

Rosemary (*Rosmarinus officinalis*) extract causes ROS-induced necrotic cell death and inhibits tumor growth *in vivo*

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Background

01.

Colorectal cancer is the third most commonly diagnosed cancer worldwide, and its incidence is increasing even in low-risk countries.

02.

Although substantial progress has been made in treatment and survival rates, new therapeutic approaches are still needed.

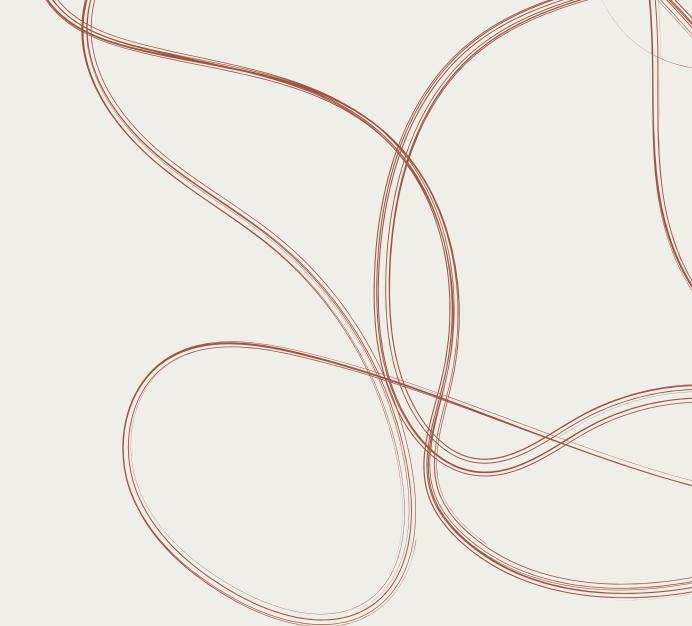
03.

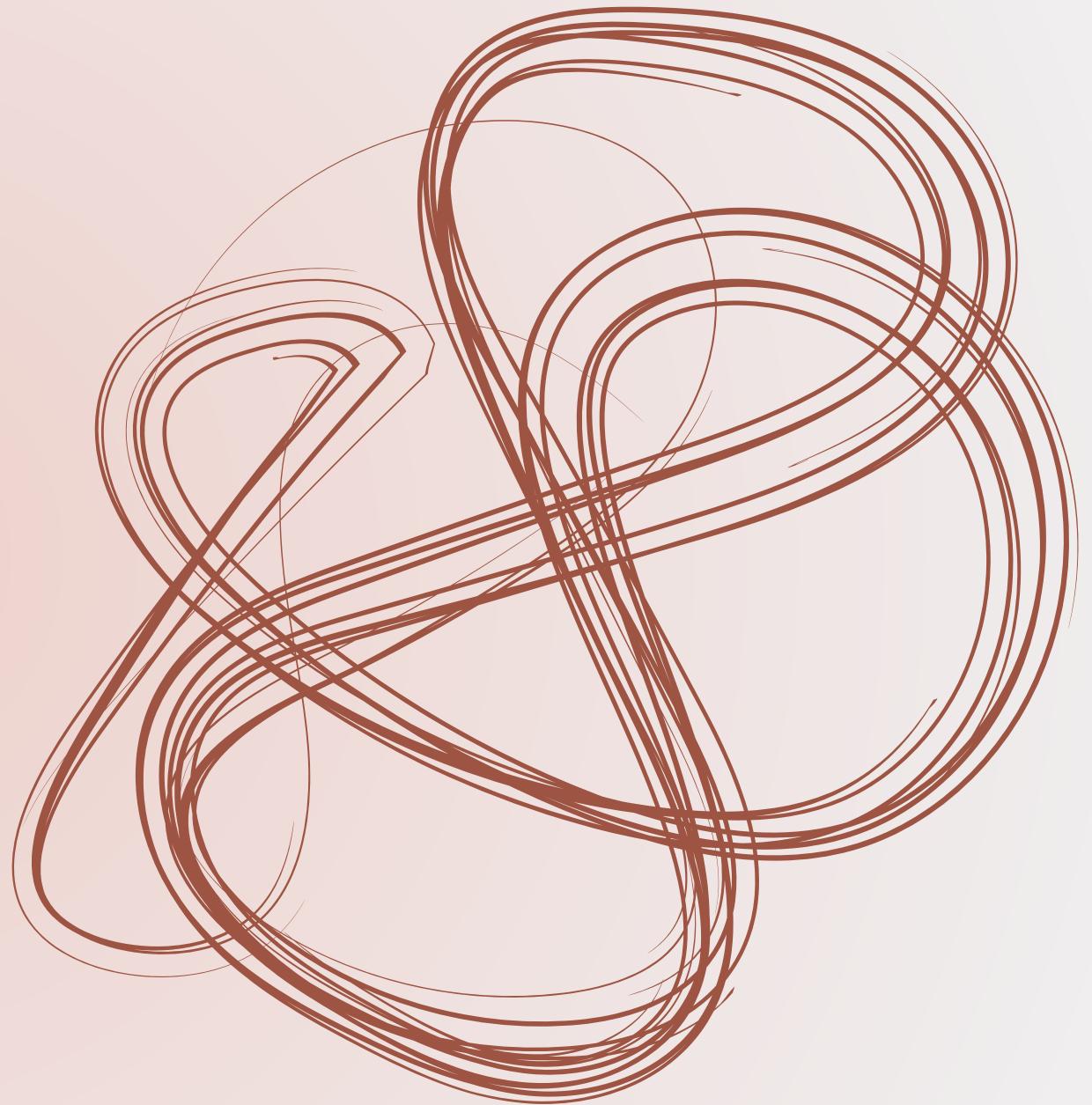
Natural compounds are a promising source of **new bioactive** compounds with anticancer properties. Among them, **rosemary polyphenols** have demonstrated **significant antiproliferative abilities** against colon cancer cells in both in vitro and in vivo models.

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Structure and Components of the Plant (Rosemary)

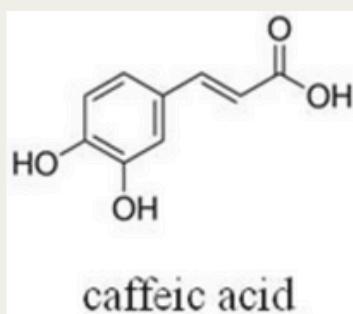
DESCRIPTION OF ROSEMARY STRUCTURE

- Rosemary (**Rosmarinus officinalis** L.) is a shrub from the Lamiaceae family, primarily **distributed in the Mediterranean region.**
- Its leaves are narrow, needle-like, leathery, and dark green with rolled edges. The stems are square-shaped, and small white flowers grow in racemes from the leaf axils, with flowers measuring about 1.2 cm in length. The flowers come in various colors such as blue, light blue, purple, pink, and white, typically blooming from December to April. The fruit is a small, spherical nut, egg-shaped or obovate, with small yellow-brown seeds.
- There are about 24 varieties of rosemary cultivated for economic purposes. Based on growth habits, rosemary is generally categorized into two types: upright and creeping.
- The English name "rosemary" is derived from its Latin name **rosmarinus**, which is composed of two roots: "ros" meaning "dew" and "marinus" meaning "of the sea," together meaning "dew of the sea." Since rosemary is especially drought-tolerant, it can survive in many places with just moisture from the sea.

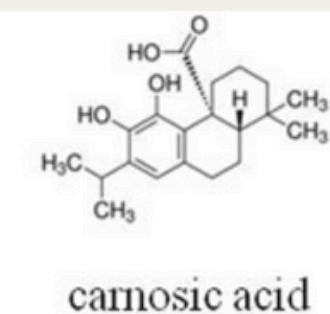


EXPLANATION OF ROSEMARY COMPONENTS

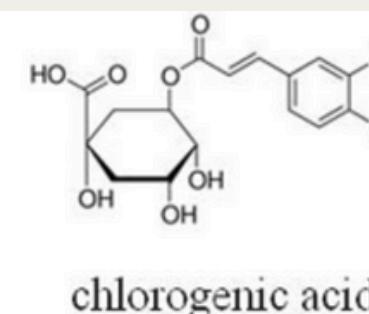
- Rosemary contains a complex mixture of chemical components, including **monoterpenes**, **sesquiterpenes**, **diterpenes**, **triterpenes**, **flavonoids**, and **fatty acids**. These components are distributed in relatively balanced proportions.
- Among them, the **diterpene phenols** present in rosemary exhibit significant antioxidant, preservative, antibacterial, anti-tumor, anti-HIV, and antimicrobial activities. The main active compounds include carnosic acid, rosmarinic acid, 7-ethoxyrosmanol, carnosol, and 7-methoxyrosmanol, with carnosic acid showing the strongest activity.
- Rosemary (**Rosmarinus officinalis L.**) is widely used as an **antioxidant** and flavoring agent in the food, cosmetics, and pharmaceutical industries.



