Anti-Cancer Characteristics of the pistachio pericarp extract in Combination with carfilzomib in SK-BR3 and MCF10A cell lines

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Abstract

Background: *Pistacia vera* is a potential source of bioactive components, particularly polyphenols known for their strong antioxidant properties. This study investigated whether pistachio pericarp extract could enhance the efficacy of carfilzomib (CFZ) while reducing its ototoxicity. We assessed the effects of carfilzomib and pistachio pericarp extract on cell viability in the breast cancer cell lines SK-BR3 and MCF10A.

Methods: SK-BR3 and MCF10A cells were cultured in RPMI1640 medium with pistachio pericarp extract (156.25 to 10,000 µg/mL) and CFZ (0.032 to 50 µg/mL) for 24 and 48 hours. The MTT assay evaluated the inhibitory effects of pistachio pericarp extract and CFZ, individually and in combination, on the proliferation of SK-BR3 and MCF10A cells.

Results: The ED50 values of pistachio pericarp extract were 0.228 mg/mL and 0.04 mg/mL for SK-BR3 cells and 0.642 mg/mL and 0.387 mg/mL for MCF10A cells at 24 and 48 hours, respectively. Carfilzomib showed ED50 values of 0.011 µg/mL and 0.00008 µg/mL for SK-BR3 cells, and 0.896 µg/mL and 0.273 µg/mL for MCF10A cells at the same time points.

Conclusion: The results demonstrated a synergistic interaction between pistachio pericarp extract and CFZ in both cell lines at both time points, indicating that the combination therapy was more effective than either treatment alone and may serve as potential molecular targets for various cancers. Future research is recommended to validate the effects in both in vitro and *in vivo*.

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1. Introduction

Female breast cancer has now overtaken lung cancer as the most frequently diagnosed cancer, with approximately 2.3 million new cases, accounting for 11.7% of diagnoses. Lung cancer follows in prevalence (11.4%) [1]. Numerous reports have emerged highlighting the development of acquired resistance to approved medications used in the treatment of breast cancer [2]. In 2008, breast cancer represented 14% of all cancer-related deaths and 23% of newly diagnosed cancer cases worldwide. It is the most common type of cancer and the leading cause of cancer mortality among women. [3, 4]. The precise origins of breast cancer remain largely unclear, even with the notable progress made in targeted therapies and early diagnostic techniques. [5]. There is an urgent necessity for research to uncover the molecular mechanisms that drive the onset and progression of breast cancer [6]. The US FDA has granted approval for CFZ as a treatment option for patients with multiple myeloma who have not responded to bortezomib, the first-generation therapy [7]. Research has shown that CFZ effectively inhibits NF-κB activation and prevents IκB degradation. Additionally, studies on CFZ's impact on a tamoxifen-resistant cell line indicate that this medication exhibits a more potent anti-proliferative effect on breast cancer (BC) cells than fluostrant [8]. A study indicates that CFZ reduces drug resistance in ovarian cancer cell lines A2780S and CisR2780A. However, in patients with advanced solid tumors, CFZ demonstrated limited effectiveness, despite the therapeutic progress achieved in the treatment of multiple myeloma [8, 9]. Studies that investigated CFZ on multiple myeloma cell lines (103), large B cell lymphoma, (104), and CLL (leukemia) (105) stated that CFZ affects Bax, Bak, and also through the Noxa pathway. Caspase 3 activation is crucial in inducing apoptosis and decreasing drug resistance. In line with the current study, there was a notable increase in p53 gene expression in both the SK-BR3 breast cancer cell line and the MCF10A normal cell line compared to the control group. Additionally, the SK-BR3 line exhibited a time-dependent increase in p53 expression relative to the MCF10A line and the combined group. Furthermore, the expression of the Caspase 3 gene was significantly elevated in the SK-BR3 breast cancer cell line compared to both the control group and the normal MCF10A line.

