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Combination effects of Pistachio hull and carfilzomib on *NF-κB p65*, *MDR1*, *MRP1*, and *Caspase3* gene expression in breast cancer cell line

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Abstract

Objective This study aimed to investigate the synergistic effects of the chemotherapy drug Carfilzomib (CFZ) and Pistachio hull extract on the SK-BR3 breast cancer cell line.

Methods In this experimental study, we evaluated the effect of Pistachio hull extract and CFZ as standalone treatments on cell viability using the MTT assay at 24- and 48-hours post-treatment. Following this, we conducted combination therapy analyses to assess the potential synergistic relationship between Pistachio hull extract and CFZ after 24- and 48-hours of treatment on both the SK-BR3 breast cancer cell line and the MCF10A normal cell line. We utilized real-time PCR to measure the expression levels of *MDR1*, *MRP1*, *NF-κB p65*, and *Caspase3* genes. Additionally, the NF-κB p65 transcription factor was evaluated using ELISA after 24- and 48-hours.

Results The MTT assay revealed IC50 values of 2.014 mg/mL and 1.031 mg/mL in the SK-BR3 cell line, and 3.265 mg/mL and 2.994 mg/mL in the MCF10A cell line at 24- and 48-hours post-treatment with Pistachio hull extract. CFZ concentrations of 0.181×10^{-3} mg/mL and 0.0057×10^{-3} mg/mL in the SK-BR3 cell line, as well as 5.54×10^{-3} mg/mL and 2.51×10^{-3} mg/mL in the MCF10A cell line, inhibited growth by up to 50%. The analysis of combination therapy indicated a synergistic effect between the two treatments after both 24- and 48-hours of exposure. Real-time PCR results demonstrated significant alterations in the expression of *MDR1*, *MRP1*, *NF-κB p65*, and *Caspase3* genes, along with changes in NF-κB p65 protein levels in both cell lines following treatment with Pistachio hull extract, CFZ, or their combination compared to the control group ($p < 0.05$).

Conclusion The findings highlight the effectiveness of CFZ as a proteasome inhibitor when used in conjunction with Pistachio hull extract in breast cancer cell lines. Therefore, both CFZ and Pistachio hull extract, whether administered alone or in combination, represent promising molecular targets for breast cancer treatment.

Keywords Pistachio hull extract, Carfilzomib, Combination therapy, Breast cancer

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Introduction

Breast cancer is the most common cancer among women worldwide and remains a leading cause of cancer-related deaths in this population. In 2020, approximately 2.3 million new cases of breast cancer were reported globally [1]. Traditional treatment options for breast cancer include chemotherapy, radiation therapy, surgical interventions, and hormone therapy. While effective medications such as tamoxifen, raloxifene, and the antitumor drug anastrozole are commonly used in conjunction with other therapeutic approaches, a significant challenge remains; drug resistance, coupled with a range of side effects that can harm healthy cells and lead to disease recurrence [2].

One of the critical issues in breast cancer treatment is that tumors can exhibit intrinsic resistance to drugs even before chemotherapy begins or can develop resistance during treatment. This resistance results in treatment failure for more than 90% of patients with metastatic breast cancer [3]. Among the genes associated with drug resistance are members of the ATP Binding Cassette (ABC) transporter family. Notable members include glycoprotein-P (MDR1/ABCB1) and Multidrug Resistance Associated Protein 1 (MRP1/ABCC1). These proteins are found on the surfaces of various cells, including normal cells; however, their increased expression in certain cancers contributes to drug resistance by actively expelling anticancer agents from the cells [3, 4]. The ABC superfamily is one of the largest protein families identified in the human genome, comprising 49 ABC genes that facilitate the efflux of multiple chemotherapeutic drugs, thereby contributing to multidrug resistance (MDR). Additionally, several ABC transporters are involved in cellular metabolism and the transport of biological molecules [5].

Nuclear factor kappa B (NF-κB) is a crucial transcription factor that plays a significant role in inflammatory responses, differentiation, tumor cell survival, invasion, metastasis, and the evasion of apoptosis in tumor tissues. Its involvement in breast cancer development and drug resistance is well-established; however, the specific molecular mechanisms by which NF-κB contributes to drug resistance are still not fully understood [6]. In most mammalian cells, NF-κB exists as an inactive heterodimer bound to an inhibitor known as IκB. When exposed to specific stimuli, IκB undergoes phosphorylation and subsequent degradation, allowing NF-κB to translocate into the nucleus where it regulates the expression of target genes. The predominant dimer in mammals is the p50/p65 heterodimer [7].

Caspase3, a member of the caspase family of proteases, plays a vital role in the execution phase of apoptosis. It cleaves various proteins, including Poly ADP Ribose Polymerase (PARP) and other structural proteins, through both mitochondrial-mediated and death receptor-mediated pathways, leading to cell death [8, 9]. In the context of apoptosis, caspase3 degrades essential cellular components required for cell survival. However, in certain cancers, including breast cancer, tumor cells may evade this apoptotic pathway, allowing them to resist treatment and persist despite therapeutic interventions [10]. Furthermore, research indicates that caspase3 may also facilitate tumor progression by creating a pro-inflammatory environment or promoting cellular changes that lead to cancer cell invasion and metastasis. This suggests a complex relationship between caspase3 activity and cancer biology, where it can function both as a tumor suppressor and as a promoter of tumor growth depending on the specific context of its activation [10, 11].

Carfilzomib (CFZ) is a second-generation proteasome inhibitor currently utilized in treating patients with multiple myeloma [12]. Notably, CFZ has demonstrated effectiveness in overcoming resistance in patients who do not respond to Bortezomib (BTZ), the first drug in the proteasome inhibitor class [13]. Proteasomes are essential protein complexes that remove unnecessary or damaged proteins within cells. In certain cancers, proteins vital for inducing cancer cell death can be rapidly inactivated and eliminated. BTZ disrupts this process, allowing these proteins to function effectively and promote cancer cell death [14–16].

One of the mechanisms through which cancer cells can develop resistance to the chemotherapy drug Doxorubicin (DOX) involves the activation of NF-κB. Research has shown that CFZ inhibits the degradation of IκB, thereby preventing NF-κB activation [17]. Studies on tamoxifen-resistant breast cancer cell lines have also indicated that CFZ is more effective than fluostrant in reducing cell proliferation. Additionally, CFZ has been found to upregulate Caspase3, enhancing apoptosis in various cancer cell lines, including those associated with breast cancer. By inhibiting the proteasome, CFZ can trigger apoptotic pathways that activate caspases, including Caspase3 [18–20].

In response to the side effects often associated with conventional chemical treatments, researchers are increasingly exploring plant-based compounds for cancer therapy [21–23]. Among these, pistachios from the Pistacia vera tree stand out as highly valued edible nuts. Their hulls are particularly rich in phenolic acids, flavonoids, fatty acids, proteins, vitamins, and polysaccharides [24, 25]. Previous studies have highlighted that pistachio hulls contain significantly higher levels of phenolic compounds compared to the seeds, underscoring their importance due to their potent antioxidant properties [26].

Recognized as a rich source of antioxidants, pistachio hulls offer easier accessibility and fewer side effects than traditional chemical drugs [27–29]. Given the roles of

genes such as *MRP1* and *MDR1* in the development of drug resistance, alongside *NF-κB p65* and *Caspase-3*'s involvement in regulating apoptosis, this research aims to investigate the effects of pistachio hull extract and CFZ-both separately and in combination-on the expression levels of these genes in a breast cancer cell line.

Methods

In this experimental study, we utilized the breast cancer cell line SK-BR3 and the normal cell line MCF10A, both sourced from the Pasteur Institute of Iran in Tehran. Both cell lines displayed characteristic epithelial cell morphology and were cultured in RPMI 1640 and DMEM culture media, respectively. The RPMI 1640 medium contained sodium bicarbonate, glutamine, penicillin, and streptomycin (with concentrations of 100 units/ml of penicillin and 100 mg/ml of streptomycin), supplemented with 10% fetal bovine serum (FBS). To inactivate the complement system, the FBS was heated to 56 degrees Celsius for 30 min in a Bain-Marie before use.

Pistachio hull extraction

For this study, we used fresh *Pistacia vera var. Ohady* that had not been exposed to harmful chemicals. This variety was identified by Dr. Ali Tajabadipour, a horticulture expert, and a voucher sample (No. O11) was preserved at the Pistachio Research Center within the Horticultural Sciences Research Institute of the Agricultural Research, Education, and Extension Organization (AREEO) in Rafsanjan, Iran. The dried hulls of *P. vera var. Ohady* were ground into a fine powder using a BOSCH TSM6A011 electric mill. We then placed five grams of this powder into a cylindrical filter paper and inserted it into a specialized Soxhlet flask. Following this, we filled the flask with 70% ethanol to about 75% of its capacity and set up the apparatus at a temperature above the evaporation point of ethanol for 100 min. The concentrated extract obtained was transferred to a Petri dish and freeze-dried for 72 h. The resulting powder was then finely shaved and dissolved in distilled water. The initial stock solution was stored at -20 °C until further use [30].

