



Article

Plant Extracts from *Origanum vulgare* and *Vaccinium macrocarpon* Induce Apoptosis of Bone Metastasized Breast Cancer Cells in a 3D Bone-Mimetic Testbed of Bone Metastasis

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Abstract

Bone metastasis remains a fatal and incurable condition for patients with breast cancer, leading to skeletal deterioration. The bone microenvironment enhances tumor proliferation and chemoresistance, necessitating novel therapeutic strategies. To investigate the cytotoxicity of two phytochemically-enriched plant extracts: *Origanum vulgare* (O.V.) and *Vaccinium macrocarpon* (V.M.) against breast cancer cells in a bone-metastatic condition. MCF-7 and MDA-MB-231 cell lines were treated with O.V. and V.M. for 24 h, in both 2D and 3D bone metastatic conditions. Live cell imaging, Alamar blue viability assay, RT-PCR, and flow cytometry analysis were used to assess cytotoxicity, apoptosis activation, and changes in oxidative stress/mitochondrial activity. Both extracts significantly inhibited cancer cell growth in a dose-dependent manner, with differential sensitivity observed between cell lines. Based on IC₅₀ analysis, O.V. demonstrated greater efficacy against the bone metastatic MCF-7 cell line, while V.M. was more effective against the bone metastatic MDA-MB-231. Apoptosis activation was confirmed via upregulation of pro-apoptotic proteins p53 and caspase-9. Importantly, we observed that normal bone cells were unaffected by the treatments. These findings elucidate the promising yet untapped potential of O.V. and V.M. extracts as robust therapies for bone metastasis.

Keywords: breast cancer; bone metastasis; 3D testbeds; apoptosis; *Origanum vulgare*; *Vaccinium macrocarpon*



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1. Introduction

By 2030, new cancer cases are expected to reach 23 million [1]. In 2024, roughly 310,720 new cases and 42,250 estimated deaths associated with breast cancer are predicted in women living in the United States [2]. From the primary site, breast cancer can eventually spread to distant organs through the process of metastasis due to factors such as attack from the immune system, lack of nutrients, and compressive stress from the microenvironment. It was reported that 70% of stage IV breast cancer patients develop bone metastasis, where patients experience side effects including bone fractures, cancer cachexia, hypercalcemia, and spinal cord compression, leading to poor quality of life and eventually death [3].

There is a necessity for new anti-cancer therapies with limited side effects to combat bone metastasis. Chemotherapy and invasive surgery have been the main treatment options for late-stage cancer. Pharmacologically, drugs such as Doxorubicin and Paclitaxel are examples of drugs used to block the formation of new tumors in diagnosed patients [4]. For late-stage pathogenesis, these options are intended to prolong life and provide palliative care. However, these options cause unwanted side effects and do not cure symptoms. As an alternative, phytochemically enriched plant or herbal extracts have been investigated as potential anti-cancer therapies [5–11].

Two phytochemically-enriched plant extracts, *Origanum vulgare* (oregano extract, O.V.) and *Vaccinium macrocarpon* (cranberry extract, V.M.), are good sources of bioactive phytochemicals [12,13]. The O.V. is derived from the aerial part/shoot of the plant. It is commonly used in alternative medicine and is currently being explored in modern therapeutic approaches [13–15]. Previous studies have demonstrated antioxidant and anti-cancer properties of O.V. and its derivatives against different types of cancer: liver, stomach, colon, skin, prostate and breast cancer [16–21]. In breast cancer-related studies, O.V. was shown to decrease cell survival of MCF-7 cells and promote cytotoxic effects against MDA-MB-231 and HCT-116 cell lines [22–24]. V.M. is derived from the pomace of the cranberry fruit and is grown in the northeastern/central regions of the United States. In health applications, V.M. has been employed to remedy urinary tract infections, kidney stones, neurogenic bladder, inflammation, type 2 diabetes, and enlarged prostate conditions [25,26]. V.M. has been considered a prospective therapeutic for cancer diseases due to its rich phenolic antioxidant profile [27,28]. This is because cranberry derivatives have properties that modulate cell growth, proliferation, adhesion, and oxidative stress regulation [29]. Prior in vitro studies have demonstrated the efficacy of cranberry derivatives on different cancer types: breast cancer, prostate cancer, glioblastoma, and colon cancer [12,30,31]. For breast cancer, an inhibition of cell viability was found in MCF-7 after being treated with cranberry solution [32]. Additionally, increased apoptosis and cell cycle arrest levels were shown to improve upon exposure to the cranberry treatment. Both of these extracts have been profiled for their phytochemical constituents and characterized in previous work [33–35].

