

Bunium Persicum Seeds Extract in Combination with Vincristine Mediates Apoptosis in MCF-7 Cells through Regulation of Involved Genes and Proteins Expression

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Abstract

Background

Seeds of Bunium persicum, a member of the Apiaceae family, are traditionally consumed in the Iranian diet. Although many of its biological properties have been fully studied, no reliable information about the anticancer/cytotoxicity of this herb is available currently.

Methods

Herein, we first determined the major bioactive compounds of *B. persicum* seeds extract (BPSE) using GC-Mass analysis. Then, the cytotoxicity of the extract alone and in combination with vincristine (VCR), a common chemotherapy drug, was investigated on Breast cancer cell lines, MCF-7 and MDA-MB-231, and a normal cell line, MCF-10A, via MTT assay. Furthermore, the anticancer activity of these compounds was evaluated on MCF-7 cells through ROS assay, Real-Time PCR, Western blot, flow cytometry, and cell cycle assay.

Results

Our results showed that the extract considerably reduced the viability of cancerous cells while was harmless to normal ones. Besides, the combination of BPSE with VCR resulted in synergistic activity. The MCF-7 cells' intracellular ROS increased more than 2 times after treatment with BPSE and/or BPSE-VCR. Moreover, the IC₃₀ of BPSE (100 µg/ml) significantly increased the *BAX/BCL-2* and *P53* genes expression, while reducing the expression of the *MYC* gene. Also, the cell cycle was arrested in the G2 phase, in treated cells. The BPSE-VCR combination synergistically reduced the NF-κB and increased the Caspase-3 proteins' expression. The percent of apoptosis in the cell treated with the extract, VCR, and their combination was 27, 11, and 50, respectively.

Conclusion

The present study demonstrated the anticancer activity of the BPSE and its potential for application in combination therapy with VCR.

Background

Recent advances in the treatment with anticancer agents have improved the quality of the treatment process and the quality of life of cancer patients [1]. According to the WHO, malignant neoplasms are the leading health risk for women, of which 19.6 million are due to breast cancer. Breast cancer, with 2.26 million new cases in 2020, is the most common cancer diagnosed in women and the leading cause

of cancer death in women worldwide [2]. One of the most important approaches to treating cancer is chemotherapy. The main problems of chemotherapy drugs are their harmful side effects and their lethal effect on healthy and proliferating cells such as stem cells [3]. Vincristine (VCR) is one of the most widely used drugs in chemotherapy. Its main molecular mechanism is disturbing the formation of microtubules, which affects various cellular and physiological processes [4]. One of the strategies for optimal use and increasing the performance of this drug to reduce its side effects is to reduce neurotoxicity by keeping the minimum dose low [5]. VCR shows a better effect when combined with other anti-tumor agents. Combination chemotherapy can enhance the destruction of tumor cells, along with reducing toxicity and drug resistance. This treatment strategy reduces the dose of VCR and its side effects [6, 7].

Apoptosis is one of the most essential programmed cell death mechanisms recently has become the mainstay of treatment for fighting cancer [8]. Numerous studies have shown that NF- κ B activation inhibits apoptosis [9]. Many factors in natural daily food, such as curcumin, genistein, silymarin, caffeine, catechins, capsaicin, etc., have been known to inhibit the expression and activation of the NF- κ B signaling pathway [10, 11].

Chemo-preventive compounds are those substances present in the daily diet, such as vegetables, fruits, spices, and condiments, and have the effect of preventing the development of chronic diseases such as cancer [12–14]. In the last decade, a lot of research has been done on these compounds and plant extracts to investigate their anti-cancer effects [15–18]. Also, the combined effects of various drugs and chemo-preventive compounds are being studied [19–21]. Whether these compounds, in addition to their chemo-preventive and anti-cancer effects, can be helpful in combination with chemotherapy drugs is being investigated. These compounds may improve treatment strategies, reduce therapeutic doses of chemotherapy drugs, and eliminate side effects of chemotherapy drugs [22].

Black cumin with the scientific name of *Bunium persicum* (Boiss.) Fedtsch. belongs to the Apiaceae family. *B. persicum* seeds produce valuable compounds with antimicrobial, antioxidant, anti-inflammatory, anti-diabetic, antihyperlipidemic, and analgesic properties [23]. Also, the seeds' essential oil contains large amounts of oxygenated monoterpenes, especially γ -Terpinene, cuminaldehyde, *p*-cymene, and limonene, which have high antimicrobial and antioxidant effects [24]. Research on the chemo-preventive effect of *Carum carvi* (a species close to black cumin) demonstrates that this plant can well prevent the formation of aberrant crypt foci (ACF) 1,2-dimethylhydrazine (DMH)-induced rat colon carcinogenesis [25]. In addition, the anti-cancer effect of *Carum carvi* extract along with several species of the Apiaceae family has been reported [26].

Given that the tumorous cells develop resistance to chemotherapy by increasing NF- κ B expression [27], it is important to keep NF- κ B expression low in combination therapy. One of the important strategies to improve the effect of VCR is to keep its dose low and increase its effectiveness through combination therapies [5]. The aim of this study was to characterize PBSE phytochemicals. Furthermore, the anti-cancer effect of the PBSE alone and in combination with VCR was evaluated on MCF-7 cells.

Material And Methods

Materials

Cell culture media, penicillin-streptomycin, and trypsin EDTA were purchased from Biosera (Ringmer, UK). Bovine Fetal Serum (FBS) was purchased from Gibco, Thermo Fisher Scientific (Germany). The MTT powder_2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide_ was purchased from MELFORD (United Kingdom). Vincristine (VCR) and Propidium iodide solution were purchased from Sigma Aldrich (Germany). Also, Caspase-3: 9662S, NF- κ Bp56: 3033T, and GAPDH: 2118S antibodies applied in western blot analysis were purchased from Cell Signaling Technology company (USA). ROS assay Kit was purchased from Teb Pazhouhan Razi Company. Annexin V apoptosis detection kit was purchased from the BD Biosciences company. RealQ plus 2x master mix green high ROX and RevertAid Reverse transcriptase (RT) were purchased from ampliqon (Denmark) and Thermo Fisher Scientific (USA), respectively. TRIzol Reagent was purchased from Zaver Zist Azma(Iran).

Preparation of the *B. persicum* seeds extract

To prepare *B. persicum* seeds' methanolic extract, the plant seeds were collected from the Jabalbarez foothills of a private land (the owner land: Mr. Mahdi Hatami) of Jiroft city in Kerman province of Iran and authenticated by Dr. Seyed Mansour Mirtadzadini (Specimen number: 3843), the botanist at the herbarium of biology department of Shahid Bahonar University of Kerman, Kerman, Iran (<https://biol.uk.ac.ir/>). The specimens were collected with the permission of the landowner. The seeds were then dried in the shade at room temperature (22-25 °C), followed by grinding with an electric milled. Next, the obtained powder was mixed with methanol (80%) and shaken at room temperature for 48 hours. The obtained extract was refined several times with Whatman filter paper (Grade1) to get a clear solution, which was then concentrated via a rotary evaporator at 40 °C to remove the organic solvent.