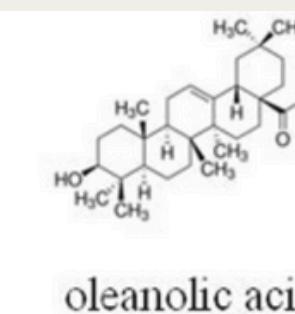
caffein acid



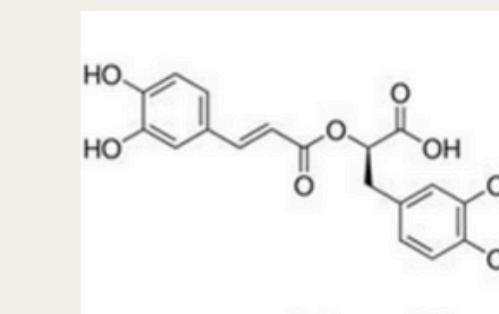
carnosic acid



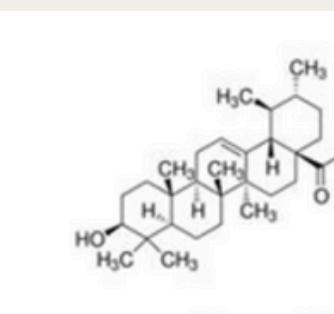
chlorogenic acid



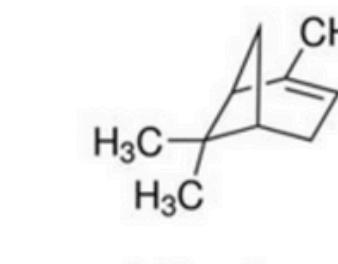
oleanolic acid



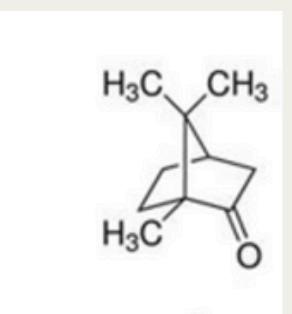
rosmarinic acid



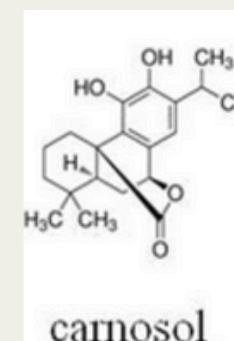
ursolic acid



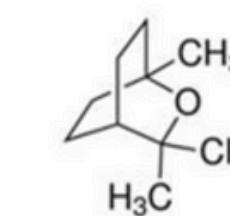
alpha-pinene



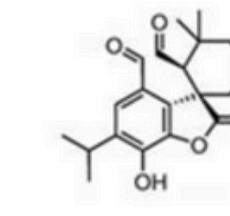
camphor



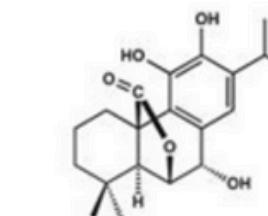
carnosol



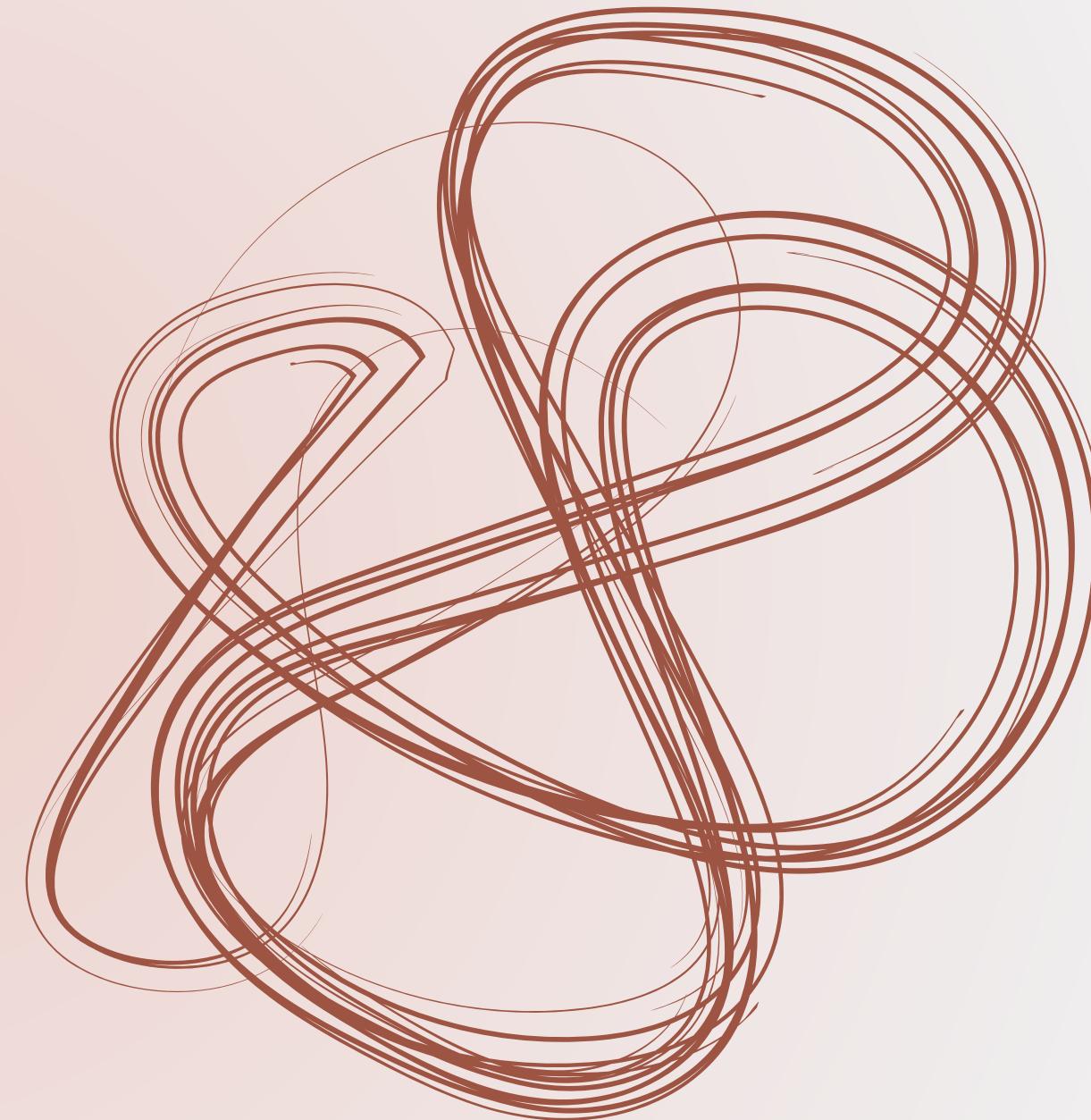
eucalyptol



rosmadial



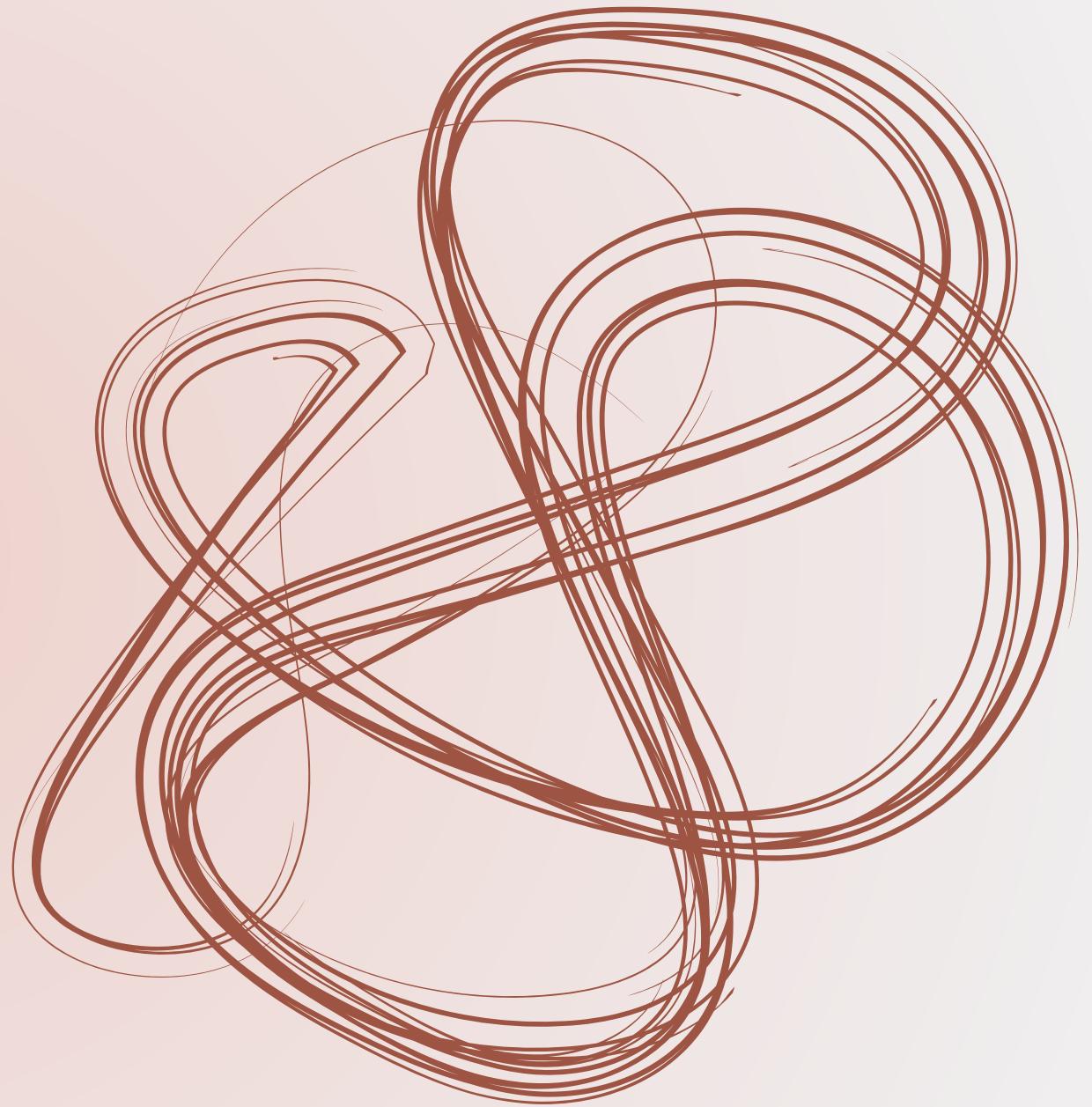
rosmanol



Functions from Past Research

FUNCTIONS OF PREVIOUS STUDIES

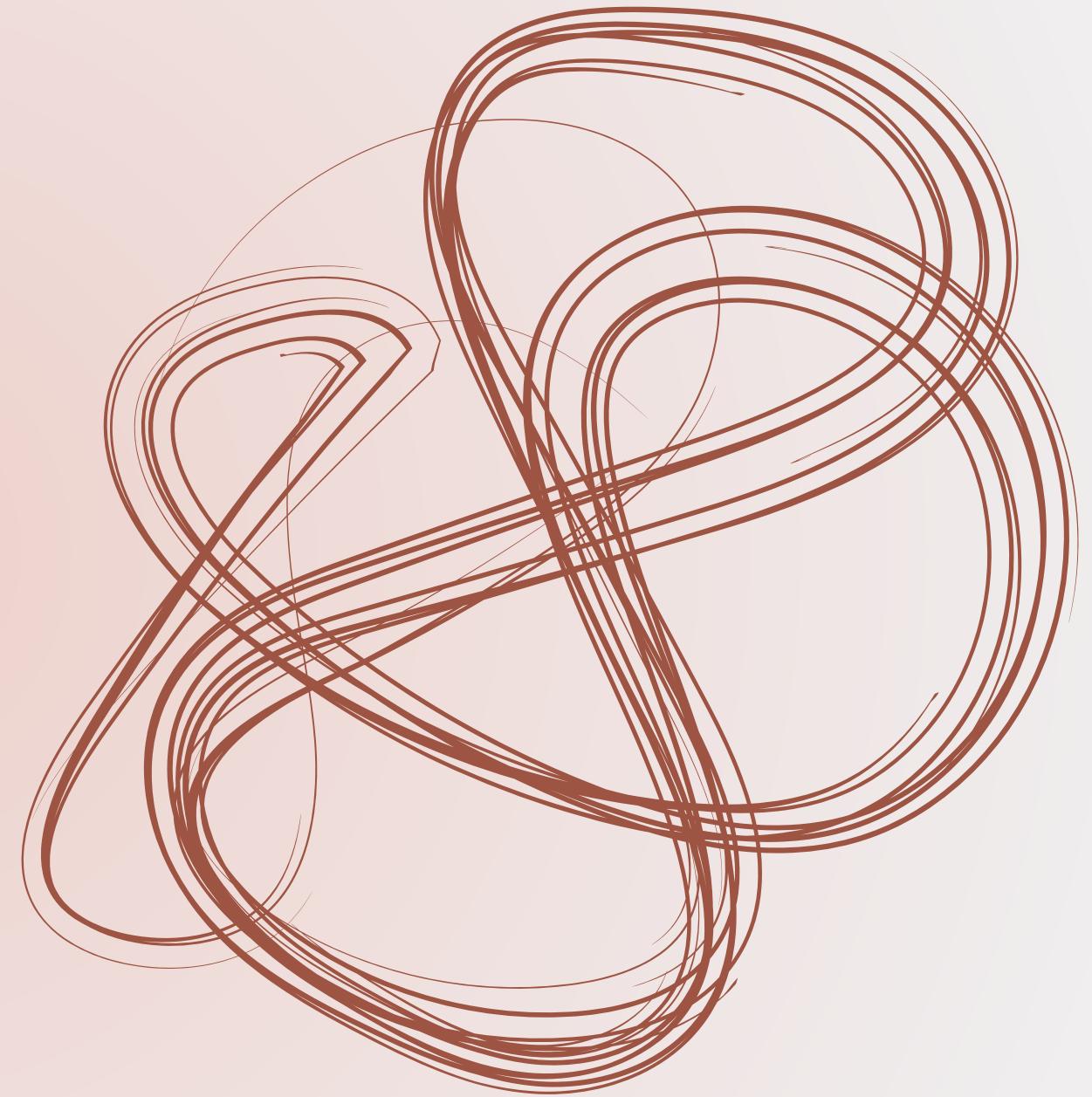
- Previous studies have investigated the detailed components of RE (rosemary extract), its anti-proliferative activity, and the purified fractions in colon cancer cells.
- It was found that there are pharmacological interactions between certain RE compounds. Transcriptomic analysis of some isolated compounds from RE, such as CA (carnosic acid) and CAR (carnosol), in colon cancer cells also indicated this.
- To elucidate the anti-proliferative effects of RE, the basic cytotoxicity studies reported earlier were further extended to show that RE can inhibit cell proliferation, colony formation, and migration in three types of colon cancer cells.
- Our previous research detailed the characterization of RE components used in this study via HPLC-ESI-QTOF-MS, showing that **diterpenes (CA and CAR) and triterpenes (UA and BA) are the most abundant compounds.**



Functions of This Article

FUNCTION OF THIS LITERATURE

