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Original Article

# Evaluation of the Combined Effects of Rosmarinic Acid and Cisplatin in Gastric Cancer Cells

## *Mide Kanseri Hücrelerinde Rosmarinik Asit ve Sisplatinin Kombine Etkilerinin Değerlendirilmesi*

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## ABSTRACT

**Objective:** Gastric cancer remains a significant global health concern, necessitating investigation into more effective treatment approaches. This study investigates the combined effects of rosmarinic acid, a polyphenolic compound with known anticancer properties, and cisplatin, a conventional chemotherapeutic agent, on human gastric carcinoma (HGC-27) cells.

**Methods:** Cell viability was evaluated at different concentrations for rosmarinic acid and cisplatin, and inhibitory concentration (IC)50, IC30, and IC10 values were subsequently determined. IC30 and IC10 doses were selected for combination experiments. Thiazolyl Blue Tetrazolium Bromide assay, colony formation assay, *in vitro* scratch assay, and 3D tumor spheroid growth assay were performed to evaluate the effects of individual and combined treatments.

**Results:** Rosmarinic acid and cisplatin individually reduced cell viability in a dose-dependent manner. Both the IC10 and IC30 dose combinations of the two agents demonstrated significant inhibitory effects on colony formation and cell motility, indicating an additive interaction compared with the control and the individual treatments. The combined treatment also inhibited spheroid growth, although the extent of the reduction was similar to that observed with the individual agents.

**Conclusions:** This study provides initial insights into the potential efficacy of the rosmarinic acid-cisplatin combination. The combination of these agents reduced cell viability, colony formation, and cell motility. The increased cytotoxicity observed in 2D models was not evident in 3D spheroid models, highlighting the importance of 3D systems that more accurately mimic the complex structure of tumors. This finding suggests that differences in drug sensitivity between 2D and 3D models should be considered when evaluating combination therapies.

**Keywords:** Cisplatin, drug combinations, multicellular spheroid, rosmarinic acid, stomach neoplasms

## ÖZ

**Amaç:** Mide kanseri, dünya çapında önemli bir sağlık sorunu olmaya devam etmektedir ve bu durum daha etkili tedavi yaklaşımlarına olan ihtiyacı artırmaktadır. Bu çalışmada, bilinen anti-kanser özelliklere sahip polifenolik bir bileşik olan rosmarinik asit ile geleneksel bir kemoterapötik ajan olan sisplatinin insan mide kanseri (HGC-27) hücreleri üzerindeki kombine etkileri araştırılmıştır.

**Yöntemler:** Hücre canlılığı, her iki ajanın farklı konsantrasyonları için değerlendirildi ve inhibitör konsantrasyon (IC)50, IC30 ve IC10 değerleri belirlendi. Kombinasyon deneylerinde IC30 ve IC10 dozları kullanıldı. Tekli ve kombine tedavilerin etkilerini değerlendirmek için Thiazolyl Blue Tetrazolium Bromide, koloni oluşumu, *in vitro* çizik deneyi ve 3D tümör sferoid büyüme deneyleri gerçekleştirildi.

**Bulgular:** Rosmarinik asit ve sisplatin, tekli kullanımlarında hücre canlılığını doza bağlı olarak azalttı. Her iki ajanın IC10 dozlarının ve IC30 dozlarının kombinasyonu, koloni oluşumu ve hücre hareketliliği üzerinde önemli bir inhibitör etki göstererek, kontrol grubu ve tekli ajan uygulamalarına kıyasla ek bir etkileşim olduğunu düşündürdü. Kombine uygulama sferoid oluşumunu da etkiledi, ancak bu etki tekli ajan uygulanan gruplardaki etkiyle benzerlik gösterdi.

**Sonuçlar:** Bu çalışma, rosmarinik asit ve sisplatin kombinasyonunun potansiyel etkisine yönelik ön bulgular sunmaktadır. Bu ajanların kombinasyonu, hücre canlılığını, koloni oluşumunu ve hücre hareketliliğini sınırlamıştır. 3D sferoid modellerde, 2D modellerde gözlenen artmış sitotoksik etkinin ortaya çıkmaması, tümörlerin karmaşık yapısını daha iyi taklit eden 3D sistemlerin önemini vurgulamaktadır. Bu sonuç, kombinasyon tedavilerinin değerlendirilmesinde 2D ve 3D modeller arasındaki ilaç duyarlılığı farklarının dikkate alınması gerektiğini göstermektedir.