GC-MS analysis

The GC-MS process and data analysis were performed according to the protocol of Sharififar *et al.* [28]. In brief, the carrier used was helium with a flow rate of 1 mL/min (split ratio 1:200) with an injection volume of 0.2 μ L. The detector temperature was 290 °C and the injector temperature was 220 °C. After keeping the oven temperature at 50 °C for 3 minutes, it gradually increased to 160 °C (with the rate of 3 °C/min) and, after ten minutes, it gradually increased to 240 °C with a 3 °C rise. GC-MS analyzes were performed using Shimadzu QP 5050 operating at 70 eV ionization energy, equipped with an HP-5 capillary column with helium as the carrier gas. The compounds were determined by comparing their relative retention time (RT) and mass spectra with those of standards, Wiley library data of the GC-MS system, and literary data. To calculate relative retention indices (RRI), alkanes were utilized.

Cell culture

To evaluate the cytotoxic effect of the extract, MCF-7, MDA-MB-231, and MCF-10A cell lines were purchased from the Iranian Genetic Resources Center, Tehran, Iran. DMEM and RPMI media containing 10% FBS and 1% antibiotic were used to proliferate MCF-7 and MDA-MB-231 cells, respectively. Also, the medium containing 5% FBS, 5% horse serum, and 1% antibiotic was used for the proliferation of MCF-10A cells. The cells were incubated at 37 °C, in 5% CO₂, with 95% humidity.

MTT assay

The viability of cells treated with the extract and drug was assessed by the MTT assay. Toward this end, at first, 5000 cells/well of each MCF-7, MDA-MB-231, and MCF-10A cell line were cultured in a 96-well plate and incubated for 24h. After this time, the cells were treated with doses ranging from 20 to 200 µg/ml of the BPSE. In addition to the extract, MCF-7 cells were exposed to a range of 50 to 500 nM of VCR, and their viability was determined. To evaluate the effect of the BPSE in combination with VCR, MCF-7 cells were treated with 100, 200, and 400 nM of VCR combined with 60, 80, 100, and 120 µg/ml of BPSE, respectively. After 24 h of treatment, 30 µl of the MTT solution (0.5 µg/ml in PBS) was added to each well, and plates were incubated for 3h. After this time, the culture medium was removed and 100 µl of DMSO was added to each well, followed by slightly shaking the plate for 40 min. Finally, the absorption values of each well were evaluated at 570 nm by an ELISA reader (Eliza MAT 2000, DRG Instruments, GmbH, Marburg, Germany), and the percentage of cell viability was calculated.

Noteworthy, after determining the effect of different concentrations of these compounds on the cells, their IC₃₀ (100 µg/ml of the extract and 200 nM of VCR) were used in further tests. These features were also combined to evaluate their combination effect.

Intracellular ROS assay

The reactive oxygen species (ROS) level in the MCF-7 cells was measured with the ROS assay Kit according to the company instructions. Briefly, the cells were cultured in a 96-well plate, and once they reached 80% confluency, treated with intended concentrations of the extract (80-200 µg/ml), VCR (200 µg/ml), and their combination (100 µg/ml of the extract combined with 200 µg/ml of VCR). After treatment, the cell culture medium was removed and 100 µl of ready assay buffer was added to each well. After removing the buffer, 100 µl of DCF staining buffer was added and the plate was held at 37 °C in the dark for 60 minutes. After that, 100 µl of stimulator buffer was added and the plate was kept at 37 °C for 20 min in the dark. Finally, the wells were drained, 100 µl of the ready assay buffer was added, and the plate was read by a plate reader at the excitation wavelength of 485 nm and the emission wavelength of 538 nm.

Gene expression assessment

The real-time PCR technique was performed to assess the effect of the compounds on the expression of MYC, BAX, BCL-2, and P53 genes. Briefly, RNA was first extracted from treated cells using TRIzol reagent according to the manufacturer's protocol. A NanoDrop™ 2000/2000c spectrophotometer was used to

measure the quality and concentration of the extracted RNA. Next, cDNA was synthesized by Thermo Scientific Kit. After cDNA synthesizing, real-time PCR was done by using the RealQ Plus 2x Master Mix (AMPLIQON). The GAPDH gene was used as a housekeeping gene. The primers designed for each gene were controlled for their specificity using BLAST on the NCBI site. The formula $2^{-\Delta\Delta CT}$ was used to estimate the percentage of the relative gene expression. The primers used in this study were as follow; GAPDH forward primer: '5CCCCAGCAAGAGCACAAAGAGG3' and reverse primer: '5AGGAGGGGAGATTCA-GTGTGG 3'; MYC forward primer: 5'CCCAAACCAGAAATGATGTTG3' and reverse primer: 5'GAC-CTACTTTGAGACTGAGAC3'; BCL-2 forward primer: 5'TGGGGTTCATGTGTGTGGAG3' and reverse primer: 5'CGGTTTCAGGTACTCAGTCATCC3'; BAX forward primer: 5'CCCGAGAGGTCTTTTCC-GAG3' and reverse primer: 5'CCAGCCCATGATGGTTCTGAT3'; P53 forward primer: 5'ACCTAAAA-GGAAATCTCACCC3' and reverse primer: 5'ACCCTGAGCATAAAACAAGTC3'.

Western blot analysis

For western blotting, firstly, MCF-7 cells were seeded on a six-well plate and allowed to attach for 24h. The cells were then treated with the intended concentrations of VCR, BPSE, and their combination, and incubated for 24h. Next, the cells were harvested and homogenized by a buffer containing 1 mM EDTA, 0.1% SDS, 1% NP-40 with protease inhibitors (1 mM phenylmethyl fluoride) Sulfonyl (2.5 mg/ml leupeptin, 10 mg/ml aprotinin) and 1 mM sodium orthovanadate, 0.1% Na deoxycholate and 10 mM Tris-HCl (pH 7.4). Next, the samples were centrifuged at 4 °C for 14 minutes at 14000 rpm, and the supernatant was retained. Then, the extracted proteins' concentration was measured using the Bradford method (Bio-Rad Laboratories, Feldkirchen, Germany). At the next step, an equal amount of protein was loaded on an SDS-PAGE gel (9%). After loading, the fractionated proteins were transferred to a nitrocellulose membrane by electrophoresis (Hybond ECL, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Next, the blocking with skim milk (5%) in TBS-T (Tris-buffered saline with Tween 20) was done for 24h at 4 °C. The membrane was then incubated overnight with rabbit monoclonal antibody Caspase-3 and NF-κB p65, at 4 °C. After washing with TBS-T (three times, 5 minutes), the blots were exposed to the secondary antibody of horseradish peroxidase mixture (GE Healthcare Bio-Sciences Corp) for 60 min at room temperature. Antibody-antigen complexes were evaluated using the ECL system and exposed to the Lumi-Film chemical detection film (Roche Applied Science, Mannheim, Germany). Image j analysis software was used to analyze the intensity of expression. Noteworthy, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control.

