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Research Article

Bergenin Synergistically Enhances the Efficacy of Cisplatin and 5-FU: Strategy to Enhance Chemotherapy in Breast and Colorectal Cancer - An *In Vitro* Study

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Abstract

Background: The cytotoxic and synergistic efficacy of bergenin, a natural compound, against MCF-7 breast cancer and HCT 116 colorectal cancer cell lines was investigated. Bergenin, combined with cisplatin (Cis) and 5-fluorouracil (5-FU), can enhance the efficacy of chemotherapy and be ultimately useful for dose reduction of chemotherapeutic agents.

Methods: The *in vitro* study involved treating MCF-7 and HCT 116 cells with varying doses of bergenin, Cis, and 5-FU for 24 and 48 hours. The cytotoxic effects were measured using MTT assay, while synergistic activity was evaluated using CompuSyn. Fluorescent staining, colony forming, wound healing, cell cycle and apoptosis assays were performed to support the findings. Statistical significance less than 0.05 was considered and assessed using ANOVA (one-way or two-way) and Tukey's test.

Results: Bergenin showed a dose- and time-dependent inhibition of cell proliferation in both MCF-7 and HCT 116 cells, at 24 and 48 hours, respectively. Combining bergenin with Cis or 5-FU led to significant reductions ($P<0.0001$) in their IC_{50} values. Multiple lines of evidence, including fluorescent staining, colony forming, wound healing, cell cycle and apoptosis assays, corroborated the statistically significant cytotoxic and synergistic effects of bergenin compared to control.

Conclusion: This study underscores bergenin's potential as a therapeutic agent in cancer treatment. Bergenin inhibits cell growth, induces apoptosis, and enhances the efficacy of Cis and 5-FU, suggesting its potential as an addition to existing treatment regimens. This necessitates further research, elucidating the clinical applications of bergenin in cancer therapy, which could improve treatment outcomes and reduce adverse effects associated with high doses of chemotherapeutic agents.

Introduction

Breast cancer, the most frequently diagnosed cancer among women globally, and colorectal cancer, which affects both men and women, pose significant public health challenges due to their widespread prevalence and profound impact on patient outcomes. Despite advancements in screening techniques and treatment modalities, breast cancer remains a leading cause of cancer-related morbidity and mortality among women worldwide.[1] Similarly, colorectal cancer, originating in the colon or rectum, ranks among the most prevalent and deadliest cancers worldwide.[1] The multifactorial nature of both breast cancer and colorectal cancer underscores the complexity of these diseases, involving genetic, environmental, and lifestyle factors.

Bergenin, a natural compound, exhibits strong antitumor effects across multiple cancer types, including cervical carcinoma, colorectal cancer, oral squamous cell carcinoma, bladder cancer, and non-small cell lung cancer. It effectively reduces cancer cell viability and colony formation and inhibits tumor growth in both *in vitro* and *in vivo* models by targeting critical signaling pathways and proteins. In 2022, Sultana et al[2] demonstrated that the aqueous extract of *E. agallocha* inhibits the growth of SiHa cervical cancer cells by inducing autophagy, apoptosis, mitophagy, and G2/M phase arrest, with bergenin identified as the key anti-cancer agent. In cervical carcinoma, bergenin specifically targets angiogenic proteins such as galectin-3 and MMP-9.[3] In colorectal cancer, it downregulates the Akt/GSK3β pathway and promotes Mcl-1 degradation.[4] In oral squamous cell carcinoma, bergenin upregulates PTEN and inhibits the AKT/HK2 axis, thereby suppressing glucose metabolism.[5] Moreover, bergenin activates the PPARγ/PTEN/Akt pathway in bladder cancer and

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reduces survivin expression in non-small cell lung cancer by inhibiting the Akt/Wee1/CDK1 pathway.[6] Through its ability to induce apoptosis, autophagy, mitophagy, and cell cycle arrest, bergenin emerges as a promising candidate for anticancer therapy across a wide range of malignancies.[7]

Although cisplatin (Cis) and 5-fluorouracil (5-FU) have demonstrated efficacy in combating breast and colorectal cancer respectively, their utility is often hindered by the occurrence of adverse side effects. Cis, a platinum-based chemotherapy drug widely used in various cancer types including breast and colorectal cancer, can induce severe adverse effects such as nephrotoxicity, neurotoxicity, gastrointestinal disturbances, and bone marrow suppression.[8] Similarly, 5-FU, a cornerstone in the treatment of colorectal cancer, is associated with gastrointestinal issues such as nausea, vomiting, diarrhea, mucositis, as well as myelosuppression, fatigue, and increased susceptibility to infections.[9] In response to these challenges, researchers are exploring innovative approaches to cancer treatment, including the investigation of adjunctive therapies. The study addresses a noted gap in the literature regarding the potential synergistic effects of combining the natural compound bergenin with standard chemotherapeutic agents, specifically Cis and 5-FU, for treating breast (MCF-7 cell line) and colorectal (HCT 116 cell line) cancer. Despite bergenin's known benefits, studies on its synergistic effects with chemotherapy are lacking. Therefore, the aim of this study is to enhance the effectiveness of Cis and 5-FU by utilizing bergenin, potentially allowing for lower dosages and a subsequent reduction in associated side effects. The underlying hypothesis is that combining bergenin with Cis or 5-FU will produce a synergistic effect. This synergy is expected to enhance the cytotoxic efficacy of the chemotherapy drugs, potentially allowing for reduced dosages, which could consequently minimize associated adverse side effects and improve treatment tolerability.

Methods

Cell culture maintenance and experimental groups

The breast cancer cell line MCF-7 and colorectal cancer cell line HCT 116 were obtained from National Centre for Cell Science (NCCS), Pune, Maharashtra, India. MCF-7 cells were grown in RPMI 1640, and HCT 116 cells in DMEM medium, both supplemented with 10% fetal bovine serum (FBS) and a 1 % pen/strep solution (100 units/mL penicillin and 100 µg/mL streptomycin). The cells were incubated with humidified 5% CO_2 at 37 °C. MCF-7 cells were subjected to 35 µM bergenin, 14 µM Cis, and a combination of 20 µM bergenin with 5 µM Cis for 24 hours. Likewise, HCT 116 cells were treated with 40 µM bergenin, 23 µM 5-FU, and a combination of 10 µM bergenin with 10 µM 5-FU for the same time period as shown in Table 1.

Table 1. Distinct groups, treatments and concentration based on analysis of combination study

Groups	Cell line	Treatment*	Concentration (µM)
I	MCF-7	Untreated	-
II		Bergenin	35 µM
III		Cis	14 µM
IV		Bergenin + Cis	20 µM + 5 µM
V	HCT 116	Untreated	-
VI		Bergenin	40 µM
VII		5-FU	23 µM
VIII		Bergenin + 5-FU	10 µM + 10 µM

* < 0.1% DMSO was maintained for all the experimental groups.