**Anahtar kelimeler:** Sisplatin, ilaç kombinasyonları, multiselüler sferoid, rosmarinik asit, mide neoplazmaları

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## INTRODUCTION

Gastric carcinoma poses a significant public health challenge, as it is one of the leading causes of cancer-related mortality worldwide[1]. The etiology of gastric cancer is associated with multiple factors, such as genetic predisposition, *Helicobacter pylori* infection, dietary behaviors, and environmental factors[2]. Since gastric cancer is often diagnosed at an advanced stage, chemotherapy is a critical component of treatment. Cisplatin (CP), a platinum-based chemotherapeutic drug, is among the most widely used antineoplastic agents in treating gastric cancer[3].

The mechanism of action of CP involves the inhibition of cell division through the formation of covalent bonds with genomic or mitochondrial DNA, which leads to DNA damage, mitochondrial dysfunction, and eventual death of tumor cells[4]. However, clinical use is significantly restricted by drug resistance and systemic toxicity. Thus, there is increasing interest in novel combination therapies to improve CP's therapeutic efficacy and mitigate its toxicity.

Combination therapies in oncology have gained considerable attention in recent years because of their potential for improved efficacy compared to monotherapies. Combining diverse agents can enhance treatment efficacy and reduce the likelihood of cancer cells developing resistance[5]. It may also lower systemic toxicity by allowing the use of reduced amounts of chemotherapy drugs. The use of natural compounds in combination with cytotoxic drugs is considered a promising strategy because it can improve treatment outcomes while minimizing side effects[6,7].

Currently, natural compounds are being studied to enhance the efficacy of chemotherapeutic agents and mitigate adverse effects in anticancer treatments. Rosmarinic acid (RA) is a polyphenolic compound present in several aromatic plants, notably Rosmarinus officinalis. Because of its strong antioxidant, anti-inflammatory, and antiproliferative effects, RA is being investigated as a potential anticancer agent, with emphasis on its cytotoxic effects in a variety of cancers[8-11]. RA is proposed to exhibit synergistic potential in chemotherapy due to its impact on oxidative stress reduction, regulation of apoptotic pathways, and modulation of the cell cycle[12-15].

The purpose of this study is to enhance scientific understanding of the development of alternative and complementary therapeutic strategies by investigating the effects of RA in combination with CP on human gastric carcinoma (HGC)-27 gastric cancer cells.

## MATERIALS and METHODS

### Reagents

Thiazolyl blue tetrazolium bromide (MTT) reagent was purchased commercially (AppliChem, Darmstadt, Germany, A2231-0001, Lot: 3103285). MTT solution was prepared using Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO, USA). RA was purchased from its commercial supplier. (Sigma-Aldrich, St. Louis, MO, USA). The stock solution of RA was prepared using dimethyl sulfoxide as the solvent and stored at -20 °C until use. CP was used as a ready-to-use solution and stored at room temperature. Drug solutions were diluted in culture media to obtain the final doses.

### Ethics Statement

Since this study does not involve human participants, human data, or animal experiments, ethical approval is not required.

### Cell Culture

HGC-27 is a human gastric cancer cell line obtained from a metastatic lesion in an adult patient with undifferentiated gastric carcinoma. This cell line demonstrates a strong capacity for proliferation and maintains the morphological and molecular characteristics typical of poorly differentiated gastric cancer. HGC-27 cells are commonly used as an in vitro model to investigate the biological properties of aggressive gastric cancer and to assess the cytotoxicity of chemotherapeutic drugs or natural substances[16-18]. HGC-27 gastric carcinoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco-Thermo Scientific, Waltham, MA, U.S.) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco-Thermo Scientific, Waltham, MA, U.S.) and 1% (v/v) penicillin-streptomycin (Pen/Strep, Gibco-Thermo Scientific, Waltham, MA, U.S.). Cells were maintained at 37 °C within a humidified incubator that contained 5% $CO_2$. Cells were subcultured when culture flasks reached approximately 80% confluency.

