

# Red Ginger (*Zingiber officinale* var. *rubrum*) Nanoparticles Induce Senescence and Apoptosis in MCF-7 Cells through Downregulation of *BCL2* and Upregulation of *TP53* and *CASP3* Genes

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## Abstract

Globally, breast cancer remains a common and serious health concern, with different incidence rates worldwide. Potential directions in cancer treatment include red ginger components and the efficient drug delivery provided by nanoemulsion technology. To assess the potential of red ginger extract nanoemulsion (RGE-NE) as a treatment for breast cancer, this study evaluated RGE-NE on the MCF-7 cell line. Flow cytometry was used to analyze the cell cycle and apoptosis. Using qRT-PCR, the expression of genes linked to apoptosis, including *TP53*, *CASP3*, and *BCL2*, was examined. Using Senescence Associated- $\beta$ -Galactosidase labeling, the MCF-7 cell senescence detection experiment was carried out. In MCF-7 cells, RGE-NE boosted necrosis, cell death, and apoptosis while decreasing the amount of surviving cells. RGE-NE was shown to upregulate *TP53* and *CASP3* expression while downregulating *BCL2* gene expression at a dose of 200  $\mu$ g/mL. Additionally, the percentage of cells in the G0/G1 phase dropped and the proportion of cells in the S phase, which indicates cell cycle arrest, increased after receiving RGE-NE treatment. Moreover, MCF-7 cells experienced senescence at exposure to 800  $\mu$ g/mL of RGE-NE. According to these results, RGE-NE may have anti-breast cancer properties by causing MCF-7 cells to undergo cell cycle arrest, senescence, and apoptosis.

**Keywords:** Anti-breast cancer, Cytotoxicity, Nanoparticles, *Zingiber officinale* var. *rubrum*.

## 1. Introduction

Cancer remains a significant global public health concern (Mahdavifar *et al.*, 2016). According to Savitri *et al.* (2023), the most common type of cancer that affects women is breast cancer. Breast cancer is the second most common cancer, affecting 2.1 million individuals (11.6%) and causing 626,679 deaths (6.6%). In Indonesia, the reported incidence of breast cancer was 16.7% (Lim *et al.*, 2022). Breast cancer is a complex disease involving various biological processes in its development and progression (Ji *et al.*, 2019). One of the key protective mechanisms employed by cells is cell cycle arrest, which allows for DNA repair before proceeding with cell proliferation. The letters M (mitosis), G1 (gap 1), S (DNA synthesis), and G2 (gap 2) stand for different cell cycle phases. The cell cycle includes several checkpoints that guarantee accurate chromosome replication and serve as a crucial defense against tumorigenesis (Thu *et al.*, 2018). In cases of severe DNA damage, alternative signal pathways

come into play to either induce cell senescence, aiming to prevent the development of malignancies (Lo *et al.*, 2015). According to Marvalim *et al.* (2023), Tumor Protein 53 (*TP53*) is an essential transcription factor that controls several biological processes, including as apoptosis, cell cycle arrest, and cellular senescence. One of the most important steps in stopping malignant growth is apoptosis (Rahman *et al.*, 2021). According to Cui *et al.* (2007), mitochondrial membrane permeability is regulated by B-cell CLL/lymphoma 2 (*BCL2*), which impacts cytochrome c release and activates Caspase 9 (*CASP9*) and *CASP3*, controlling the process of apoptosis. Caspases, which are cysteine proteases specific to aspartate, destroy hundreds of cellular proteins, including important structural elements, to facilitate cell destruction during apoptosis (Valente *et al.*, 2013).

Various types of chemotherapy have been unsuccessful due to side effects, drug resistance, and the selective targeting of certain drugs (Nindrea *et al.*, 2023). Currently, researchers are focused on developing medications that use natural ingredients to address these issues. These

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substances may have a multiplicity of benefits, reduce adverse reactions, and be useful in treating different kinds of cancer (Aung *et al.*, 2017). There are various studies that report on natural compounds that have anticancer effects and modulate the immune system (Subramaniam *et al.*, 2019). Ginger is a widely used natural spice available in both powdered and fresh root forms. It comprises various bioactive compounds, including gingerol, resin, zingerone, volatile oil, paradol and vitamins A and C. These compounds endow ginger with a range of pharmacological properties, such as antimicrobial, antiviral, antioxidant, antihypertensive, gastroprotective, cardioprotective, antidiabetic, anticancer, and immunomodulatory effects (El-Borm *et al.*, 2023). Recent research has brought considerable focus to ginger due to its potential anti-cancer characteristics, particularly in its capacity to combat several malignancies, including colorectal, prostate, breast, and cervical cancers (Mao *et al.*, 2019). Numerous compounds in ginger, such as vanilloids (e.g., 6-gingerol and 6-paradol), shogaol, zingerone, and galanal A and B, are recognized for their anticancer properties. These elements play therapeutic roles in disease control by altering a variety of biological functions (Rahmani *et al.*, 2014). However, red ginger contains higher concentrations of vanilloid compounds compared to regular ginger (Zhang *et al.*, 2022). Traditional medicine uses red ginger extensively, particularly in China, Malaysia, and Indonesia (Suciwati *et al.*, 2017). Red ginger rhizome extract's active ingredients include 6-shogaol, which can impede the development of breast and colon cancer cells (Tan *et al.*, 2013), and 6-gingerol, which can cause the LNCaP human prostate cancer cell line to undergo apoptosis (Kim *et al.*, 2011). However, the therapeutic potential of these compounds is limited by poor solubility and bioavailability, which can be overcome by nanotechnology-based delivery systems such as nanoemulsions. Nanotechnology has evolved into one of the quickest, inventive, and adaptive technologies in modern science and cancer therapy (Chaturvedi *et al.*, 2023). Nanoemulsion is a widely applied nanodrug delivery system for bioactive compounds and nutraceuticals (Amalraj *et al.*, 2019). Nanoemulsion have the capability to encapsulate inadequately water-soluble drugs, enhancing their solubility, bioavailability, and targeted delivery to cancer cells (Sánchez-López *et al.*, 2019). Encapsulating plant extracts is essential for protecting bioactive compounds from degradation (Noore *et al.*, 2021). MCF-7 cells were chosen in this research because these cells retain several ideal properties specific to the breast epithelium. These characteristics include the capability to metabolize estrogen, specifically estradiol, through estrogen receptors located within the cell cytoplasm (Camarillo *et al.*, 2014). In vitro tests were used in this work to investigate the anticancer properties of red ginger extract nanoemulsions (RGE-NE) on the MCF-7 breast cancer cell type. *BCL2*, *TP53*, and *CASP3* gene quantification, senescence, cell cycles, and apoptosis assessment are among the methods used to assess the RGE-NE potential.

