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Research article

Rosmarinic acid enhances 5-fluorouracil sensitivity in HGC27 gastric cancer cells

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Abstract

Gastric cancer remains a prominent contributor to global cancer-related mortality, even though major advances in treatment have been made. Its high mortality rate is associated with resistance to chemotherapy and the limited clinical efficacy of commonly used drugs, such as 5-fluorouracil (5-FU). Rosmarinic acid (RA), a polyphenol found in high concentrations in Lamiaceae species, has antioxidant and anti-cancer properties. However, its potential to improve the effectiveness of 5-FU in poorly differentiated gastric cancer has not been adequately investigated. The objective of this study was to assess the cytotoxic effects, apoptosis-inducing potential, and underlying molecular alterations of RA and 5-FU, administered individually or in combination, in HGC27 gastric cancer cells. The effects on cell viability were assessed using the MTT assay, and apoptotic cell death was analyzed by Annexin V-FITC flow cytometry. The expression of the genes associated with apoptosis, stemness, and proliferation was evaluated by qRT-PCR. The IC_{25} and IC_{50} values of 5-FU were approximately 1.25 µM and 3.62 µM, respectively, whereas those of RA were approximately 23.2 µM and 50.7 µM, respectively. Both RA and 5-FU reduced cell viability in a concentration-dependent fashion and induced apoptosis at their respective IC_{50} concentrations. Specifically, the combined use of RA and 5-FU resulted in a much greater decrease in cell viability. A significant increase in apoptotic cell death was also observed compared with either treatment alone. This was accompanied by an augmentation in the pro-apoptotic genes *APAF1* and *BCL2L11*, a reduction in the anti-apoptotic genes *XIAP* and *NOL3*, reduced expression of the proliferation-related marker MKI67, and suppression of the stemness-associated transcription factors *OCT4* and *NANOG*. These results demonstrate that RA enhances the anticancer effects of 5-FU in gastric cancer cells through molecular alterations that may contribute to improved chemosensitivity. These results suggest that RA may improve the therapeutic response to 5-FU and represents a promising candidate for combination treatment of gastric cancer.

Keywords: *Apoptosis; chemosensitivity; fluorouracil; gastric cancer; rosmarinic acid*

1. Introduction

Gastric cancer continues to be associated with substantial cancer-associated morbidity and mortality. This is mainly due to late diagnosis, aggressive tumor behavior, and the development of resistance to conventional chemotherapy (Van Cutsem et al., 2016). One of the major chemotherapeutic agents for the clinical management of gastric cancer is 5-fluorouracil (5-FU), but its effectiveness in the clinical setting is often diminished by inherent (primary) or acquired (secondary) resistance to

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chemotherapy, as well as systemic toxicity related to the use of this drug (Lordick et al., 2024).

The major problem in the therapy of gastric cancer is that cancer cells can evade apoptosis, alter their response to oxidative stress, and induce several pro-survival signaling pathways. All these adjustments are interrelated and are associated with reduced sensitivity to chemotherapy, resulting in poor therapeutic response (Hu et al., 2019; Mao et al., 2025). Thus, it is clinically crucial to identify drugs that improve chemosensitivity.

Rosmarinic acid (RA) is an ester of caffeic acid and is widely distributed in the members of the Lamiaceae family, such as *Rosmarinus officinalis*, *Melissa officinalis,* and *Basilicum polystachyon* (Villegas et al., 2024). RA has gained significant interest owing to its anti-inflammatory, antioxidant, and neuroprotective properties. Mounting evidence has shown that RA also exhibits anti-cancer activity in various tumor types, such as colorectal, breast, liver, and gastric cancers (Villegas et al., 2024; Xu et al., 2025).

RA exhibits potent antiproliferative and pro-apoptotic effects and inhibits tumor cell migration and invasion. RA has shown selective cytotoxicity toward cancer cells in some experimental models. This renders it a promising candidate for combination therapy (Noor et al., 2022; Huang et al., 2024).