Our understanding of the metastatic cascade is necessary for developing effective therapeutics against breast cancer bone metastasis. Previously, researchers used two-dimensional monolayer systems to investigate signaling pathways and cellular behavior. However, the constraint of 2D (two-dimensional) cell models is the inability to mimic the 3D microenvironment. In a 3D (three-dimensional) microenvironment, cell-matrix and cell-cell communication regulate genotype and phenotype [36,37]. In prior studies, in vivo animal models were used to recapitulate different stages of pathogenesis by creating metastatic xenografts. However, limitations of in vivo models are the inconsistency of cells metastasizing within the animal and the difficulty of connecting data from clinical trials to animal studies. 3D in vitro models bridge this gap, where the 3D microenvironment is recreated while being relevant to human physiology, which is crucial in drug development and design, allowing us to observe precise drug responses. Previous studies reported various 3D cancer models for screening anti-cancer drugs [38–40]. Ongoing studies further improve the efficacy of in vitro systems towards immune, vascular, endocrine and mechanical factors.

Previously, we have also reported a bone-mimicking 3D testbed of breast and prostate cancer metastasis. The nano clay-based PCL (polycaprolactone) scaffold allows mesenchymal stem cells to osteogenically differentiate without needing osteogenic supplements [41–45]. Our group also optimized the tunability of in situ HAPclay (hydroxyapatite clay) scaffolds by modifying montmorillonite clay with different amino acids [46]. Further, the formation of mineralized bone tissue on nano clay-based PCL scaffolds by vesicular delivery by the osteogenically differentiated hMSCs (human mesenchymal stem cells) was observed. Addi-

tionally, a sequential culture method was noticed using osteogenically differentiated hMSCs with human breast cancer cells (commercial and patient-derived) and commercial human prostate cancer cells, mimicking late-stage pathogenesis [47–51]. Further studies reported the alterations in breast cancer biomechanics during cancer progression [52]. Prostate cancer to bone has been reported in dynamic culture conditions using bioreactor systems [53,54]. The impact of breast cancer on osteogenesis was found to be mediated by Wnt/beta-catenin signaling at bone metastasis [55]. We have reported the potential of this testbed to accurately screen commercial drugs and phytochemical-enriched compounds [56–58].

Although O.V. and V.M. plant extracts have been investigated against various cancer cell lines, their effect against bone metastasis has never been reported. This research study presents the evaluation of two phytochemically enriched plant extracts, O.V. and V.M., as therapies against bone metastasized breast cancer. Concurrently, for the first time, we also explored the effects of both extracts on healthy bone tissue.

2. Results

2.1. Bone Metastatic Breast Cancer Cells Require Higher Dosages to Attain Equivalent Decrease in Cell Viability

Cell viability assay was employed to observe the cytotoxic effects of O.V. and V.M. treatment on 2D and 3D BM cultures. Cells were treated with 100, 200, 400, and 800 ppm of both phytochemically-enriched plant extracts for 24 h. The IC₅₀ concentration was determined from the dose-response study using curve fitting. Treatments of O.V. and V.M. induced various responses in 2D and BM cultures. After treatment of O.V., BM MM231 and BM MCF-7 experienced a reduction in viability with increasing dosages. However, BM cultures had a higher live cell percentage compared to 2D cultures. The IC₅₀ drug concentrations of BM MM231 and BM MCF-7 are 767.4 ppm and 590.3 ppm, respectively (Figure 1a,b).