## 2. Materials and Methods

### 2.1. Red ginger extract nanoemulsion preparation

The materials were red ginger extract (RGE) with ethanol 70% as the solvent, obtained from PT FAST, Depok, Indonesia (No. Batch 001.07.25.ERJM.01). RGE (15 mL) was added to 100 mL ddH<sub>2</sub>O and mixed with a solvent consisting of propylene glycol, 70% ethanol, and 10% DMSO (60 mL). 1% chitosan (40 mL) (Phy Edumedia) was added to the extract solution and stirred using a magnetic stirrer (1500 rpm). Na-TPP 0.4% was added at 1 drop per 3 seconds as much as 20 mL and stirred at 300 rpm. After that, stirred for 15 minutes using a magnetic stirrer (Widowati *et al.*, 2023). Nanoparticles were confirmed successfully after the formation of turbidity and sediment. The pellet from this process was a red ginger extract, while the supernatant was RGE-NE. The size of nanoparticles was analyzed by Particle Size Analyzer (PSA) (Beckman Coulter LS 13 320). The results of PSA showed that the RGE-NE had a diameter of less than 1000 nm, which was 773 nm. Thus, that the production of red ginger nanoparticles was successful and can be continued with further testing.

### 2.2. MCF-7 Cell Line Cell Culture

The ATCC HTB-22 human breast cancer MCF-7 cell line was provided from PT Aretha Medika Utama, Bandung, Indonesia. Using the protocol from Widowati *et al.* (2018), the media used to culture MCF-7 cells was high-glucose DMEM (Biowest, L0103-500) with all necessary components. The cell suspension was seeded into the T25 flask (SPL, 70025). MCF-7 cells were cultivated with 5% CO<sub>2</sub> at 37°C in an incubator (Thermo IH3543). The cells cultivated in the T25 flask were observed using an inverted microscope (Olympus, CKX41-F32FL) until they reached around 70–80% confluence (Widowati *et al.*, 2018).

### 2.3. Apoptosis Assay

Flow cytometry was performed to assess the live cell populations, apoptotic, and necrotic using the Apoptosis Kit (Elabscience, E-CK-A211), as directed by the manufacturer. Six-well plates with five million cells each were incubated for twenty-four hours, different doses of RGE-NE (200, 400, and 800 µg/mL) were applied. After five minutes of centrifugation (1600 rpm), the growing media was discarded and the cells were recovered. After the cell pellet has been resuspended in 500 µL of FACS buffer, an additional centrifugation was carried out. Before analysis, after resuspended in 500 µL of Annexin binding solution, the final pellet, stained with FITC and propidium iodide (PI), and incubated for one hour (Girsang *et al.*, 2023; Widowati *et al.*, 2018; Widowati *et al.*, 2019).

### 2.4. Cell Cycle Analysis

To analyze the cell cycle, the procedure outlined in the instructions for the Cell Cycle Assay Kit (Red Fluorescence) (Elabscience, E-CK-A351) was followed. Following plating, MCF-7 cells were subjected to doses of 200, 400, and 800 µg/mL of RGE-NE at a density of  $5 \times 10^5$  cells per well. The control and treated cells were allowed to incubate for one hour after the addition of cold 70% ethanol to prevent cell clumping. Samples were mixed with 100 µL of RNase A after the cells were washed with

PBS. Thirty minutes were spent incubating the cells in a water bath at 37°C after staining them with 50 µg/mL of PI solution. The MacSQuant Analyzer 10 (Miltenyi Biotec) was then used to perform a flow cytometry analysis (Kalamegam *et al.*, 2018).

### 2.5. Senescence Assay

The analysis of senescent cells was conducted using the Senescence Cells Histochemical Staining Kit (Sigma, CS0030). After plating on 6-well plates, the cells were exposed to 200, 400, or 800 µg/mL of RGE-NE for a whole day. The cell was rinsed with 1x PBS solution. After that, it was fixed using a 1x fixation buffer for 7 minutes, followed by another wash using a 1x PBS solution. Following the addition of the staining mixture to each well, the cells were incubated overnight at 37°C without CO<sub>2</sub>. The outcomes of cell staining can be seen through a light microscope (Priyandoko *et al.*, 2019).