In vitro cytotoxicity assay using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The *in vitro* anticancer activity of bergenin was assessed using the MTT assay on MCF-7 and HCT 116 cell lines, as previously outlined by Mosmann.[10] MCF-7 and HCT 116 cells were seeded in 96-well plates (10^4 cells/well in 200 µL of media) and allowed to adhere for 24 hours. MCF-7 cells were treated with bergenin and Cis, and HCT 116 cells with bergenin and 5-FU, each at concentrations of 5-50 µM. After a 24- and 48-hours treatment with the test compounds, the culture medium was removed, and the cells were rinsed with PBS. Subsequently, 50 µL of MTT reagent (5 mg/mL in PBS) was added into each well and incubated for 4 hours at 37 °C in a humidified incubator with 5% CO_2, shielded from light, to facilitate the formation of formazan crystals. Afterward, MTT was removed, and 100 µL of DMSO was added to dissolve the formazan crystals. The plate was further incubated for 30 minutes, and the absorbance was measured at 570 nm using the BioTek Epoch Microplate Spectrophotometer (Agilent, USA). Cell viability (%) was calculated using the given formula:

$$\text{Cell viability (\%)} = \frac{A_t}{A_c} \times 100$$

Where, A_t is the absorbance of test samples and A_c is the absorbance of untreated control.

Analysis of combination and dose reduction index

The combination index (CI) is a graphical measure of drug interactions, indicating synergism, additivity, or antagonism based on whether the CI is less than, equal to, or greater than 1, respectively. For combination studies, bergenin was tested at concentrations of 10, 20, and 30 µM in combination with varying concentrations of Cis (2.5-20 µM) and 5-FU (5-40 µM) for MCF-7 and HCT 116 cell lines, respectively for 24 hours. Combination analysis was conducted using CompuSyn 1.0[11] based on Chou-Talalay method.[12] To observe the gradual decrease in cell viability among the combination groups, the percentage cell viability of different combinations was plotted against varying concentrations of Cis and 5-FU. Additionally, the CI was calculated at different combination concentrations

to identify specific doses that exhibit high synergism with a 50% effect. Normalized isobologram, a dose-oriented graph based on the CI equation at non-constant ratios of different combination groups was constructed. Finally, the dose reduction index (DRI) was calculated at 50% effect level for lowest CI. This dose-reduction defines the combination doses for each positive control (i.e., Cis and 5-FU) with bergenin.

Fluorescent staining

For examining induction of apoptosis using fluorescence staining, MCF-7 and HCT 116 cells were seeded in 12-well plates (1×10^5 cells/well in 1 mL of media) and allowed to adhere for 24 hours. Following exposure to test compounds for 24 hours, the culture medium was removed, and cells were washed with PBS before fixation with 4% paraformaldehyde for 10-12 minutes. The cells were treated with respective fluorescence stains, rinsed with PBS, and then examined using a ZOE fluorescent cell imager (Bio-Rad) at 20× magnification.

Dual (AO/EtBr) fluorescent staining is employed to detect cellular alterations associated with apoptosis and to distinguish cells at various stages of apoptosis.[13,14] The cells were stained with AO/EtBr (100 µg/mL) solution for 10 minutes, followed by another PBS wash.

Hoechst 33342 effectively labelled the nuclei of both viable and non-viable cells, while propidium iodide (PI) specifically labelled the nuclei of non-viable cells.[15] The cells were stained with PI (10 µg/mL) solution for 10 minutes, followed by a PBS wash and then stained with Hoechst 33342 (10 µg/mL) solution for 10 minutes.

DAPI, a fluorescent dye that specifically binds to DNA, is commonly used to evaluate DNA fragmentation during apoptosis. Staining was conducted based on the method outlined by Atale et al[16] using a 25 µg/mL DAPI solution for 10 minutes.

Colony forming assay

MCF-7 and HCT 116 cells were seeded at a density of 3×10^5 cells per well in 6-well plates, allowed to adhere for 24 hours, and then treated with the test compounds for an additional 24 hours. After the treatment duration, cells were trypsinized and 1×10^3 cells were seeded into each well and maintained in a 5% CO_2 environment at 37 °C for two weeks, with media refreshed every 72 hours. Following this, colonies were immobilized using 500 µL/well chilled aceto-methanol solution (1:3 ratio) for 20-30 minutes and then stained with 0.5% crystal violet. Subsequent to capturing images, analysis was conducted using ImageJ software (version 1.54f). The survival fraction (percentage of colonies) was calculated as per the Kabakov et al[17] using the given formula:

$$\text{Colonies or Survival fraction (\%)} = \frac{C_t}{C_c} \times 100$$

Where, C_t is the number of colonies formed in treatment groups, C_c is the number of colonies formed in untreated control group.

Wound healing assay

To explore the effect of test compounds on cancer cell migration, the wound healing assay, also known as the scratch assay[18] was performed. MCF-7 and HCT 116 cells were seeded in 6-well plates at a density of 3×10^5 cells/well. Upon reaching 80% confluence, a gentle scratch was made across the center of each well using a sterile 200 µL pipette tip. Following the removal of detached cells by washing with PBS, the cells underwent treatment with various test compounds for 24, 48, and 72 hours. Throughout the exposure period, the cells were cultured in medium supplemented with 5% serum. The closure of the wound was monitored at 24-hours intervals from 0 to 72 hours. Photographs were captured using a Zeiss Primovert inverted microscope at 10× magnification, and the images were analyzed using ImageJ software (version 1.54f).

Cell cycle analysis

The cell cycle was evaluated using a DNA-based flow cytometry method, following the protocol outlined by Chung et al.[19] MCF-7 and HCT 116 cells were cultured in 6-well plates at a density of 3×10^5 cells/well and allowed to adhere for 24 hours. Following this, cells were treated with test compounds for an additional 24 hours. Cell harvesting was conducted by incubating them with 300 µL of hypotonic staining solution, comprising 100 mg sodium citrate, 30 µL Triton-X 100, 5 mg PI, and 0.002 mg Ribonuclease A in 100 mL distilled water, for 15 minutes. Analysis of the samples was conducted using the BD FACSAria™ Fusion Flow Cytometer (BD Biosciences, USA). Data analysis and results were obtained using FCS Express™ 7 (Dotmatics, USA).

Cell apoptosis analysis

Early apoptosis involves various morphological and biochemical changes, such as membrane blebbing and the externalization of phosphatidylserine (PS) on the cell surface, which can be detected through Annexin V binding. Through the application of flow cytometric analysis and Annexin V coupled with FITC, apoptotic cells can be effectively identified. Apoptosis in cells was evaluated using the BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I, following the method described by Jin et al., with slight modifications.[20] MCF-7 and HCT 116 cells were seeded in 6-well plates at a density of 3×10^5 cells/well and treated with different concentrations of the test compounds for 24 hours. After washing with cold PBS, cells were resuspended in 1× binding buffer, and a mix of 5 µL of FITC Annexin V and 5 µL of PI was added. The mixture was then incubated for 15 mins at room temperature in the dark before analysis using the BD FACSAria™ Fusion Flow Cytometer (BD Biosciences, USA). Data analysis and results were obtained using Floreada (https://floreada.io, accessed 26th March 2024).