Flow cytometry analysis

The incidence of apoptosis and necrosis in the cells exposed to the extract, VCR, and their combination was examined using the Annexin-V PE/7-ADD apoptosis diagnosis kit based on the manufacture gaudiness. Toward this end, after treating the cells with the compounds for 24h, the cells were washed with PBS and suspended in 100 µl of Annexin V binding buffer, 5 µl of PE Annexin V, and 5 µl of 7-AAD, followed by keeping in the dark for 15 minutes. Finally, after adding 400 µl of binding buffer, the samples were assayed using a flow cytometer (Becton Dickinson, Franklin Lakes NJ).

Cell cycle assay

To assess the effect of the compounds on the cell cycle, the cells were treated with the compounds for 24h. Next, they were detached from the plate bottom by trypsin and washed twice with PBS. Cells were then fixed with 70% ice-cold ethanol for 30 minutes, treated with 20 µg/ml of propidium iodide and 10 µg/ml of RNase, and incubated in the dark at 37 °C for one hour. Finally, the samples were evaluated by BD FACSCalibur flow cytometer at a wavelength of 488 nm, and analyzed by Cell Quest 3.3 software.

Statistical analysis

Data were analyzed using SPSS software. Analysis of variance (ANOVA) and the Tukey test were used to evaluate the significant differences among groups. All experiments were performed with at least three independent repetitions. The results were shown as mean ± SEM, and $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant values.

Results

GC/MS analysis

GC/MS analysis was done to determine the bioactive compounds of the extract. Based on the GC/MS chromatogram (Fig. 1), the BPSE consisted of over 30 compounds. 74% of the components were cuminaldehyde, Cis-Linoleic acid, 1H-indene, apiol, and stearin. Other identified compounds were Pinene, o-Cymol, Sitosterol, Epiglobulo, methyl octadeca-10,13-dienoate, 3-(3-Methoxyphenyl) propionic acid, β-Cubebene, Cuminyll acetate, p-Vinylguaicol, and Stearic acid, which each one made up less than 2% of the BPSE (Table 1).

Table 1 Chemical composition of BPSE determined by GC-MS analysis.

Compound Name	RT (min)	Molecular Formula	Peak Area%
Cuminaldehyde	19.3	C ₁₀ H ₁₂ O	14.2
cis-Linoleic acid	39.8	C ₁₈ H ₃₂ O ₂	11
1H-indene	30.07	C ₁₃ H ₁₆ O ₂	7.4
Apiol	29.1	C ₁₂ H ₁₄ O ₄	4.9
γ-Terpinen	13.45	C ₁₀ H ₁₆	3.5
Thymol	20.8	C ₁₀ H ₁₄ O	2.4
Cumic acid	25.06	C ₁₀ H ₁₂ O ₂	2.2
Dehydrosabinene	25.25	C ₁₀ H ₁₄	2.5
Myristicin	26.7	C ₁₁ H ₁₂ O ₃	2.8
Cuminol	20.7	C ₁₀ H ₁₄ O	3
2,3-2H-Benzofuran-2-one, 3,3,4,6-tetramethyl	31.4	C ₁₂ H ₁₄ O ₂	5.7
Palmitic acid	36.47	C ₁₆ H ₃₂ O ₂	5.2
Stearin	48.4	C ₂₁ H ₄₀ O ₄	5.7
i-Propyl 9,12-octadecenadienoate	52.7	C ₂₁ H ₃₈ O ₂	3.5
Total			74%

Cytotoxicity of BPSE and VCR

MTT assay was used to evaluate the cytotoxicity of BPSE and its combination with VCR against two breast cancer cell lines, MCF-7 and MDA-MB-231, and a normal cell line, MCF-10A. Based on the results (Fig. 2A-C), BPSE showed significant toxicity toward all cell lines in a dose-dependent manner. The highest cytotoxicity was observed against MCF-7 cells (IC₅₀: 160 µg/ml and IC₃₀: 100 µg/ml), followed by MDA-MB-231 (IC₅₀: 180 µg/ml). The extract showed the lowest toxic effect on normal MCF-10A cell line with the IC₅₀ of 210 µg/ml. VCR also showed considerable cytotoxicity against MCF-7 cells with the IC₅₀ of 400 nM and IC₃₀ of 200 nM (Fig. 2D). Fig. 2E represents the effect of the extract combined with VCR on MCF-7 cells. Based on the results, combining these agents resulted in an additive cytotoxic impact in most combined ratios. A combination index of 1±0.08 was obtained for these compounds.

Considering their significant activity, the combined ratio consisting of the IC₃₀ of each compound (100 µg/ml of BPSE and 200 nM of VCR) was chosen for further evaluation and compared with each separate ratio. Noteworthy, the effects of 160 and 200 µg/ml of BPSE were evaluated in further molecular studies, and results have been presented in the supplementary file.

Intracellular ROS assay

As Fig. 3A shows, although the amount of intracellular ROS in MCF-7 cells after 4 hours of treatment with BPSE was slightly reduced, more prolonged incubation resulted in an increased ROS amount, especially at doses higher than 120 µg/ml. The increasing trend peaked at 24h when this feature was twice as high as the control. Also, both VCR (200 nM) and BPSE (100 µg/ml) increased the intracellular ROS by approximately 1.5-fold, yet their combination increased the ROS by approximately 2.2-fold (Fig. 3B).

The effect of BPSE on the genes' expression

Based on the results obtained from real-time PCR, the treatment of cells with both BPSE and VCR significantly increased the expression of *BAX* and *P53* genes. On the contrary, the expression of *BCL-2* and *MCY* genes decreased in the treated cells (Fig. 4). The combination of BPSE and VCR showed similar behavior in the genes' expression, but its activity was significantly higher than that of each one alone. The combined agent, for example, increased the *P53* expressions by about 4 folds, while this feature in cells treated with each of BPSE and VCR was 2.5 fold and 1.5 fold, respectively.

Western blot analysis

Western blot results showed that BPSE significantly caused the fission of caspase-3 protein (Fig. 5A). This extract could also decrease the expression of NF-κB (Fig. 5B). Likewise, VCR increased the levels of cleaved caspase-3; though, its effect on the NF-κB levels was not significant. Furthermore, our results revealed that the extract-VCR combination affected these features more considerably than the extract and/or VCR on their own, at the same concentration.

