. Data represent mean ± SD of three independent experiments (n=3). Statistical analysis was performed using one-way ANOVA (*P*<0.05 versus control). EGCG: Epigallocatechin-3-gallate



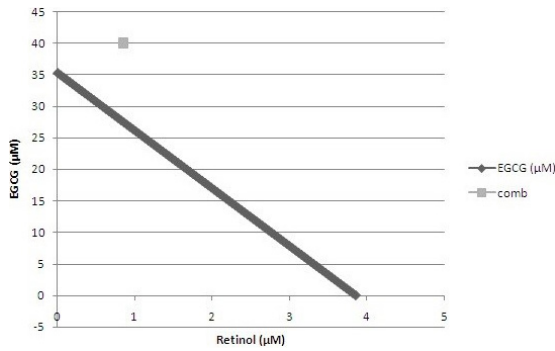
**Figure 2.** EGCG, retinol, vRetinol/cEGCG, and cRetinol/vEGCG cytotoxic activity in Caco-2 and HCT-116 colon cancer cell lines. Data represent mean ± SD of three independent experiments (n=3). Statistical analysis was performed using one-way ANOVA (*P*<0.05 versus control). EGCG: Epigallocatechin-3-gallate

values of 35.26 μM for EGCG and 3.855 μM for retinol, compared with 67.73 μM and 7.839 μM, respectively, in HCT-116 cells (Figure 2).

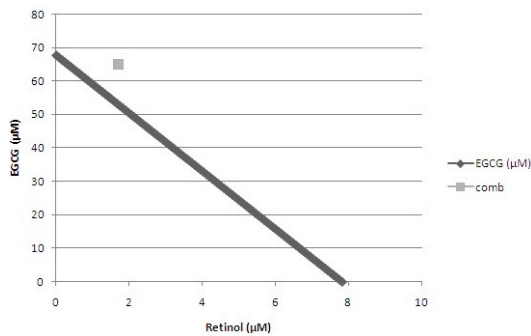
Combination index (CI) analysis showed CI values exceeding 1 across multiple concentration combinations, indicative of an antagonistic interaction between EGCG and retinol in both cell lines (Figure 3). Consistently, isobologram analysis (Figures 4 and 5) positioned the combination points above the theoretical line of additivity, further supporting an



**Figure 3.** Combination index (CI) values for EGCG and retinol in Caco-2 and HCT-116 cells, indicating antagonism (CI > 1). EGCG: Epigallocatechin-3-gallate



**Figure 4.** Isobologram analysis of EGCG and retinol in Caco-2 cells, indicating antagonistic interaction above the additive line EGCG: Epigallocatechin-3-gallate

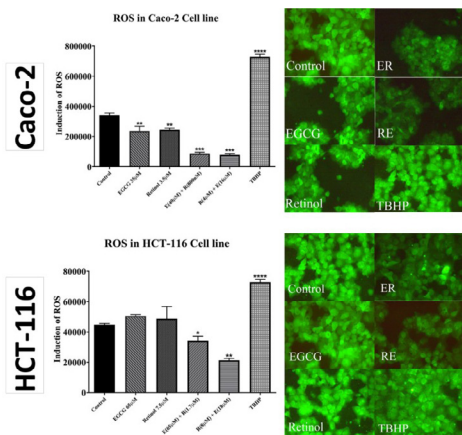


**Figure 5.** Isobologram analysis of EGCG and retinol in HCT-116 cells, showing antagonistic interaction above the additive line EGCG: Epigallocatechin-3-gallate

antagonistic rather than synergistic effect.

**ROS generation following treatment**

To explore the role of oxidative stress in the cytotoxic effects, intracellular ROS levels were measured using the DCFDA assay after 24 hr of treatment with EGCG, retinol, and their combination. TBHP was used as a positive control (Figure 6). The results showed that the combination treatment significantly boosted the cells’ ability to scavenge free radicals compared to either treatment alone. This synergistic antioxidant effect was especially noticeable in Caco-2 cells compared to HCT-116 cells.