GC/MS analysis

Gas chromatography-mass spectrometry (GC/MS) analysis was performed by the Analytical Chemistry Unit at Vali Asr University in Rafsanjan, following established protocols. This analytical technique enables detailed identification and quantification of compounds present in the extracted sample [31].

Initial stock preparation

CFZ (CFZ) was obtained from AdooQ BioScience (CA, USA) and prepared as an initial stock by dissolving 2.88 mg of the compound in 2 ml of dimethyl sulfoxide (DMSO). Additionally, a separate stock solution was created by dissolving 25 mg of pistachio hull extract in 1 ml of culture medium. The CFZ stock was stored at -20 °C, while the extract was freshly prepared each day. To achieve the desired concentrations for treatment, both stock solutions were diluted with culture medium.

Cell preparation for treatment

Cells were cultured in 25 cm^2 flasks and reached approximately 80% confluence before being trypsinized, counted, and seeded into 96-well plates at a density of 10^3 cells per well. The plates were then placed in a CO_2 incubator for a period ranging from 24- to 48-hours, depending on the specific experimental requirements. This incubation allowed the cells to adhere to the bottom of the wells and continue growing. After this period, the culture medium in the wells was replaced with fresh medium containing various concentrations of the treatments. Control wells received only complete culture medium with a maximum of 1% DMSO.

Evaluation of cytotoxicity

MTT assay for individual treatments

To assess cytotoxicity, the cells were initially trypsinized, counted, and transferred to a 96-well plate at a density of 10^3 cells per well. Each well was supplemented with 200 µl of complete culture medium and incubated for at least 24 h to ensure proper attachment and entry into the logarithmic growth phase. Following this incubation, the medium was replaced with fresh medium containing different concentrations of pistachio hull extract (ranging from 0.156 to 10 mg/mL) and CFZ (ranging from 0.0032 to 0.05 mg/mL). After designated time points of either 24- or 48-hours, 35 µl of MTT solution (final concentration of 5 mg/ml in PBS) was added to each well and incubated for an additional 4 h. Following incubation, 165 µl of DMSO was added to each well to dissolve the formed formazan crystals, and the absorbance was measured at a wavelength of 570 nm using an ELISA reader. The absorbance values are directly correlated with the number of viable cells present. The percentage of viable cells compared to the control was calculated using the following formula:

Percentage of viable cells = 100 × (mean absorbance of control cells)/(mean absorbance of treated cells).

Analysis of combination treatment using the MTT assay

The combination treatment of pistachio hull extract and CFZ was evaluated through the MTT assay on two cell lines: the SK-BR3 breast cancer line and the MCF10A normal cell line. Treatments were administered for both 24- and 48-hours. To explore potential interactions between the two substances-whether synergistic, additive, or antagonistic-various concentrations were tested.

Specifically, four doses below the IC50 of each drug and two doses above the IC50 were added to the culture medium. After exposure to these combinations for the designated time periods, optical density (OD) was measured, and cell viability percentages were calculated using the established formula.

Data analysis was performed using CompuSyn software (Version 1.0, Combo-Syn Inc., US). The combination index (CI) values for effective doses were determined based on different fractions of the cell population, following the principles laid out by Chou and Talalay for assessing drug interactions in combination therapies [32]. In this context, CI values greater than 1.1 indicate antagonism, values between 0.9 and 1.1 suggest additivity, and values less than 0.9 reflect synergism. According to the Chou-Talalay method, the CI value for each fraction of cells affected by a cytotoxic agent (termed Fraction affected or Fa) is calculated using the equation [33]:

$CI = (D_1)/ (Dx)_1 + (D_2)/ (Dx)_2$.

In this equation, D_1 and D_2 represent the concentrations of each drug when administered individually, while $(Dx)_1$ and $(Dx)_2$ denote their concentrations when combined.

Real-time PCR

For RNA extraction, the RNXTm-Plus extraction kit was utilized according to the manufacturer's protocol from Pars-Toos. The purity and concentration of the extracted RNA were assessed using a NanoDrop spectrophotometer (NanoDrop 2000, Wilmington, DE, USA), and RNA quality was confirmed by running a 1.5% agarose gel. Following extraction, complementary DNA (cDNA) synthesis was performed using the Pars-Toos kit and a Bio-Rad thermocycler model S1000. The resulting cDNA was stored at -20 °C for future experiments.

For Real-Time PCR analysis, two microtubes were prepared for each sample. One microtube contained primers specific to target genes (*MDR1* (*ABCB1*), *MRP1*(*ABCC1*), *NF-kB p65*, and *Caspase3*), while the other contained GAPDH primers as a housekeeping control (Table 1). After adding 5 µl of synthesized cDNA to each microtube, the contents were briefly centrifuged according to the specified temperature program. Gene expression analysis was conducted using the $2^{-\Delta\Delta Ct}$ method after constructing standard curves for each gene in the respective cell lines [34].

Table 1 Sequences of primers used in qPCR

Genes	Forward	Reverse
MDR1(*ABCB1*)	5′-ACAGTGGAATTGGTGCT GGG-3′	5′-TAAGCTCCCCA ACATCGTGC-3′
MRP1(*ABCC1*)	5′-TGTTGGTTGCTTACAGT GTTG-3′	5′-TGGGAGGTATT TCGTGTTCTT-3′
NF-kB p65	5′-AGCCCACAAAGCCTTA TC-3′	5′-AATGCCAGT-GCCAATAVAG-3′
Caspase3	5′-GGAAGCGAATCAATGGAC TCTGG-3′	5′-GCATCGACATC TGTACCAGACC-3′
GAPDH	5′-CTCATTTCCTGGTATGAC AACGA-3′	5′-TCTTCCTCTTGT GCTCTTGCTG-3′

ELISA

To evaluate the effects of pistachio hull extract and CFZ on NF-κB p65 protein levels, we employed the ZellBio kit (Cat No: ZB-1512930-H9648) in a colorimetric assay. SK-BR3 and MCF10A cells were plated at a density of 2.5×10^5 cells per well in a 6-well plate, using 200 µl of complete medium. After allowing the cells to incubate for 24 h, they were treated with the IC50 values of each drug, both individually and in combination, for durations of 24- and 48-hours. Following these treatment periods, we conducted an ELISA according to the manufacturer's protocol to measure the levels of NF-κB p65.

Statistical analysis

Statistical analyses were performed using T-tests, non-parametric one-way ANOVA, and post hoc Tukey tests. The results are expressed as Mean ± SD, with statistical significance determined at $P < 0.05$. All analyses were conducted using SPSS-18 software.

Ethical considerations

This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the Institutional Review Board under code IR.KMU.AH.REC.1400.164.

Results

Compounds identified in pistachio hull variety Ohady

The analysis of the pistachio hull variety Ohady using Gas Chromatography-Mass Spectrometry (GC-MS) revealed several compounds, as shown in Fig. 1 (Chromatogram) and detailed in Table 2. The three predominant components identified were phthalic acid (53.10% with a retention time of 29.59 min), 4-bromobutyric acid (12.53% with a retention time of 26.98 min), and octanoic acid (10.82% with a retention time of 30.70 min).

Toxicity assessment of carfilzomib (CFZ) and pistachio hull extract

We evaluated the toxicity of CFZ and pistachio hull extract by determining their IC50 values for various exposure durations using GraphPad Prism software (Version 6.07). The MTT assay results indicated that the IC50 values for pistachio hull extract were 2.014 mg/ml and 1.031 mg/ml after 24- and 48-hours of treatment in the SK-BR3 cell line, respectively. In the MCF10A cell line, the IC50 values were found to be 2.994×10^{-3} mg/ml and 3.265×10^{-3} mg/ml for the same time points.