### 2.6. Quantification of CASP3, TP53, BCL2 Gene Expression

RNA was extracted and purified from the samples using TRI Reagent (R2050-1-200, Zymo Research) and the Direct-zol RNA Miniprep Plus kit (R2073, Zymo Research) following the manufacturer's protocol. The RNA concentration was then measured using a microdrop plate, with absorbance readings taken at 260/280 nm using a Multiskan™ GO Thermo Scientific spectrophotometer (Model 51119300), as shown in Table 1. A three-step PCR

procedure was used for cDNA synthesis using the SensiFAST cDNA Synthesis Kit (BIO-65054, Meridian Bioscience): reverse transcription at 46°C for twenty minutes, priming at 25°C for five minutes, then inactivating the reverse transcriptase at 95°C for one minute (Prasetyo *et al.*, 2019; Girsang *et al.*, 2023).

**Table 1.** RNA purity of MCF-7 cells treated with RGE-NE.

No.	Sample	Concentration (ng/µL)	Purity (λ260/λ280 nm)
1	I	603.36	1.997
2	II	549.68	2.089
3	III	792.08	2.022
4	IV	639.92	2.017
5	V	755.20	2.024

\* I: untreated breast cancer cells; II: breast cancer cells+DMSO 1%; III: breast cancer cells+200 µg/mL RGE-NE; IV: breast cancer cells+400 µg/mL RGE-NE; V: breast cancer cells+800 µg/mL RGE-NE.

Real-time qPCR was used to examine constitutively expressed β-Actin genes and the expression of *TP53*, *CASP3*, and *BCL2* genes (Table 2). Widowati *et al.* (2020) employed β-Actin, a housekeeping regulatory gene, as an internal control. Using the temperature, time, and RT-qPCR cycle settings listed in Table 3, the AriaMx Real-time PCR System (Agilent, G8830A) was used to amplify the PCR.

**Table 2.** Primers that are utilized in RT-qPCR.

Genes	Primer	References
Human <i>CASP3</i>	F 5'-AGAACTGGACTGTGGCATTGAG -3'	NM_032991.3
	R 5'-GCTTGTCGGCATACTGTTTCAG -3'	
Human <i>TP53</i>	F 5'-AGAGTCTATAGGCCACCCC-3'	NM_000546.6
	R 5'-GCTCGACGCTAGGATCTGAC-3'	
Human <i>BCL2</i>	F 5'-GGTCATGTGTGTGGAGAGCG-3'	NM_000657.3
	R 5'-GGTGCCGGTTCAGGTACTCA	
Human β-Actin	F 5'-TCTGGCACCACCTTCTACAATG-3'	NM_001101.5
	R 5'-AGCACAGCCTGGATAGCAACG-3'	

\* R: Reverse primer; F: Forward primer

**Table 3.** Temperature, time, and RT-qPCR cycle settings.

Genes	Temperature; Time; Cycle					
	Pre-denaturation	Denaturation	Annealing	Pre-elongation	Elongation	~
<i>CASP3</i>	95°C; 5'	95°C; 30"; 40 cycles	58°C; 50"; 40 cycles	72°C; 50"	72° C; 5'	4°C
<i>TP53</i>	95°C; 5'	95°C; 30"; 40 cycles	58°C; 50"; 40 cycles	72°C; 50"	72° C; 5'	4°C
<i>BCL2</i>	95°C; 5'	95°C; 30"; 40 cycles	58°C; 50"; 40 cycles	72°C; 50"	72° C; 5'	4°C
β-Actin	95°C; 5'	95°C; 30"; 40 cycles	58°C; 50"; 40 cycles	72°C; 50"	72° C; 5'	4°C