### Cell Viability Assay

Cells were seeded at a density of $5 \times 10^3$ cells per well and incubated overnight at 37 °C in a humidified incubator with 5% $CO_2$. After incubation, cells were treated with different concentrations of RA (25, 50, 100, 200, 400, 800 µM) or CP (2.5, 5, 10, 20, 40, 80 µM) for 48 hours. At the end of the incubation period, MTT dye was added, and the cells were incubated for an additional 3 hours. Spectrophotometric analysis was performed at 570 nm using a microplate reader (Varioskan Lux, Thermo Fisher Scientific).

### Colony Formation Assay

Cells cultured in 6-well plates were treated for 48 hours with IC10 and IC30 doses of RA and CP, and with combined doses (IC10 of RA together with IC10 of CP, or IC30 of RA together with IC30 of CP). After trypsinization, cells were harvested, seeded at a density of 8×10^2 cells/well in 6-well plates, and maintained at 37°C with 5% $CO_2$ for 10 days. Upon completion of the incubation, cells were washed with phosphate-buffered saline (PBS) and fixed in methanol:acetic acid:water (1:1:8 v/v). Following fixation, cells were stained with crystal violet for 20 minutes and then rinsed with distilled water to remove residual dye. Spots with more than 50 cells were considered colonies and were analyzed[19]. Treated cell groups were normalized to the untreated control groups. The colony formation rate was determined using the formula (number of colonies / number of seeded cells) ×100%.

### *In Vitro* Scratch Assay (Wound Healing)

Wound healing was evaluated using 24-well plates seeded with 1×10^5 cells per well and incubated overnight at 37 °C in a 5% $CO_2$ incubator. Following incubation, cells were serum starved in fresh medium containing 0.5% FBS for 19 hours. Cell monolayers were carefully scratched the next day using sterile 200-µl pipette tips, and cellular debris was removed by washing with PBS. Cells were grown in serum-reduced medium (containing 0.5% FBS) with or without RA, CP, or their combination for 48 h. Imaging was performed at 0 and 48 h. The distribution of cells in the scratch area was evaluated via microscopic imaging (Labscope software, Primovert, Zeiss). ImageJ, with the MRI Wound Healing Tool (RRID:SCR_025260), enables quantitative measurement of wound closure.

### Tumor Spheroid Growth Assay

Spheroids were formed by seeding HGC-27 cells at a density of 5 × 10^3 cells in 200 µl of DMEM into 96-well U-bottom spheroid plates (Nunclon Sphera, Thermo Scientific, Waltham, MA, U.S.). Spheroids were formed by incubating cells at 37°C in 5% $CO_2$ for 72 hours. Spheroids were exposed to RA, CP, or their combination at doses of IC10 and IC30. Images were acquired on days 0, 1, 2, and 3 using a Zeiss Primovert microscope (4x objective) with Labscope and Zen software. The spheroid core area was measured using ImageJ software.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism 10 software. Student's t-test with Welch correction was applied to compare two groups. All analyses were conducted using three replicates from independent experiments. The error bars show the mean ± SEM of at least three independent experiments. Treated cell groups in all experiments were normalized to the untreated control groups.

## RESULTS

### RA and CP Reduced Cell Viability in HGC-27 Cells

The MTT assay was employed to assess the cytotoxicity of RA and CP in HGC-27 cells. Cell viability decreased in RA- and CP-treated cell groups in a dose-dependent manner (Figure 1A-B). The IC50, IC30, and IC10 values were determined from the cell viability analysis. The IC50, IC30, and IC10 values for RA were found to be 52 µM, 27 µM, and 9 µM, respectively. Furthermore, CP exhibited IC50, IC30, and IC10 values of 11 µM, 5 µM, and 2 µM, respectively.

The anticipated increase in cytotoxic effect of the combined treatment was assessed by administering combinations of the determined IC10, IC30, and IC50 doses. Therefore, the combinations of IC10, IC30, and IC50 doses of RA and CP were analyzed separately. Compared with single treatments, IC10, IC30, and IC50 combinations showed greater cytotoxicity (Figure 1C). The combined IC10 doses showed efficacy comparable to the single IC30 doses, and the combined IC30 doses closely replicated the effects observed with IC50 doses. Thus, IC10 and IC30 doses were selected for combined use in subsequent experiments.