- Several dietary components and their bioactive compounds found in plant-based foods and medicines have shown chemoprotective effects against various cancers, including colon cancer, and possess anti-cancer and anti-bacterial properties. **These compounds exhibit antioxidant and anti-diabetic effects both in vitro and in vivo.** Therefore, identifying new bioactive foods or components with anti-cancer potential to **prevent and/or treat human cancers is related to this research.**
- In this paper, the **antiproliferative** effect of **terpenoid-rich rosemary extract (RE)**, obtained through supercritical extraction technology, has been demonstrated in a colon cancer cell model. Experimental studies have confirmed the pharmacological potential of rosemary and some of its major compounds, such as the diterpenes **carnosic acid (CA) and carnosol (CAR)**, further expanding their possible therapeutic applications.
- These findings suggest that targeting colon cancer cells by increasing intracellular ROS and reducing cell survival mechanisms might provide a treatment strategy by combining rosmarinic compounds with chemotherapy drugs for the treatment of colon cancer.



Research Evidence

RESEARCH EVIDENCE

- The supercritical extraction of a terpene-rich fluid extract has demonstrated antiproliferative effects in colon cancer cell models. Transcriptomic and metabolomic analyses show that RE treatment activates genes involved in cell cycle progression and phase II antioxidant enzyme activity.
- Bio-guided fractionation suggests that **CA and CAR are the main active compounds, though the higher activity in the full extract points to potential synergistic effects between diterpenes and triterpenes.** The exact molecular mechanisms and interactions between RE components remain unclear.
- **Diterpenes (CA, CAR) and triterpenes (BA, UA)** were selected for individual or paired treatments. IC50 measurements in HT-29 cells showed dose-dependent antiproliferative effects, with UA and BA exhibiting stronger activity than CA and CAR. All isolated compounds had lower IC50 values compared to the RE extract.