Statistical analysis

All experiments were conducted separately in triplicate and are expressed as mean ± SD. The statistical significance was evaluated using one-way or two-way analysis of variance (ANOVA), and the individual comparison was obtained by Tukey's multiple comparison test using GraphPad Prism (version 9.4.0). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

In vitro cytotoxicity assay using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The results revealed a dose- and time-dependent inhibition of cell growth in both the MCF-7 and HCT 116 cell line, as depicted in Figure 1. In MCF-7 cells, bergenin demonstrated an IC_{50} of 34.16 ± 0.57 µM and 20.60 ± 0.60 µM after 24 and 48 hours, respectively as shown in Table 2. Comparatively, the positive control Cis exhibited an IC_{50} of 14.43 ± 0.37 µM and 7.86 ± 0.74 µM at 24 and 48 hours. In the case of HCT 116 cells, bergenin displayed an IC_{50} of 40.93 ± 0.40 µM and 25.50 ± 0.69 µM after 24 and 48 hours, while the positive control 5-FU demonstrated an IC_{50} of 22.87 ± 0.46 µM and 15.04 ± 0.98 µM at 24 and 48 hours, respectively (Table 2). These findings underscore the cytotoxic efficacy of bergenin against both breast and colorectal cancer cell lines.

Analysis of combination and dose reduction index

The combination strategy involving bergenin along with the drug molecules used in the treatment of breast and colon cancer i.e., Cis and 5-FU respectively (positive controls) demonstrated a synergistic effect in inhibiting the growth and proliferation of both MCF-7 and HCT 116 cells (Supplementary file 1, Figures S1 and S2). After evaluation of CI, it was observed that among the 15 data points representing various combinations of bergenin with Cis, two of them exhibited antagonism (CI > 1), two were nearly additive, while the rest demonstrated synergism (CI < 1). Conversely, when combined with 5-FU, only one data point indicated antagonism, with all other points displaying synergistic effects Figure S3 (Supplementary file 1). Notably, both bergenin + Cis and bergenin + 5-FU combinations exhibited synergism at a 50% effect level. These groups revealed the dose dependent inhibitory effects.

Analysis through the DRI revealed significant reductions in the IC_{50} values for the combination groups. DRI > 1 indicates favorable and DRI < 1 indicates unfavorable dose-reduction. For the combination of bergenin with Cis, the DRI indicated a substantial decrease in IC_{50} values. Specifically, the combination of 10, 20, and 30 µM bergenin with Cis resulted in dose reductions of 1.45, 4.01, and 19.13-fold ($P < 0.0001$), respectively, while the combination of 10, 20, and 30 µM bergenin with 5-FU resulted in dose reductions of 1.93, 3.05 and 22.29-fold ($P < 0.0001$) as highlighted in Table 3. This suggests a synergistic enhancement of cytotoxic effects when bergenin is combined with Cis and 5-FU, emphasizing the potential for dose optimization in cancer treatment.

The normalized isobologram in Figure S4 (Supplementary file 1) illustrates the interaction of bergenin combined with Cis and 5-FU at non-constant ratios. This analysis maps out various points, representing different combination groups, which exhibit antagonistic, additive, or synergistic effects. Isobolograms are typically used to visualize drug interactions, where points below the line indicate synergy, points on the line suggest additivity, and those above the line indicate antagonism. This isobologram reveals that most of the combination groups fall below the line, showing a predominantly synergistic interaction of bergenin in combination with Cis and 5-FU in both MCF-7 and HCT 116 cell lines, respectively. Combination groups as shown in Table 4, were finalized based on the lowest Combination index at 50% inhibition effect. Based on the CI value, it can be inferred that both groups show synergistic behavior in reducing cell viability and proliferation.

Fluorescent staining

Dual AO/EtBr (acridine orange/ethidium bromide) staining

Acridine orange has the ability to permeate normal cell

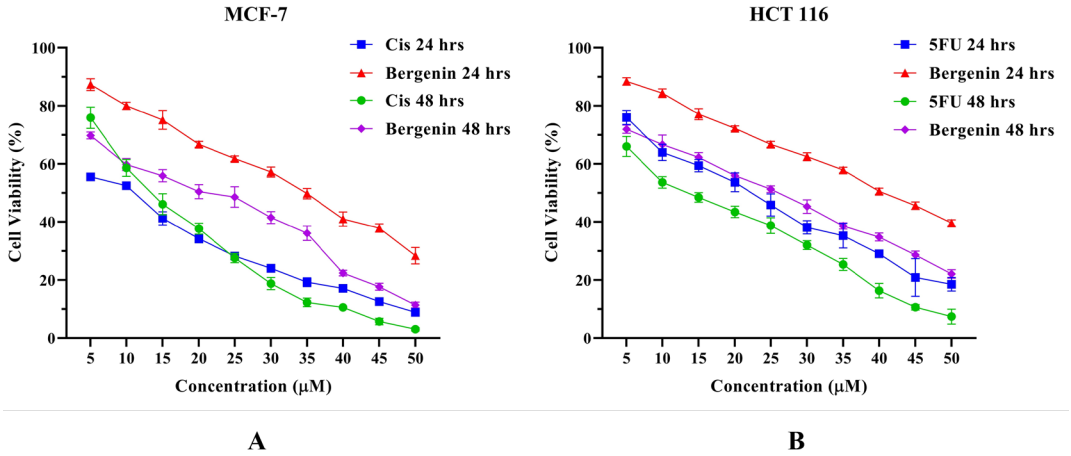

Figure 1. Cell viability (%) of (A) MCF-7 cells and (B) HCT 116 cells treated with Bergenin compared to positive control Cis and 5-FU, respectively, at different concentrations for 24 and 48 hrs. Values are expressed in mean ± SD

Table 2. IC_{50} of bergenin for MCF-7 and HCT 116 cells compared to positive control Cis and 5-FU, respectively for 24 and 48 hours

IC_{50} in µM	MCF-7		HCT 116	
	24 hours	48 hours	24 hours	48 hours
Bergenin	34.16 ± 0.57	20.60 ± 0.60	40.93 ± 0.40	25.50 ± 0.69
Cis	14.43 ± 0.37	7.86 ± 0.74	-	-
5-FU	-	-	22.87 ± 0.46	15.04 ± 0.98

Values are expressed as mean ± SD.

membranes. In the absence of any treatment, cells exhibit a bright green fluorescence, highlighting a well-organized structure. Conversely, early apoptotic cells display a yellow-orange hue, while late apoptotic cells, marked by nuclear shrinkage, damage, and blebbing, exhibit an orange-red color. Cells with red-colored nuclei at higher concentrations suggest late-stage apoptosis. Untreated cells, maintain their healthy appearance with a vibrant green fluorescence. Compared to the untreated control, the 24 hour treated groups (bergenin and Cis alone as well as in combination) exhibited distinctive apoptotic features such as blebbing, chromatin fragmentation, condensed chromatin, and nuclear shrinkage (Figure 2). Untreated HCT 116 cells when compared with treated groups (bergenin and 5-FU alone as well as in combination), also showed similar alterations after 24 hours treatment as highlighted in Figure 3.

Dual propidium iodide/Hoechst 33342 staining

The Hoechst 33342 dye, which emits blue fluorescence, serves as a cell-permeable nucleic acid stain commonly used for identifying chromatin condensation and fragmentation. It specifically stains the fragmented nuclei of cells undergoing apoptosis. Conversely, the pink fluorescent PI functions as a DNA-binding dye that selectively enters cells with compromised plasma membrane integrity, indicating instances of increased permeability. Treatment-induced apoptosis in MCF-7 and HCT 116 cells were characterized by morphological features of apoptosis, including chromatin condensation and nuclear fragmentation (marked by arrows in Figures 2 and 3).

DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) staining

Untreated MCF-7 and HCT 116 cells showed faint, homogenous blue fluorescent nuclei, suggesting intact nuclear structure. The signs of apoptosis were then observed when MCF-7 cells treated bergenin and Cis alone as well as in combination for 24 hours, the blue emission was much brighter than in the unaffected cells, indicating features of apoptosis like chromatin fragmentation and condensation (Figure 2). Moreover, as shown in Figure 3, the treated HCT 116 cells (bergenin and 5-FU alone and their combination) displayed nuclear fragmentation, confirming the induction of apoptosis compared to untreated cells.

Table 3. IC_{50} of positive control Cis and 5-FU alone and in combination with bergenin (10, 20 and 30 µM) and Drug reduction indices of combination groups

Combination group	IC_{50} in uM	DRI (Dose reduction index)
Cis alone	15.38 ± 0.31	
10 µM Bergenin + Cis	10.64 ± 0.17****	1.45
20 µM Bergenin + Cis	3.84 ± 0.29****	4.01
30 µM Bergenin + Cis	0.80 ± 0.06****	19.13
5-FU alone	25.17 ± 0.01	
10 µM Bergenin + 5-FU	13.02 ± 0.41****	1.93
20 µM Bergenin + 5-FU	8.23 ± 0.97****	3.05
30 µM Bergenin + 5-FU	1.13 ± 0.22****	22.29

Results are expressed as mean ± SD. Statistical comparisons were conducted to assess the significance of differences in IC_{50} values between single-agent (Cis or 5-FU) and combination treatments. A highly significant difference (****$P < 0.0001$) was observed, indicating a strong level of statistical confidence in the observed effects.

Colony forming assay

Treatment with bergenin, both individually and in conjunction with Cis and 5-FU, resulted in significant inhibitory effects on colony formation. Figure 4 depicts a notable reduction in colony formation of MCF-7 and HCT 116 cancer cells following 24 hours of treatment with bergenin alone and in combination with Cis and 5-FU, respectively. In MCF-7 cells, the combined treatment of bergenin and Cis exhibited a substantial decrease in colony formation (53.57%; $P < 0.0001$) compared to untreated groups. Similarly, bergenin and Cis monotherapies independently prevented colony formation, 67% ($P < 0.0001$) and 64% ($P < 0.0001$) area of colonies observed, respectively, when compared to the untreated group. In HCT 116 cells, as shown in Figure 4, combined treatment with bergenin and 5-FU significantly inhibited colony formation, reducing the number of colonies to 33.46% ($P < 0.0001$) compared to the untreated group. Treatment with bergenin alone resulted in a significant reduction to 83.58% ($P < 0.0001$), while treatment with 5-FU alone reduced the colony count to 55.93% ($P < 0.0001$), both compared to the untreated group.

Wound healing assay

The results revealed a noteworthy reduction in the cell migration capacity of MCF-7 and HCT 116 cells following a 72 hours treatment, in comparison to the untreated group. Figure 5 displays images of wound healing assay for MCF-7 and HCT 116 cells at 0, 24, 48, and 72 hours for group I to VIII.

MCF-7

In the untreated group, the wound completely closed within 72 hours as shown in Figure 5, with the wound area decreasing from 53.72% (0 hours) to 4.949% (72 hours). In contrast, the groups treated with Cis alone, bergenin alone, or the combination of bergenin and Cis showed only a minimal reduction in wound area.

Table 4. Doses (μM) of combination groups and combination indices at ≈50% effect

Combination groups	Combination doses	Fa (≈ 50 % effect)	Combination index (CI)	Description based on CI
Bergenin + Cis	20 μM + 5 μM	0.537	0.897	Synergism
Bergenin + 5-FU	10 μM + 10 μM	0.495	0.684	Synergism

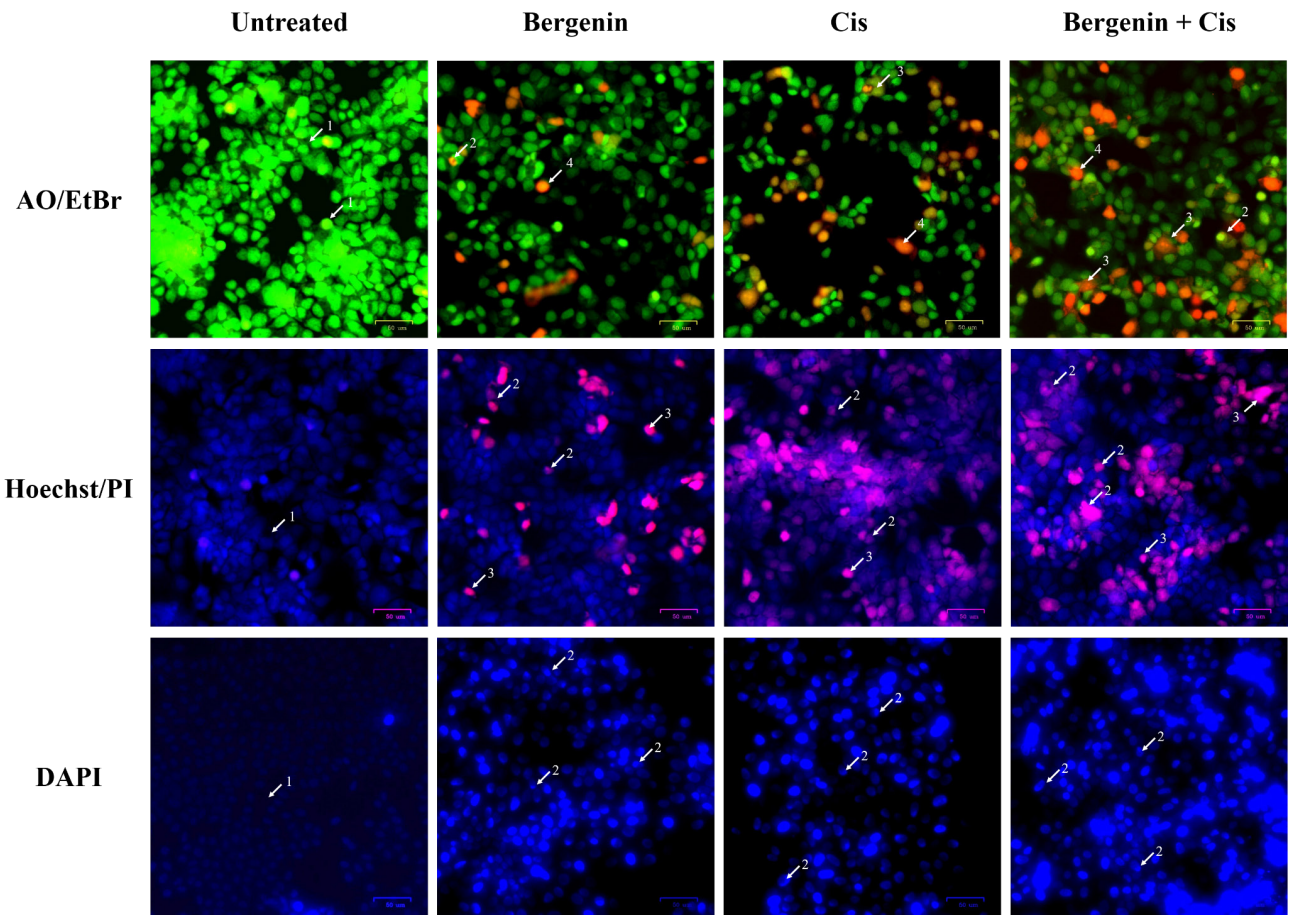

Figure 2. Fluorescent staining micrographs of treated MCF-7 cells compared to untreated cells. Morphological changes are highlighted using white arrows for AO/EtBr staining (1. Viable cells with bright green nuclei, 2. Early apoptotic cells with fragmented green nuclei, 3. Late apoptotic cells with red fragmented nuclei, 4. Dead cells, 5. Apoptotic bodies); Hoechst/PI staining (1. Viable cells with intact blue nuclei, 2. Apoptotic cells with pink fragmented nuclei and apoptotic bodies, 3. Dead cells); and DAPI staining (1. Viable cells with intact nuclei, 2. Apoptotic cells with fragmented bright blue nuclei)