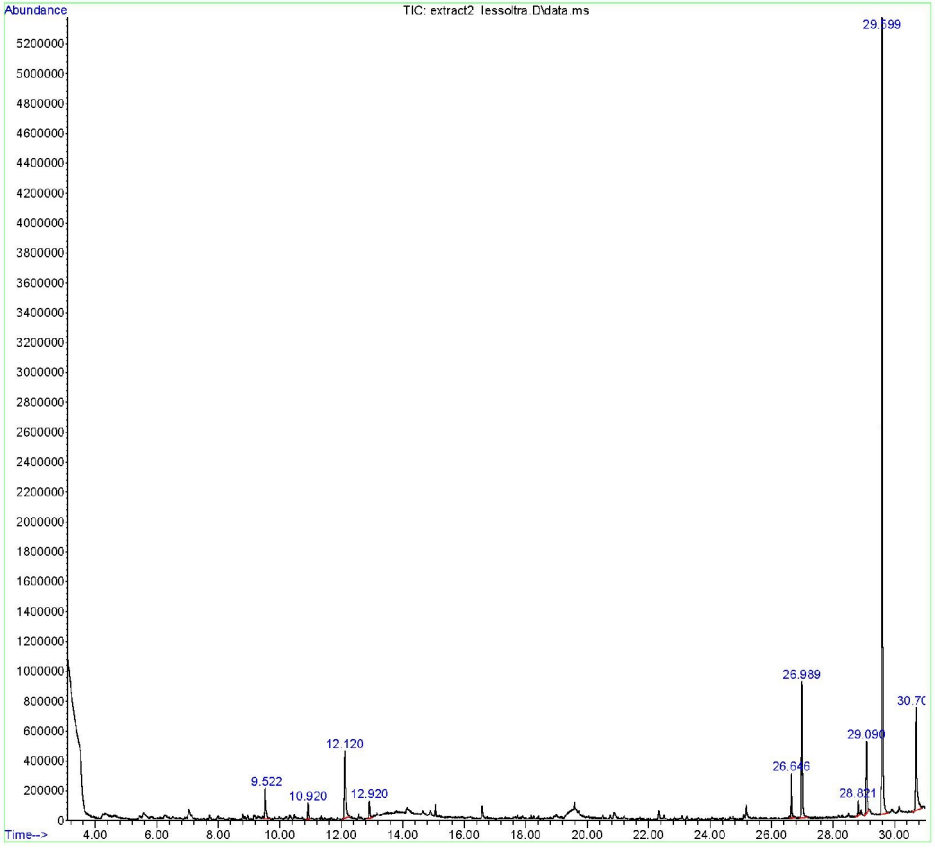

Fig. 1 Chromatogram from GC-MS analysis of pistachio hull variety Ohady

Table 2 Compounds identified in pistachio hull variety ohady through GC-MS analysis

Number	Compound Name	%	RT (min)
1	Pyrocatechol	1.52	9.52
2	Camphor	0.66	10.92
3	Pyrogallic acid	6.22	12.12
4	2,7-ditert-butylnaphthalene	0.71	12.92
5	Butanoic acid	5.18	26.64
6	4-Bromobutyric acid	12.53	26.98
7	α-Fluorocinnamic acid	0.43	28.82
8	Hydrocardanol	7.15	29.09
9	Phthalic acid	53.10	29.59
10	Octanoic acid	10.82	30.70

RT: Retention Time

In addition, CFZ exhibited IC50 values of 0.181×10^{-3} mg/ml and 0.0057×10^{-3} mg/ml in the SK-BR3 cell line, and 5.54×10^{-3} mg/ml and 2.51×10^{-3} mg/ml in the MCF10A cell line after 24- and 48-hours, respectively, demonstrating its capacity to inhibit cell growth by approximately 50%. The percentage of viable cells was calculated relative to the control group, revealing a dose- and time-dependent decrease in cell growth and proliferation rates following treatment with both pistachio hull extract and CFZ. Each experimental condition included three replicates for each concentration (Fig. 2).

Combination treatment of carfilzomib (CFZ) and pistachio hull extract

In this study, we administered CFZ and pistachio hull extract at four doses below the IC50 threshold and two doses above it, as illustrated in Fig. 3. Both cell lines were subjected to these treatments, and after 24- and 48-hours of incubation, we analyzed the results using the MTT assay alongside dose-response graphs (Fig. 3). Additionally, we generated Combination Index (CI) plots (Fig. 4) and Median Effect plots (Fig. 5) using CompuSyn software to further assess the interaction between the treatments.

As depicted in Fig. 3, the effective dose (ED50) values for pistachio hull extract were found to be 0.228×10^{-3} and 0.04×10^{-3} mg/ml in the SK-BR3 cell line, while in the MCF10A cell line, they were 0.642×10^{-3} and 0.387×10^{-3} mg/ml after 24- and 48-hours of combination treatment, respectively. Furthermore, CFZ exhibited concentrations of 0.011×10^{-3} and 0.00008×10^{-3} mg/ml in SK-BR3 cells, and 0.896×10^{-3} and 0.273×10^{-3} mg/ml

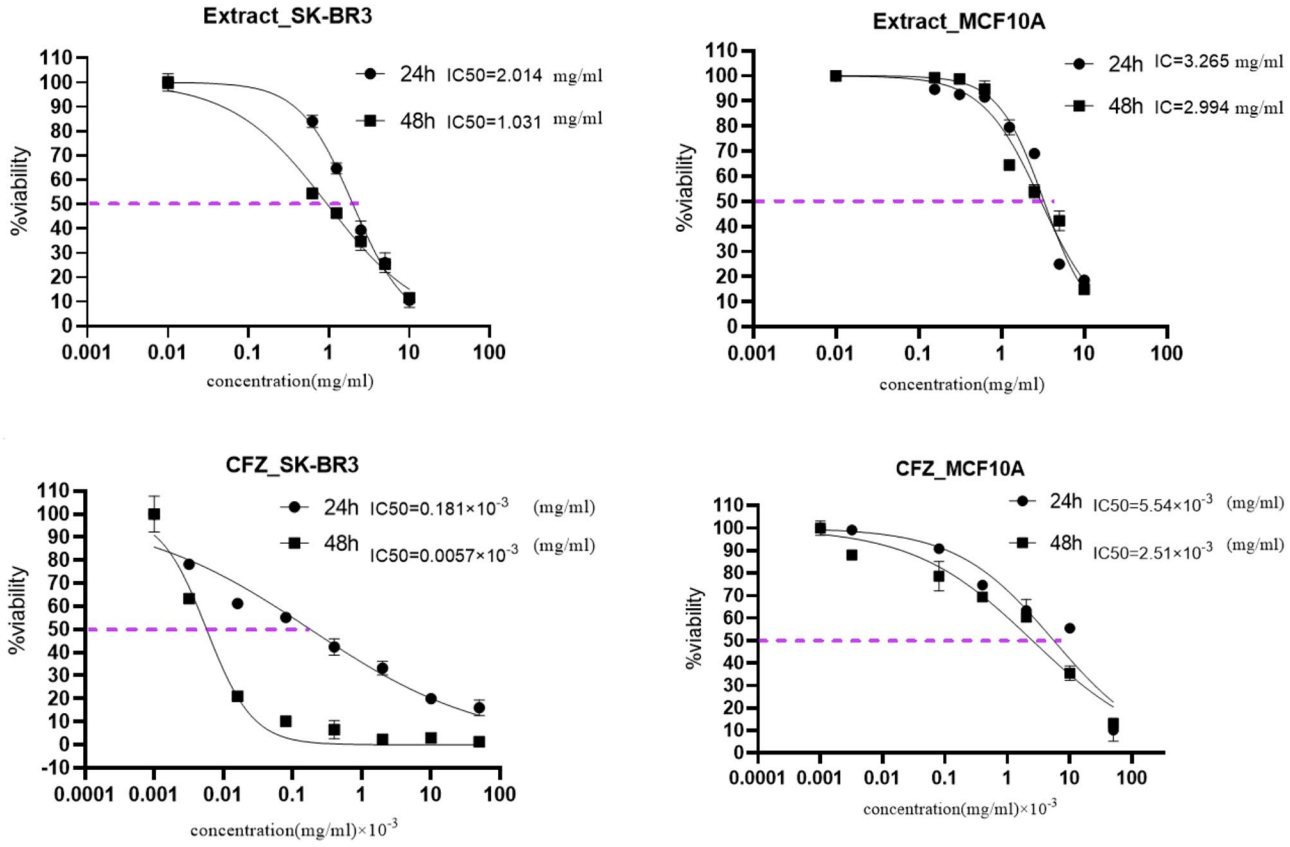

Fig. 2 Effects of varying concentrations of pistachio hull extract and carfilzomib (CFZ) on the growth of SK-BR3 and MCF10A cells over 24- and 48-hours. Results are presented as Mean ± SD compared to control

in MCF10A cells at the same time points, demonstrating its capacity to inhibit cell growth by approximately 50%.

The analysis of the combination treatment shown in Fig. 4 reveals that the CI values for the ED50 in SK-BR3 cells were 0.102 and 0.022, while in MCF10A cells, they were 0.261 and 0.165 at 24- and 48-hours, respectively. The Log CI values provide insights into the nature of the interaction: values greater than zero (Log CI > 0) indicate antagonism, values equal to zero (Log CI = 0) suggest an additive effect, and values less than zero (Log CI < 0) imply a synergistic effect. Notably, in SK-BR3 cells, a fraction affected (Fa) of 0.5 indicates a more favorable synergistic interaction between the two treatments (Fig. 5).