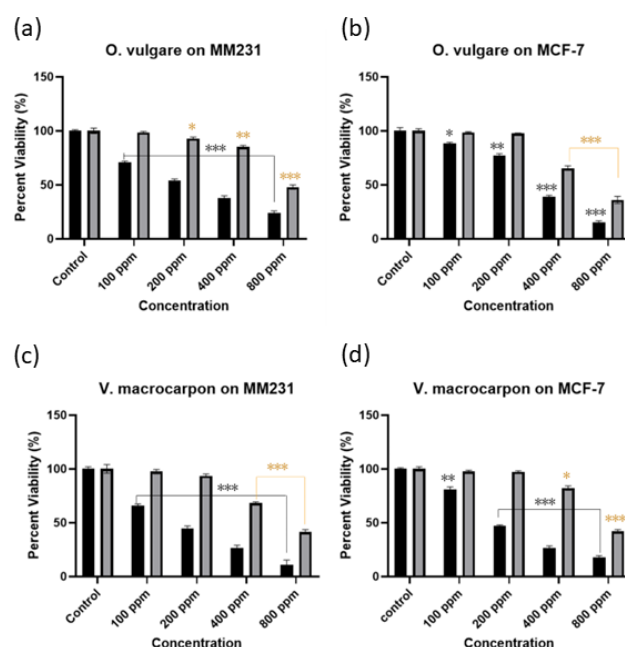


Figure 1. Viability of 2D (Black) and BM (Gray) breast cancer cultures after 24 h of treatment. Viability of 2D and BM breast cancer cultures after 24 h of treatment. (a–d). O.V. (a,b) and V.M. (c,d) cytotoxic effects were observed. Both cultures of breast cancer (MM231 and MCF-7) were evaluated. The concentrations of 0, 100, 200, 400, and 800 ppm were used. Cell viability was determined using the Alamar blue assay. The asterisk symbol (*) indicates a statistically significant difference between drug-treated and non-treated samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In contrast, the IC₅₀ concentration of MCF-7 and MM231 cancer cells in 2D cell cultures was reduced in comparison, with values of 310.7 ppm and 240.2 ppm, respectively (Figure 1a,b). Alternatively, post-treatment of V.M., BM MM231, and BM MCF-7 experienced opposite responses compared to O.V. treatment. BM MCF-7 required a higher IC₅₀ dosage of V.M. compared to BM MM231. Even though BM cultures had a higher viable cell percentage compared to 2D cultures. The IC₅₀ (half maximal inhibitory concentration) drug concentrations of BM MM231 and BM MCF-7 are 660.8 ppm and 735.7 ppm, respectively (Figure 1c,d). In contrast, the IC₅₀ concentrations of MCF-7 and MM231 cancer cells in 2D cell cultures were smaller compared with values of 194.2 ppm and 185.5 ppm, respectively (Figure 1c,d). Overall, our results indicate that 3D BM cultured breast cancer cells commanded higher dosages of O.V. and V.M. compared to 2D cultured breast cancer cells. Furthermore, treatment with O.V. was more effective against BM MCF-7, whereas V.M. was more effective against BM MM231.

2.2. Bone Cells Are Unaffected After *Origanum vulgare* and *Vaccinium macrocarpon* Treatment

We evaluated the cytotoxic response of bone (hMSCs grown on a 3D testbed for 33 days). Bone cells were treated for 24 h with select concentrations of 100 ppm, 200 ppm, 400 ppm, and 800 ppm of both O.V. and V.M. We observed no reduction in cell viability in all concentrations for both treatments (Figure 2a). Furthermore, live-dead images confirmed that bone cells were unaffected after 24 h of treatment with 800 ppm of both extracts (Figure 2b). Overall results indicate that both phytochemical-enriched plant extracts are non-lethal toward normal bone cells.