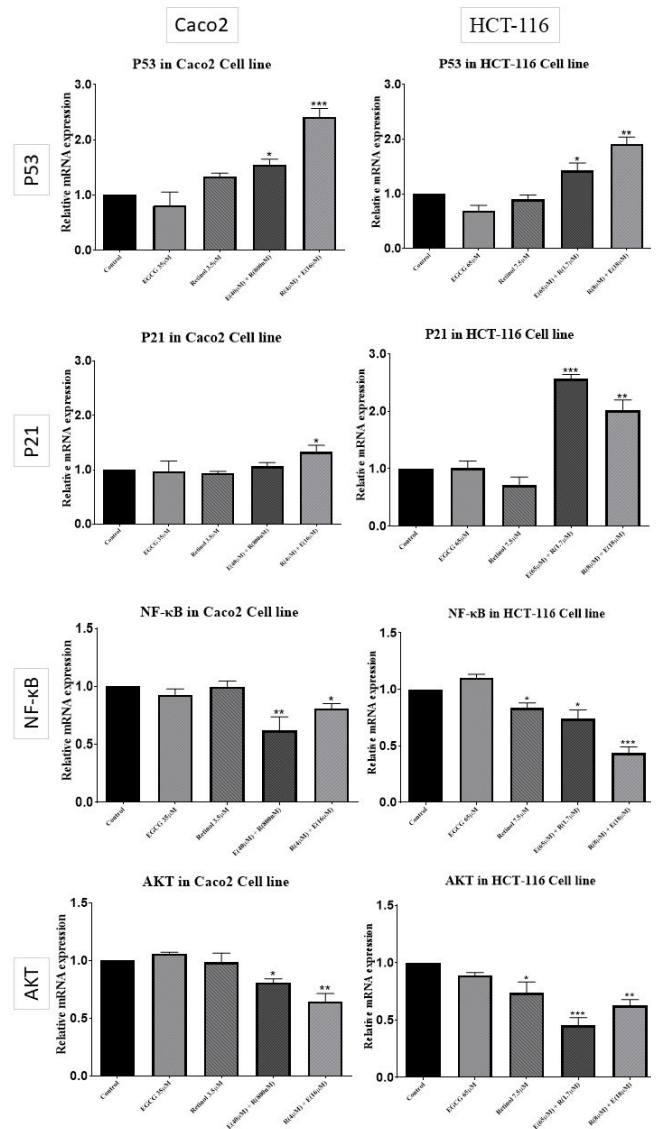
**Figure 6.** Intracellular ROS production mediated by different concentrations of EGCG and retinol in Caco-2 and HCT-116 colon cancer cell lines Data represent the mean ± SD of three independent experiments (n = 3). Statistical analysis was performed using one-way ANOVA, with significance indicated as \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, and \*\*\*\*P<0.0001 compared with the control group. EGCG: Epigallocatechin-3-gallate; ROS: Reactive oxygen species

**Alterations in gene expression**

The expression of genes related to apoptosis and cell survival (p53, p21, NF-κB, and AKT) was measured using RT-qPCR after treating the cells with EGCG, retinol, and their combination at concentrations close to their IC<sub>50</sub> values (Figure 7).

In Caco-2 cells, the combined treatment with EGCG (16 µM) and retinol (4 µM) significantly increased the expression of p53 and p21 compared to treatments with each compound alone. Additionally, EGCG (40 µM) together with retinol (0.8 µM) lowered NF-κB levels, while the combination of EGCG (16 µM) and retinol (4 µM) reduced AKT expression.

In HCT-116 cells, co-treatment with EGCG (65 µM) and retinol (1.7 µM) caused a significant rise in p21 expression and a decrease in AKT levels. Moreover, the combination of EGCG (18 µM) and retinol (8 µM) produced the most notable increase in p53 and the strongest reduction in NF-κB expression.



**Figure 7.** Gene expression profile following treatment with different concentrations of EGCG and retinol in Caco-2 and HCT-116 colon cancer cell lines is shown Data represent the mean ± SD of three independent experiments (n = 3). Statistical analysis was performed using one-way ANOVA, with significance indicated as \*P<0.05, \*\*P<0.01, and \*\*\*P<0.001 compared with the control group. EGCG: Epigallocatechin-3-gallate

## Discussion

This study examined the interaction between EGCG and retinol in two CRC cell lines, Caco-2 and HCT-116, focusing on their effects on cell viability, oxidative status, and gene expression. As expected, both compounds reduced cell viability in a dose-dependent manner, and Caco-2 cells were more sensitive than HCT-116 cells (19, 20). However, despite the clear effects of each compound alone, the combination treatments exhibited a weaker impact on cell viability compared to single-compound treatments. Furthermore, combination index (CI) analysis and isobologram plots indicated an antagonistic interaction in both cell lines, with CI values consistently greater than 1 (20). Under the tested conditions, the combined treatment with EGCG and retinol did not demonstrate an enhanced effect compared to individual treatments.