### Combination Therapy Decreased The Formation of Cancer Cell Colonies

A colony formation assay was performed to examine the combined effects of RA and CP on continuous cell growth and colony formation. When tested alone, RA significantly reduced colony formation at IC10 and IC30 concentrations (18% and 40%, respectively). However, CP showed a much stronger inhibitory effect at its IC10 and IC30 concentrations (82% and 89%, respectively). Nevertheless, the cell groups treated with the combination doses exhibited significantly decreased colony formation compared to the groups treated with the individual drugs (Figure 2). Specifically, the combination of IC10 concentrations led to a 94% decrease in colony formation, while the combination of IC30 concentrations resulted in a 99% decrease in colony formation.

### Combination Therapy Decreased Cell Movement

The effects of a combination of RA and CP on cell motility were evaluated using an in vitro scratch assay. Following a 48-hour incubation, the untreated cell group

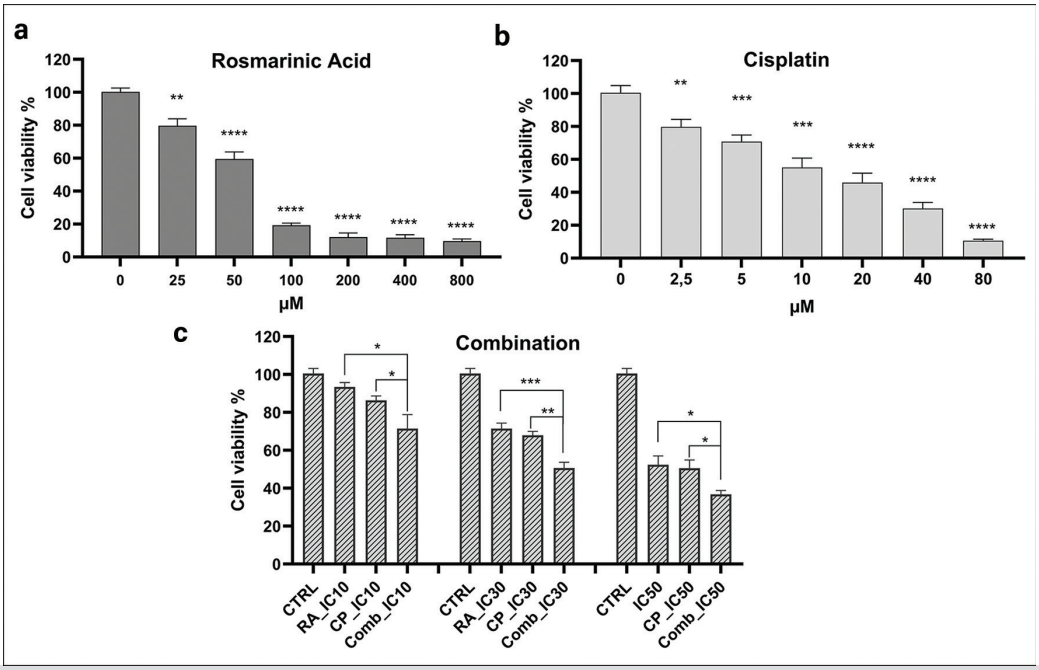


**Figure 1.** Cytotoxic effects of RA, CP and their combination against HGC-27 cell line. a) RA treatment, b) CP treatment, c) Combination treatment. Statistical analysis was performed using Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

RA: Rosmarinic acid, CP: Cisplatin , HGC-27: Human gastric carcinoma cells

fully covered the scratched area. At IC10 doses of RA and CP, the scratch area showed near-complete closure; at IC30 doses, it was wider. Administration of a single IC10 dose of RA resulted in approximately 85% closure of the scratch area, while a single IC10 dose of CP resulted in 95% closure. Co-administration of IC10 doses of RA and CP resulted in the suppression of cellular motility, leading to a 65% closure of the scratch area. Using an IC30 dose of RA alone resulted in approximately 70% closure of the scratch area, whereas a single IC30 dose of CP resulted in 71% closure. When IC30 doses of RA and CP were combined, cellular motility decreased further, resulting in only 46% of the area being covered by cells. Thus, the scratch areas were found to be wider than those observed with individual treatments (Figure 3).