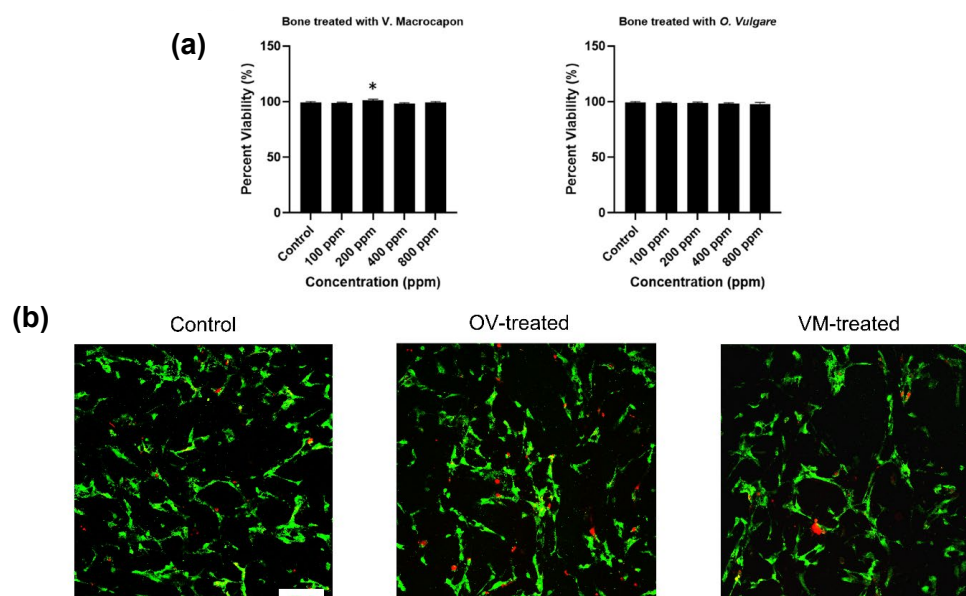


Figure 2. Viability of bone cells after treatment of V.M. and O.V. (a). The effects of O.V. and V.M. on bone (33-day hMSC) after 24 h. The concentration dosages used were 100, 200, 400, and 800 ppm. Alamar blue assay was used to evaluate cell viability. (b) Live/dead imaging of 33-day bone untreated and treated with 800 ppm O.V. and V.M. for 24 h (scale bar: 250 μ m). Live cells are indicated by a green stain (FITC), and dead cells are indicated with a red stain (TRITC). The asterisk symbol (*) indicates a significance between drug-treated and non-treated samples. * $p < 0.05$.

2.3. O.V. and V.M. Induce Apoptosis in 2D and 3D BM Cultures of Breast Cancer Cells Within 12 h

BM breast cancer cells were incubated with their corresponding IC₅₀ dosages of O.V. and V.M. to confirm the resulting cytotoxicity (Figure 3a,b). Live-dead imaging indicated that with treatment, the percentage of live cells decreased and the percentage of dead cells

increased. Images indicated that BM MM231 had a similar number of live cells (green stain) after O.V. and V.M. treatment. Meanwhile, BM-MCF-7 had more dead cells after O.V. treatment. Overall, treatment of O.V. and V.M. induced death in BM breast cancer cells.