Flow cytometry

Fig. 6 shows the results of flow cytometry analysis for evaluating apoptosis. We found that the incidence of apoptosis in the cells treated with BPSE, and VCR was 27 % and 11%, respectively. While the rate of apoptosis observed in the combined dose was considerably higher and resulted in 50% apoptosis, indicating their synergic effect on the apoptosis induction.

Cell cycle assay

Examination of the effect of BPSE, VCR, and their combination on the cell cycle of MCF-7 cells indicated that after treatment with VCR, the cells accumulated more in phases S and G2 compared to the control group so that, in total, 88% of cells entered these two phases. In the BPSE treatment, the cells tended to stop in phase G2 compared to the control group. And, in combined dose, the cells accumulated in G2 and S phases (Fig. 7).

Discussion

Since cancer is one of the leading causes of death in human societies, finding the right strategy to treat cancer is essential. In the present study, we evaluated the anticancer potential of the *B. persicum* seeds extract both in its pure form and in combination with the vincristine, as a chemotherapy drug, and promising results have been achieved.

GC-MS analysis indicated that a variety of monoterpenes exists in the *B. persicum* seeds extract. The seed is widely consumed due to its biological properties, such as amelioration of digestive disorders, treat obesity, diarrhea, indigestion, and abdominal pain. These biological activities are attributed to the presence of phenolic compounds [29]. Also, previous studies have shown the anti-cancer effects of these compounds [30, 31]. No study, however, investigated the seeds' anticancer activities in detail.

In the present study, we evaluated the cytotoxicity of PBSE against cancerous (MCF-7, MDA-MB-231) and normal cells (MCF-10A). We found that the extract was more active against cancerous cells than normal cells. Similar to our results, previous studies have demonstrated that plant extracts are mainly innocent toward normal cells, while they are toxic against cancerous cells [32, 33]. Researchers attribute such observations to the presence of polyphenols, including flavonoids, tannic acid, ellagic acid, and ellagitannin, which are known to be safe and health beneficial [34–36]. Considering its side effects, especially at high concentrations, reducing the dose of VCR and increasing its effectiveness by combining the drug with other drugs or plant extracts is one of the important new strategies for cancer treatment [5, 37]. To our knowledge, this is the first study investigating the effect of BPSE combined with VCR. Our results indicated that their combined effect on cell viability is additive with a combination index of 1 ± 0.08 .

The ROS level is one of the important factors determining cell fate [38]. Plant compounds with antioxidant properties can reduce the amount of ROS [39, 40]. However, some plant compounds can increase the amount of ROS, inducing apoptosis in cancer cells [41–43]. The effect of BPSE on the ROS level of MCF-7 cells can be one of the causes of induction of apoptosis. Also, in the combined dose, the rate of ROS induction increased up to 2.2 times compared to the control, which can justify its higher cytotoxicity.

During the apoptosis process, many changes occur in cell morphology, such as losing cells' connection with the surrounding area, chromatin condensing, and blebbing cell membranes [44]. Caspase-3 is an executive protease in apoptosis that leads to the finalization of apoptosis and subsequent cell death [45]. Furthermore, BCL-2 family proteins play an essential role in protecting and permeability of the mitochondrial outer membrane. The increased expression of BCL-2 protein enhances resistance to cell death, while its decrease, along with increased BAX protein, induces apoptosis through the mitochondrial outer membrane permeabilization (MOMP) process [46]. Apparent changes were observed in the cells treated with the extract, VCR, and their combination, indicating their effectiveness in the apoptosis induction.

We evaluated the expression of some key genes and proteins involved in the apoptosis pathway. The caspase-3 protein is one of the primary markers of the internal pathway apoptosis; its levels significantly

increased in the presence of the BPSE, based on the western blotting analysis. Flow cytometry results also confirmed the evidence of western blotting and the incidence of apoptosis in the treated cells. The increased *BAX/BCL-2* genes' expression also supported the above results. Based on the mentioned evidence, it seems that BPSE activates the internal pathway of apoptosis. Changes in the expression of *BAX* and *BCL-2* in the combined group can also indicate the activation of the internal pathway of apoptosis.

Currently, combination therapy is one of the treatment strategies to increase the effectiveness of some drugs, such as VCR, by reducing the utilized drugs' dose, and subsequently reducing the side effects of this drug [7]. Herein, we showed that the combination of VCR and BPSE (at their IC_{30}) induced considerably more cleavage of caspase-3 and activation of the internal apoptotic pathway. Annexin V staining confirmed BPSE and VCR separately induced 27% and 11% of apoptosis (in total 38%), respectively. Yet, in the combined dose, this amount reached 47%, indicating a synergistic effect on apoptosis induction.

Numerous studies have shown that NF- κ B activation in cancer cells promotes resistance to induction of apoptosis. The BPSE could reduce the expression of the NF- κ B protein in MCF-7 cells. In addition, in the combined dose of VCR and BPSE, NF- κ B decreases significantly, while in the dose treated with VCR, it shows a slight increase in expression. It could be concluded that, at the combined dose, BPSE by inhibiting NF- κ B leads the MCF-7 cells to apoptosis, which may explain the mechanism of action of BPSE to stimulate apoptosis at the combined dose. Other studies have shown that chemopreventive agents such as genistein, resveratrol, diallyl sulfide, S-allyl cysteine, lycopene, capsaicin, curcumin, catechins, and eugenol can reduce NF- κ B [47].

Up to now, various studies have shown that anti-cancer agents inhibit the cell cycle at cell checkpoints by inducing the *P53* gene expression, thereby causing apoptosis [48]. On the other hand, overexpression of the oncogene *MYC* in many cancers promotes cell proliferation and even resistance to chemotherapy [49, 50]. The results of our study implied that the combination of BPSE and VCR regulates the expression of *P53* and *MYC* in favor of apoptosis induction. This impact was highly more than that of each compound on its own. Recently, researchers showed that resveratrol could induce apoptosis by reducing *MYC* expression and stopping the cell cycle [51], which is similar to the results of our study.

Regulation of the cell cycle plays a vital role in the natural growth and proliferation of cells in the body. One of the essential functions of chemotherapy drugs is inhibition of the cell cycle and then inducing apoptosis in cancer cells [52]. Chemotherapy drugs, especially alkaloid compounds derived from plants, affect the destruction of microtubules. Among alkaloid compounds, VCR, vinblastine, paclitaxel, and recently colchicine have been shown to stop the cell cycle in phase G2/M and induce apoptosis in PC3 prostate cancer cells [53]. In this study, examination of the cell cycle indicated that VCR disrupted the S phase and cell division, and MCF-7 cells accumulated in this phase. Those that passed through this phase stopped and accumulated in the G2 phase. BPSE also stopped cells in the G2 phase. At the combined dose, cell arrest was also observed in phases G2 and S. It seems that both the extract and the

drug reach a good harmony to stop cells in phases G2 and S. Previous studies have shown that arresting the cell cycle in phase G2 by drug treatment can induce apoptosis [54, 55] As a result, the combination of the BPSE and VCR can induce apoptosis by arresting the cell cycle in phase G2.