### The Combination of RA and CP Suppressed Tumor Spheroid Growth

A spheroid growth assay was conducted to further investigate the combined effects of RA and CP on tumor growth in a 3D setting. Measurements of spheroid core area from the 3D spheroid growth assay revealed that RA and CP, at IC10 and IC30 doses, significantly reduced spheroid size over time under both individual and combined treatment conditions (Figure 4A-C). On day 3, CP alone exhibited the highest inhibition of spheroid growth (42%), followed by the combination treatment (34%) and RA alone (25.7%) (Figure 4D). Although the combination treatment showed the most statistically significant effect compared with the control group (p<0.0001), this inhibition was not greater than that observed with CP alone. Moreover, no significant difference was observed in spheroid size between the combined and individual treatments (Figure 4D), indicating no additive or synergistic effect between RA and CP. This highlights the need for further optimization of dosing in 3D tumor models.

## DISCUSSION

Combination therapies are now widely favored in cancer treatment to increase efficacy and reduce adverse effects. Specifically, combining chemotherapy with natural compounds may improve treatment outcomes while permitting reduced doses. Our study examines the cytotoxic and antiproliferative effects of RA and CP on the HGC-27 gastric cancer cell line, both individually and in combination, to advance this approach.

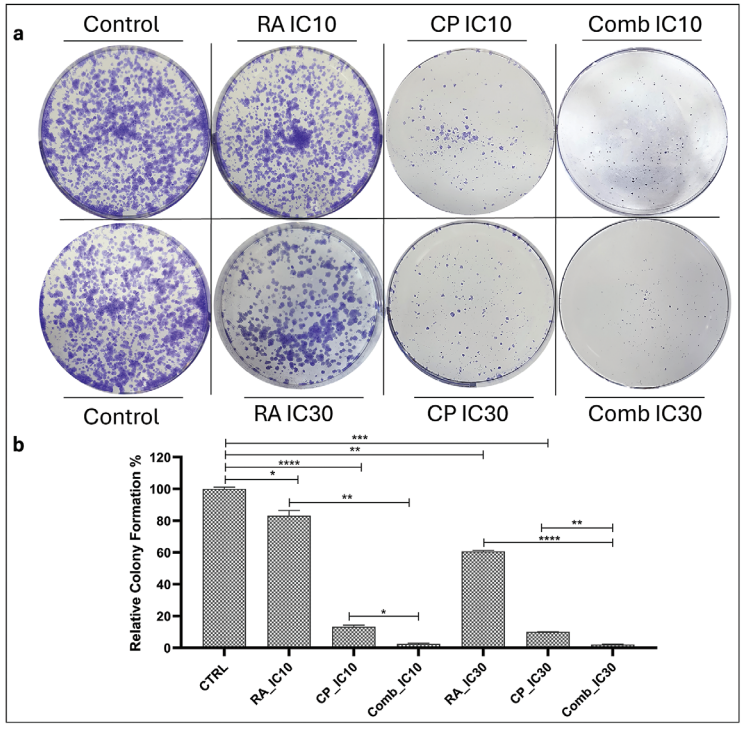


**Figure 2.** The colony formation of HGC-27 cells following RA, CP and combination treatments. a) Colony formation of HGC-27 cells, b) Statistical analysis (Student's t-test, *p<0.05, **p<0.01, ***p<0.001).