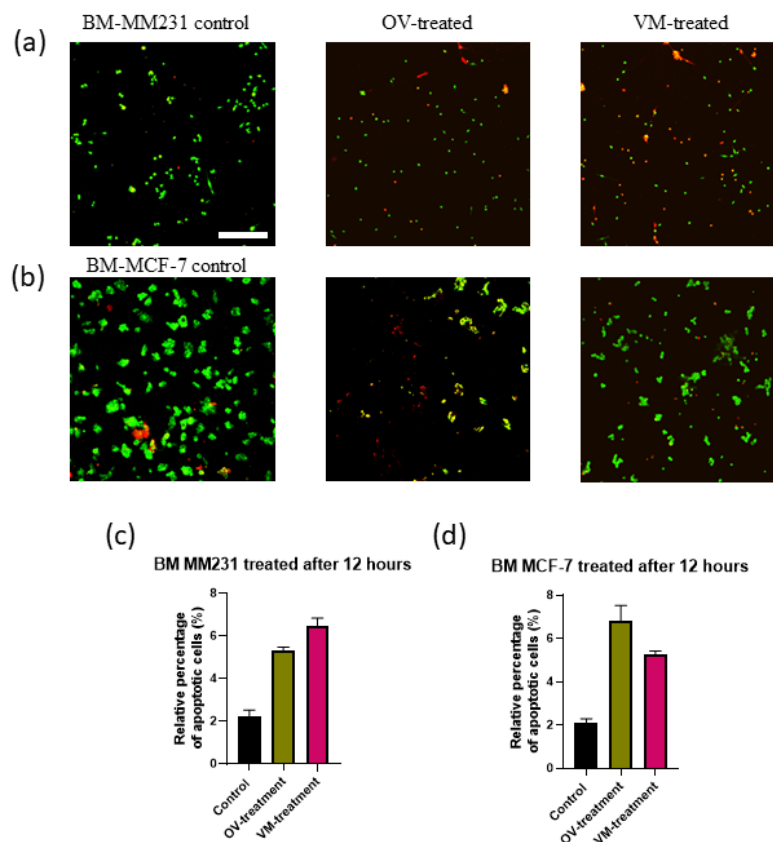


Figure 3. Effect of IC₅₀ concentrations assessed using live-dead imaging and flow cytometric analysis. (a,b) The cytotoxic effects of O.V. and V.M. were observed in 3D BM culture of breast cancer cells by live-dead staining. (c) Bar plots representing the percentages of apoptotic cells for 3D BM cultures of MM231. (d) Bar plots representing the percentages of apoptotic cells for 3D BM cultures of MCF-7. (Scale bar: 250 μ m).

Additionally, breast cancer cell BM cultures were exposed to their particular IC₅₀ concentrations of O.V. and V.M. for 12 h. Screening of the drug paclitaxel for 48 h utilizing the breast cancer bone metastatic testbed was reported earlier [15]. Annexin V and propidium iodide stains were used to evaluate the percentage of cells that become apoptotic. We found that treated samples had an increased apoptosis rate compared to non-treated samples in 3D BM cultures (Figure 3c,d). As indicated, the results showed a significant difference between control and treated samples for 3D BM cultures. The percentage of the cell population that became apoptotic, induced by O.V. and V.M., was ~5.33 % and ~6.25% in 3D BM MM-231, respectively. The early-stage percentage of apoptosis was ~6.32% and ~5.7% in 3D BM MCF-7, respectively, demonstrating an increase in apoptotic cells to both O.V. and V.M. in 3D BM cultures.

2.4. 3D Bone-Metastatic Cells Are Resistant to Cell Death After Treatment with O.V. and V.M.

To confirm the cell death initiation, we evaluated Bcl-2 and p53 biomarkers in treated cultures. We compared the fold-change of mRNA expression levels between treated and untreated after 24 h of O.V. and V.M. treatment. We observed that 2D MCF7 cultures experienced a ~5.99-fold and ~7.13-fold change in p53 expression, compared to untreated cultures, respectively (Figure 4a). MCF-7 BM cultures experienced ~2.15-fold and ~1.78-fold increase in p53

expression, compared to untreated cultures. Alternatively, Bcl-2 levels were observed in treated 2D MCF-7 cultures and BM MCF-7 cultures. In 2D MCF7 treated cultures experienced a ~3.31-fold and ~4.07-fold reduction in Bcl-2 expression, respectively (Figure 4b). MCF-7 BM cultures experienced ~1.53-fold and ~1.75-fold reduction in bcl-2 expression, compared to untreated cultures. A similar trend was observed with MM231 2D and BM MM231 cultures. After 24 h of O.V. and V.M. treatment, MM231 2D cultures experienced a ~5.71-fold and ~6.22-fold increase in p53 expression compared to untreated cultures, respectively (Figure 4b). At the same time, MM231 BM cultures experienced ~1.84-fold and ~1.99-fold increase in p53 expression, compared to untreated cultures. Additionally, Bcl-2 levels in treated 2D MM231 cultures and BM MM231 cultures were observed. We observed that 2D-treated cultures experienced a reduced ~3.125-fold change and ~3.74-fold change in Bcl-2 expression, respectively (Figure 4b). Whereas MCF-7 BM-treated cultures experienced a reduced ~1.19-fold change and ~1.23-fold change in bcl-2 expression. Overall, breast cancer cells in bone metastatic conditions experience increased resistance to apoptosis after treatment with O.V. and V.M.