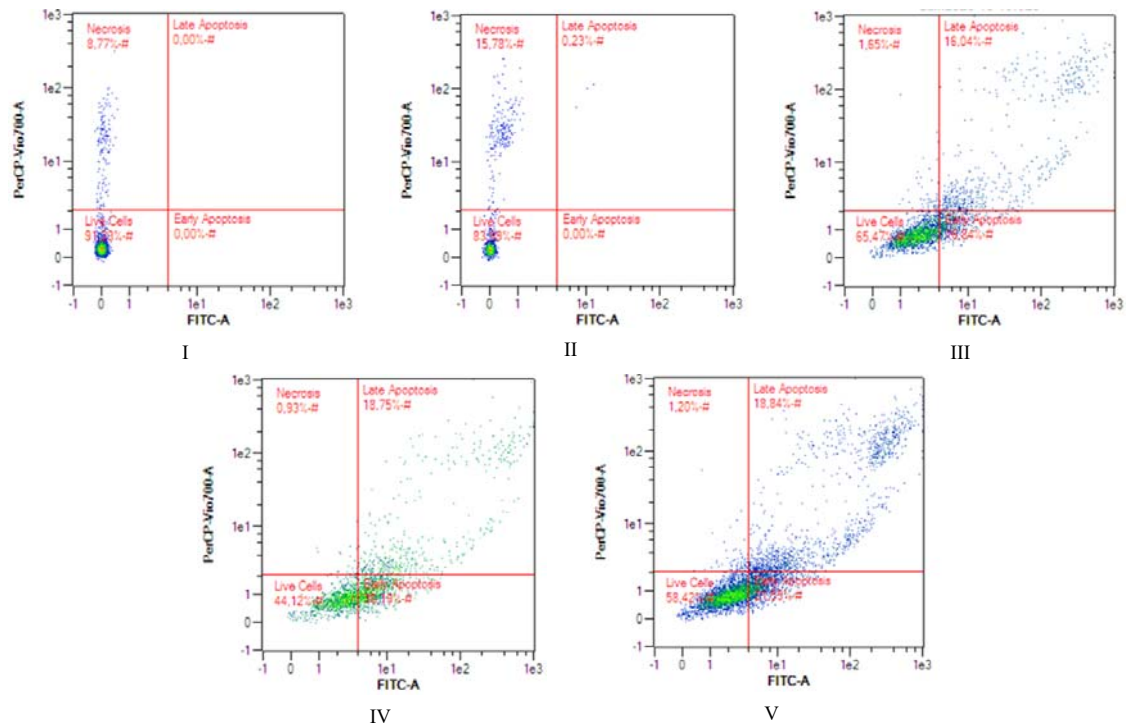
### 2.7. Statistical Analysis

SPSS software (version 20.0; SPSS Inc., USA) was used to perform the statistical analysis. The data was analyzed using One-Way ANOVA. The Tukey HSD post-hoc test was utilized for data that met the homogeneity and normality criteria, whereas the Dunnett's T3 post-hoc test was employed for not homogeneous and normally distributed data. A threshold of P < 0.05 was set for significance. Using GraphPad Prism (version 8.0.244), histograms displaying the mean ± standard deviation were produced.

## 3. Results

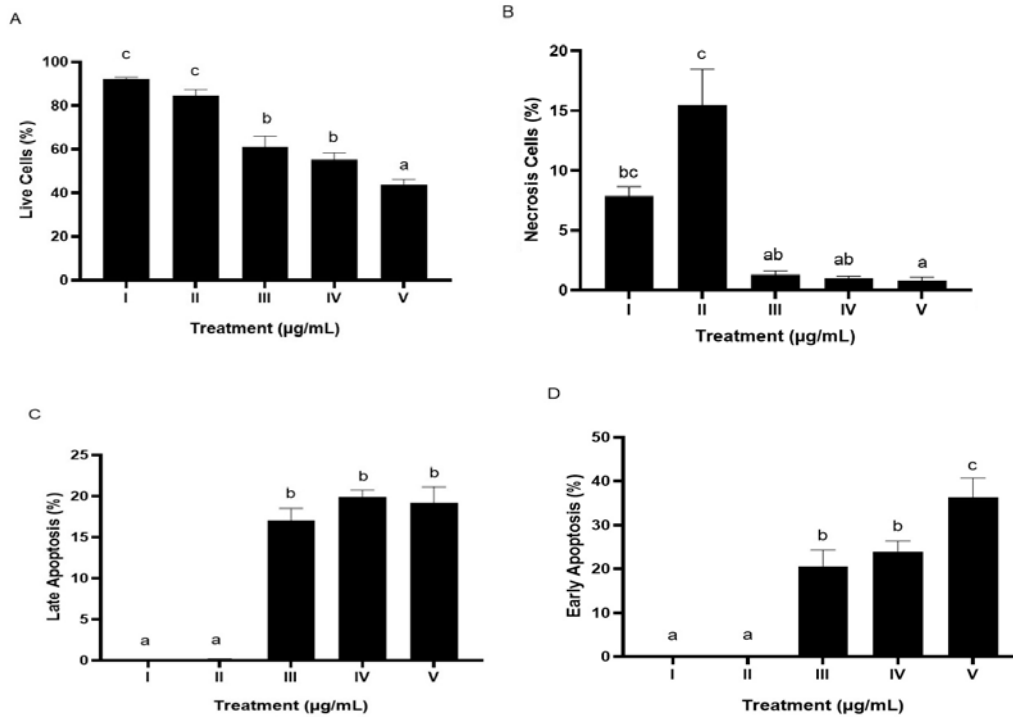
### 3.1. RGE-NE Impact on MCF-7 Cells Apoptosis

Using flow cytometry, the amount of apoptosis in MCF-7 cells treated with RGE-NE was assessed. The relative distribution of necrotic, viable, early apoptotic, and late apoptotic cells is shown. The comparison of each therapy on viable cells, necrotic cells, late apoptosis, and early apoptosis is displayed in Figures 1 and 2. RGE-NE treatment caused necrosis and apoptosis, as well as a reduction in viable cells. The degree of apoptosis rose as the nanoemulsion concentration increased.



**Figure 1.** Dot plots showing different RGE-NE concentrations in relation to breast cancer cells' apoptosis

\* I: untreated breast cancer cells; II: breast cancer cells+DMSO 1%; III: breast cancer cells+200 µg/mL RGE-NE; IV: breast cancer cells+400 µg/mL RGE-NE; V: breast cancer cells+800 µg/mL RGE-NE.



**Figure 2.** Effect of various concentration of RGE-NE toward apoptosis in breast cancer cells

\*(A) live cells, (B) necrosis cells, (C) late apoptosis, and (D) early apoptosis.