- Since migratory ability is one of the core characteristics of metastatic cells, a wound healing assay (Figure 1C) was used to **evaluate the inhibitory effect of RE on the migratory ability of HGUE-C-1, HT-29, and SW480 cells.**

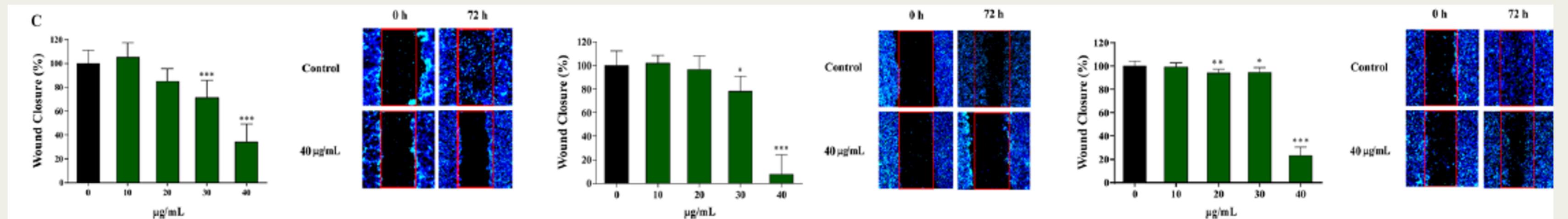


Figure 1.RE extract inhibits the proliferation, migration, and colony formation of human colon cancer cells.

(C) The wound closure percentage in the wound healing migration assay was analyzed through fluorescence imaging in the three cell lines. Representative microscopic images were also shown, indicating the effect of increasing RE extract concentrations.

- A cyclic growth pattern (troughs) was observed in the cell index parameters of HGUE-C-1 and HT-29 cells, which is **related to the morphological changes that occur during the mitotic process.**
- A colony formation assay was used to test whether RE could achieve the following: inhibit the unlimited proliferation ability of cancer cells, thereby preserving their ability to reproduce and form large colonies.
- Cultivate colorectal cancer cell lines with different concentrations of RE (20 or 40 µg/mL) for 7 days. RE inhibited colony formation in three colon cancer models in a dose-dependent manner (Figure 1B).

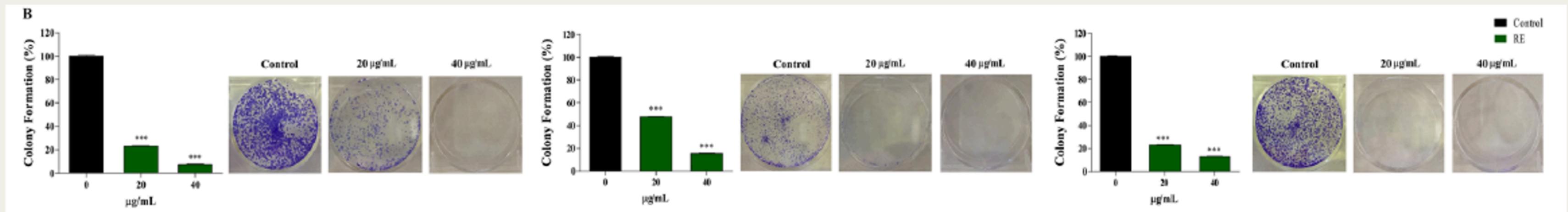


Figure 1. RE extract inhibits the proliferation, migration, and colony formation of human colon cancer cells.

(B) Inhibition of colony formation in three colon cancer cell lines without (control) or in the presence of 20 µg/mL or 40 µg/mL of RE.

-
- Treatment with 20 and 40 µg/mL of RE inhibited colony formation by x% compared to the control group

RE		HGUE-C-1 cell	SW480 cell	HT-29 cell
	20µg/mL	76.9%	76.9%	53.3%
	40µg/mL	92.3%	87.1%	84.5%

- It was further confirmed that **HGUE-C-1 and SW480 cells were more sensitive to RE than HT-29 cells.**

STUDY ON THE MECHANISM OF RE'S ANTIPROLIFERATIVE EFFECT ON COLON CANCER CELLS

- A significant decrease in the G0/G1 phase was observed, accompanied by an accumulation of cells in the G2/M phase in SW480 cells (observed at 30 and 40 µg/mL RE treatment, $P < 0.05$), and in HT-29 cells (with 40 µg/mL RE treatment, $p < 0.01$).
- In contrast, in HGUE-C-1 cells, RE reduced the G0/G1 phase, with an accumulation of cells in the SubG1 phase, increasing from 2.2% in the control group to a maximum of 15.0% at the measured concentration ($P < 0.01$).

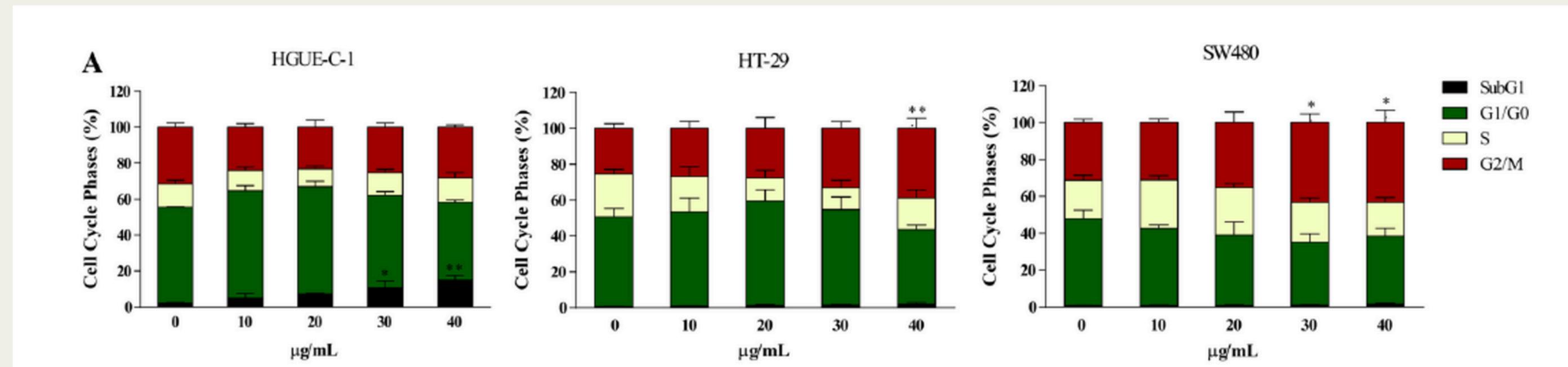


Figure 2.RE induces cell cycle arrest and cell death in human colon cancer cells.

(A) Cell cycle phase (%) analysis of three colon cancer cell lines in the absence or presence of increasing concentrations of RE using the Muse cell analyzer.

- Then, the impact of RE on apoptosis induction was specifically assessed by detecting Annexin V-positive cells. HGUE-C-1, HT-29, and SW480 cell lines were stained with Annexin V/7-AAD and analyzed using a Mus flow cytometer (Figure 2B).
- After 24 hours of RE treatment, there was a significant increase in the percentage of Annexin V/7-AAD double-positive cells in a concentration-dependent manner, indicating late apoptosis or necrosis in all cell lines. While late apoptotic and necrotic cells are both Annexin V and 7-AAD positive, the absence of early apoptotic cells through cell cycle analysis suggests the presence of necrosis rather than apoptosis.

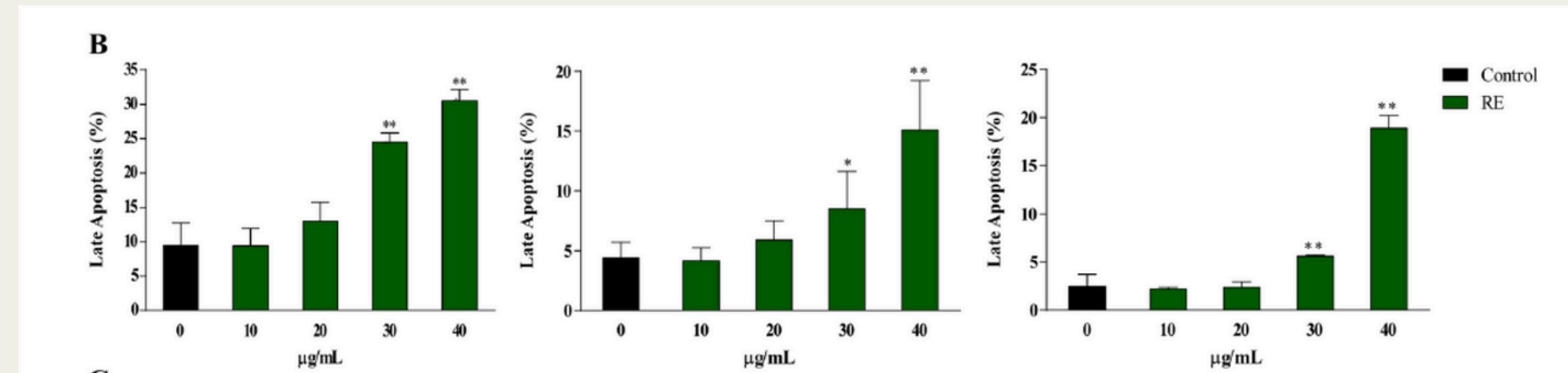
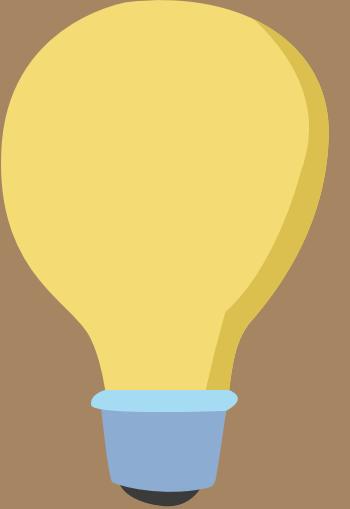


Figure 2. RE induces cell cycle arrest and cell death in human colon cancer cells.