A part of the ineffectiveness may stem from the distinct molecular and cellular characteristics of solid cancer cells compared to those of multiple myeloma (MM) cells [10], as well as the short circulation duration and poor metabolic stability of CFZ in vivo, which restrict the availability of active medication in solid tumor tissues [11, 12]. Because proteasomes are widely distributed in all cell types, adverse effects resulting from on-target suppression in non-malignant cells make it impossible to resolve these problems by simply increasing CFZ doses [11, 12].

Herbal remedies are frequently utilized in contemporary medicine to mitigate the side effects associated with various illnesses. Beyond aiding in disease management, these natural treatments can also reduce the risk of adverse drug reactions linked to pharmaceutical prescriptions [13]. For many years, medicinal herbs have been utilized to address various illnesses. Recently, researchers have turned their attention to pistachio hulls due to their natural antioxidant and phenolic compounds. Recent studies have demonstrated that extracts from pistachio husks exhibit antibacterial, antioxidant, and antimutagenic properties.

Numerous reports have validated the pharmacological activity and therapeutic benefits of pistachio hulls [14-16]. A report by Tomaino revealed that polyphenol extracts from naturally shelled pistachios (NP) exhibit notable antioxidant activity. In the study, rats treated with NP demonstrated significant reductions in CAR-induced histological paw damage, nitrotyrosine production, and neutrophil infiltration. These results indicate that the polyphenols possess antioxidant properties even at lower concentrations [14]. Limited research has been conducted on the impact of pistachio on the SK-BR3 breast cancer cell line and the MCF10A normal breast cell line. Consequently, this study aims to explore the anti-tumor effects of pistachio skin extract, particularly in conjunction with CP, in the treatment of breast cancer, as evaluated through the MTT assay.

2. Material and methods

Cell Lines

The SK-BR3 breast cancer cell line and the MCF10A normal cell line, both sourced from the Tehran Pasteur Institute, were utilized in this study. These cell lines display epithelial morphology and adhere to the flask's bottom during culture. SK-BR3 cells were cultured in RPMI 1640 medium, while MCF10A cells were grown in DMEM..

Cell Culture

The culture media utilized included RPMI 1640 for SK-BR3 cells and DMEM for MCF10A cells. Both media were supplemented with $NaHCO_3$, glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml). Additionally, 10% Fetal Bovine Serum (FBS) was incorporated, which had been heat-inactivated at 56°C for 30 minutes.

Cell Counting

Cells were counted using a hemocytometer (Neubauer slide) along with a 0.4% trypan blue solution prepared in PBS. The procedure included the following steps: First, a suspension of trypsinized cells was mixed with trypan blue in a 1:1 ratio. Next, 20 µL of this mixture was placed into the hemocytometer. The sample was then incubated for 2 minutes to facilitate staining. Finally, both viable (unstained) and dead (stained) cells were counted, and the percentage of viable cells was calculated using:

100 x (total cells/number of living cells) = percentage of living cells

Determining the cell concentration using:

The average number of cells counted in the four outer squares × dilution factor × 10^4 = number of cells

Extraction of Pistachio Pericarp

The dried skins of the Ohady variety of pistachio (P. vera) were ground into a fine powder using an electric mill. A quantity of five grams of this powder was placed in a filter paper cylinder and subjected to extraction with 70% ethanol in a Soxhlet apparatus for a duration of 100 minutes. The resulting extract was then concentrated and freeze-dried for 72 hours, after which it was redissolved in distilled water and stored at a temperature of -20°C..