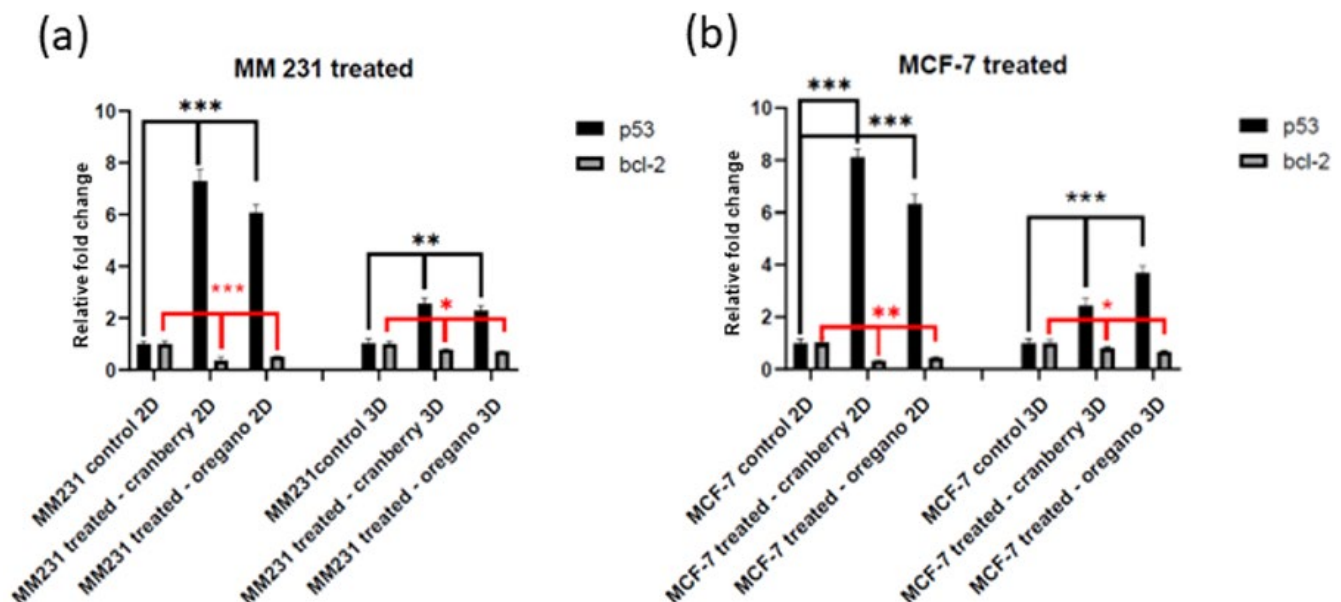


Figure 4. Pro- and anti-apoptotic markers p53 and bcl-2 were analyzed using RT-PCR. (a) Analysis of p53 and bcl-2 expression in 2D MCF7 cultures and BM MCF7 cultures. (b) Analysis of p53 and bcl-2 expression in 2D cultures of MM231 and BM cultures of MM231. As seen, there is a significant difference in p53 and bcl-2 expression between treated and untreated cultures of breast cancer. Cultures were treated with O.V. and V.M., the significance is presented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.5. O.V. and V.M. Treatment Does Not Induce Apoptosis in Bone Cells

We evaluated the apoptotic response of bone cells when treated for 24 h and compared the relative fold change of treated and non-treated samples for bone and 3D BM culture of breast cancer cells, MM231 and MCF-7. Samples were treated with O.V. and V.M. for 24 h. Firstly, Bcl-2 expression was compared between bone and 3D BM breast cancer cultures. In healthy bone, we observed no significant change in either treated conditions versus non-treated samples (Figure 5A–D). The same trend was observed, where there is a significant upregulation of p53 expression in treated samples on 3D BM culture of breast cancer cells. However, no significant change in treated versus non-treated bone cells was observed (Figure 5A–D).