Conclusion

In the present research, for the first time, we studied the anticancer activity of the *B. persicum* seed extract (BPSE). BPSE predisposed MCF-7 to apoptosis by decreasing NF- κ B expression and increasing P53 expression and finally cleaved the caspase-3. In addition, the extract increased intracellular ROS, which was also observed in combination with VCR at considerably higher levels. It was revealed that during the treatment of MCF-7 with the VCR-BPSE combination, VCR reduced the growth rate and stopped the MCF-7 cells in S and G2 phases, facilitating the effect of BPSE on the advancing the process of apoptosis. Overall, it is concluded that the seed extract of this plant has a good potential for cancer treatment and combined therapy.

Abbreviations

VCR

Vincristine

BPSE

Bunium persicum seed extract.

Declarations

Ethics approval and consent to participate

The experimental research on *Bunium Persicum* seeds was carried out according to relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not Applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing Interests

The authors declare that they have no conflict of interest.

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Authors' Contributions

Mohammad Rasoul Samandari-Bahraseman: Conceptualization, Investigation. Ahmad Ismaili: Supervision. Saeed Esmaeili-Mahani: Methodology, Resource. Esmaeil Ebrahimie: Software. Evelin Loit: Writing-Review& editing.

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Figures

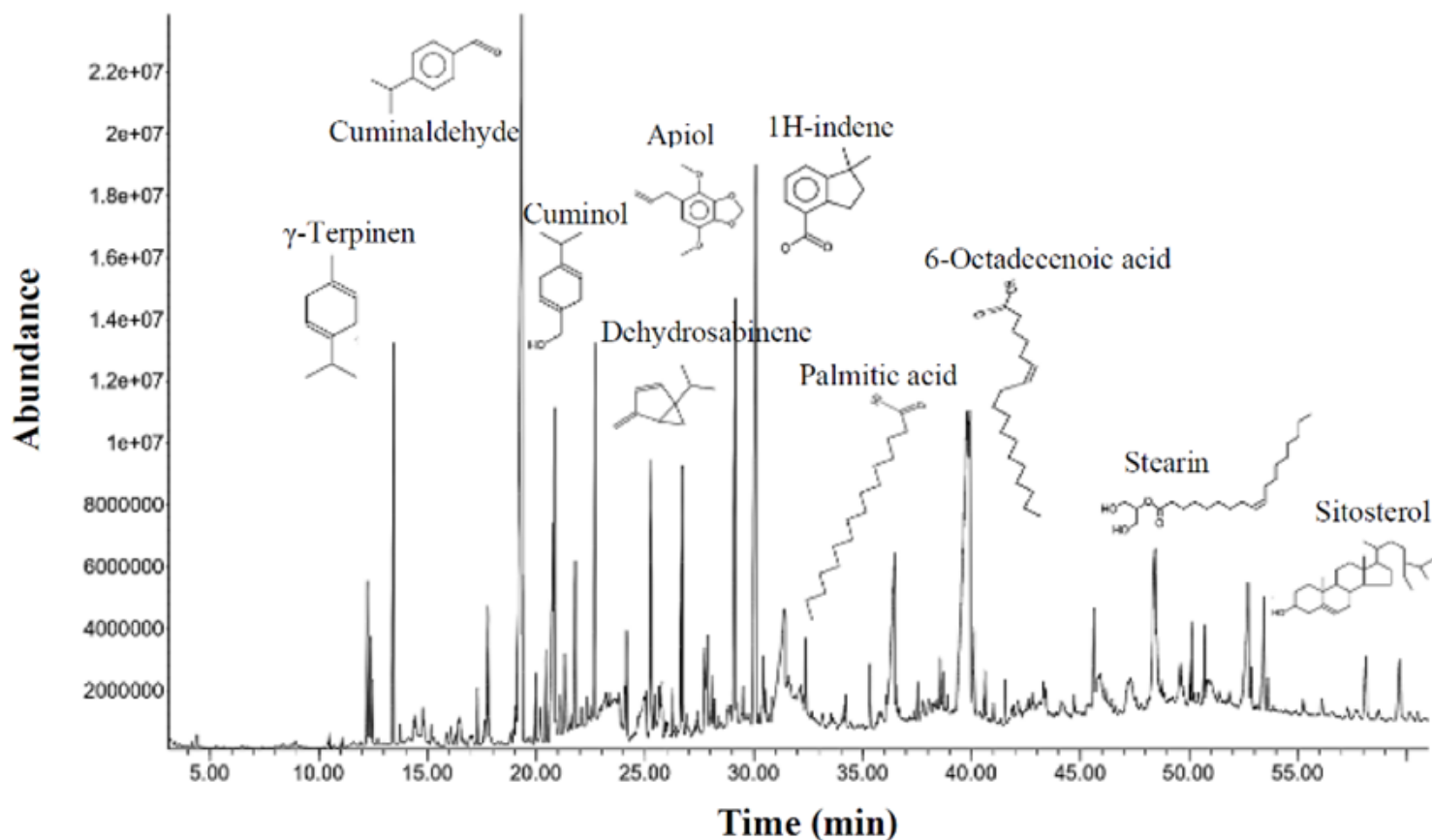


Fig 1

Figure 1

GC-MS chromatogram and chemical structures of main compounds of BPSE.