Accumulating evidence suggests that disruption of redox homeostasis together with persistent activation of pro-survival signaling pathways contributes to gastric cancer progression and promotes resistance to anticancer therapy (Chen et al., 2025). Elevated ROS levels promote genomic instability and tumor heterogeneity while activating pro-survival signaling pathways, such as NF-κB and PI3K/Akt, thereby facilitating cancer progression and therapeutic resistance (Akter et al., 2026). These pathways enable cancer cells to tolerate the cytotoxic stress induced by chemotherapy (Reczek and Chandel, 2017; Liu et al., 2020). Natural polyphenolic compounds with antioxidant and signaling-modulatory properties have attracted increasing interest as adjuvants to conventional chemotherapy. RA has been shown to attenuate oxidative stress and modulate key survival pathways, thereby promoting apoptosis in cancer cells (Nadeem et al., 2019; Wang et al., 2019; Villegas et al., 2024). This gives a robust mechanistic rationale for its use as a chemosensitizing agent.

Previous studies have shown that RA enhances 5-FU sensitivity in gastric cancer cells through FOXO4-related mechanisms (Yu et al., 2019). However, the potential of RA to potentiate the effects of 5-FU on the poorly differentiated HGC27 gastric cancer cell line has not yet been adequately evaluated. We aimed to evaluate the cytotoxic effects of RA and 5-FU, administered individually or in combination, and to investigate selected molecular responses associated with RA-mediated chemosensitization in HGC27 gastric cancer cells.

2. Materials and methods

2.1. Reagents

Thiazolyl blue tetrazolium bromide (MTT; AppliChem, Darmstadt, Germany; A2231-0001) was used for cell viability assays. Prior to use, the MTT working solution was prepared in Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO, USA). Rosmarinic acid (RA; Sigma-Aldrich, St. Louis, MO, USA) was initially dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution, which was stored at -20°C until further use. A stock solution of 5-FU (Sigma-Aldrich, St. Louis, MO, USA) was prepared in sterile distilled water.

2.2. Cell culture

HGC27 (accession number CVCL_1279) was established from a metastatic lymph node in a patient with gastric cancer. HGC27 cells were kindly provided by Prof. Dr. Ömer Faruk Bayrak (Yeditepe University, Istanbul, Türkiye). Cell culture was carried out in RPMI-1640 medium enriched with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine (Diagnovum GmbH, Greifswald, Germany). Incubation was performed in a humidified atmosphere adjusted to 37°C and 5% CO_2.

2.3. Cytotoxicity assay

MTT assay was employed to assess cell viability and cytotoxicity. The principle of the assay is based on viable cells with active metabolism that reduce MTT to insoluble formazan crystals. A total of 3×10^3 cells per well were seeded in 96-well culture plates containing 100 µL of complete RPMI-1640 medium and cultured overnight to permit adherence.

After an overnight attachment period, the cells were treated with increasing concentrations of RA or 5-FU to determine the dose-dependent effects. Cells treated with 0.01% DMSO were used as the vehicle control. For subsequent experiments, the cells were exposed to IC_{25} and/or IC_{50} concentrations of RA and/or 5-FU for 48 h. All treatments were carried out in triplicate wells. After treatment, cells were incubated with MTT reagent at 37°C for 2h. The formazan crystals formed by viable cells were then dissolved in DMSO prior to absorbance measurement.

Synergy Neo2 multimode microplate reader (BioTek Instruments, Winooski, VT, USA) measured absorbance at 570 nm. The viability of treated cells was calculated relative to the vehicle control (0.01% DMSO) and reported as a percentage. The half-maximal inhibitory concentration (IC_{50}), or the concentration required to cause a 50% decrease in cell viability, was calculated from the data of three independent experiments by linear regression analysis of dose-response curves (Cifci et al., 2026).

2.4. RNA isolation and quantitative real-time PCR analysis

A total of 4.5×10^5 HGC27 cells were plated in each well of six-well culture plates. They were maintained under standard culture conditions for 48h to allow cell attachment and growth. The cells received the IC_{50} concentration of the corresponding compound and were maintained for another 48h.

Following treatment, the adherent cells were collected by trypsinization. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. The yield and purity of the RNA were then assessed using a spectrophotometer. First-strand cDNA synthesis was then performed using the OneScript® Plus cDNA Synthesis Kit (Applied Biological Materials Inc., Richmond, BC, Canada).