Overall, we observed a decrease in cell viability across both cell lines that was dependent on both the dose and duration of treatment. Interestingly, a lower dose of the combination treatment resulted in a higher percentage of cell death in SK-BR3 cells compared to MCF10A cells.

Real-time PCR analysis

Figure 6 presents the effects of pistachio hull extract, CFZ, and their combination on the expression levels of the *MDR1* gene in SK-BR3 and MCF10A cell lines after 24- and 48-hours of treatment.

After treating both SK-BR3 and MCF10A cell lines for the specified durations (indicated with *), we observed a significant reduction in *MDR1* gene expression across all treatment groups when compared to the control group. Notably, in the SK-BR3 cell line, all treatment groups demonstrated a marked decrease in *MDR1* expression relative to the MCF10A cell line (highlighted with ⬧). Furthermore, there was a significant difference between the combined treatment group at 48 h and the same group at 24 h (denoted with †), underscoring the effectiveness of the drug-extract combination.

In the 48-hour treatment group, the cancer cells receiving combination therapy exhibited an impressive decrease of approximately 95% in *MDR1* gene expression compared to the control group. In contrast, those treated with CFZ and the pistachio hull extract alone showed reductions of about 73% and 70%, respectively.

Figure 7 illustrates the impact of pistachio hull extract, CFZ, and their combination on *MRP1* gene expression in SK-BR3 and MCF10A cell lines at both 24- and 48-hours. Similar to the findings for *MDR1*, a significant reduction in *MRP1* gene expression was noted across all treatment groups compared to the control group after 24- and 48-hours of treatment (indicated with *). In the SK-BR3

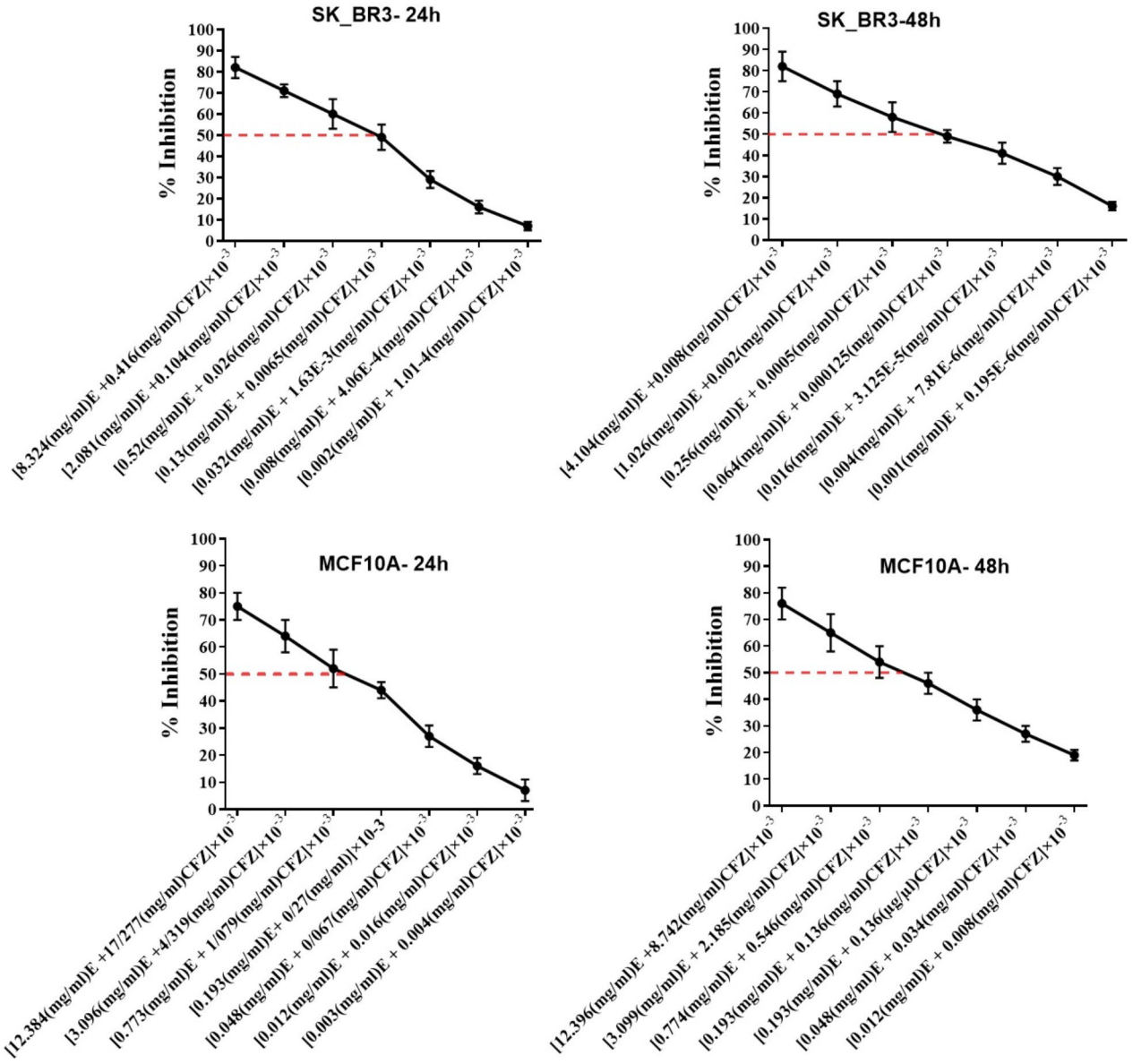

Fig. 3 Dose-Response curve showing the effects of pistachio hull extract (E) (mg/ml) and carfilzomib (CFZ) (mg/ml) on SK-BR3 and MCF10A cell lines at 24- and 48-hours post-treatment. Results are presented as Mean ± SD compared to control

cell line, significant decreases in MRP1 expression were observed in the combined treatment group at 24 h and in all treatment groups at 48 h when compared to the MCF10A cell line (marked with •). Additionally, a notable decrease was found between the combined treatment at 48 h and that at 24 h (denoted with †), indicating that the drug-extract combination's efficacy improves over time.

In the 48 h treatment group, cancer cells receiving combination therapy showed a substantial reduction of approximately 98% in *MRP1* gene expression compared to the control group. In contrast, those treated individually with CFZ and pistachio hull extract exhibited reductions of around 85% and 80%, respectively.

Figure 8 depicts the effects of pistachio hull extract, CFZ, and their combination on the expression levels of the *NF-κB P65* gene in SK-BR3 and MCF10A cell lines after 24- and 48-hours of treatment

Following treatment of both cell lines, a notable reduction in *NF-κB p65* gene expression was observed at both time points (indicated with *), particularly in the SK-BR3 cell line, which showed a significant decrease compared to the MCF10A line (highlighted with •). Additionally, there was a marked decline in *NF-κB p65* expression at 48 h across all treatment groups when compared to the 24 h results (noted with †). This pattern suggests that the