(B) Measurement (%) of late apoptosis in three cell lines treated with the same concentration of RE.



*In summary, all these results indicate that apoptosis
is not the primary mechanism of cell death.*

INTRACELLULAR ROS GENERATION AND MITOCHONDRIAL MEMBRANE POTENTIAL MEASUREMENT

- Rosemary compounds have shown the ability to **modulate oxidative stress** in various in vitro and cellular systems. Furthermore, **apoptosis and necrosis** have both been demonstrated to be **triggered by ROS**. Therefore, we aimed to determine whether treating colon cancer cells with RE could regulate intracellular ROS. The accumulation of intracellular ROS was assessed using three colorectal cancer cell lines with the non-polar, cell-permeable probe H2DCFDA.
- Treatment with RE induced an increase in fluorescence intensity in all colorectal cancer cell lines, indicating an increase in ROS generation in a concentration-dependent manner (Figure 3A).

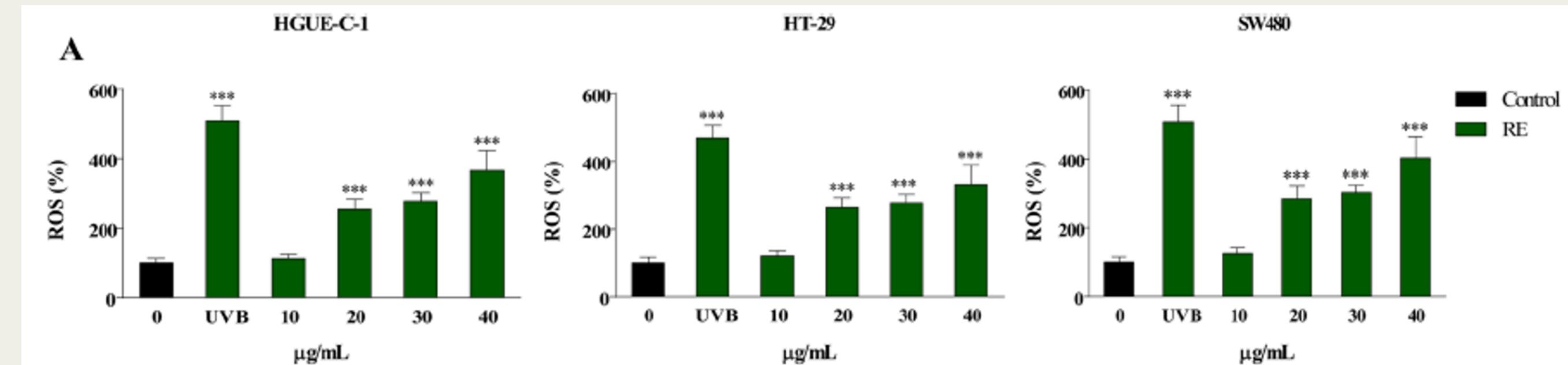


Figure 3. RE induces ROS and mitochondrial membrane depolarization in human colon cancer.**

(A) Intracellular ROS levels (%) were measured in three colon cancer cell lines using H2DCFDA dye, with or without 10, 20, 30, or 40 $\mu\text{g/mL}$ of RE, following UV radiation.

- Rosemary compounds have shown the ability to **modulate oxidative stress** in various in vitro and cellular systems. Furthermore, **apoptosis and necrosis** have both been demonstrated to be **triggered by ROS**. Therefore, we aimed to determine whether treating colon cancer cells with RE could regulate intracellular ROS. The accumulation of intracellular ROS was assessed using three colorectal cancer cell lines with the non-polar, cell-permeable probe H2DCFDA.
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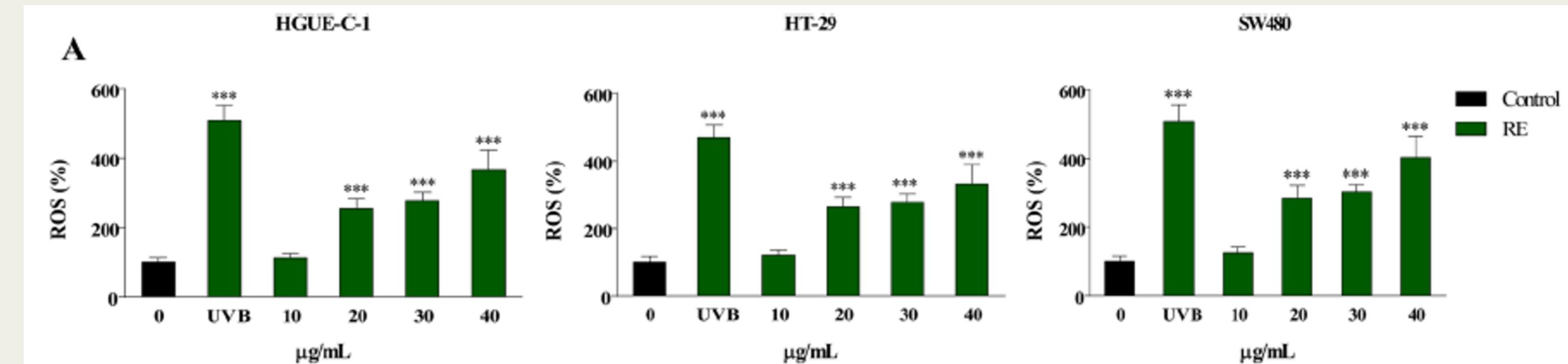


Figure 3. RE induces ROS and mitochondrial membrane depolarization in human colon cancer.**

(A) Intracellular ROS levels (%) were measured in three colon cancer cell lines using H2DCFDA dye, with or without 10, 20, 30, or 40 $\mu\text{g}/\text{mL}$ of RE, following UV radiation.

- Additionally, **MitoTracker Red CMXRos** and **MitoTracker Green** fluorescent probes were used to investigate whether mitochondrial membrane potential in colorectal cancer cells was affected by RE treatment.
- A decrease in the red-to-green fluorescence ratio indicates a loss of mitochondrial membrane potential. When all colorectal cancer cells were treated with RE in a dose-dependent manner, especially in SW480 cells (Figure 3C, D), this confirmed the results obtained in Figure 3B.