The BlasTaq™ 2X qPCR MasterMix (Applied Biological Materials Inc., Richmond, BC, Canada) was used for quantitative real-time PCR (qRT-PCR). After verifying amplification specificity via melt curve analysis, relative gene expression was normalized to *GAPDH* and determined using the $2^{(-\Delta\Delta Ct)}$ approach (Table 1). The PCR amplification profile

Table 1
The primer sequences for qRT-PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
OCT4	CCTGAAGCAGAAGAGGATCACC	AAAGCGGCAGATGGTCGTTTGG
NANOG	CTCCAACATCCTGAACCTCAGC	CGTCACACCATTGCTATTCTTCG
APAF1	GCCAAGCAGGAGGTCGATAATG	GACCATCCTCAGAAAAGCAGGC
NOL3	TGCCAGGAGCTGCTACGCTGT	TGGGCATGGAGGGTCATAGCTG
BCL2L11	CAAGAGTTGCGGCGTATTGGAG	ACACCAGGCGGACAATGTAACG
XIAP	TGGCAGATTATGAAGCACGGATC	AGTTAGCCCTCCTCCACAGTGA
MKI67	GAAAGAGTGGCAACCTGCCTTC	GCACCAAGTTTTACTACATCTGCC

was programmed as follows: initial denaturation at 95°C for 5 min, succeeded by 40 cycles of 95°C for 10s (denaturation) and 60°C for 30s (annealing). Relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

2.5. Apoptosis analysis by annexin V-FITC staining

Apoptosis was analyzed using the Annexin V-FITC Apoptosis Detection Kit (Elabscience, Houston, TX, USA). HGC27 cells (8×10^5 cells/well) were seeded in six-well plates and allowed to attach for 24 hours. The cells were then treated with the compounds for 48h. Following treatment, both the floating cells and the detached cells after trypsinization were collected to recover the entire cell population before staining.

Centrifugation at 300× g for 5 min was performed to pellet the cells. Then, cells were washed twice using ice-cold PBS. The pellet was carefully mixed with 500 µL of 1× Annexin V binding buffer. After being stained with Annexin V-FITC in the dark at room temperature for 20 min, the samples were analyzed within 1 h using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). Apoptosis was assessed by the number of Annexin V-positive cells using CytExpert software (Kim et al., 2023).

2.6. Statistical analysis

GraphPad Prism version 10.2.3 (GraphPad Software, San Diego, California, USA) was used for statistical analyses. Statistical differences among the experimental groups were examined using the Kruskal-Wallis test, with Dunn's post hoc multiple-comparisons test applied when appropriate. Statistical comparisons were performed between each treatment group and the control group, and between the combined treatment group and each single-agent group. All experiments were conducted independently on three separate occasions, with each condition tested in triplicate. Results are depicted as mean ± standard deviation (SD). Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. The effects of 5-FU and RA on the viability of the HGC27 cells

The cytotoxic effects of 5-FU and RA on HGC27 cells were assessed by culturing cells at increasing drug concentrations for 48 hours. It was found that there is a concentration-dependent reduction in viability of cells after treatment with 5-FU ($p=0.021$ at 12.5 µM and $p=0.008$ at 25 µM) (Fig. 1A).

Similarly, RA treatment led to a concentration-dependent decline in HGC27 cell viability (Fig. 1B; $p=0.034$ at 80 µM and $p=0.006$ at 160 µM). The viability of cells decreased at moderate doses while cytotoxicity increased significantly at higher doses.

IC_{25} and IC_{50} values of 5-FU were approximately 1.25 µM and 3.62 µM, respectively. Similarly, IC_{25} and IC_{50} values after treatment with RA were 23.2 µM and 50.7 µM, respectively.

To test the combined effects of 5-FU and RA, the cells were treated with both compounds at their own IC_{25} and IC_{50} concentrations, individually and in combination. Combined treatment resulted in a significantly greater decrease in cell viability than single-agent treatments at the same concentrations (Fig. 1C; $p=0.018$ vs RA IC_{50}; $p=0.012$ vs 5-FU IC_{50}). The greatest decrease in viability was observed with the combination of RA and 5-FU at IC_{50} concentrations, suggesting an increased antiproliferative effect.