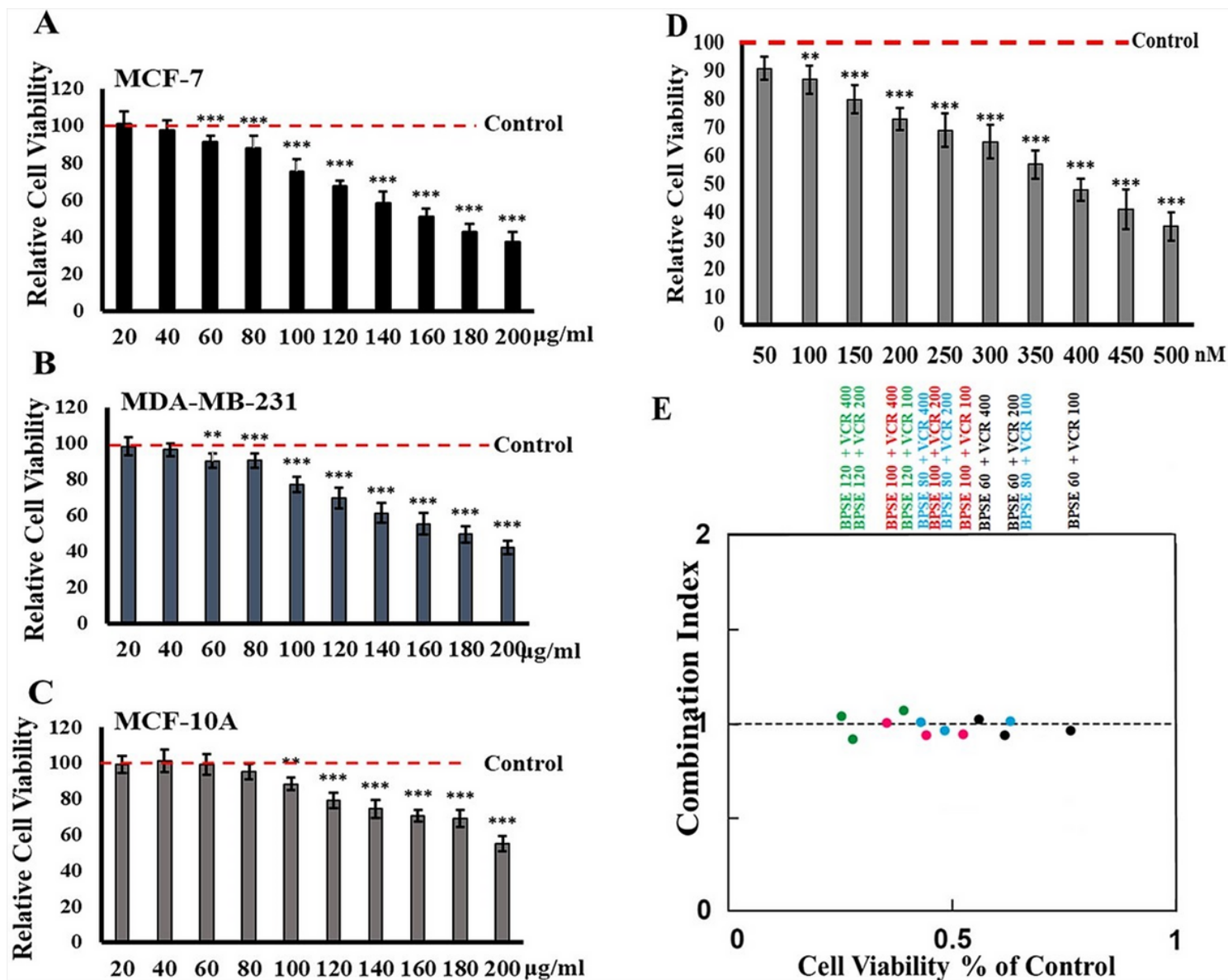


fig.2

Figure 2

The MTT assay results of the BPSE extract, VCR, and their combination. The cytotoxicity of different concentrations of BPSE on MCF-7 (A), MDA-MB-231(B), and MCF-10A cells (C) after 24h treatment. D) The cytotoxicity of VCR on MCF-7 cells after 24h. E) The cytotoxic effect of combined doses of BPSE and VCR on MCF-7 cells after 24h, indicating a cumulative effect with a combination index of 1 ± 0.08 . **: p 0.01, ***: p 0.001, indicate levels of significance.

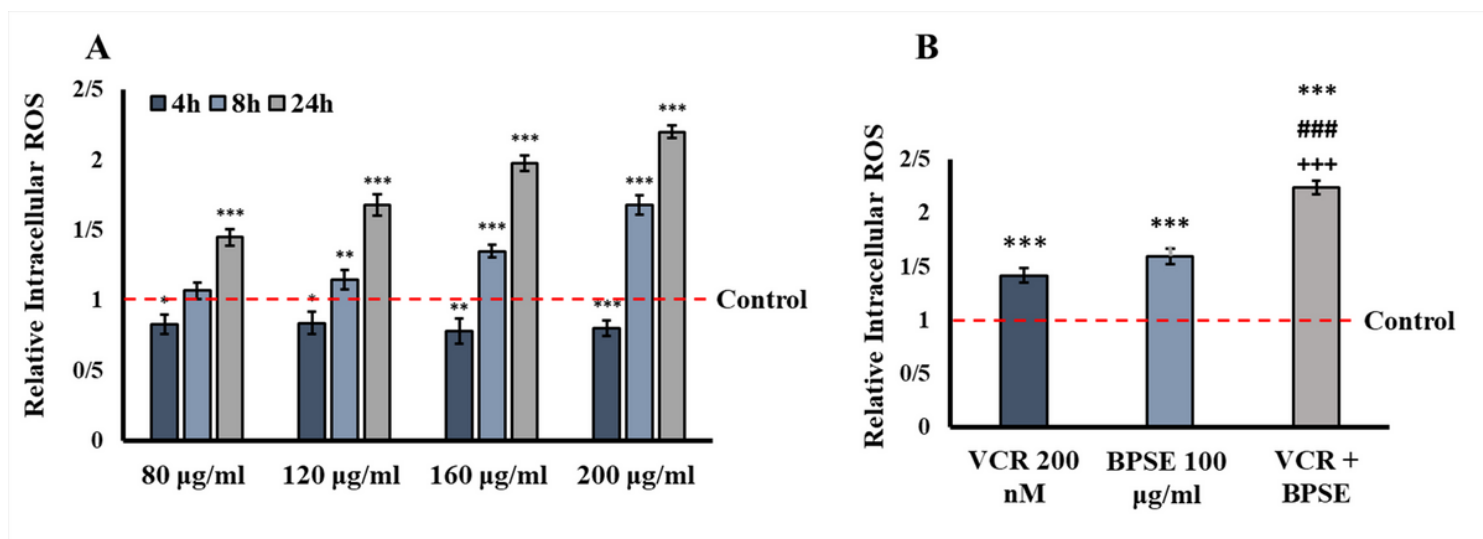


fig.3

Figure 3

The changes in intracellular ROS levels of MCF-7 cells after treatment with BPSE, VCR, and their combination. A) The ROS level after treatment with different concentrations of BPSE at different times. B) Changes in ROS level of cells treated with VCR, BPSE, and their combined dose (200 nM:100 µg/ml) after 24h. *, **, and *** show significant differences compared to the control with $p = 0.05$, 0.01 , and 0.001 . ### indicates a significant difference compared to VCR at $p = 0.001$. +++ shows a significant difference with BPSE at $p = 0.001$.