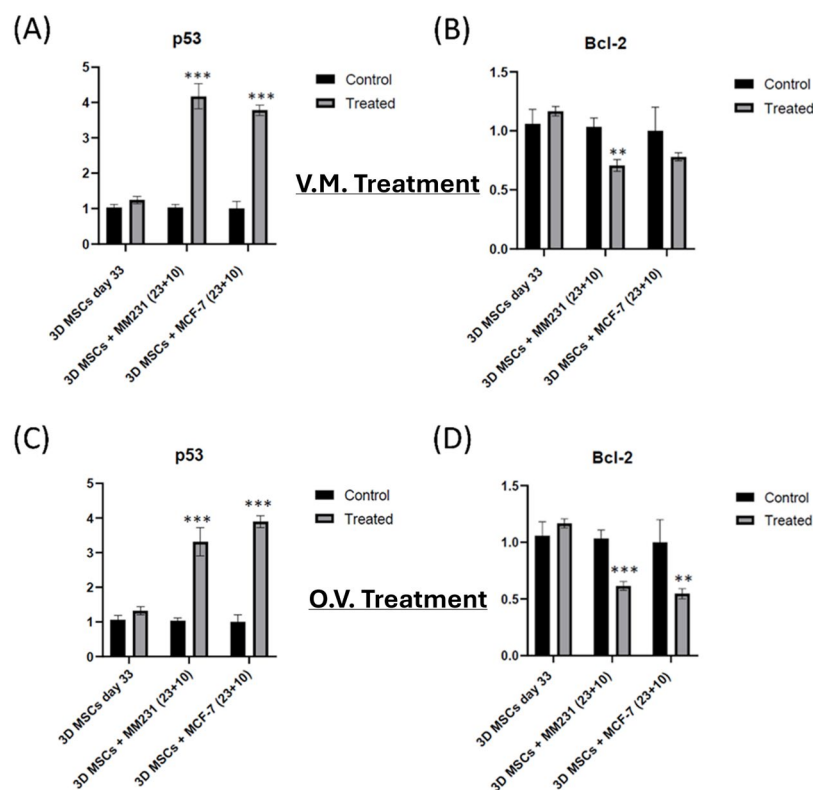


Figure 5. Apoptotic biomarkers experience no significant changes when a healthy bone is treated with O.V. and V.M. (A–D). (A,B) Expressions of p53 and bcl-2 are represented by bone compared to the 3D cultures (MM231 and MCF-7) after V.M. treatment. (C,D) p53 and bcl-2 expression is represented by bone compared to 3D BM cultures (MM231 and MCF-7) after O.V. treatment. Significance (*) indicates the significance of relative expression between treated and non-treated cultures. ** $p < 0.01$, *** $p < 0.001$.

2.6. Activation of Intrinsic Apoptosis in Bone Metastatic Breast Cancer, Induced via PE-Plant Extracts

Live cell imaging of bone metastatic cultures gave insight into the redox health of cancer cells after treatment with phytochemically enriched plant extracts. Both ROS levels and mitochondrial membrane potential of BM cultures were determined after treatment of both plant extracts: O.V. and V.M. We observed that the mitochondrial membrane potential staining was reduced in BM MM231 cultures after treatment of the three PE-plant extracts (Figure 6A). Consequently, we observed the ROS levels to be unchanged after the treatment in BM MM231. Alternatively, BM-MCF7 cultures experienced reduced mitochondrial membrane potential (Figure 6B). Live cell staining also showed reduced ROS levels after treatment.