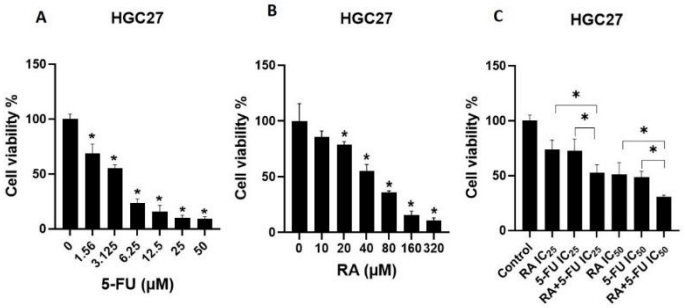


Fig. 1. The effects of 5-FU and RA, administered individually or in combination, on HGC27 cell viability. A) The cytotoxic effects of 5-FU (1.56-50 µM) on cells after 48 h of exposure were assessed using the MTT assay. B) Dose-dependent effects of RA (10-320 µM) on cell viability following 48 h of treatment. C) Cells were treated with RA or 5-FU alone at their respective IC_{25} (RA: 23.2 µM; 5-FU: 1.25 µM) and IC_{50} (RA: 50.7 µM; 5-FU: 3.62 µM) concentrations, or in combination (RA+5-FU), and cell survival was evaluated after 48 h. Results are presented as mean ± SD. Brackets indicate statistical significance across the highlighted treatment cohorts (*$p<0.05$).

3.2. Combined treatment with 5-FU and RA enhances apoptosis in HGC27 cells

To determine whether the decrease in cell viability induced by 5-FU and RA was associated with apoptosis, Annexin V-FITC-labeled cells were subjected to flow cytometric analysis. HGC27 cells were treated with each agent at its respective IC_{50} concentration, either alone or in combination, for 48h.

Control cells exhibited a low basal level of apoptosis. The use of 5-FU alone resulted in a significant increase in the proportion of Annexin V-positive cells compared to the control group ($p=0.031$) (Fig. 2). Treatment with RA alone resulted in a modest increase in apoptotic cells relative to the control group. The combined treatment of RA and 5-FU increased the number of Annexin V-positive cells compared to treatments with either