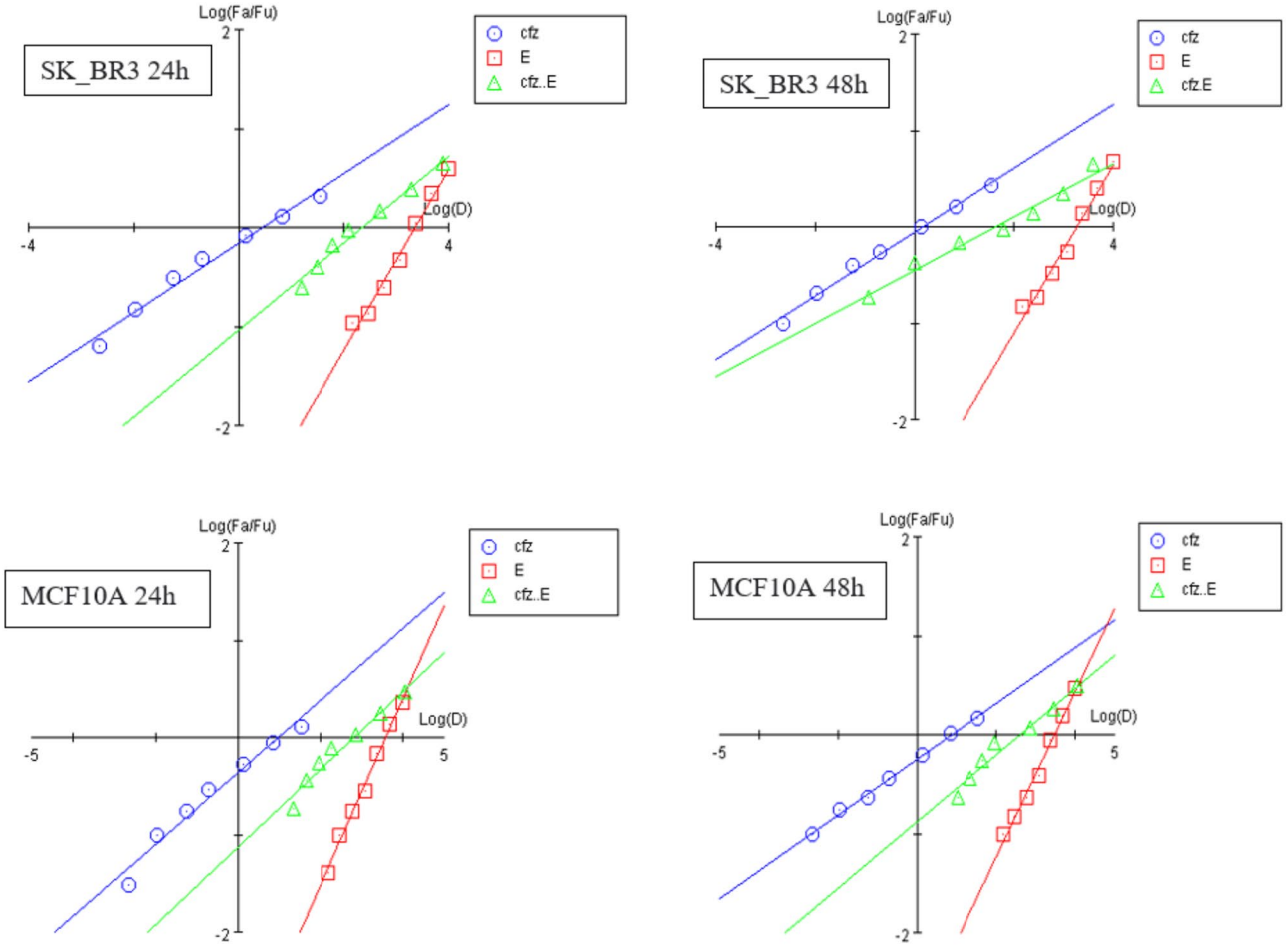

Fig. 4 Combination Index plot illustrating the effects of pistachio hull extract (E) and carfilzomib (CFZ) on SK-BR3 and MCF10A cell lines at 24- and 48-hours post-treatment. Fa (Fraction affected)/Fu (Fraction unaffected) = (D/Dm) m, where D represents the dose required to achieve Fa, Dm is the median-effect dose, and m is the slope

combination of the drug and extract becomes increasingly effective over time.

In the cancer group receiving the combined treatment for 48 h, *NF-κB P65* gene expression dropped dramatically by approximately 91% compared to the control group. In contrast, those treated with CFZ and the extract alone exhibited reductions of about 71% and 69%, respectively.

According to Fig. 9, treatment of the SK-BR3 cell line for both 24- and 48-hours resulted in a significant increase in *Caspase3* gene expression across all groups, while in MCF10A cells, significant increases were noted at 24 h for both the combined group and CFZ (marked with *). Notably, the SK-BR3 cell line exhibited a significantly higher level of *Caspase3* expression compared to the MCF10A line (marked with ⬧) at 24 h in both the combination and CFZ groups, as well as at 48 h in the CFZ group.

In the cancer group treated with combination therapy for 48 h, *Caspase3* gene expression increased approximately 2.4-fold compared to the control group. In contrast, the groups treated with CFZ and pistachio hull extract alone showed increases of about 1.9-fold and 1.6-fold, respectively.

NF-κB p65 transcription factor

To evaluate the effects of CFZ and pistachio hull extract, both individually and in combination, on the intracellular levels of the NF-κB p65 transcription factor, we treated SK-BR3 and MCF10A cell lines with various concentrations of the extract and CFZ, determined based on their IC50 values. Following treatment periods of 24- and 48-hours, we generated a standard curve using at least six data points from the provided kit solutions. The concentration of the NF-κB p65 transcription factor was quantified according to the Zellbio kit protocol (Fig. 10).

Analysis of the treatment effects on both cell lines after 24- and 48-hours revealed a significant reduction in NF-κB p65 protein levels compared to the control group (indicated by *). Notably, the SK-BR3 cell line showed a

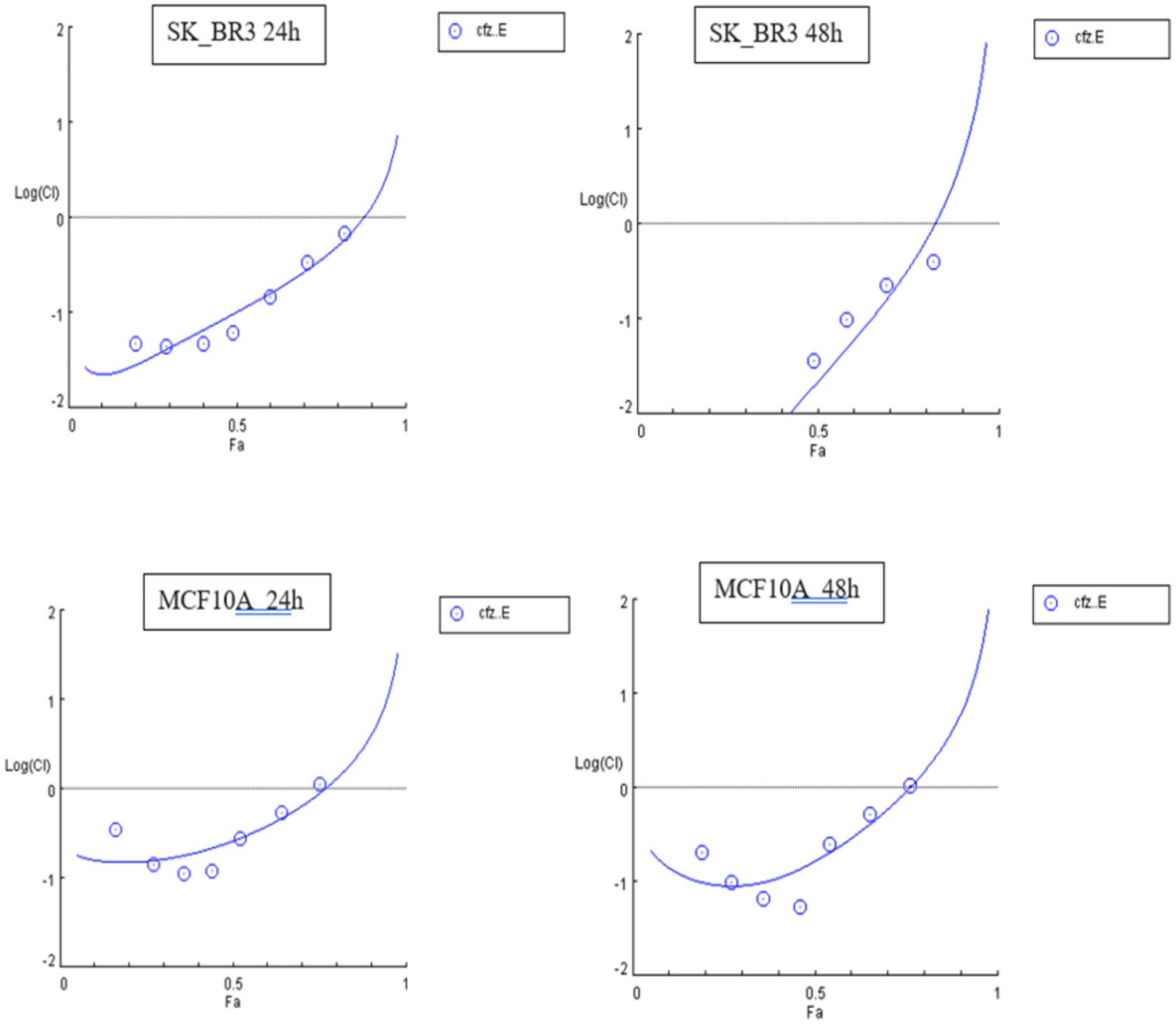

Fig. 5 Median Effect plot resulting from the combination treatment of pistachio hull extract (E) and carfilzomib (CFZ) on SK-BR3 and MCF10A cell lines at 24- and 48-hours post-treatment. The values of Log CI > 0 indicate antagonism, Log CI = 0 denotes an additive effect, and Log CI < 0 signifies synergy. A fraction affected (Fa) of 0.5 represents the effective dose that kills 50% of the cells. CI = Combined Index

more substantial decrease in NF-κB protein levels than the MCF10A cell line (highlighted with •). Furthermore, the combined treatment group exhibited a greater reduction in NF-κB p65 protein levels compared to the individual treatment groups (indicated by the c symbol). This observation may account for the enhanced effectiveness of the combined treatment in modulating protein levels.