Statistical analysis revealed a significant inhibition in cell migration for all three treatment groups. The wound area in cells treated with bergenin alone decreased to 39.65% ($P<0.001$) and to 48.94% ($P<0.001$) when combined with Cis after 48 hours, compared to the untreated group. After 72 hours, cells treated with bergenin alone showed a wound area of 38.44% ($P<0.01$), while those treated with the bergenin-Cis combination had a wound area of 42.51% ($P<0.0001$), showing significant inhibition of cell migration compared to the control group.

HCT 116

As shown in Figure 5, the untreated HCT 116 cells exhibited a substantial decrease in width of the wound, declining from 52.27% (0 hours) to 19.14% after 48 hours and further to 10.13% after 72 hours. The positive control, 5-FU impeded wound closure, resulting in a reduction of gap size only from 51.86% at 0 hours to 29.94% after 48 hours and to 28.58% after 72 hours. Notably, when compared to both bergenin alone and its combination with 5-FU, there was a significant reduction in wound gap to 27.43% ($P<0.001$) and 34.17% ($P<0.0001$) after 48 hours, and further to 22.91% ($P<0.01$) and 33.17% ($P<0.0001$) after 72 hours, respectively.

Cell cycle analysis

Flow cytometry was utilized to analyze the cell cycle of MCF-7 and HCT 116 cells, aiming to ascertain whether cell cycle arrest contributed to the reduction in cell density.

MCF-7

As depicted in Figure 6, after 24 hours the cell cycle retardation was observed during the culture period, in both the treated and untreated cells of MCF-7. Results showed that 72.43%, 6.61% and 20.96% of untreated cells were in G0/G1, S and G2/M phase, respectively. Treatment of bergenin and Cis alone significantly retarded the

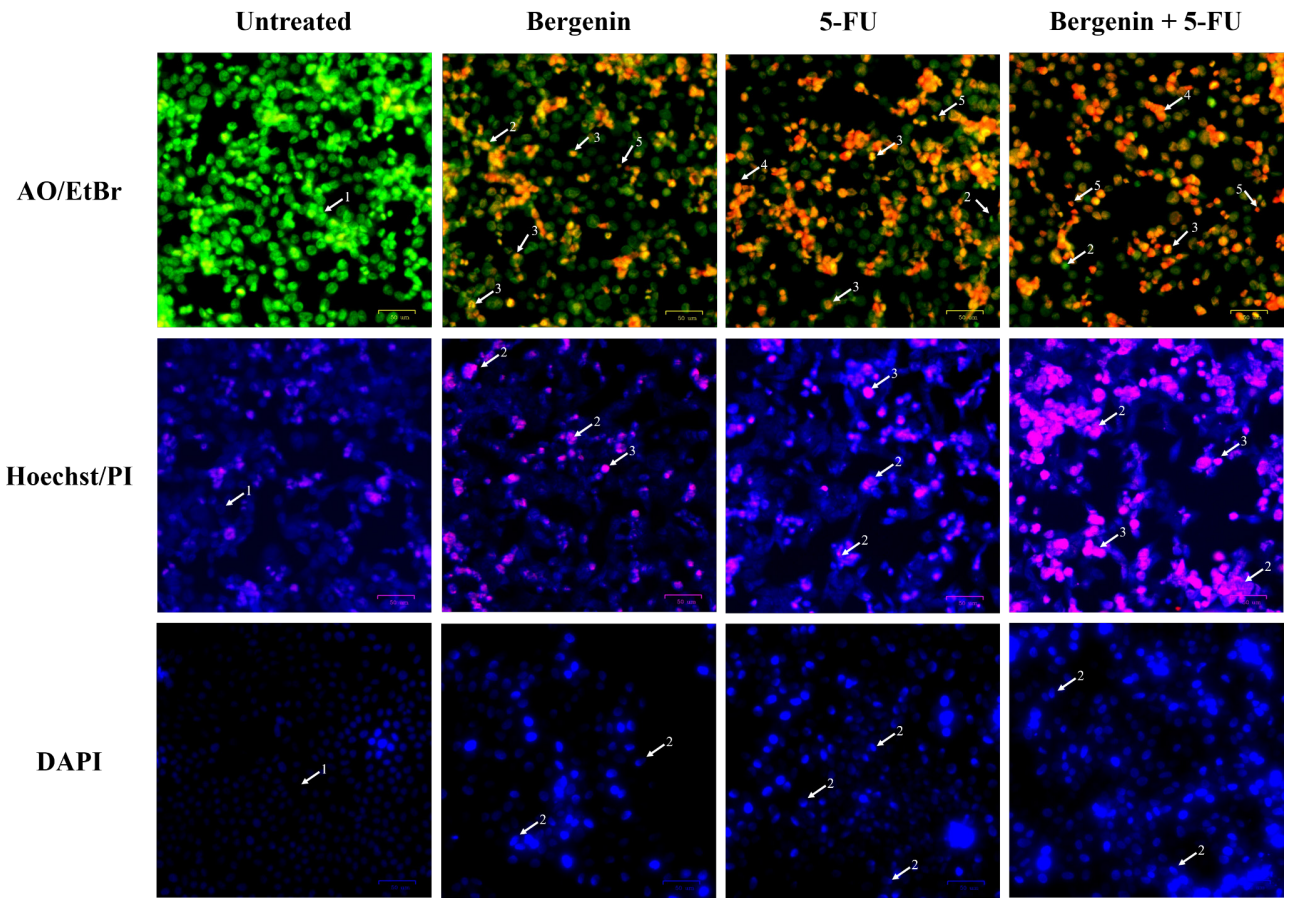

Figure 3. Fluorescent staining micrographs of treated HCT 116 cells compared to untreated cells. Morphological changes are highlighted using white arrows for AO/EtBr staining (1. Viable cells with bright green nuclei, 2. Early apoptotic cells with fragmented green nuclei, 3. Late apoptotic cells with red fragmented nuclei, 4. Dead cell, 5. Apoptotic bodies); Hoechst/PI staining (1. Viable cells with intact blue nuclei, 2. Apoptotic cells with pink fragmented nuclei, 3. Dead cells); and DAPI staining (1. Viable cells with intact nuclei, 2. Apoptotic cells with fragmented bright blue nuclei)