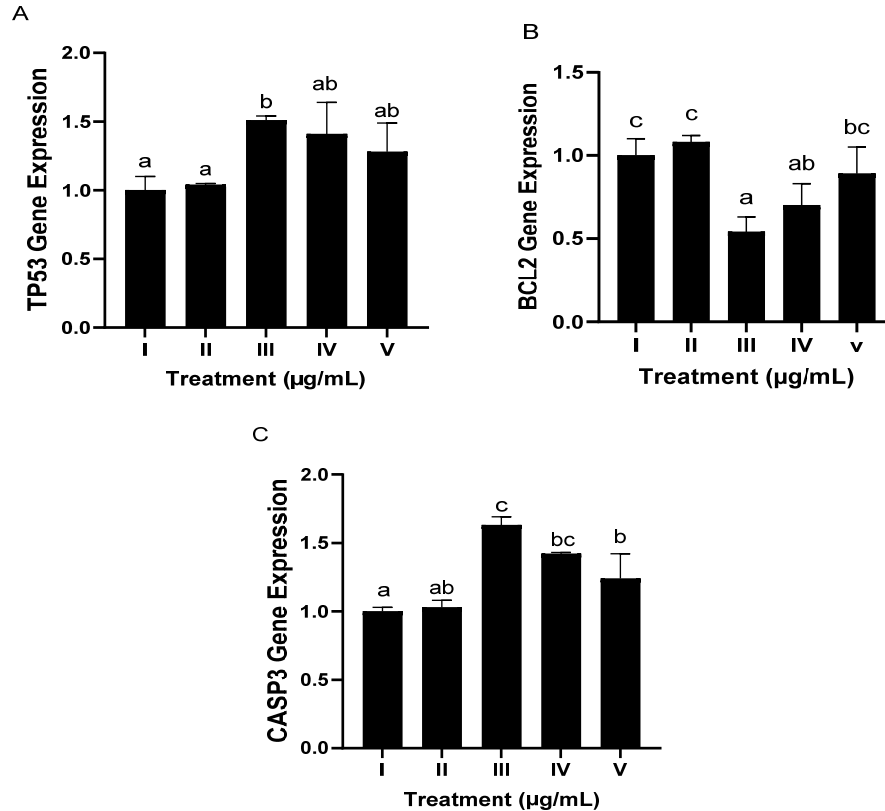
\* I: untreated breast cancer cells; II: breast cancer cells+DMSO 1%; III: breast cancer cells+200 µg/mL RGE-NE; IV: breast cancer cells+400 µg/mL RGE-NE; V: breast cancer cells+800 µg/mL RGE-NE.

\* Data are presented as mean ± SD from three independent replicates. Different letters indicate significant differences (p<0.05, Tukey's HSD): live cells (A), necrotic cells (B), late apoptosis (C), and early apoptosis (D).

### 3.2. Effect of RGE-NE on the Apoptosis Gene Expression in MCF-7 Cells

Gene expression was investigated using RT-PCR in order to evaluate the apoptosis-related gene expression that RGE-NE produced in MCF-7 cells. Proapoptotic genes like *TP53* and *CASP3* as well as antiapoptotic genes like *BCL2* were assessed in this study. Figure 3 illustrates that

in MCF-7 cells, RGE-NE at 200 µg/mL (group III) exhibited the highest expression of *TP53* and *CASP3* and the lowest expression of the *BCL2* gene. Comparing RGE-NE at 200 µg/mL concentration (group III) to the negative control (group I), it can dramatically boost *TP53* and *CASP3* gene expression and decrease *BCL2* gene expression.



**Figure 3.** Effect of various concentrations of RGE-NE toward *TP53*, *BCL2*, and *CASP3* genes expression in breast cancer cells

\* (A) *TP53*, (B) *BCL2*, (C) *CASP3* genes expression in breast cancer cells

\*I: untreated breast cancer cells; II: breast cancer cells+DMSO 1%; III: breast cancer cells+200 µg/mL RGE-NE; IV: breast cancer cells+400 µg/mL RGE-NE; V: breast cancer cells+800 µg/mL RGE-NE.

\* Data are presented as mean ± SD from three independent replicates. Different letters indicate significant differences (p < 0.05, Tukey's HSD test) for *TP53* (ab, abc, bc), *BCL2* (a, ab, bc, c), and *CASP3* (ab, abc, b, c).

### 3.3. Effect of RGE-NE on the MCF-7 Cell Cycle

Flow cytometry was employed to examine how RGE-NE affects cell cycle distribution and to determine if alterations in cell cycle distribution are associated with the observed reduction in cell viability. PI fluorescent dye was used to determine DNA content and analyze cell cycle distribution. Every therapy in the G2/M, S, and G0/G1 stages is compared in Table 4. As concentration rose, the proportion of cell cycle distribution increased in the S phase and reduced in the G0/G1 phase. RGE-NE at 800 µg/mL demonstrated the biggest reduction in the G0/G1 phase (90.30±2.90%) of the cell cycle. Furthermore, it showed the largest rise in the G2/M phase (1.86±1.55%) and S phase (7.27±1.67%) cell counts. Consequently, MCF-7 cells were arrested in the S phase rather than the G0/G1 phase upon treatment with RGE-NE. This finding suggests that the S phase, where fewer cells progress through the cycle, is the site of cell cycle arrest or delay.