Discussion

In this study, we explored the synergistic effects of pistachio hull extract and CFZ on the expression of genes related to drug resistance and apoptosis pathways, specifically focusing on *NF-κB p65*, *MDR1*, *MRP1*, and *Caspase3* in breast cancer cells (SK-BR3) and normal cells (MCF10A). Our results indicate that the combination of pistachio hull extract and CFZ at the effective dose (ED50) produced notable synergy, with combination index (CI) values of 0.102 and 0.0221 for the SK-BR3 cell line at 24- and 48-hours, respectively. For the MCF10A cell line, the CI values were 0.261 and 0.165 at the same time points. This pronounced synergy in SK-BR3 cells aligns with CFZ's role as a proteasome inhibitor, which disrupts protein degradation in cancer cells, leading to increased intracellular stress and enhanced apoptosis [35].

To further investigate this synergy, we employed Real-Time PCR to quantify changes in gene expression. Our analysis confirmed significant modulation of *MDR1*, *MRP1*, *NF-κB p65*, and *Caspase3* levels-key genes involved in drug resistance and apoptosis.

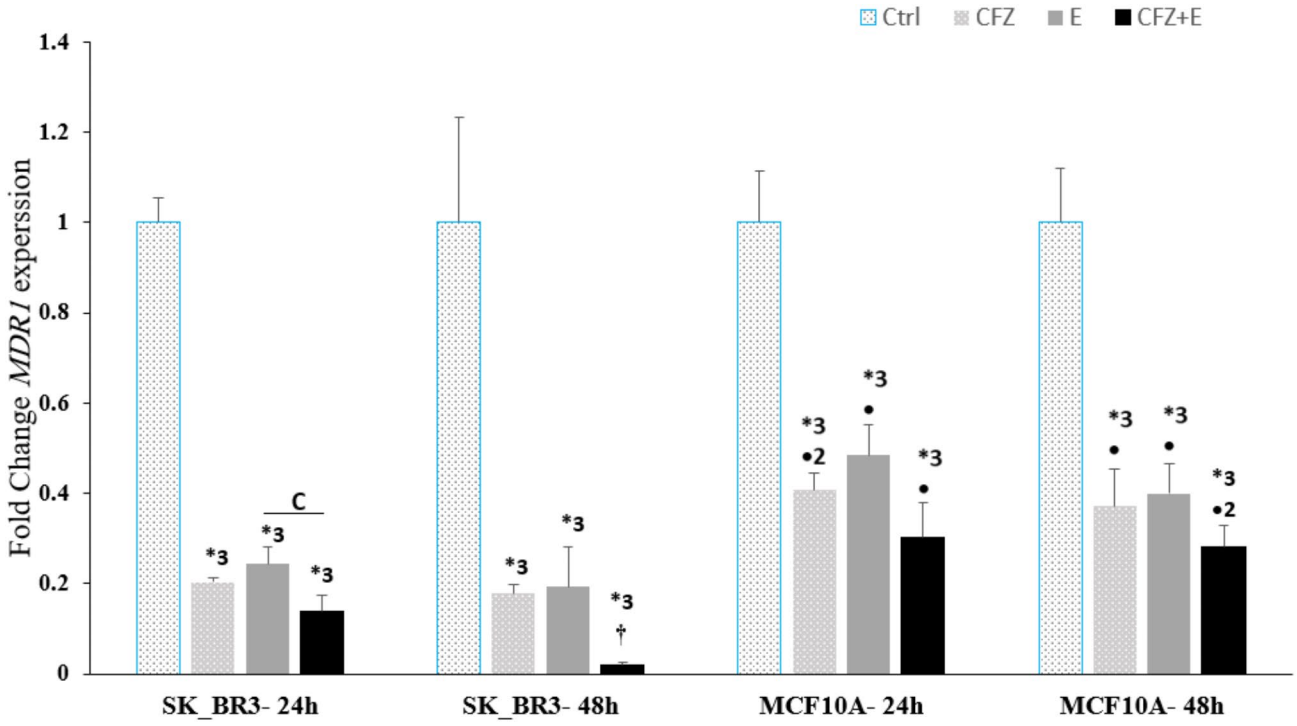

Fig. 6 The effects of pistachio hull extract (E), carfilzomib (CFZ), and their combination on *MDR1* gene expression levels in SK-BR3 and MCF10A cell lines. Statistical significance is indicated as follows: $P < 0.05$ * for all treatment groups compared to the control group (Tukey-HSD test); • $P < 0.05$ for tumor cell line compared to normal cell line (T-test); c $P < 0.05$ for comparisons among treatment groups (Tukey-HSD test); † $P < 0.05$ for comparisons between 24- and 48-hours within each category (T-test); ^{2} $P < 0.005$; and ^{3} $P < 0.001$

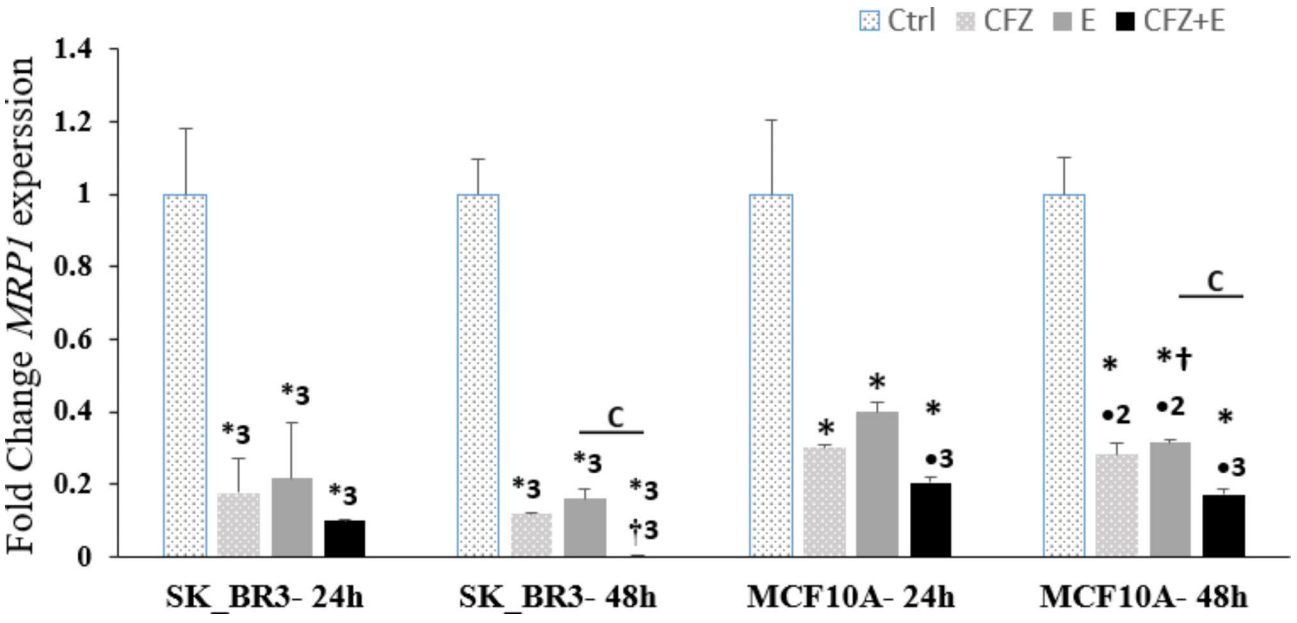

Fig. 7 The effects of pistachio hull extract (E), carfilzomib (CFZ), and their combination on *MRP1* gene expression levels in SK-BR3 and MCF10A cell lines. Statistical significance is indicated as follows: $P < 0.05$ * for all treatment groups compared to the control group (Tukey-HSD test); • $P < 0.05$ for tumor cell line compared to normal cell line (T-test); c $P < 0.05$ for comparisons among treatment groups (Tukey-HSD test); † $P < 0.05$ for comparisons between 24- and 48-hours within each category (T-test); ^{2} $P < 0.005$; and ^{3} $P < 0.001$