MTT Assay for Single Drugs

Cells were trypsinized, counted, and seeded at a density of 10,000 cells per well in a 96-well plate, each containing 200 µL of culture medium. After 24 hours to facilitate attachment and growth, the medium was replaced with a formulation containing varying concentrations of pistachio extract (ranging from 156.25 to 10,000 µg/mL) and CFZ (from 32 x 10^-4 to 50 µg/mL). Following incubation for either 24 or 48 hours, 35 µL of MTT solution (5 mg/mL in PBS) was introduced to each well and incubated for an additional 4 hours. Subsequently, 165 µL of DMSO was added to dissolve the formazan

crystals, and the absorbance was measured at 570 nm. Cell viability was then calculated as:

100 x (average uptake of control cells/average uptake of treated cells) = percentage of live cells

Combination Therapy Analysis

The combination therapy was evaluated through the MTT assay for both cell lines at 24 and 48 hours. Various doses of pistachio extract and CFZ were administered individually and in combination. Cell viability data were processed using CompuSyn software (Version 1.0, Combo-Syn Inc., US), and the Combination Index (CI) was determined following the Chou-Talalay method. The CI serves to assess the interaction between the two treatments, categorizing it as synergistic, additive, or antagonistic.

For CI Calculation: The CI is computed for the effective dose that impacts 50% of the cells (ED50). A CI greater than 1 (Log CI > 0) indicates antagonism between the treatments, a CI equal to 1 (Log CI = 0) signifies an additive effect (no interaction), and a CI less than 1 (Log CI < 0) suggests synergy between the treatments. The CI is calculated using the formula:

$CI = (D)1/(Dx)1 + (D)2/(Dx)2.$

In this relation, D1 and D2 are the concentration of each of the factors alone, and (Dx)1 and (Dx)2 represent their concentration in a combined state [17].

Statistical Analysis

Data were analyzed using T-test, one-way ANOVA, and Tukey's post-hoc test. Results are presented as Mean ± SD, with a significance level of ($P < 0.05$). SPSS version 18 was used for the analysis.

Ethical Approval

The study was conducted by the Declaration of Helsinki and received approval from the Institutional Review Board (code: IR.KMU.AH.REC.1400.164).

3. Results

The results of investigating the toxicity of carfilzomib and pistachio pericarp extract alone

Carfilzomib and pistachio pericarp extract were tested in combination at four concentrations below their IC50, at their IC50, and at two concentrations above their IC50, as illustrated in Diagram 1. These combinations, along with each drug administered individually, were applied to SK-BR3 and MCF10A cell lines. Following incubation periods of 24 and 48 hours, the outcomes were evaluated using the MTT assay. The ED50 values for pistachio pericarp extract were determined to be 0.228 mg/mL and 0.04 mg/mL for SK-BR3 cells, and 0.642 mg/mL and 0.387 mg/mL for MCF10A cells at the 24 and 48-hour marks, respectively. In contrast, Carfilzomib demonstrated ED50 values of 0.011 µg/mL and 0.00008 µg/mL for SK-BR3 cells and 0.896 µg/mL and 0.273 µg/mL for MCF10A cells at the same time intervals.

Dose-response curve resulting from the therapeutic combination of Extract (E)(mg/ml) and CFZ (µg/ml) in SK-BR3 and MCF10A cell lines at 24 and 48 hours after treatment. Results are presented as mean ± SD compared to control.