RA: Rosmarinic acid, CP: Cisplatin , HGC-27: Human gastric carcinoma cells

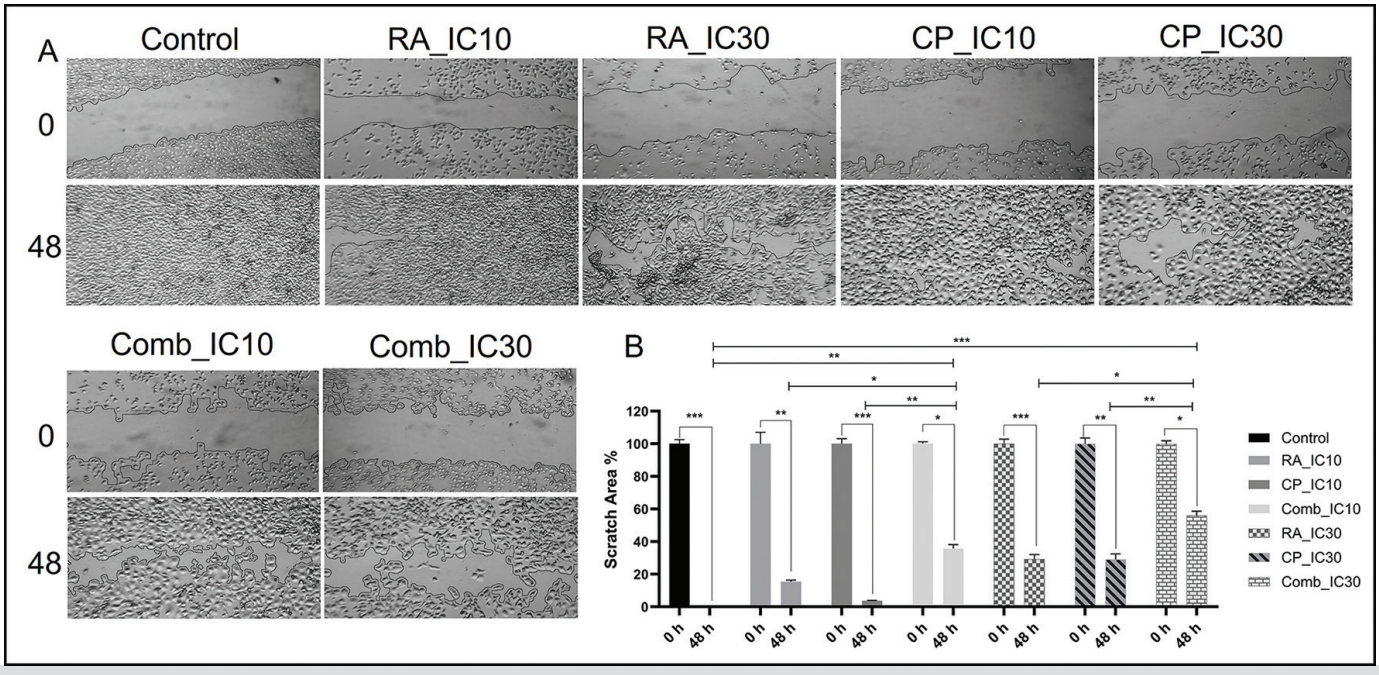


**Figure 3.** *In vitro* scratch assay of HGC-27 cells following RA, CP and combination treatments. A) Scratched regions of cells, B) Statistical analysis (Student's t-test, *p<0.05, **p<0.01, ***p<0.001).