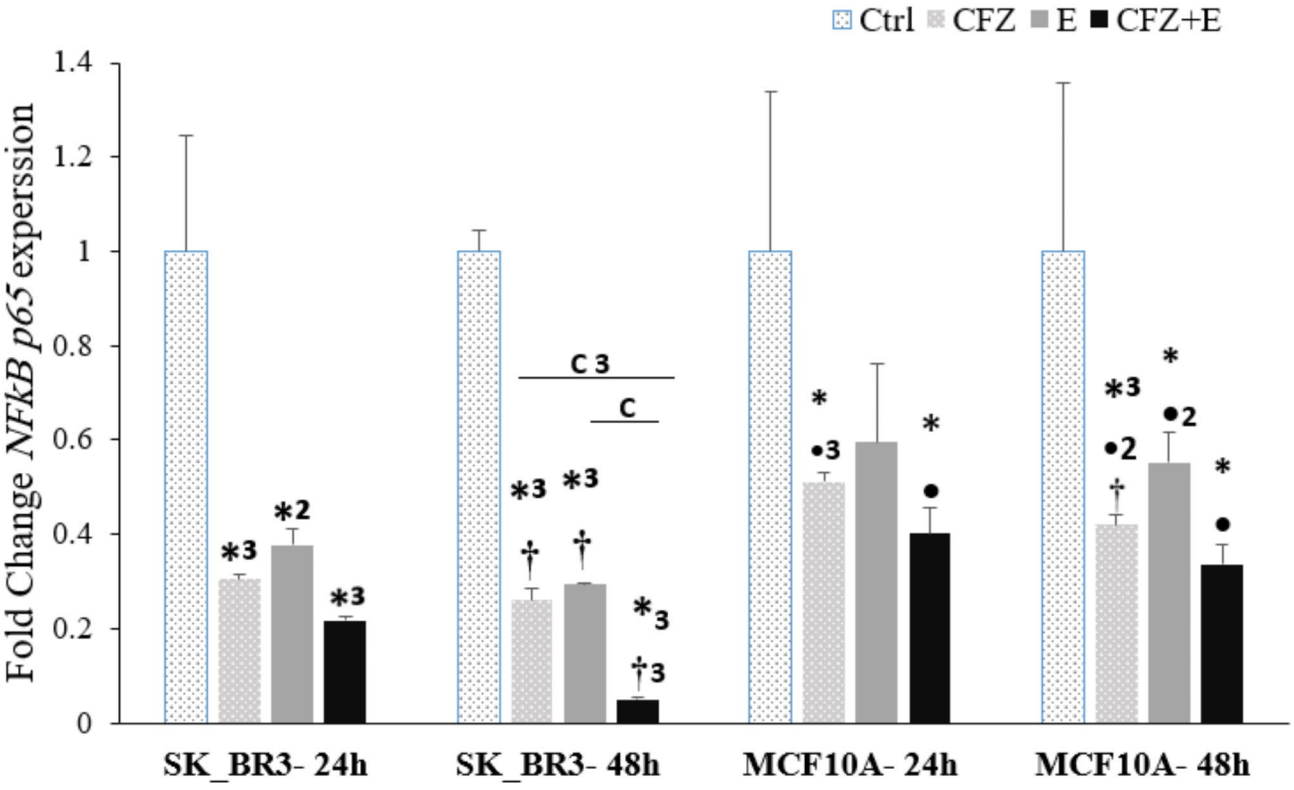

Fig. 8 The effects of pistachio hull extract (E), carfilzomib (CFZ), and their combination on *NF-κB P65* gene expression levels in SK-BR3 and MCF10A cell lines. Statistical significance is indicated as follows: $P < 0.05$ * for all treatment groups compared to the control group (Tukey-HSD test); • $P < 0.05$ for tumor cell line compared to normal cell line (T-test); c $P < 0.05$ for comparisons among treatment groups (Tukey-HSD test); † $P < 0.05$ for comparisons between 24- and 48-hours within each category (T-test); [2] $P < 0.005$; and [3] $P < 0.001$

When comparing our findings to those of Ashley et al., who examined the synergistic effects of CFZ combined with Doxorubicin (Dox), we noted similar synergistic outcomes at the 24-hour mark (CI: 0.981) [36]. Importantly, our study revealed a substantial reduction in *MDR1* and *MRP1* gene expression in both SK-BR3 and MCF10A cells; however, the effects were more pronounced in the cancer cell line. Specifically, under combination therapy, *MDR1* expression decreased by approximately 95% in SK-BR3 cells, compared to reductions of 73% and 70% observed with CFZ or pistachio hull extract alone, respectively. This finding is consistent with research by Clemens et al., which demonstrated that CFZ similarly downregulated *MDR1* expression in other cancer cell lines, highlighting its potential to mitigate multidrug resistance in tumor cells [37].

For MRP1, the reduction was even more significant, with combination treatment resulting in a decrease of around 98% in SK-BR3 cells. This observation aligns with findings from Zarei et al., who reported substantial MRP1 downregulation when CFZ was used alongside other chemotherapeutic agents. These results further support CFZ's ability to inhibit drug efflux mechanisms by targeting both MRP1 and MDR1 pathways, suggesting a promising avenue for enhancing the efficacy of cancer treatments [38].

The effects on NF-κB p65 expression were notably significant in both cell lines, with a more pronounced decrease observed in the SK-BR3 cancer cells compared to the MCF10A normal cells. Following combination treatment, NF-κB p65 expression in SK-BR3 cells was reduced by approximately 91%. This substantial reduction indicates a strong inhibition of the NF-κB pathway, which plays a critical role in cancer cell survival and the modulation of immune responses. These findings are consistent with previous research demonstrating that treatments involving CFZ, either alone or in combination with agents like curcumin, resulted in decreased nuclear accumulation of NF-κB. This reduction, in turn, diminished the transcription of survival genes dependent on NF-κB [39].

Moreover, inhibiting NF-κB in cancer cells is associated with increased sensitivity to apoptosis. This suggests that the combination of CFZ and pistachio hull extract may selectively disrupt survival signaling in breast cancer cells, presenting a promising therapeutic strategy. Preclinical studies have shown that CFZ can act independently in hematopoietic malignancies and specific solid tumors, such as head and neck cancer, by inhibiting

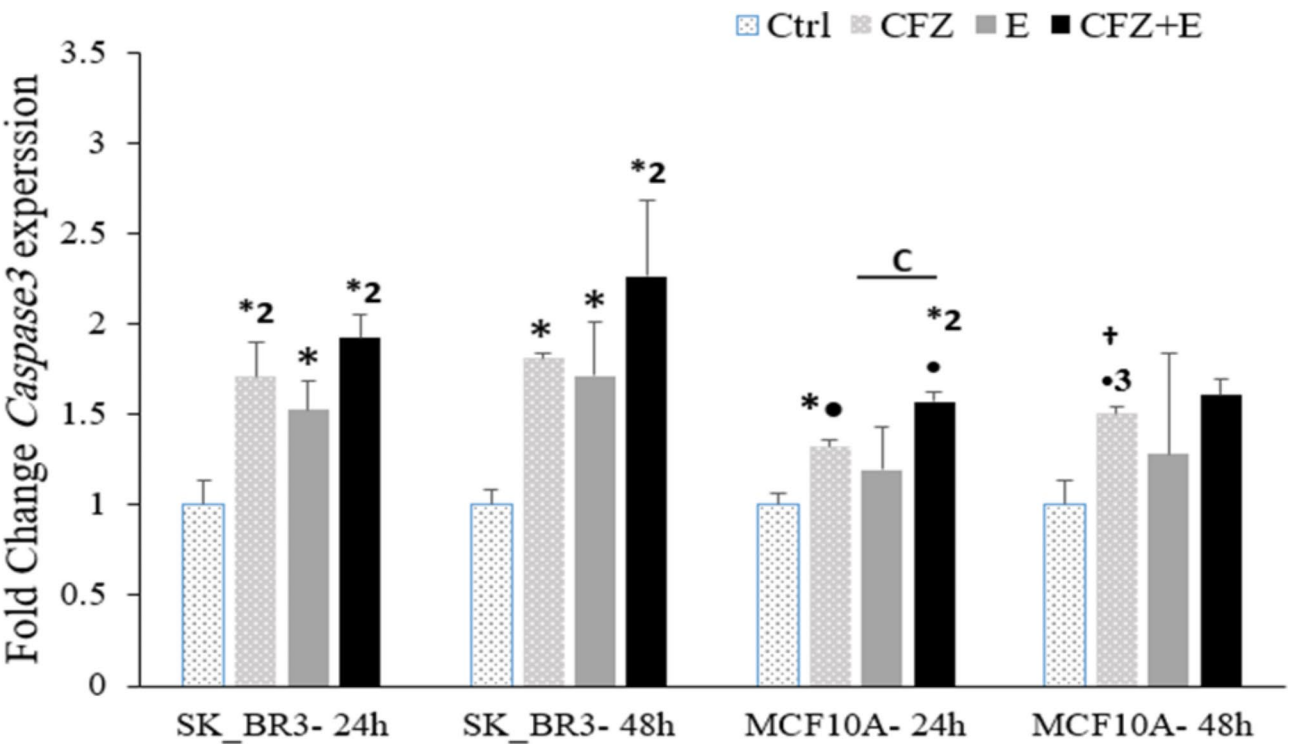

Fig. 9 The effects of pistachio hull extract (E), carfilzomib (CFZ), and their combination on *Caspase3* gene expression levels in SK-BR3 and MCF10A cell lines. Statistical significance is indicated as follows: $P < 0.05$ * for all treatment groups compared to the control group (Tukey-HSD test); • $P < 0.05$ for tumor cell line compared to normal cell line (T-test); c $P < 0.05$ for comparisons among treatment groups (Tukey-HSD test); † $P < 0.05$ for comparisons between 24- and 48-hours within each category (T-test); ^{2} $P < 0.005$; and ^{3} $P < 0.001$