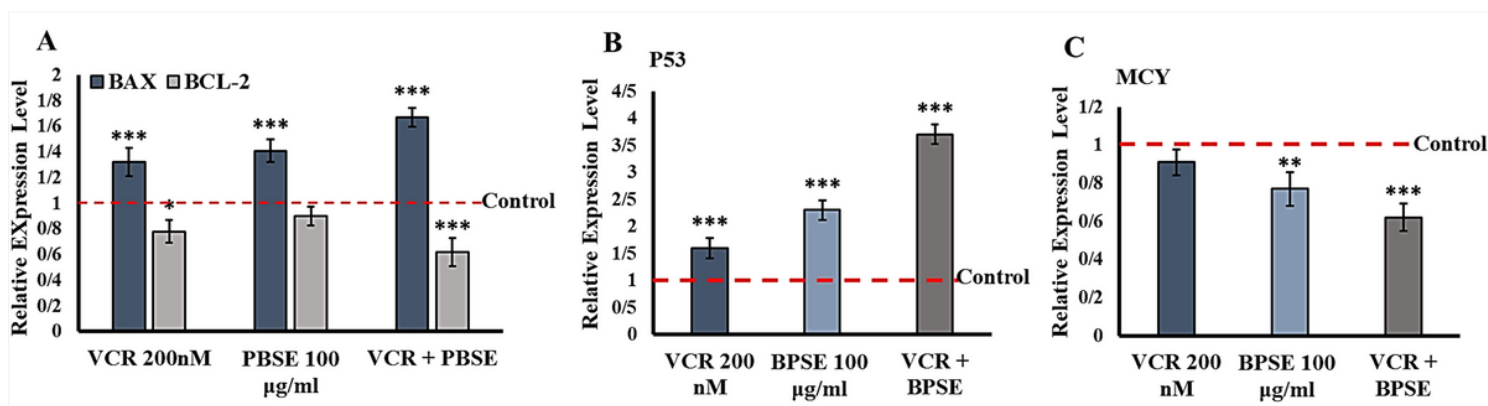


fig.4

Figure 4

The effects of BPSE, VCR, and their combination on the expression of *BAX*, *BCL-2*, *P53*, and *MCY* genes.

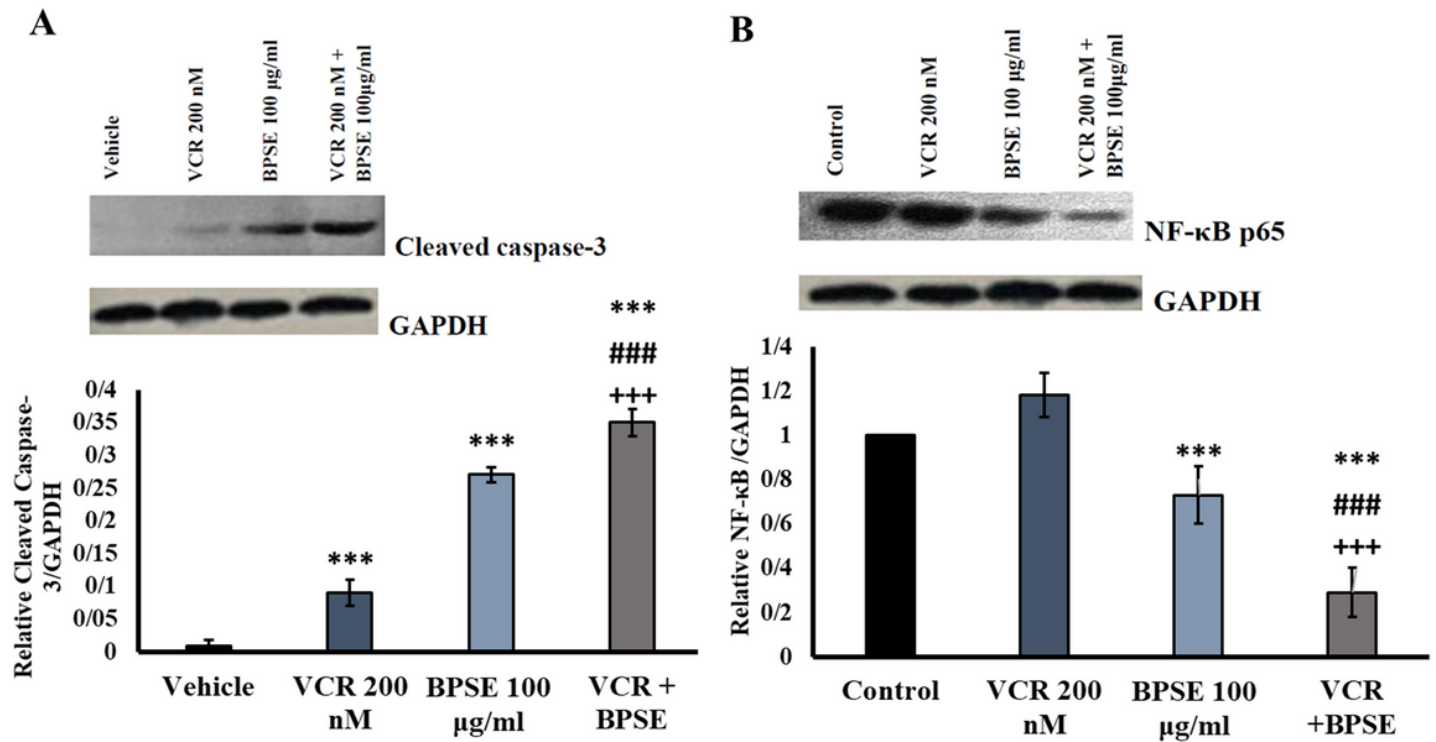


fig.5

Figure 5

Western Blot analysis of NF-κB expression (A) and cleavage of caspase-3 (B) in MCF-7 cells exposed to the VCR, the extract, and their combination. ***indicates a significant degree of control ($p < 0.001$). ### indicates a significant degree of VCR ($p < 0.001$). +++ indicates a significant degree of BPSE ($p < 0.001$).