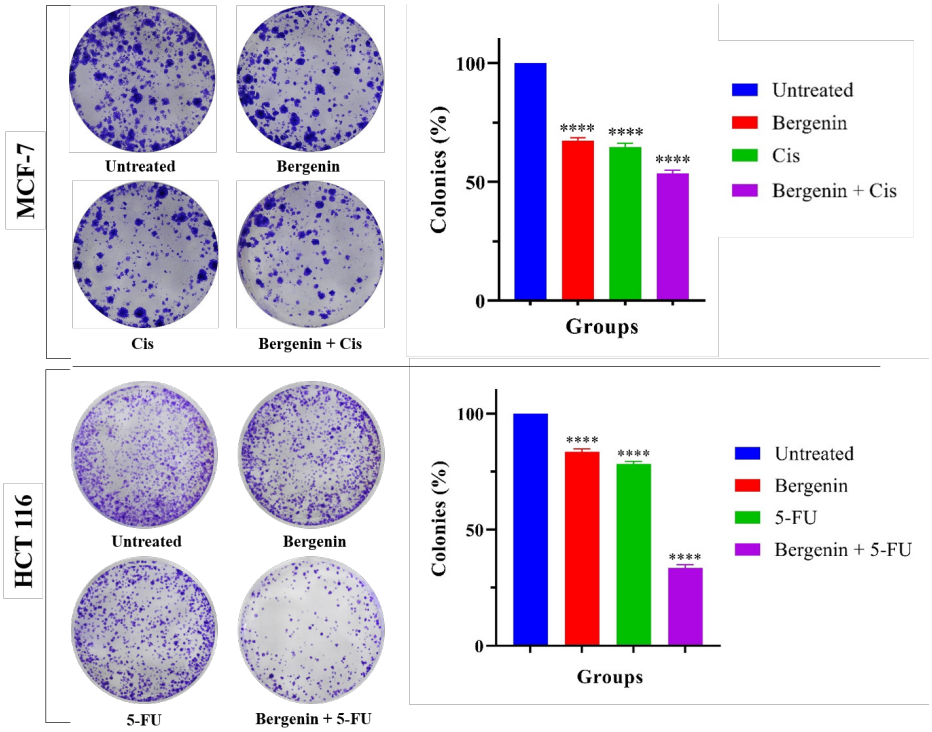

Figure 4. Colony forming assay on MCF-7 and HCT 116 cells, significant reduction in colony number was observed in treated groups when compared to untreated; Images of colonies and bar graph showing the number of colonies as survival fraction compared to untreated groups. All experiments were performed independently in triplicate. Values are expressed in mean ±SD. Statistical analysis identified a highly significant difference between the treated and untreated groups (****$P<0.0001$), demonstrating a strong level of confidence that the observed effect is unlikely to be attributable to chance

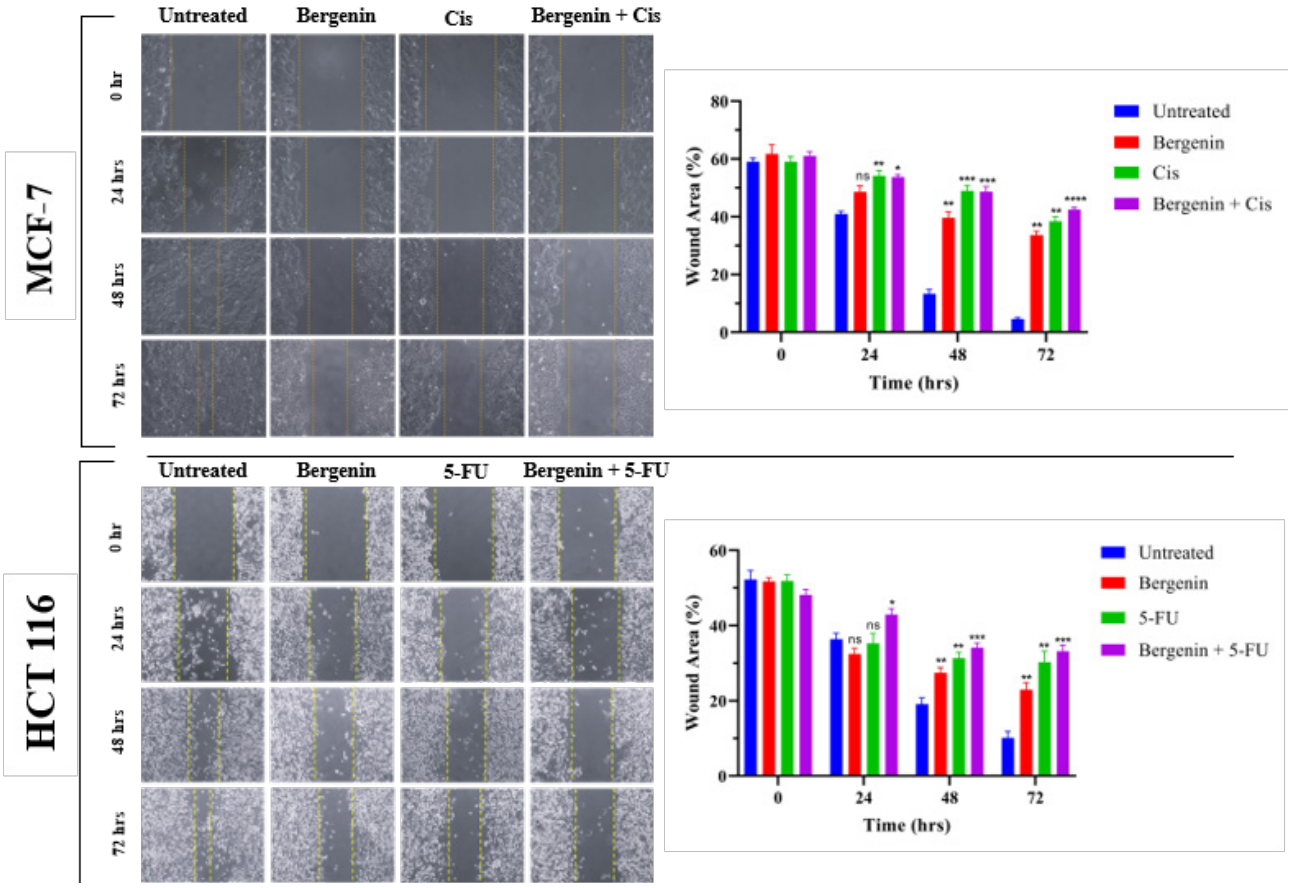

Figure 5. The wound healing assay conducted on MCF-7 and HCT 116 cells included - capturing images of wound closure at 24-hrs intervals over a 72-hrs period, and bar graphs depicting the relationship between wound area (%) and time. The results indicated a significant decrease in cell migration for treated groups when compared to untreated groups. All results were averaged for triple independent experiments. Values are expressed in mean ±SD. Statistical significance was considered as *$P<0.05$; **$P<0.01$; ***$P<0.001$; and ****$P<0.0001$ when all treated groups compared to untreated group