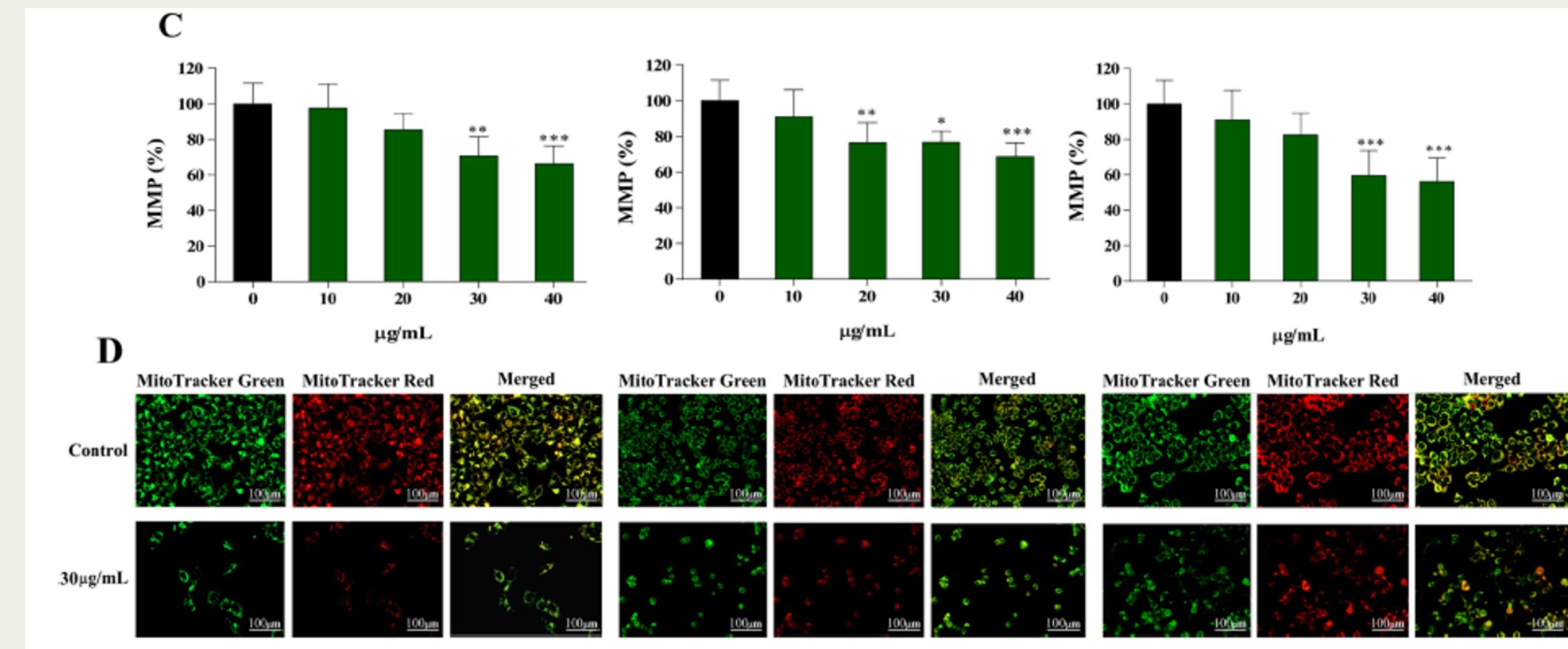


Figure 3. RE induces ROS production and mitochondrial membrane depolarization in human colon cancer.

(C) Fluorescence measurement of MMP (%) using MitoTracker Green FM and MitoTracker Red CMXRos dyes.

(D) Representative fluorescence images of HGUE-C-1, HT-29, and SW480 cells.

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- **Regulation of colorectal cancer cells by Nrf2 through RE treatment.** It is well known that the production of intracellular ROS affects the function of various redox-sensitive transcription factors and leads to the upregulation of antioxidant genes.
 - The cellular antioxidant defense mechanisms include ROS scavenging molecules, phase II detoxifying enzymes, and other detoxifying proteins.
 - The transcription factor Nrf2 is a key regulator of many detoxification and antioxidant genes, and it is activated during oxidative and electrophilic stress responses. **Its mechanism in relation to RE was studied by silencing Nrf2 using specific siRNA.**

- Silencing of the Nrf2 gene in HGUE-C-1, HT-29, and SW480 cells did not alter cell viability or ROS production in the absence of RE (Figure 4).**
- However, when the cells were treated with RE, Nrf2 silencing induced a significant decrease in cell viability (Figure 4A) and an increase in ROS production (Figure 4B), both of which were dose-dependent.

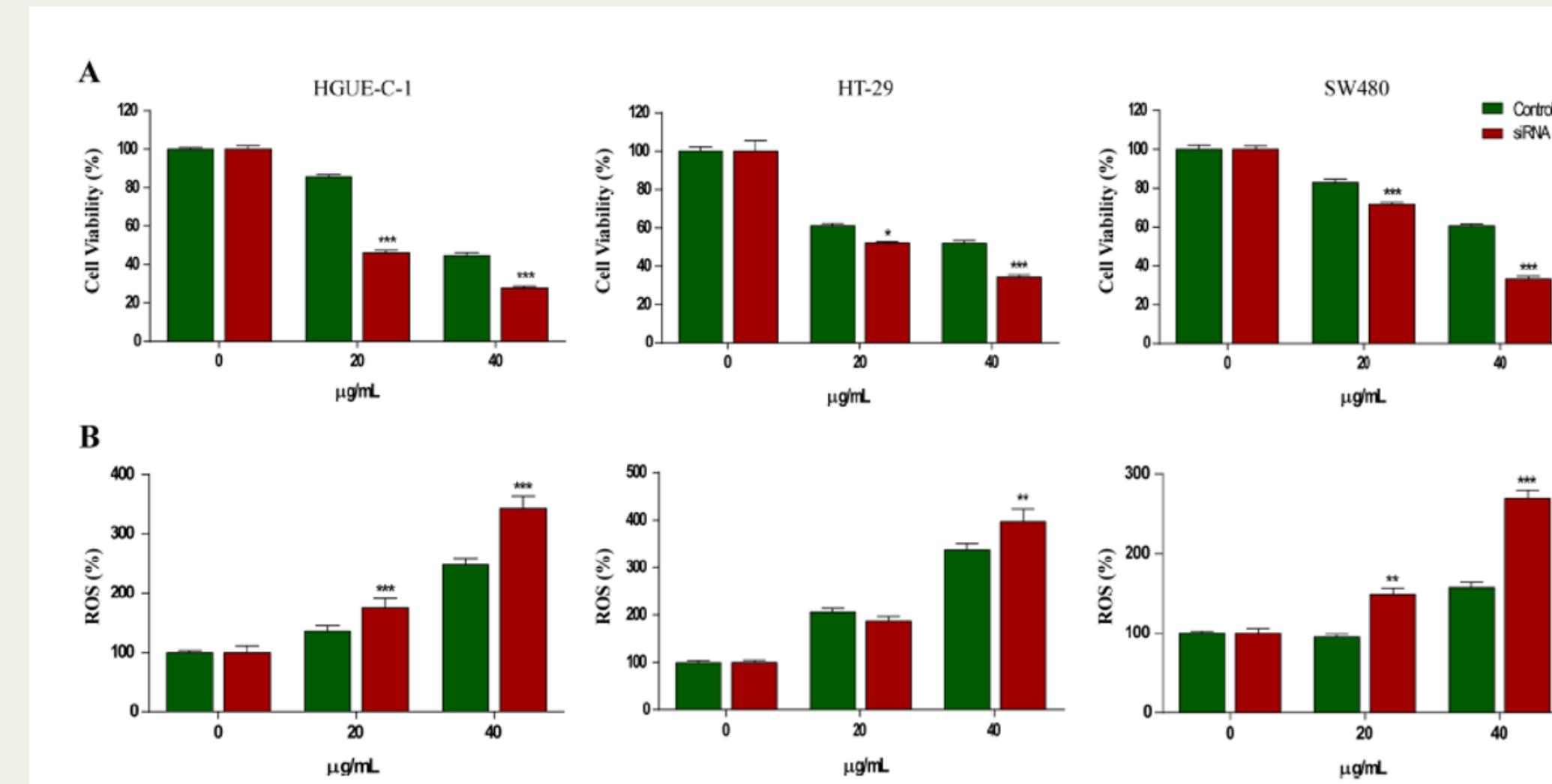


Figure 4. Silencing of the Nrf2 transcription factor increases RE-induced cell death in colon cancer cells.**

(A) Measurement of cell viability (%) in three colon cancer cell lines (HGUE-C-1, HT-29, and SW480) without (control) or in the presence of 20 $\mu\text{g}/\text{mL}$ or 40 $\mu\text{g}/\text{mL}$ of RE, either with or without Nrf2-specific siRNA.

(B) Measurement of intracellular ROS levels (%) using the H2DCFDA dye in the same three cell lines under the same treatment conditions.

IN VIVO EFFECTS OF RE ON THE TUMORIGENICITY OF HT-29 CELLS IN EXERCISE-INDUCED MICE

- After confirming RE's significant in vitro antiproliferative activity, the next step was testing its effects in vivo.
- HT-29 cells were implanted into exercise-induced nude mice, with two oral RE treatment methods:
 - a) Pre-treatment for 2 weeks + cell inoculation + treatment
 - b) Cell inoculation + treatment
- Body weight and food intake were monitored weekly, showing no signs of toxicity or weight loss. In the control group, tumor volume increased over 35 days. **Both treatments significantly reduced tumor size:** pre-treatment reduced tumor volume by 34.1%, and post-inoculation treatment reduced it by 27.5% ($p < 0.001$ for both).