**Table 4.** Effect of various concentrations of RGE-NE toward cell cycles in breast cancer cells

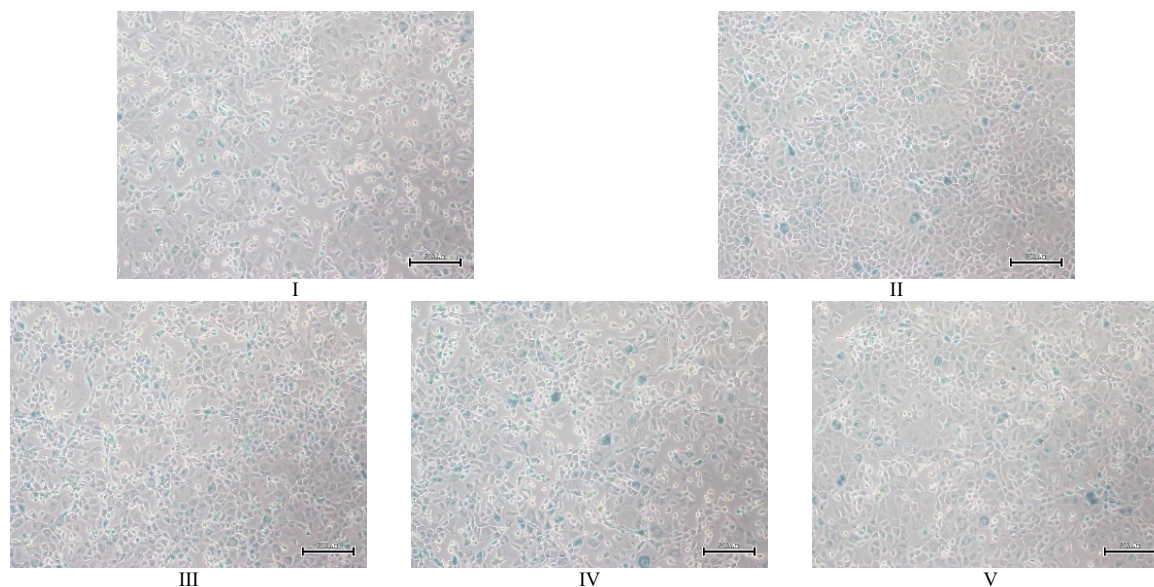
Sample	Cell Cycle		
	G0/G1 phase (%)	S phase (%)	G2/M phase (%)
I	91.43±0.54	4.83±0.70	0.64±0.35
II	92.49±1.23	6.02±0.98	0.42±0.14
III	92.04±1.34	6.09±1.10	0.60±0.14
IV	91.87±0.56	6.64±0.75	0.69±0.26
V	90.30±2.90	7.27±1.67	1.86±1.55

\*I: untreated breast cancer cells; II: breast cancer cells+DMSO 1%; III: breast cancer cells+200 µg/mL RGE-NE; IV: breast cancer cells+400 µg/mL RGE-NE; V: breast cancer cells+800 µg/mL RGE-NE.

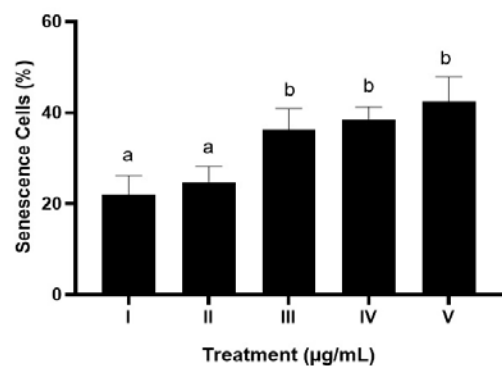
### 3.4. RGE-NE at Various Concentrations' Impact on MCF-7 Cell Senescence

In this study, we used the senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining to identify cells undergoing senescence in MCF-7 cell culture. Beta-galactosidase activity is one of the characteristic signs of cells that have reached the senescence stage. As a result, senescent cells were identified by blue staining. The senescence morphology of MCF-7 cells treated with RGE-NE can be observed in Figure 5. In addition to visual observation, we performed quantitative analysis to

measure the percentage of cells showing beta-galactosidase activity. The senescence staining measurements indicated a notable impact of RGE-NE treatment on MCF-7 breast cancer cells in comparison to the negative control (group I) (Figure 6). The induction of senescence increased as the concentration rose, reaching its maximum at 800  $\mu$ g/mL (group V) RGE-NE. These findings suggest that RGE-NE can cause MCF-7 breast cancer cells to undergo senescence.



**Figure 4.** Effect of various concentration of RGE-NE toward senescence morphology in breast cancer cells. The magnification used was 40x. \*I: untreated breast cancer cells; II: breast cancer cells+DMSO 1%; III: breast cancer cells+200  $\mu$ g/mL RGE-NE; IV: breast cancer cells+400  $\mu$ g/mL RGE-NE; V: breast cancer cells+800  $\mu$ g/mL RGE-NE.



**Figure 5.** Effect of various concentration of RGE-NE toward senescence in breast cancer cells

\*I: untreated breast cancer cells; II: breast cancer cells+DMSO 1%; III: breast cancer cells+200  $\mu$ g/mL RGE-NE; IV: breast cancer cells+400  $\mu$ g/mL RGE-NE; V: breast cancer cells+800  $\mu$ g/mL RGE-NE.

\* Data are presented as mean  $\pm$  SD from three independent replicates. Different letters (a, b) indicate significant differences between treatments ( $p < 0.05$ ) according to Tukey's HSD post hoc test.