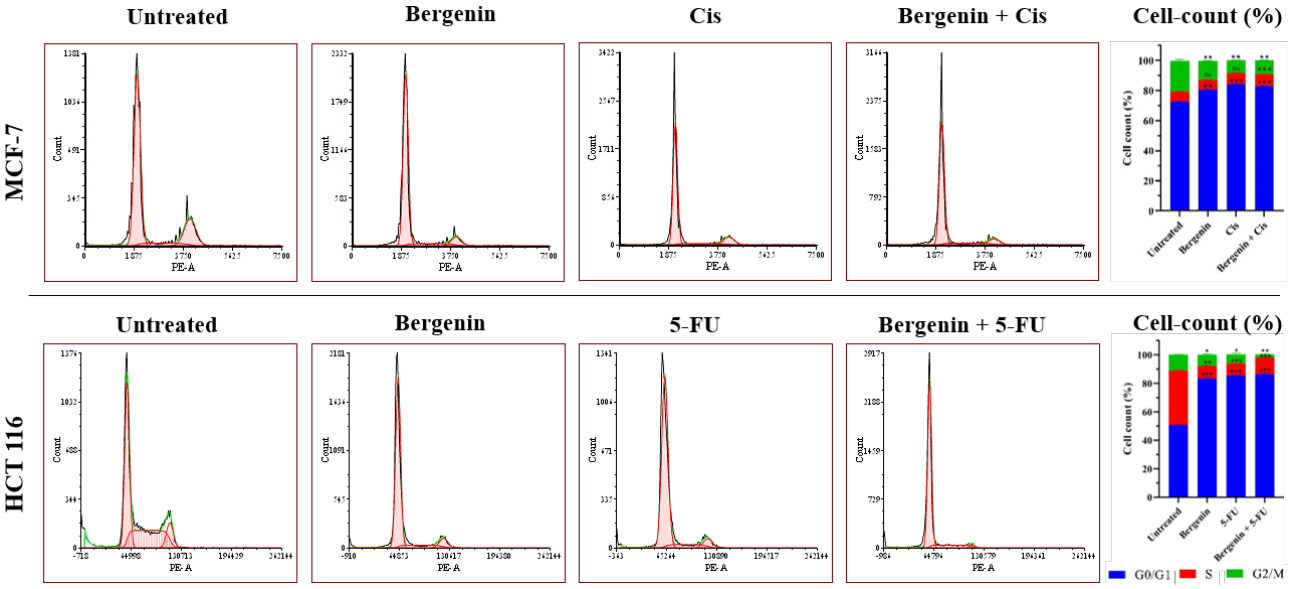

Figure 6. Results of cell cycle analysis for groups I to IV and groups V to VIII on MCF-7 and HCT 116 cells respectively, represented as cell count plotted against DNA content (PI fluorescence/cell); and percent cell count, where cell count (%) at G2/M stage is significantly low in treated groups compared to the untreated groups (*$P<0.05$; **$P<0.01$ and ***$P<0.001$). Values are expressed in mean ±SD

cells at G0/G1 phase with 80.66% ($P<0.01$) and 84.19% ($P<0.001$) cells respectively compared to untreated. When exposed to bergenin, 6.53% and 12.81% of cells were found to be confined in the S and G2/M phases, respectively. Conversely, treatment with Cis resulted in 7.36% and 8.45% of cells categorized in the S and G2/M phases, respectively. While treatment of bergenin and Cis in combination significantly retarded 83.10% ($P<0.001$)

cells in G0/G1, 7.75% in S and 9.15% in G2/M phase.

HCT 116

As depicted in Figure 6, both treated and untreated HCT 116 cells exhibited a delay in cell cycle progression after 24 hours. The findings revealed that, 50.55% of the untreated cells were in G0/G1 phase, while 38.19% were in S phase, and 11.27% were in G2/M phase. Separate treatment of bergenin and 5-FU led to significant cell cycle arrest with 83.14% ($P<0.001$) and 85.18% ($P<0.001$) of cells primarily in the G0/G1 phase, respectively. Exposure to bergenin resulted in 8.77% of cells in the S phase and 8.09% in the G2/M phase while 5-FU treatment led to 8.32% of cells in the S phase and 6.50% in the G2/M phase. The combination dose of bergenin and 5-FU significantly arrested 86.44% ($P<0.001$) cells at G0/G1 phase, and only a few cells (11.54% and 2.02%) were in S and G2/M phase, respectively. These findings suggest that bergenin may inhibit cell cycle progression of both MCF-7 and HCT 116 cells, primarily by arresting cells at the G0/G1 phase.

Cell apoptosis analysis

MCF-7

It was observed (Figure 7) in flow cytometry analysis for apoptosis that 87.66% of cells in the untreated MCF-7 cell group were live, with only 12.33% identified as early apoptotic cells. Very few cells were detected in the other two quadrants, which represent dead and late apoptotic cells. Upon treatment with the positive control Cis, there was a significant shift in cell distribution towards the early apoptotic quadrant, with 75.42% ($P<0.0001$) of cells falling into this category, while the percentage of live cells decreased to 18.93% ($P<0.0001$) as compared to untreated control group. Similar induction of apoptosis was observed in the group treated with bergenin alone, with 67.21% ($P<0.0001$) of cells in early apoptosis and 9.72% ($P<0.0001$) in late apoptosis, while the remaining 23.08% ($P<0.0001$) were live as compared to untreated control group. Furthermore, when cells were treated with both bergenin and Cis in combination, there was a significant increase in early apoptotic cells, accounting for 81.89% ($P<0.0001$) of the total, while 7.78% ($P<0.0001$) were in the late apoptotic stage when compared to untreated control group.

HCT 116

Flow cytometric analysis, as shown in Figure 7, demonstrated that 99.17% of cells in untreated HCT 116 cells were viable, with only 0.42%, 0.04%, and 0.38% in early apoptotic, late apoptotic, and dead states, respectively. Upon treatment with 5-FU and bergenin individually, there was a significant increase in early apoptotic cells to 40.44% ($P<0.0001$) and 23.87% ($P<0.0001$), respectively, compared to the untreated group. Similarly, the group treated with both bergenin, and 5-FU exhibited a significant increase in apoptotic cells to 40.87% ($P<0.0001$) compared to the untreated group. This data suggests that bergenin alone and in combination with Cis or 5-FU has the potential to induce apoptosis in MCF-7 and HCT 116 cells, respectively.

Discussion

Bergenin can be useful to reduce the cancer cell viability.[21,22] The present study on breast and colorectal cancer cells (MCF-7 and HCT 116 respectively) also demonstrated the concentration and time-dependent significant reduction in cell viability. Combination analysis established initial dosage parameters for its synergistic use with Cis and 5-FU. CompuSyn analysis[12] confirmed that bergenin co-administration reduced the required doses of these