RA: Rosmarinic acid, CP: Cisplatin , HGC-27: Human gastric carcinoma cells

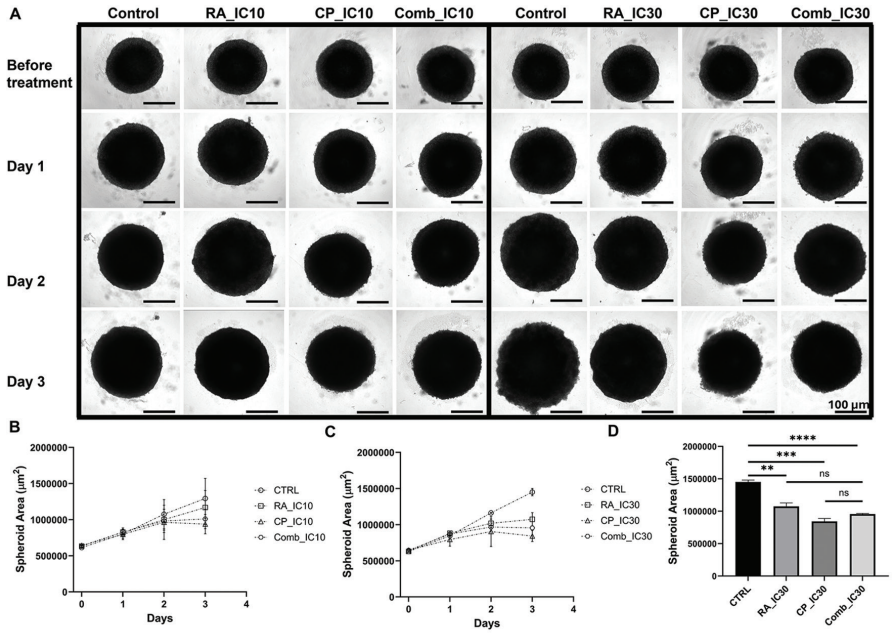


**Figure 4.** Spheroid growth assay of HGC-27 cells following RA, CP and combination treatments. A) Microscopic images of cells, B) Spheroid core area measurement following IC10 treatments up to day 3, C) Spheroid core area measurement following IC30 treatments up to day 3, D) Spheroid core area measurement on day 3 following IC30 treatments (Student's t-test, **p<0.01, ***p<0.001, ****p<0.0001).

HGC: Human gastric carcinoma, RA: Rosmarinic acid, CP: Cisplatin, IC:Inhibitory concentration

After determining the appropriate IC values for each agent, the combination groups (RA+CP IC10 and RA+CP IC30) exhibited greater cytotoxicity than the groups treated with each agent individually. The results indicate that the combination therapy's impact on cell viability may be additive or synergistic. Prior investigations have documented the synergistic impact of polyphenols in conjunction with chemotherapeutic agents[20]. Analysis across a broader dose range may reveal more significant synergistic effects.

Prior studies have indicated that various polyphenols can inhibit the colony-forming ability of cancer cells [20-22]. Thus, we utilized colony formation assays to assess how RA and CP, alone or in combination, influenced the long-term growth potential of HGC-27 cells. Individual administration of RA and CP significantly inhibited colony formation, although CP exerted a more substantial effect than RA. Nevertheless, the IC10 and IC30 combinations exhibited a marked reduction in colony numbers, suggesting that these combinations possess more potent antiproliferative effects than the individual treatments.

Cellular motility is essential to the pre-metastatic process and poses a major challenge to cancer treatment[23-25]. For this reason, the development of therapeutic strategies that can impact cell motility is of the utmost importance. In addition to affecting cell viability and proliferation, combining polyphenols with chemotherapeutic agents may inhibit cell motility. Our study revealed that after 48 hours of incubation, the scratched region in the control group had completely closed. Although the scratched region was almost closed at IC10 doses, complete closure was not achieved at IC30 doses. At IC10 concentrations, cell motility was largely unaffected, permitting cells to move and close the scratch region. In contrast, following treatment with IC30 doses of both agents, cell motility was considerably reduced, and a larger scratch area remained after 48 hours. These findings align with the dose-dependent inhibitory effects of RA and CP; specifically, higher concentrations demonstrate more pronounced cytotoxic effects, which impede scratch closure. Therefore, the observed "near-complete closure" at IC10 doses appears indicative of preserved basal motility under minimal toxicity, whereas the "larger scratch area" at IC30 doses reflects suppression of cell motility due to cytotoxic stress. The scratched region, however, revealed a significant gap when IC10 and IC30 doses were used in combination. This indicates that the simultaneous administration of RA and CP suppressed cellular migration to a greater extent

than individual treatments. These findings suggest that this combination has the potential to reduce cell motility and indirectly inhibit metastasis.