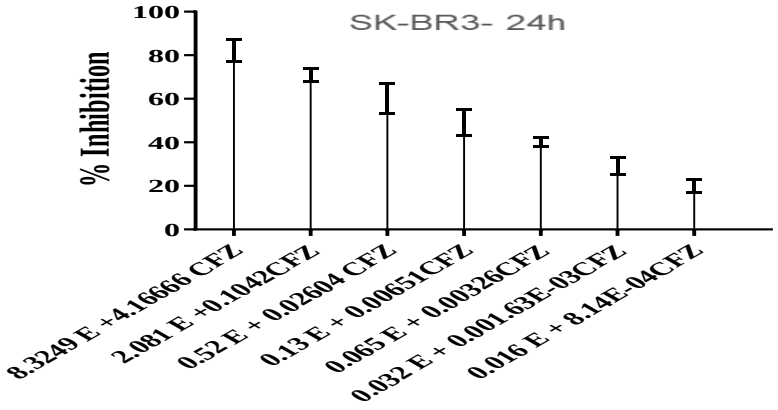

Fig. 1. Drug and pistachio peel extract (four doses lower than the IC50 dose of both drugs, the IC50 dose of both drugs, and two doses higher than the IC50 dose of both drugs are combined in the concentration ranges mentioned in the diagram, and the form of a single drug was exposed to two cells and after 24 hours of incubation, the results of the therapeutic combination were analyzed by MTT test and shown in the Dose-Response diagram, pistachio peel extract at a concentration of 0.228 (mg/ml) and carfilzomib drug. At a concentration of 0.011 (µg/ml) in the SK-BR3 cell line, it was able to inhibit the growth up to 50%.
E= Pistachio skin extract, CFZ= Carfilzomin

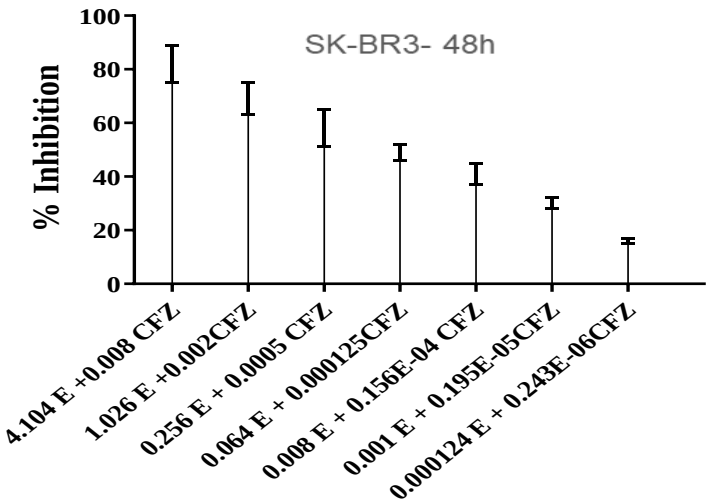

Fig. 2. Carfilzomib drug and pistachio peel extract (four doses lower than the IC50 dose of both drugs, the IC50 dose of both drugs and two doses higher than the IC50 dose of both drugs is combined in the concentration ranges mentioned in the diagram, and the form of a single The drug were exposed to two cells and after 48 hours of incubation, the results of the therapeutic combination were analyzed by MTT test and shown in the Dose-Response diagram, pistachio peel extract at a concentration of 0.04 (mg/ml) and carfilzomib drug. At a concentration of 0.00008 (µg/ml) in the SK-BR3 cell line, it was able to inhibit the growth up to 50%.
E= Pistachio skin extract, CFZ= Carfilzomin

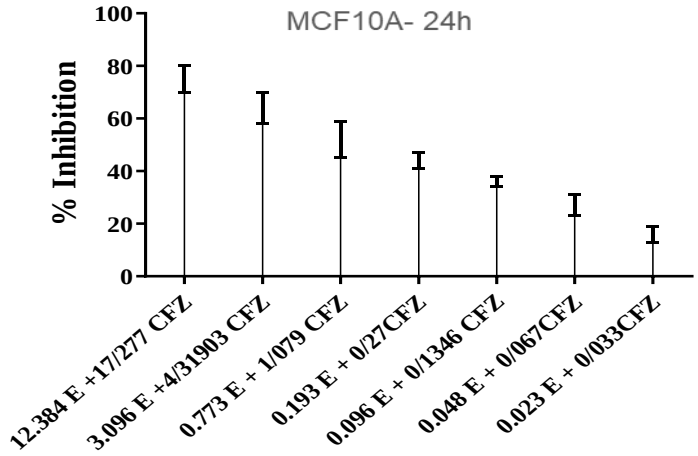

Fig. 4. Carfilzomib drug and pistachio peel extract (four doses lower than the IC50 dose of both drugs, the IC50 dose of both drugs, and two doses higher than the IC50 dose of both drugs are combined in the concentration ranges mentioned in the diagram, and the form of a single The drug was exposed to two cells and after 24 hours of incubation, the results of the therapeutic combination were analyzed by the MTT test and shown in the Dose-Response diagram, pistachio peel extract at a concentration of 0.642 (mg/ml) and carfilzomib drug. At a concentration of 0.896 (µg/ml) in the MCF10A cell line, it was able to inhibit growth by 50%.
E= Pistachio skin extract, CFZ= Carfilzomin