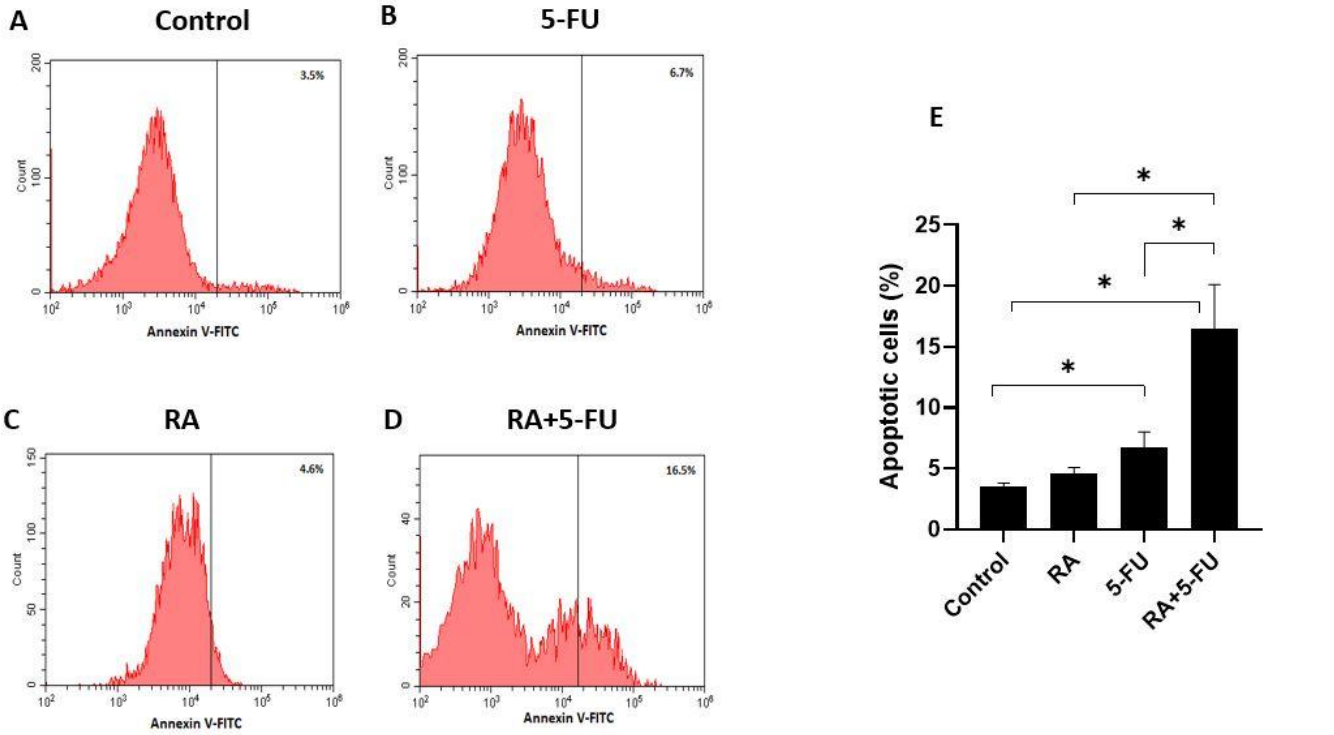


Fig. 2. The effects of 5-FU and RA, administered individually or in combination, on apoptosis. The individual and combined effects of 5-FU and RA at the determined IC_{50} concentrations were evaluated in HGC27 cells after 48 hours of incubation. Programmed cell death was quantified utilizing Annexin V-FITC staining followed by flow cytometry. Representative flow cytometric histograms are provided for A) control, B) 5-FU, C) RA, and D) combination (RA+5-FU) groups. The proportion of Annexin V-positive cells is highlighted in each corresponding panel. E) The percentage of apoptotic cells relative to total cells is summarized quantitatively. All data are expressed as mean ± SD. Brackets indicate statistical significance across the highlighted treatment cohorts (*p<0.05).

agent alone (p=0.001 vs 5-FU; $p<0.001$ vs RA). The findings indicate that apoptotic cell death increases following administration of both drugs in combination. The findings correlate with the decreased viability found in the combined treatment group.

3.3. Combined treatment alters the expression of apoptosis-, stemness-, and proliferation-related genes in HGC27 cells

To better understand the mechanisms of action of 5-FU and RA, gene expression analyses of genes involved in apoptosis, stemness, and proliferation were performed in HGC27 cells. As illustrated in Fig. 3, treatment with either 5-FU or RA alone resulted in moderate shifts in the expression of apoptosis-related genes. Combined treatment significantly upregulated the pro-apoptotic genes apoptotic peptidase activating factor 1 (*APAF1*) (p=0.014 vs 5-FU; p=0.021 vs RA) and BCL2-like 11 (*BCL2L11*, also known as *BIM*) (p=0.018 vs 5-FU; p=0.027 vs RA), while markedly downregulating the anti-apoptotic genes X-linked inhibitor of apoptosis protein (*XIAP*) (p=0.02 vs 5-FU and p=0.012 vs RA) and nucleolar protein 3 (*NOL3*, also known as *ARC*) (p=0.016 vs 5-FU).

Similarly, the stemness-associated transcription factors *OCT4* (p=0.019 vs 5-FU) and *NANOG* (p=0.013 vs 5-FU) were significantly reduced following combined treatment compared with single-agent treatments. Furthermore, the expression of the proliferation-related marker *MKI67* decreased markedly in the combination group (p=0.009 vs control). The observed changes suggest that treating HGC27 gastric cancer cells with a combination of RA and 5-FU enhances apoptotic signaling and suppresses stemness-related features and proliferative capacity.