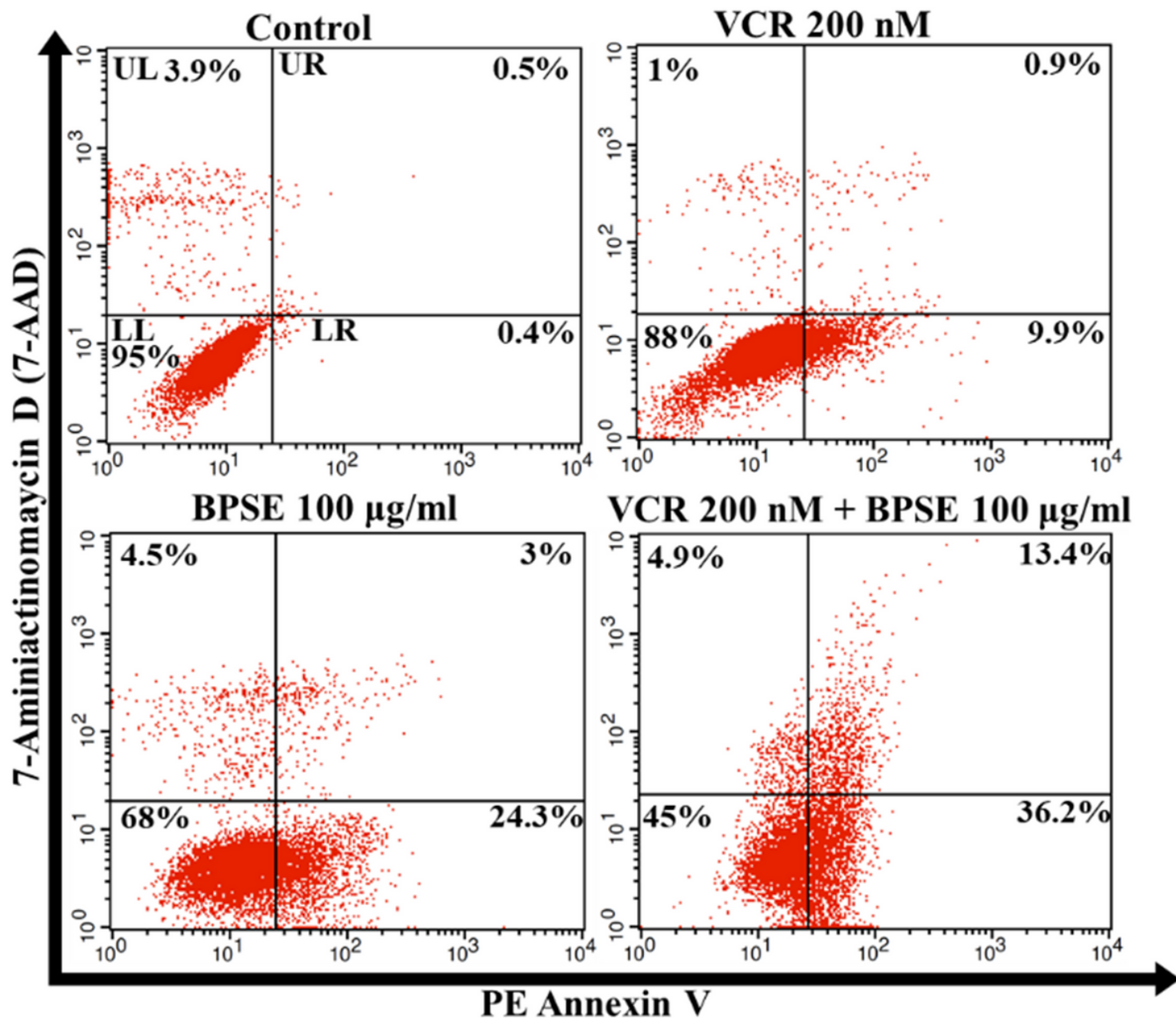


fig.6

Figure 6

The flow cytometry analysis of the apoptotic effect of BPSE, VCR, as well as their combination on MCF-7 cells for 24h. UL: necrosis, LR: primary apoptosis, UR: secondary apoptosis, LL: living cells.

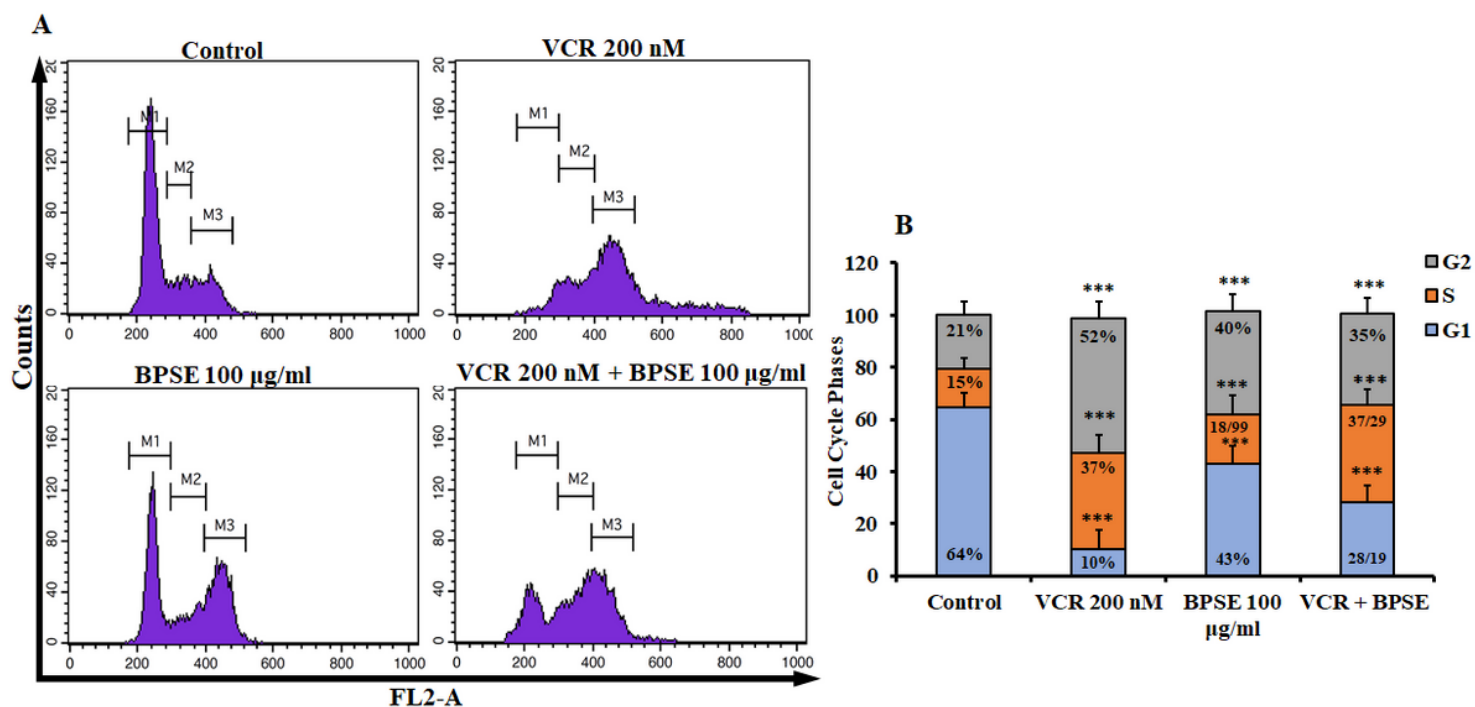


fig.7

Figure 7

Cell cycles assay of MCF-7 cells after 24h treatment. A: the effect of the BPSE (100 µg/ml), VCR (200 nM), and their combined dose on the cell cycles of MCF-7. (M1: G1 phase. M2: S phase. M3: G2 phase). B: Chart of relative cell cycle phases in different treatments. ***: significance levels compared to the control group (p 0.001).

Supplementary Files

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