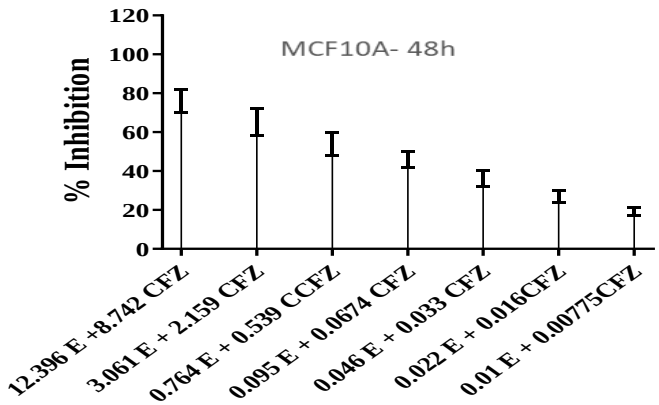

Fig. 4. Carfilzomib drug and pistachio peel extract (four doses lower than the IC50 dose of both drugs, the IC50 dose of both drugs, and two doses higher than the IC50 dose of both drugs are combined in the concentration ranges mentioned in the diagram, and the form of a single The drug was exposed to two cells and after 48 hours of incubation, the results of the therapeutic combination were analyzed by the MTT test and shown in the Dose-Response diagram, pistachio peel extract at a concentration of 0.387 (mg/ml) and carfilzomib drug. At a concentration of 0.273 (µg/ml) in the MCF10A cell line, it was able to inhibit the growth up to 50%.
E= Pistachio skin extract, CFZ= Carfilzomin

The therapeutic combination's effectiveness was evaluated by calculating the Combination Index (CI) for the effective dose affecting 50% of the cells (ED50, the concentration of a drug that achieves a 50% reduction in cell viability. It is a standard measure used to assess the potency of a therapeutic agent) [18].

The CI values for SK-BR3 and MCF10A cells were assessed after 24 and 48 hours of treatment with pistachio pericarp extract and CFZ. For SK-BR3 cells, the CI values were recorded as 0.102 at 24 hours and 0.022 at 48 hours. In contrast, MCF10A cells exhibited CI values of 0.261 at 24 hours and 0.165 at 48 hours. The interpretation of the CI values is as follows: a CI greater than 1 (Log CI > 0) indicates antagonism, a CI equal to 1 (Log CI = 0) signifies an additive effect, and a CI less than 1 (Log CI < 0) suggests synergy. The findings demonstrated a synergistic interaction between the pistachio pericarp extract and CFZ in both cell lines at both time points, indicating that the combination therapy was more effective than either treatment administered alone.

Additionally, cell viability was measured using the MTT assay, and the IC50 values were reported in Table 1, further supporting the synergy observed in the combination therapy.

Table 1. Investigating the effect of IC50 values of the hydroalcoholic extract of pistachio pericarp and CFZ against SK-BR3 and MCF10A cell lines in 24 h and 48 h after treatment

The Hydroalcoholic	IC50, 24h	IC50, 48h
E (SK-BR3)	2.014(µg/µl)	1.031(µg/µl)
CFZ (SK-BR3)	0.181 (µg/µl)	0.0057 (µg/µl)
E (MCF10A)	2.994 (µg/µl)	5.624 (µg/µl)
CFZ (MCF10A)	5.54 (µg/µl)	2.51 (µg/µl)