3D tumor models provide improved tools to more fully recapitulate the complex architecture of tumors and to enhance drug-screening processes, thus facilitating the identification of more effective therapeutic candidates[26-28]. Therefore, to better mimic the tumor microenvironment and to obtain more physiologically relevant drug responses, we employed a 3D spheroid growth assay. Our findings demonstrated that RA and CP, both individually and in combination, significantly inhibited spheroid growth in HGC-27 cells compared with the untreated control group. RA alone reduced spheroid size by 34% and CP reduced it by 42%, whereas the combination treatment reduced size by only 25.7%. Although all treatments significantly suppressed spheroid growth relative to the control, no significant difference was observed between individual treatments and the combined treatment. Interestingly, although our study observed a combinatorial efficacy of RA and CP in 2D cultures, this interaction was not reproduced in the 3D spheroid model. This discrepancy may be attributed to the structural and physiological differences between 2D and 3D culture systems. Unlike 2D cultures, tumor spheroids, with diameters greater than 500 µm, typically develop gradients of oxygen, nutrients, and waste, leading to the formation of hypoxic and necrotic zones that are observed in solid tumors. Furthermore, in 3D cultures, tight cell-cell and cell-matrix interactions, along with diffusion barriers, can limit drug penetration, thereby altering cellular drug responses[28,29]. Consistent with the literature, the spheroids established in our study, each larger than 500 µm, are expected to reflect the architectural complexity of solid tumors. Therefore, the doses that were effective in 2D in our study might not be optimal in the 3D setting. Although no study to date has reported on CA's efficacy in HGC-27-derived spheroids, it has been tested in 3D spheroid models of several cancer types. These studies provide evidence of differences in drug sensitivity between 2D and 3D cell culture systems, indicating that 3D models are more drug-resistant than 2D systems[30-33]. For instance, Baek et al.[30] directly compared CA's cytotoxicity in 2D and 3D models and reported that IC50 values for all tested 3D spheroids were higher than previously reported 2D results in different cancer types. Inducing cytotoxicity in 3D spheroids would require higher concentrations than in 2D systems, suggesting that each system may require different treatment optimization. This emphasizes the importance of using 3D tumor models, which more accurately recapitulate tumor architecture and therapeutic resistance, to evaluate combination strategies.

### Study Limitations

This study revealed that the combined use of RA and CP elevated cellular cytotoxicity and limited cell motility in HGC-27 gastric cancer cells. Despite this, the underlying molecular mechanisms of these effects remained unexplored. While reduced cell motility could influence metastatic processes, the underlying mechanisms and metastasis-related parameters (e.g., signaling pathways and gene/protein expression profiles) were not assessed. Thus, the research findings lack full mechanistic support, and *in vitro* outcomes must be validated in 3D and *in vivo* models. To this end, we attempted to establish spheroid cultures to better reflect the tumor microenvironment. However, the effects observed in 2D in vitro assays, particularly those of the combination treatment, were not detected in 3D models. This discrepancy suggests that 3D spheroid models, which better recapitulate the native tumor, may require further dose optimization.

Additional research is necessary to elucidate the molecular processes driving the observed cytotoxicity and motility inhibition, with particular regard to their potential impact on metastasis, and to validate the results in different experimental models. These studies are important for evaluating the clinical significance of the combination strategy and its potential translation into practical therapeutic methodologies.

## CONCLUSION

In this study, we demonstrated that both RA and CP exhibit inhibitory effects on the proliferation, colony-forming capacity, and motility of HGC-27 gastric cancer cells. The drug combination showed enhanced efficacy compared with individual treatments in 2D assays, suggesting that combining these agents is a promising approach. However, this additive effect was not observed in our 3D spheroid models, highlighting the need to consider the biological complexity and therapeutic resistance reproduced by 3D tumor models when evaluating drug combination approaches. Ultimately, our study provides insight into the efficacy of the combination of RA and CP in gastric cancer therapy and emphasizes the importance of integrating 3D culture systems into preclinical testing to obtain more physiologically relevant results.

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### Ethics

**Ethics Committee Approval:** Since this study does not involve human participants, human data, or animal experiments, ethical approval is not required.

**Informed Consent:** Not appreciable.

### Footnotes

### Author Contributions

Concept: C.S., C.Sü., S.K.A., B.Y., Design: C.S., C.Sü., S.K.A., B.Y., Data Collection or Processing: C.S., C.Sü., S.K.A., B.Y., Analysis or Interpretation: C.S., C.Sü., Literature Search: C.S., C.Sü., S.K.A., B.Y. Writing: C.S., C.Sü.

**Conflict of Interest:** The authors declare no conflicts of interest.

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