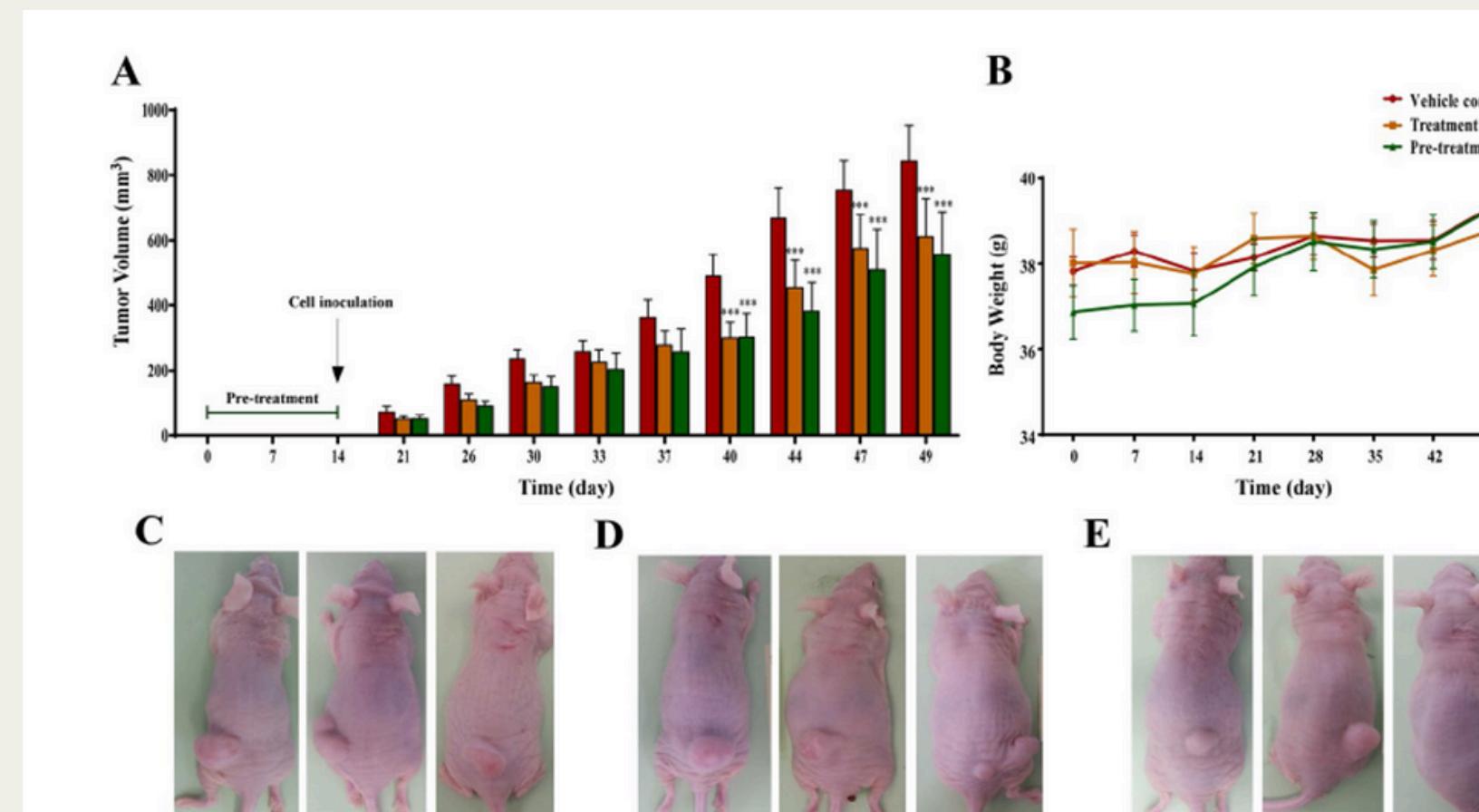


Figure 5. Oral administration of RE reduces tumor volume in athletic nude mice.

(A) In a xenograft model of colon cancer cells using robust nude mice: Groups include no treatment (control), pretreatment (oral RE for two weeks before cell inoculation), or treatment (RE administered after cell inoculation). Tumor volume (mm³) was measured with an oral dose of 200 mg/kg RE. Animals were monitored twice a week over a 35-day period.

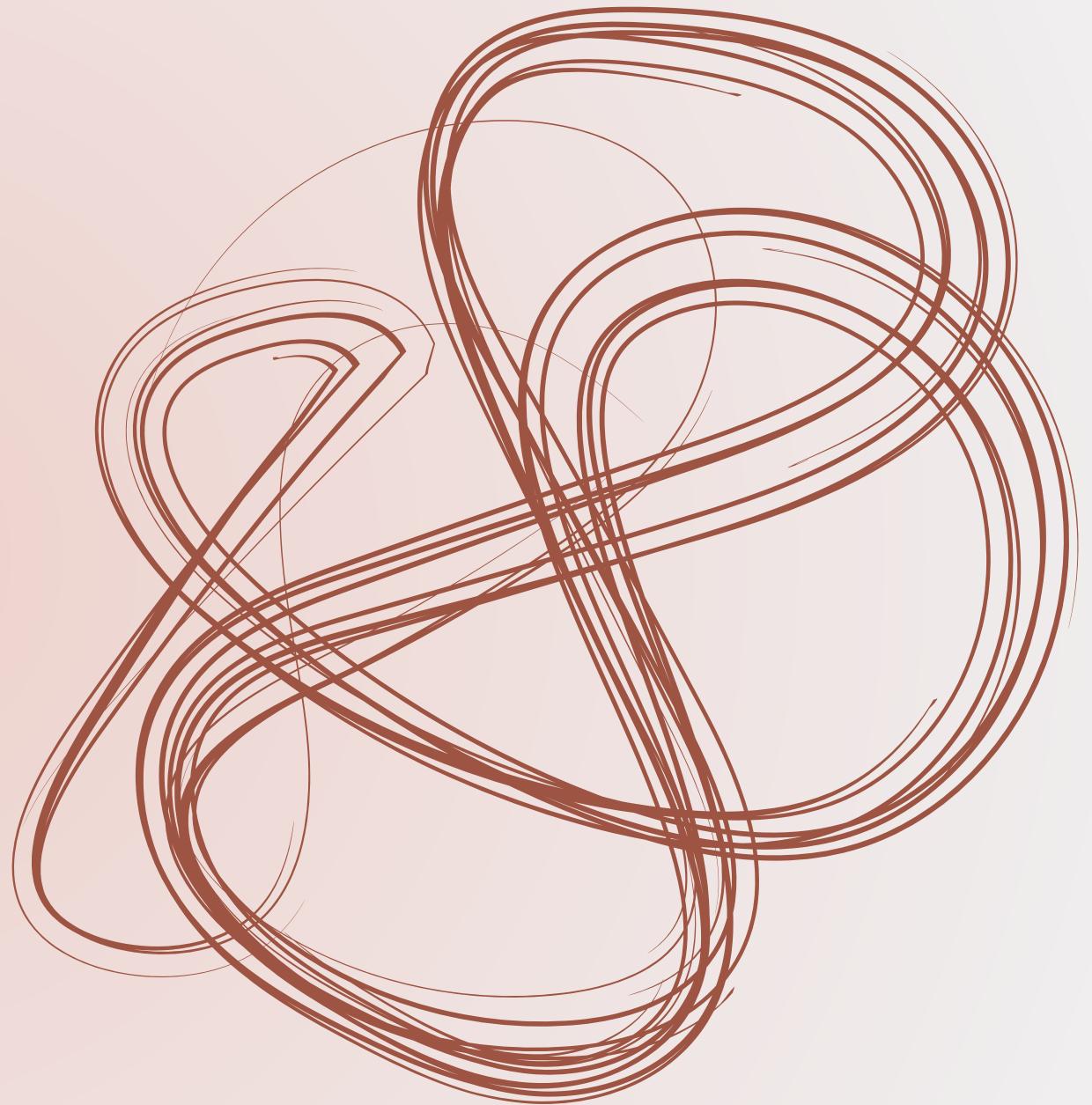
(B) Body weight (g) of animals from all three groups was recorded during the experiment, along with representative images of tumor formation.

(C) Control group.

(D) Treatment group.

(E) Pretreatment group.