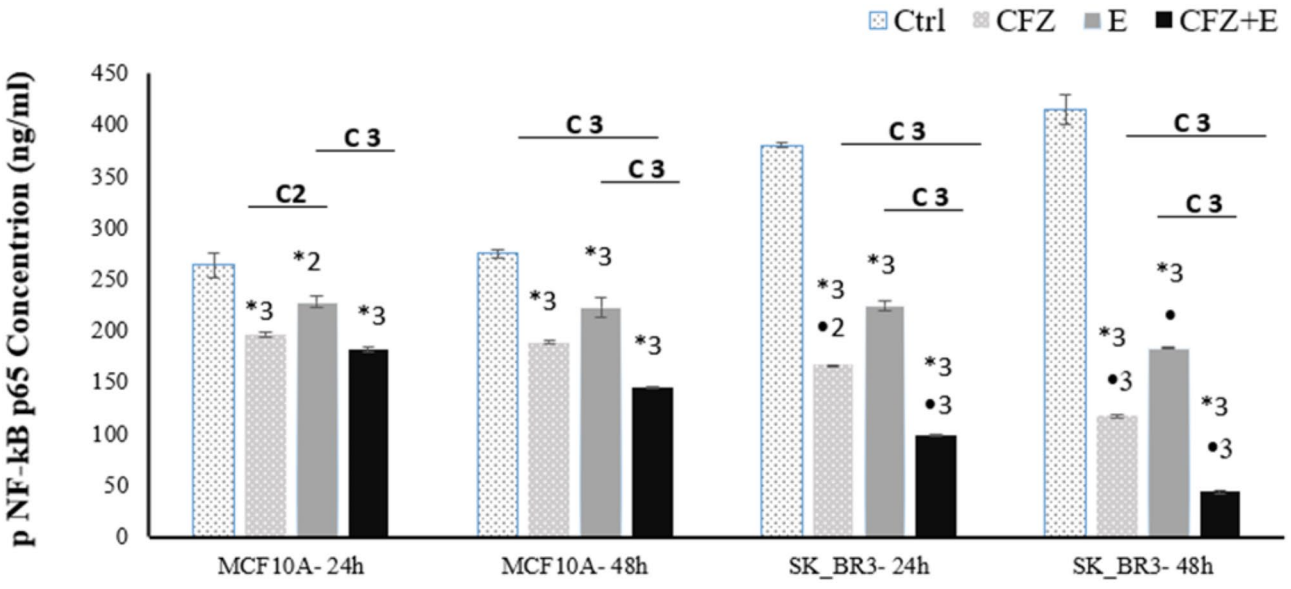

Fig. 10 The effects of pistachio hull extract (E), carfilzomib (CFZ), and their combination on NF-κB p65 transcription factor levels in SK-BR3 and MCF10A cell lines. Statistical significance is indicated as follows: $P < 0.05$ * for all treatment groups compared to the control group (Tukey HSD test); • $P < 0.05$ for tumor cell line compared to normal cell line (T-test); c $P < 0.05$ for comparisons among treatment groups (Tukey HSD test); ^{2} $P < 0.005$; and ^{3} $P < 0.001$

NF-κB activation through the prevention of IκBα ubiquitination [40–42]. Research conducted by Zanotto Filho et al. indicated that targeting the NF-κB pathway is a primary mechanism through which proteasome inhibitors induce apoptosis selectively in glioblastoma cells [43].

In another study, Li et al. examined the effects of curcumin, the active compound in turmeric, on NF-κB and its target genes-including interleukin 8 (IL-8) and cyclooxygenase 2 (COX-2)-in various pancreatic cancer cell lines. Their results revealed that inhibiting NF-κB, IL-8, and COX-2 activity led to reduced cell growth and increased apoptosis [44].

Regarding apoptosis, we observed a significant increase in *Caspase3* expression in SK-BR3 cells treated with the combination therapy. As an effector caspase, Caspase3 plays a crucial role in executing cell death by cleaving proteins vital for maintaining cellular integrity [8]. The elevated levels of *Caspase3* suggest that the combination treatment may enhance apoptosis in SK-BR3 cells by activating the caspase pathway. This finding aligns with studies indicating that proteasome inhibition can facilitate apoptotic cell death through the upregulation of *Caspase3* [45].

Furthermore, this highlights the interplay between the NF-κB and caspase pathways; the inhibition of NF-κB by CFZ and pistachio hull extract likely sensitizes cancer cells to apoptosis. By concurrently downregulating NF-κB and drug resistance markers such as MDR1 and MRP1 while upregulating Caspase-3, this combined treatment disrupts both survival and resistance mechanisms. As a result, it enhances the vulnerability of cancer cells to therapy-induced apoptosis [46].

Our study highlights the promising therapeutic potential of combining pistachio hull extract with CFZ to influence various pathways involved in cancer cell survival and drug resistance. Rich in bioactive compounds, pistachio hull extract enhances the action of CFZ on the proteasome, targeting key players such as MDR1, MRP1, NF-κB, and Caspase-3. The significant reduction in MDR1 and MRP1 gene expression indicates a marked decrease in the cells' ability to expel drugs, which could potentially lower chemoresistance and improve treatment efficacy [47, 48].

Furthermore, the downregulation of NF-κB p65 destabilizes mechanisms that promote cancer cell survival, making these cells more susceptible to apoptosis [49]. The increased expression of *Caspase3* further suggests a heightened apoptotic response [8]. Collectively, these findings support the idea that dual inhibition of drug resistance and survival pathways, alongside enhanced apoptosis, may offer a comprehensive strategy for more effectively targeting breast cancer cells.

Future research should delve into the intricate molecular interactions that contribute to this synergistic effect, particularly examining specific signaling pathways and post-translational modifications within the NF-κB and Caspase3 pathways. Additionally, exploring the application of this combination therapy in various cancer cell lines could shed light on its broader therapeutic potential. This multifaceted approach holds promise for developing more effective targeted therapies for breast cancer, aimed at minimizing resistance and enhancing apoptotic outcomes, ultimately improving clinical results for patients facing resistant cancer types.

However, it is important to note that this study is limited by its focus on only two cell lines, SK-BR3 and MCF10A, which may not fully capture the heterogeneity of breast cancer. Moreover, the in vitro nature of our experiments may not entirely reflect the complexities of in vivo tumor environments and systemic responses, which could impact the translational applicability of our findings. Further studies are essential to validate these results in clinical settings and to investigate the effects on other cancer types.

Conclusion

This study highlights the potential anticancer effects of pistachio hull extract in combination with CFZ. The findings indicate that this combination can modulate the expression of genes associated with drug resistance (*MDR1* and *MRP1*), as well as *NF-κB p65* and *Caspase3*. These results suggest a promising new approach for breast cancer treatment.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12906-024-04716-7.

Supplementary Material 1

Acknowledgements

Thanks to guidance and advice from the "Kerman University of Medical Sciences". BMC Complementary Medicine and TherapiesDear EditorWe would like to request your consideration for a full waiver of publication for our manuscript entitled as "Combination Effects of Pistachio Hull and Carfilzomib on NF-κB p65, MDR1, MRP1, and Caspase3 Gene Expression in Breast Cancer Cell Line". Please note that this request is due to several reasons that make us incapable of paying the fees. The authors not received any financially support for publication from university or other resources. Also, note that the authors of this manuscript are college students and government employees and they receive no extra found to pay the article processing charges. Therefore, your kind attention would be highly appreciated and it will contribute to us in whole. Also, recently Iran involved in economic crisis and regarding to the currency devaluation against US dollar in Iran "the rial has been on a downward spiral, falling to an all-time low against the US dollar" we can't afford the APC for this manuscript. Therefore, your kind attention would be highly appreciated and it will contribute to us in whole.Sincerely Mehdi Mahmoodi, Professor of Clinical Biochemistry, Dept. of Clinical Biochemistry, Afzalipoor Faculty of Medicine, Kerman University of Medical Sciences, Kerman, IranPostal Code: 7616914115 E-mail: mahmoodies@yahoo.com Tel: +98 34 33257319 Fax: +98 34 33257671.

Author contributions
ZM, SZ and MM were responsible for study concept and design. SZ, RH and MNK led data collection. ZM, SZ and MM were responsible for the analysis and interpretation of data. ZM wrote the first draft. SZ to the writing of the second and third draft. AS, RH, MNK, and MM provided comments on initial drafts and coordinated the final draft. All authors read and approved the final manuscript. All authors take responsibility for the integrity of the data and the accuracy of the data analysis.

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Data availability
The data used in this study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki and Institutional Review Board approval (code: IR.KMU.AH.REC.1400.164) has been obtained.

Consent for publication
By submitting this document, the authors declare their consent for the final accepted version of the manuscript to be considered for publication.

Competing interests
The authors declare no competing interests.

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