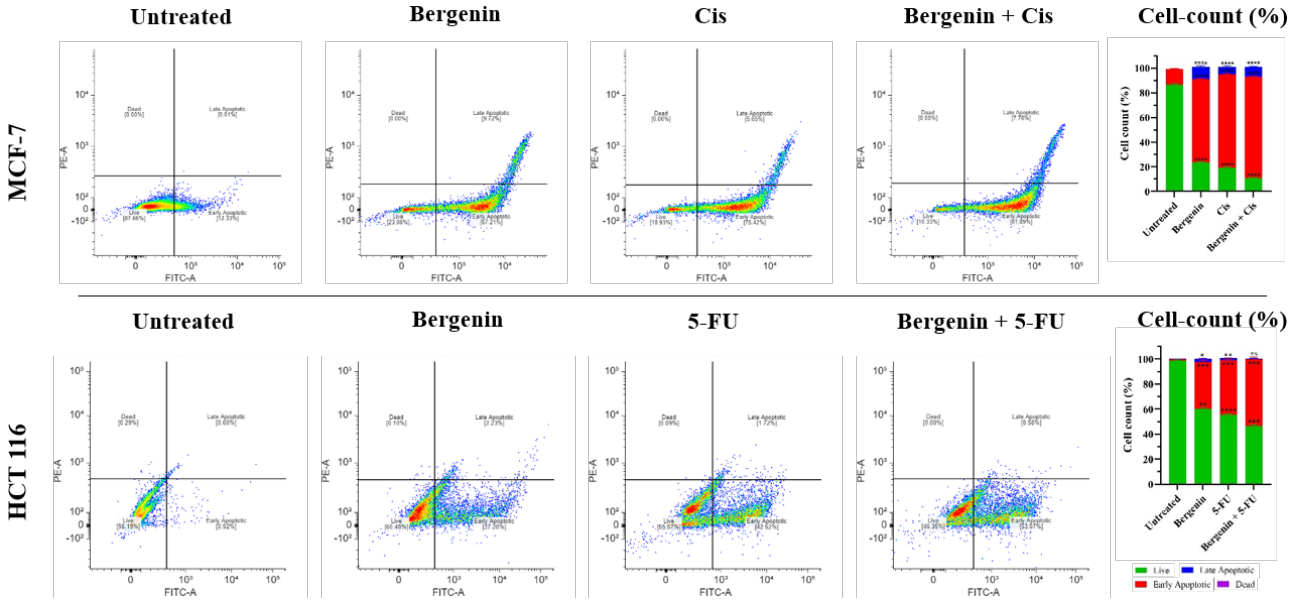

Figure 7. Apoptosis detection assay on MCF-7 and HCT 116 cells revealed that percent of early apoptotic cells increased when treated with Cis or 5-FU and Bergenin alone and in combination respectively; increased percent of early apoptotic cells as shown in bar graphs was statistically significant (*$P<0.05$; **$P<0.01$; ***$P<0.001$ and ****$P<0.0001$) in all treatment groups when compared to untreated groups. Values are expressed in mean ± SD

chemotherapeutic agents while enhancing their efficacy, achieving optimal inhibition at lower concentrations.

For MCF-7 cells, a 50% viability (IC_{50} value) reduction required 34.16 µM of bergenin or 14.23 µM of Cis alone, while in best reduced combination dose, only 20 µM of bergenin and 5 µM of Cis were needed. Similarly, in HCT 116 cells, 40.93 µM of bergenin or 22.87 µM of 5-FU alone achieved a 50% reduction, while the best reduced combination dose required just 10 µM of each. This synergy reduces the need for higher chemotherapy doses, potentially mitigating side effects. Similar synergistic effects were observed in other studies combining Cis with piperine[23] and curcumin[24] in MCF-7 cells and 5-FU with diosmetin[25], apigenin[26], and zerumbone[27] in HCT 116 cells.

Various *in vitro* tests were conducted to evaluate the inhibitory effects of bergenin, Cis, 5-FU, and their combinations on MCF-7 and HCT 116 cells. The analysis revealed that bergenin, both alone and in combination with 5-FU or Cis, effectively halted cell cycle progression at the G0/G1 phase. This interruption is critical as it prevents cancer cells from synthesizing DNA and progressing to mitosis, thereby inhibiting their proliferation. This effect is likely due to the downregulation of cyclins and cyclin-dependent kinases needed for the G1 to S phase transition,[28] underscoring bergenin's potential to disrupt cell cycle dynamics in cancer cells.

The colony formation and wound healing assays further showed the reduction in cells' capacity for unlimited proliferation and migration potential post-treatment. Notably, treatment with bergenin, both alone and in combination with Cis or 5-FU, significantly suppressed the clonogenic formation of MCF-7 and HCT 116 cells, respectively. The findings of this study also supported previous experiments that showed reduced colony formation and inhibited wound closure in human cancer cells treated with various combinations.[29-33] These inhibitory effects are likely mediated through the induction of apoptosis, which is important for eliminating cancer cells. Apoptosis acts as a crucial defense mechanism against carcinogenesis, eliminating genetically defective cells.[34,35] Therefore, inducing apoptosis is highly desirable in cancer management.[36-38] The present study not only proves the apoptosis of cell using fluorescent staining (AO/EtBr, DAPI, and Hoechst/PI staining) but also proves it using flow cytometry study. AO/EtBr staining differentiated between live, early apoptotic, and late apoptotic cells, while DAPI was used to observe nuclear integrity and fragmentation in the treatment groups. Hoechst/PI dual staining further differentiated nuclear damage by identifying condensed pyknotic nuclei with Hoechst and distinguishing late apoptotic from normal cells using PI in treated cells. To further quantify the apoptotic potential of bergenin and the combination treatments, the expression of annexin V, a protein that binds tightly to PS in a calcium-dependent manner, was evaluated using flow cytometry. These fluorescent staining and flow cytometry analysis prove that bergenin, alone and in combination with 5-FU or Cis, notably increased the proportion of apoptotic cells in both MCF-7 and HCT 116. Similar study carried out using bergenin and other phytocomponents also support these findings and prove that apoptosis is the major activity to eliminate the compromised cells.[39-41]

Cancer drugs induce cell death through apoptosis, with their efficacy directly related to the level of apoptosis induced.[42,43] This pro-apoptotic effect may involve intrinsic pathways characterized by mitochondrial dysfunction and caspase activation, as well as extrinsic pathways involving death receptors.[44-46] Recent studies emphasize combining natural compounds with conventional chemotherapy to enhance efficacy and reduce toxicity. Gano and colleagues[46] demonstrated synergistic phytochemical combinations in prostate cancer, while Hakeem et al[47] reported piperine's ability to enhance Doxorubicin's effects in breast cancer. Detailed *in vivo* and *in vitro* phytochemical studies against breast and colorectal cancers carried out by Talib and co-workers[48] also corroborate the findings of present study. Clinical investigations on tamoxifen-thymoquinone[49] and on sulforaphane-docetaxel,[50] further support the role of phytochemicals in chemotherapy enhancement. More recently, Li et al[51] demonstrated that ginsenosides synergize with Cis, improving efficacy and reducing toxicity. These findings align with broader research on Cis-based combination therapies, which enhance anticancer effects while mitigating drug resistance and adverse effects. Collectively, these insights support bergenin as a promising candidate for combination therapy, reducing chemotherapy-associated toxicity while maintaining efficacy and helping to improve patient outcomes and quality of life. By effectively reducing the required dosages of traditionally applied chemotherapeutic agents without compromising their efficacy, bergenin could potentially be helpful to reduce the adverse side effects associated with high-dose chemotherapy.

Conclusion

These findings show that bergenin plays a dual role, inhibiting the progression of cancer cells while promoting apoptosis. Furthermore, when combined with 5-FU or Cis, it synergistically inhibits cancer cell proliferation and enhances the apoptotic potential of Cis and 5-FU, even at lower doses. This combination approach alleviates the adverse effects linked to higher doses of Cis and 5-FU in the treatment of breast and colorectal cancer. Therefore, utilizing bergenin alongside chemotherapy drugs can minimize their dose and side effects and improve treatment outcomes through synergistic effects.

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Competing Interests

The authors declare that they have no conflict of interest.

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Supplementary Files

Supplementary file 1 contains Figures S1-S4.

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