Although the combined treatment led to reduced ROS levels, inhibition of AKT and NF- $\kappa$ B signaling, and induction of p53 and p21 expression, the results of the viability assay together with isobologram and IC analyses pointed to an overall antagonistic interaction under the tested conditions. This pattern suggests that, during short-term exposure, modulation of redox status and survival pathways mainly drives p53/p21-dependent cell-cycle arrest rather than efficiently triggering apoptotic mechanisms, which may explain the limited cytotoxic effect observed. Accordingly, the convergence of overlapping or competing signaling events between the two compounds appears to shift the cellular response toward growth arrest and adaptive processes rather than synergistic cell death.

There are plausible biological explanations for this antagonism. EGCG regulates several signaling pathways linked to proliferation and apoptosis, including MAPK, PI3K/AKT, and NF- $\kappa$ B (20), while retinol and its metabolites activate RAR and RXR receptors that also control genes involved in cell cycle arrest and apoptosis (21). When used together, their effects may overlap or interfere with one another. In addition, previous studies have shown that retinol and its derivatives, such as all-trans retinoic acid, can modulate the effects of EGCG (22). Such a change may alter EGCG's ability to influence DNA structure or gene regulation, helping to explain why the combined treatment does not provide additional effects beyond those observed with each compound alone.

Gene expression results provided further insight into the underlying mechanisms. Co-treatment increased the expression of the tumor suppressor genes p53 and p21, concomitant with reduced expression of the survival-related genes AKT and NF- $\kappa$ B (23–25). The up-regulation of p21, a key regulator of cell cycle progression, together with increased p53 expression, suggests a shift toward cell cycle arrest and activation of stress-responsive pathways during the treatment period. While apoptosis was not directly confirmed using functional assays such as Annexin V/PI staining or caspase activity measurements (23,26), the observed transcriptional changes are consistent with growth-inhibitory and pro-apoptotic signaling, and should therefore be interpreted within the context of mRNA-level analyses.

ROS analysis showed that combined treatment lowered intracellular ROS more effectively than individual treatments, especially in Caco-2 cells (27). This decrease may reduce ROS-dependent activation of survival pathways, such as NF- $\kappa$ B and AKT (26,28), which is consistent

with the observed gene expression changes. However, the antagonism does not seem to arise from oxidative stress; instead, it likely results from overlapping and potentially competing signaling effects of EGCG and retinol.

The concentration ranges selected for EGCG and retinol were based on earlier studies in CRC models (19,20,29), allowing comparison with previous findings and ensuring that the doses were biologically relevant. Using both low and high concentrations made it possible to evaluate sub-cytotoxic effects, determine IC<sub>50</sub> values, and analyze interactions (30).

Although our results indicate induction of apoptosis, future studies could employ complementary methods such as Annexin V/PI staining, caspase activity assays, or TUNEL assays to more comprehensively confirm apoptotic mechanisms.

## Conclusion

This study shows that EGCG and retinol, when used together, produce antagonistic rather than synergistic effects in CRC cells. Although gene expression changes point to modulation of key pathways such as p53/p21, AKT, and NF- $\kappa$ B (23–25), functional validation is still needed. The findings contribute to a better understanding of how EGCG and retinol interact and can help guide the design of future combination strategies for CRC research.

## Acknowledgment

Not applicable.

## Authors' contributions

S G, J C, M M, and Z K designed the experiments; S G performed experiments and collected data; J C, M M, and Z K discussed the results and strategy; J C supervised, directed and managed the study; S G, J C, M M, and Z K Final approved of the version to be published.

## Conflicts of Interest

The authors declare that no conflict of interest exists.

## Declarationon

We have not used any AI tools or technologies to prepare this manuscript.

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