E= Pistachio Pericarp Extract, CFZ= Carfilzomib

4. Discussion

Herbal remedies are gaining popularity in contemporary medicine as a means to alleviate the side effects linked to numerous health conditions. Beyond managing diseases, these natural treatments may also lower the risk of adverse reactions caused by pharmaceutical drugs [13]. This study, similar to Ashley's, investigated the combined effects of carfilzomib (CFZ) on cell lines. Both studies identified a synergistic effect when CFZ was combined with doxorubicin (Dox) [17]. The efficacy of combination therapy in this study demonstrated synergistic effects in the effective dose (ED50). The combination index (CI) values for the SK-BR3 cell line were 0.102 and 0.0221, while for the MCF10A line, they were 0.261 and 0.165 at 24 and 48 hours, respectively. These findings align with the principles of drug combination analysis as outlined by the Chou-Talalay method. Notably, the SK-BR3 cell line exhibited greater efficacy, suggesting that proteasome inhibitor medications, such as CFZ, play a crucial role in inhibiting cancer cell proliferation. This is achieved by preventing the degradation of damaged proteins, which leads to intracellular accumulation and heightened proteotoxic stress, accompanied by an increase in free radicals [11, 12]. This study corroborated

the synergistic effects of CFZ observed in Ashley's study. Ashley et al. reported a synergistic interaction between CFZ and Dox over 24 hours (CI: 0.981) [17]. This investigation, akin to Ashley's methodology, concurrently administered two drugs to cell lines, leading to a synergistic effect in both. A time-dependent decrease was observed in the breast cancer cell line SK-BR3 and the normal line MCF10A within the combination group when compared to the control group. Additionally, another study examining the influence of curcumin on CFZ efficacy revealed that treatment with CFZ-curcumin compounds significantly diminished nuclear NF-κB accumulation [18]. Preclinical research suggests that CFZ plays a crucial role in independently inhibiting NF-κB activation in both hematological cancers and specific solid tumors, such as head and neck cancer, by delaying the ubiquitination of IκBα. Additionally, Zanotto Filho and colleagues have shown that the proteasome inhibitor selectively induces apoptosis in glioblastoma cells through the inhibition of the NF-κB pathway [22]. Studies on CFZ's effects on multiple myeloma cell lines [23], large B cell lymphomas [24], and chronic lymphocytic leukemia (CLL) [25] suggest that CFZ affects Bax and Bak activation through the Noxa pathway, leading to apoptosis and reduced drug resistance. In a Phase 1 study of 18 patients with relapsed/refractory multiple myeloma (RRMM) refractory to carfilzomib, 75% of the 16 evaluable patients showed at least a minimal response when treated with carfilzomib combinations. Combining SKd with carfilzomib-refractory myeloma, particularly in well-pretreated patients, demonstrated favorable efficacy and safety [26, 27]. The results are encouraging, particularly for patients in areas where the combination of carfilzomib and alkylating agents may be advantageous for those newly diagnosed with myeloma, especially in cases where immunomodulatory drugs (ImiDs) are infrequently utilized. Regarding quality of life, safety, and tolerability, carfilzomib combination therapy is generally well-received by patients with relapsed/refractory multiple myeloma (RRMM). The MTT assay indicated that 24 and 48 hours post-treatment of the SK-BR3 cell line with pistachio hull extract resulted in IC50 values of 2.014 and 1.031 mg/mL, respectively, while the MCF10A cell line showed values of 3.265 and 2.994 mg/mL. Additionally, carfilzomib was found to inhibit growth by up to 50% in the SK-BR3 cell line at dosages of 0.181 and 0.0057 µg/mL, and in the MCF10A cell line at 5.54 and 2.51 µg/mL. After 24 and 48 hours of treatment, the combination therapy demonstrated a synergistic effect between the two medications.

5. Conclusion

The study found that carfilzomib, a second-generation proteasome inhibitor, effectively acts as an anti-proliferative agent when combined with pistachio hull extract in both cell lines. Therefore, carfilzomib and pistachio hull extract may serve as potential molecular targets for various cancer treatments. Future research is needed to validate the effects of this combination in both in vitro and in vivo contexts.

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Conflict of Interest

None of the authors of the present study declared a conflict of interest.

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