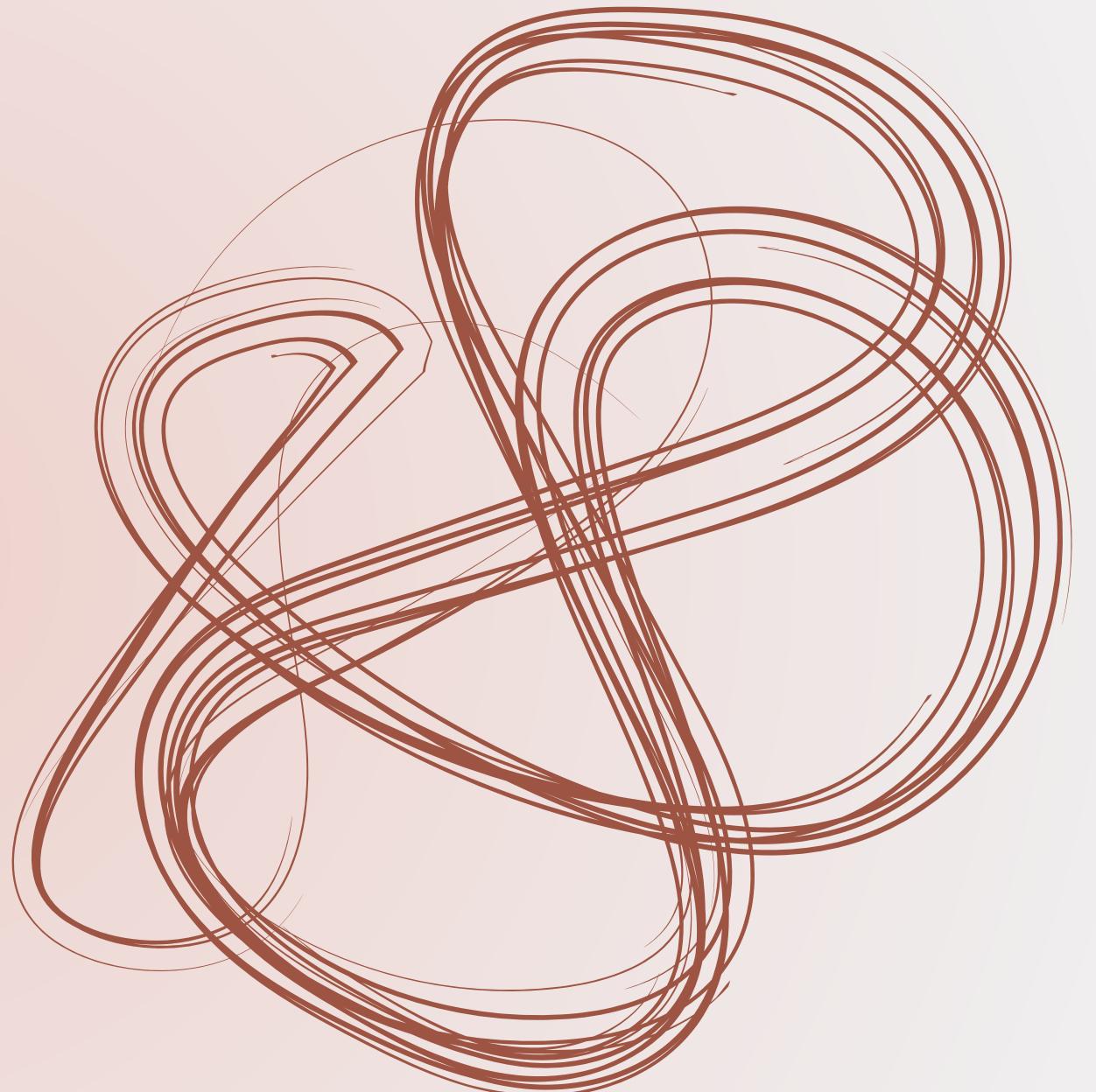
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- Apoptosis and cell cycle arrest have been proposed as part of the antiproliferative mechanism. However, our study suggests that necrosis, rather than apoptosis, is the mechanism responsible for the death of colon cancer cells.
 - Consistent with our results, a recent proteomic analysis in colon cancer cells also found that **RE altered proteins involved in the activation of the Nrf2 transcription factor and the unfolded protein response (UPR)**. In this work, we have confirmed the antioxidant effect of rosemary compounds, as recently reported by our group, as a mediator of its antiproliferative effect.
 - Based on our results and those from previous reports, we speculate that ROS produced by RE in colon cancer cells may be responsible for aggravating the UPR response and endoplasmic reticulum stress (ERS), leading to the activation of Nrf2, as well as the defense mechanisms of apoptosis and autophagy.



Mechanism of Action

MECHANISM OF ACTION

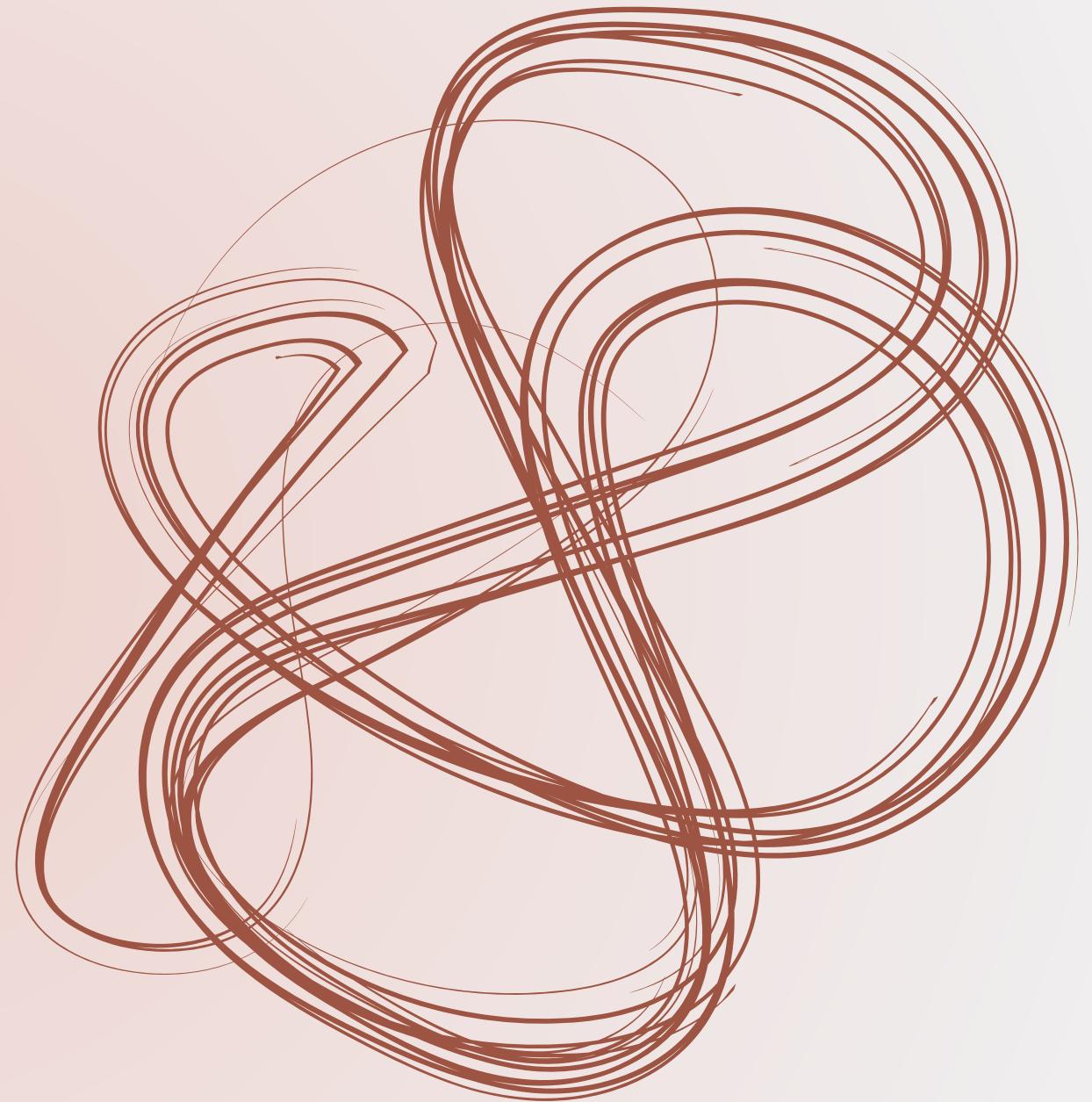
- The IC₅₀ values of each combination were obtained, and three methods were used to study synergistic effects: FIC value calculation, isobolographic analysis, and Compusyn software.
- **Most combinations showed additive or indifferent effects, except for BA-UA, which displayed antagonism.** Compusyn software indicated potential synergistic effects, especially in CA-CAR, CA-BA, CAR-CA, and CAR-BA combinations.
- However, BA-UA consistently showed antagonism in all methods. Despite testing, the complete extract showed no significant improvement in antiproliferative activity compared to isolated compounds. Therefore, subsequent studies focused on using the whole RE extract.



Conclusion



In this work, we have demonstrated the antioxidant effects of rosemary compounds, recently reported by us, as mediators of their antiproliferative activity. In summary, it has been revealed that RE compounds exhibit the ability to inhibit cell proliferation in vitro, as well as the migration and invasiveness of colon cancer cells. Treatment of cancer cells with RE greatly increases intracellular ROS levels, leading to necrotic cell death. According to our findings, the Nrf2 transcription factor pathway appears to be involved in cell survival following RE treatment. These in vitro results align with the reduction of tumor growth in colon cancer cells in a xenograft model in athymic mice treated with RE. Whether a similar antiproliferative mechanism occurs in vivo requires further preclinical studies to link the presence of rosemary's metabolic biomarkers.



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Thank you

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