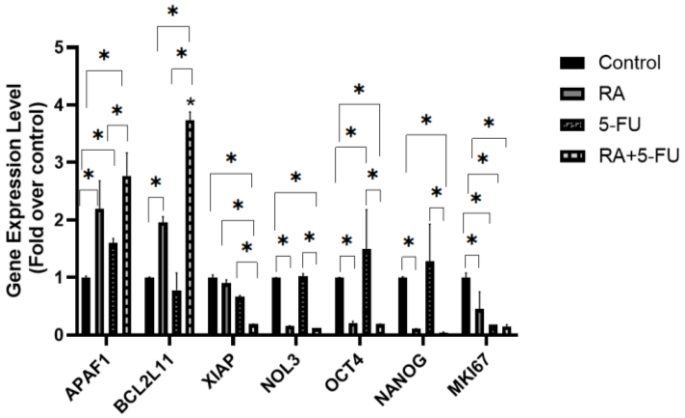


Fig. 3. Effects of 5-FU, RA, and their combined treatment on the expression of apoptosis-, stemness-, and proliferation-associated genes in HGC-27 cells. Cells were exposed to the IC_{50} concentration of 5-FU, RA, or their combination for 48 h. mRNA expression levels of genes involved in the apoptotic process (*APAF1, BCL2L11, XIAP, NOL3*), stem cell-associated markers (*OCT4*, *NANOG*), and the proliferation-related marker *MKI67* were analyzed by qRT-PCR. The gene expression data were normalized using *GAPDH* as the reference gene, and are presented as relative fold changes compared to the control. Results are presented as mean ± SD. Brackets show statistically significant differences among the indicated treatment groups (*p<0.05).

4. Discussion

This study shows that RA increases the cytotoxicity of 5-FU in HGC27 gastric cancer cells. These results add to a growing body of evidence suggesting that RA acts as a cytotoxic compound and an agent that augments sensitivity to

chemotherapy (Yu et al., 2019; Huang et al., 2024; Sari et al., 2025).

The effect of RA on sensitization to 5-FU treatment is consistent with previous reports showing that RA can reverse 5-FU resistance in gastric cancer by regulating FOXO4 signaling via miR-6785-5p (Yu et al., 2019). FOXO transcription factors have important functions in the regulation of cell cycle arrest and apoptosis (Farhan et al., 2017). Hence, alteration in FOXO signaling might explain the enhanced drug response in RA-treated cells. Although FOXO signaling was not directly investigated in the present study, the enhanced apoptotic response observed following the combined RA and 5-FU treatment may be associated with modulation of this pathway and its downstream apoptotic regulators, which needs further investigation.

Additionally, an increasing body of evidence indicates the involvement of RA in maintaining intracellular redox balance. Oxidative stress is known to have important roles in the cellular response to 5-FU treatment. However, high levels of antioxidants in cancer cells may contribute to chemotherapy resistance (Zhou et al., 2023; Czerwińska et al., 2024). It was shown that RA fine-tunes redox signaling rather than simply acting as a radical scavenger. Consequently, it promotes apoptotic signaling and enhances the anticancer efficacy of chemotherapeutic agents (Ijaz et al., 2023; Kowalczyk et al., 2024).

Our results revealed that treatment with either 5-FU or RA led to a decrease in the viability of HGC27 cells in a dose-dependent fashion. This reduction was accompanied by a significant increase in apoptosis following 5-FU treatment, whereas RA alone produced a modest increase. These effects of 5-FU in causing decreased viability and inducing apoptosis at their individual IC_{50} concentration values are in line with its role as a major chemotherapeutic agent that causes DNA damage and apoptosis (Longley et al., 2003; Lordick et al., 2022). Similarly, RA has been reported to suppress proliferation and promote apoptosis in gastric and other solid tumors through multiple molecular mechanisms, including the modulation of oxidative stress, redox signaling, and survival pathways (Yesil-Celiktas et al., 2010; Wang et al., 2019; Czerwińska et al., 2024; Kowalczyk et al., 2024).