## 4. Discussion

This study demonstrated that red ginger extract nanoemulsions (RGE-NE) exerts anticancer effects on MCF-7 cells by inducing apoptosis, disrupting the cell cycle, and promoting senescence, highlighting its therapeutic potential against breast cancer. Bioactive substances with anticancer properties, including flavonoids, gingerol, and shogaol, are found in red ginger (*Z. officinale* var. *rubrum*) (Semwal *et al.*, 2015). Some pungent vanilloids are primarily responsible for the anticancer properties of red ginger (Zhang *et al.*, 2022). Red ginger's predominant vanilloids, 6-gingerol and 6-shogaol, contribute to its piquant flavor (Semwal *et al.*, 2015). Strong anti-cancer activities of ginger have previously been confirmed for 6-shogaol and 6-gingerol (Panyajai *et al.*, 2022; Ghasemzadeh *et al.*, 2015). 6-Shogaol prevented the growth of tumors and specifically caused the death of leukemia cells in the model of U937 xenograft mice, according to Lee *et al.*, (2008). Adding 6-gingerol inhibits the human breast cancer cell line from reproducing MDA-MB-231. The extract from the rhizome of red ginger, according to Ghasemzadeh *et al.* (2015), exhibited anticancer effects on the HeLa cancer cell line

without harming normal cells. The use of nanoemulsions to deliver bioactive compounds from red ginger extract has shown promising results in enhancing their anticancer effects and modulating biological processes, including apoptosis, cell cycle, and senescence cells.

Nanoemulsions, which are emulsions on a nanoscale, enhance the delivery of active compounds, and they have proven to be useful in enhancing the apoptotic and antiproliferative impacts of ginger extracts across different cancer models (Panyajai *et al.*, 2022; Alharbi *et al.*, 2023). This study's results align with the overall advancement in research exploring ginger and its nano-based formulations for cancer treatment. Multiple research experiments have validated ginger's anti-tumor characteristics. The utilization of nano-based treatments, like nanoemulsions, has shown promise in enhancing the anticancer effects of ginger and its bioactive constituents (Chen *et al.*, 2023). Among the various stabilizing and delivery agents, chitosan has attracted particular attention due to its biocompatibility and ability to improve nanoparticle stability and drug delivery efficiency. Over the years, because of its possible use in biomedicine, chitosan has been extensively studied, especially in relation to the creation and application of medication delivery systems (Guarín-González *et al.*, 2022). Chitosan is biodegradable polysaccharide that has been widely used as coating material for various type of nanoparticles due to its biodegradable, biocompatible and non-toxic properties, in addition to its capacity to improve penetration (Nallamuthu *et al.*, 2015). Additionally, the bioactivity of chitosan includes anti-inflammatory, blood anti-coagulant, antibacterial, and antioxidant activities, further contributing to its versatility and effectiveness in biomedical applications (Hassan *et al.*, 2021). In addition, mechanical properties of nanoemulsion are enhanced by the addition of chitosan. The enhancement is attributed to increased stability and electrostatic interaction within the emulsion. Chitosan insertion also facilitates controlled release (Diedrich *et al.*, 2023).

RGE-NE appears to exert its anticancer effects primarily by influencing cell death mechanisms. Lack of cell death characterizes cancer (Kadam *et al.*, 2016), whereas in normal physiology, apoptosis is essential for growth, development, senescence, and ultimate demise (Yuan *et al.*, 2022). Each cell in the body carries a latent mechanism that triggers its own self-destruction through apoptosis (Shahouzehi *et al.*, 2023). Apoptosis has been defined as an active, programmed mechanism of self-cell dismantling that prevents inflammation, whereas necrosis is portrayed as passive, involuntary cell death triggered by external disturbances, leading to the unregulated release of inflammatory cell components (Fink *et al.*, 2005). Flow cytometry analysis revealed that RGE-NE induced both apoptosis and necrosis in MCF-7 cells in a dose-dependent manner (Figures 1 and 2). Ginger extracts at varying concentrations induced dose-dependent apoptosis in the HT 29 and HCT 116 colon cancer cell lines, according to research done by Abdullah *et al.* (2010). According to studies, 6-shogaol promotes apoptotic pathways, which in turn prevents lung, colon, and osteosarcoma from growing. Pro-apoptotic protein expression increases as a result, while anti-apoptotic protein expression decreases (Nguyen *et al.*, 2019).

Cancer cells often evade apoptosis by disrupting apoptotic triggers or inhibiting caspase function. Two major mechanisms include the suppression of pro-apoptotic proteins such as BAX and BAK, and the overexpression of the anti-apoptotic protein BCL2. Although BCL2 itself is not classified as an oncogene, its dysregulation or mutation can increase the risk of tumor initiation and progression (Pfeffer *et al.*, 2018). According to Perri *et al.* (2016), mutant *TP53* also hinder the development of cancer by interfering with processes that improve DNA repair, stop the cell cycle, and trigger apoptosis in response to oncogenic stimuli. Based on the results, RGE-NE can decrease *BCL2* gene expression while increasing *TP53* and *CASP3* expression (Figure 3). Apoptosis-related genes, such as *BCL2*, are activated by *TP53*, which also initiates caspase activation through signaling complexes that cause apoptosomes to form in the mitochondria or cell death at the cell membrane (Wiman *et al.*, 2006; Pashaei-Asl *et al.*, 2017). Consequently, by removing *BCL2* and turning on *CASP3*, *TP53* may promote apoptosis. Using Real-Time PCR, Pashaei-Asl *et al.* (2017) examined how ginger affected the expression of the *BCL2* and *TP53* genes in ovarian cancer cells (SKOV-3). The findings demonstrated that adding ginger extract to ovarian cancer cell lines reduced the expression of the *TP53* and *BCL2* genes. Ginger can boost the expression of *CASP9* mRNA in Caspase family members as well as *CASP3* and BAX proteins, according to in vitro cell research conducted by Luo *et al.* (2023). We also looked at the mechanism via which RGE-NE influences the development of MCF-7 cells. The G1, S, and G2 interphases and mitosis (M) comprise the cell cycle. In the G1 phase, cells divide, assemble proteins for DNA synthesis, and create RNA. Numerous signals, such as stress, metabolic, and environmental stimuli, take place during the G1 phase and affect the cell's developmental pathway. The ability of the cells to differentiate, self-renew, or even perish is controlled by these signals. DNA replication usually occurs during the S phase. Decreased gene expression activity is another characteristic of the S phase. In the meantime, the G2 phase is when new proteins are synthesized and cells prepares for division. The M stage is characterized by nuclear and cytoplasmic divisions. A cell uses its cytoplasm to split into two daughter cells during the process known as mitosis (Moghaddam *et al.*, 2017; Wang, 2021).