These findings are consistent, demonstrating that RA alone produced a modest increase in apoptotic cells in HGC27 cells. This supports its potential role as an anti-cancer compound. Furthermore, combination therapy with RA and 5-FU at IC_{50} concentrations led to a stronger inhibitory effect on cell viability. Compared with either single-agent treatment, a significantly higher augmentation in the proportion of Annexin V-positive apoptotic cells was observed. This enhanced apoptotic response supports that the addition of RA may sensitize gastric cancer cells to 5-FU-mediated cell death. An expanding body of research highlights the potential of plant-derived polyphenols to enhance chemosensitivity by targeting multiple resistance-associated pathways, including in gastric cancer (Jakobušić Brala et al., 2023; Niu et al., 2025). Natural compounds, including RA, have been shown to enhance the efficacy of conventional chemotherapeutic agents by modulating diverse signaling pathways associated with cell survival and the cellular stress response (Ijaz et al., 2023; Reytor-González et al., 2025; Hashemi et al., 2026). The increased apoptotic response observed after combinational therapy suggests that RA may enhance the susceptibility of gastric cancer cells to the cytotoxic effects of 5-FU. However, this needs further mechanistic and translational investigation.

One of the key mechanisms underlying chemotherapy resistance in gastric cancer is the dysregulation of apoptosis and survival signaling pathways (Hu et al., 2019; Chen et al., 2025). Studies have shown that RA can lead to the restoration of susceptibility to apoptosis through the regulation of mitochondrial integrity, a decrease in anti-apoptotic Bcl-2 expression, and activation of caspases (Chen et al., 2023; Czerwińska et al., 2024; Yumrutas et al., 2025). Our results show that using RA along with 5-FU augmented the levels of *APAF1* and *BCL2L11*. It reduced the levels of the apoptosis inhibitors *XIAP* and *NOL3*. The observed changes in apoptosis-related gene expression suggest possible involvement of the intrinsic mitochondrial apoptotic pathway, which is known to contribute to chemotherapy-induced cell death and the cytotoxic effects of 5-FU (Longley et al., 2003; Fulda, 2015). Our observations of increased apoptosis are consistent with previous reports. Moreover, the simultaneous downregulation of *OCT4* and *NANOG* suggests a reduction in stem cell-like properties, which are recognized as important contributors to chemotherapy resistance and tumor recurrence in gastric cancer (Takaishi et al., 2009; Phi et al., 2018).

There is an increasing body of evidence supporting the role of plant-derived polyphenols as chemosensitizing agents targeting multiple resistance-associated pathways, including those implicated in gastric cancer (Zhao et al., 2022; Rabbani et al., 2026). It has been shown that RA modulates oxidative stress and multiple survival-related signaling pathways, thereby enhancing apoptotic responses in several types of cancer (Ijaz et al., 2023; Czerwińska et al., 2024; Kowalczyk et al., 2024; Villegas et al., 2024). Emerging evidence suggests that suppression of stemness-associated transcription factors, including *OCT4* and *NANOG*, may reduce tumor plasticity and therapy resistance (Phi et al., 2018; Esfini Farahani et al., 2025). The present findings suggest that the chemosensitizing effect of RA may be associated with both enhanced apoptotic signaling and reduced expression of stemness-related transcription factors. This emphasizes the potential role of RA in combination-based therapeutic strategies for chemoresistant gastric cancer. Furthermore, the downregulation of *MKI67*, a well-established marker of cellular proliferation, is consistent with the enhanced antiproliferative effect observed following combined treatment (Iuchi and Paulo, 2025; Ma et al., 2026). Natural substances such as RA have been found to enhance the effects of chemotherapy by modulating survival and stress-response pathways, while exerting antiproliferative and pro-apoptotic effects in cancer cells (Yesil-Celiktas et al., 2010; Liu et al., 2021; Sari et al., 2025; Hashemi et al., 2026).

These findings suggest that RA may sensitize gastric cancer cells to 5-FU. This effect can be achieved by driving apoptosis and altering the expression of apoptosis-, survival-, and stemness-associated molecular markers. However, further evidence from experimental animal studies is needed to validate the therapeutic value of combining RA and 5-FU. Clinical studies are needed to evaluate the translational effectiveness of this combination approach in gastric cancer patients.

Conflict of interest: The authors declare that they have no conflict of interests.

Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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