The data presented (Table 4) shows that as the concentration of RGE-NE increases, less cells are in the G0/G1 phase, which may indicate that RGE-NE is causing cells to leave this phase early or is keeping them from staying in it. This premature movement out of G0/G1 could indicate that the cells are being forced into an abnormal state, where they are unable to properly complete their growth and preparation for DNA replication. After RGE-NE treatment, the percentage of S phase cells significantly increased, suggesting that RGE-NE creates a bottleneck at this stage, which causes an accumulation of S phase cells as a result of inefficient DNA replication. This "arrest" or delay in the S phase is a hallmark of a disruption in the DNA replication process, which is often targeted by anti-cancer therapies to prevent cancer cells from multiplying. In line with this observation, MCF-7 cells treated to RGE-NE had a marked increase in the S phase and a decrease in the

G0/G1 phase cell percentage, signifying the beginning of the S phase cell cycle arrest brought on by fewer cell division cycles, as shown in Figure 4. This result aligns with the observations of Bernard *et al.* (2017), who found comparable effects in MDA-MB-231 cells treated with (10)-gingerol. According to their research, the beginning of S phase arrest was indicated by a rise in S phase cells and a decrease in G1 phase cells.

Moreover, the data (Table 4) shows a modest increase in cells in the G2/M phase with higher RGE-NE concentrations, indicating that some cells are being arrested or delayed in the final stages before cell division. This effect might suggest that RGE-NE causes cells to either pause before entering mitosis due to DNA damage or insufficient preparation for division, or it could directly impair the mitotic machinery, thereby preventing cell division. Studies where MDA-MB-231 and MCF-7 breast cancer cells treated with 6-shogaol underwent different cell cycle arrests in the G2/M phase have shown similar cell cycle disturbances (Ray *et al.*, 2015). Both monolayer and stem cell-like spheroid cultures demonstrated this arrest, with 6-shogaol further obstructing the stem cell renewal route. A decrease in levels of Cdk1, Cdc24c, and Cyclin B, which are crucial for M/G2 phase progression, was observed as cells accumulated in this phase. The downregulation of Cyclin B notably suppressed cell proliferation, especially in MCF-7 and HeLa cell lines. Furthermore, T47D breast cancer cells were shown to perish when their cell cycle was suppressed in the G1/G0, S, and M/G2 phases. This collective evidence suggests that RGE-NE, like other anti-cancer agents, may exert its effects by disrupting the normal cell cycle, thereby inhibiting cancer cell proliferation, and potentially inducing cell death.

When cells in cell culture undergo cell cycle arrest, they are often overstimulated by serums, nutrients, oncogenes, and other factors, which results in senescence. As a pro-senescent state in cancer characterized by an overactive mTOR-centric network, cell cycle arrest frequently leads to senescence in cancer (Blagosklonny, 2011). Cell cycle arrest just permits the emergence of this situation. The findings of this research validate that RGE-NE has the capability to trigger senescence in MCF-7 cells (Figure 6). Blue-stained cells signify  $\beta$ -galactosidase positive cells. As illustrated in Figure 5, MCF-7 cells treated with RGE-NE showed a higher number of blue-colored cells compared to untreated controls. Previous research has identified ginger and its components as having potential benefits in addressing cellular aging. Research by Moaddel *et al.* (2022) identified extract of ginger, specifically gingerenone A, as a promising natural senolytic compound, which exhibits high selectivity to promote senescent cell death (Moaddel *et al.*, 2022). Another study on ginger extracts containing 6-gingerol and 6-shogaol showed their potential to prevent myoblast cell senescence due to their antioxidant properties (Sahardi *et al.*, 2020). Furthermore, studies by Kaewtunjai *et al.* (2018) demonstrated that in A549 lung cancer cells, ginger extract promoted telomere length reduction and the beginning of cellular senescence.

## 5. Conclusion

In summary, RGE-NE showed potential as an anticancer agent in MCF-7 breast cancer cells by specifically enhancing the activity of the *CASP3* and *TP53* genes while reducing the activity of the *BCL2* gene, as well as by inducing apoptosis and necrosis and modifying the expression of apoptosis-related genes. Additionally, RGE-NE affected the cell cycle by causing MCF-7 cells to enter the S phase, suppressing the G0/G1 phase, and promoting cell senescence. These results demonstrate that RGE-NE may interact with important systems such as apoptosis, cell cycle regulation, and cellular senescence to cure breast cancer. However, there are limitations that must be acknowledged, namely that this study only focused on in vitro analysis, and the findings may not be fully applicable to the in vivo system due to the complexity of the tumor microenvironment. Future studies incorporating animal models and detailed toxicological assessments are recommended to validate the findings and advance the application of RGE-NE in clinical settings.

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