Consequently, we confirmed the initiation of apoptosis by evaluating the expression of caspase-9. Our results show that when 3D BM cultures were treated with O.V. and V.M., caspase-9 expression levels were upregulated significantly in treated samples compared to non-treated samples (Figure 6C). Furthermore, BM MCF-7 cells experienced higher fold expression compared to BM MM231 cells. MM231 expressed more caspase-9 when treated by V.M., while MCF-7 expressed more caspase-9 when treated by O.V. Specifically, MCF-7 BM cells treated with O.V. experienced nearly ~5.89-fold upregulation in caspase-9 levels, respectively. Meanwhile, the MM-231 BM cells experienced nearly a ~3.97-fold activation in caspase-9 levels. Alternatively, MCF-7 BM cells treated with V.M. experienced nearly ~4.15-fold upregulation in caspase-9 levels, respectively. MM-231 BM cells experienced nearly ~4.31-fold activation in caspase-9 levels.

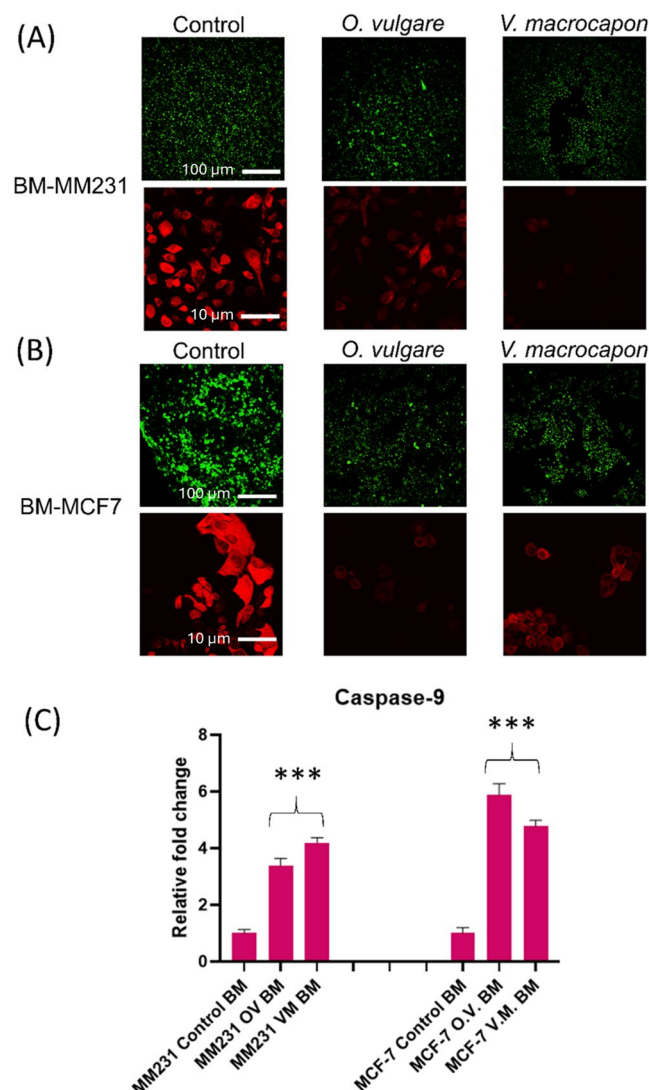


Figure 6. Alterations in ROS/MtMP levels and caspase-9 in bone-metastatic breast cancer. **(A,B)** Live cell imaging of BM breast cancer cells (MM231 and MCF-7). ROS assay (green staining) and MtMP assay (red staining) were performed for treated and untreated samples. **(C)** O.V. and V.M. treatment activates caspase-9 in bone metastatic breast cancer (MM231 and MCF-7). Caspase-9 expression was measured after 24 h of treatment. To indicate the significant difference in expression of caspase-9 between non-treated cultures and treated breast cancer cultures (MM231 and MCF-7), *** $p < 0.001$. Scale bar for ROS staining: 100 µm. Scale bar for MTMP staining: 10 µm.

3. Discussion

Breast cancer cells experience altered growth and chemoresistance when arriving at the bone site. Bone-like ECM formation is observed from MSCs, which differentiate osteogenically on nano-clay scaffolds, and enhance the movement of cancer cells toward the bone microenvironment. Two phytochemically-enriched plant extracts, O.V. and V.M., contain many different bioactive phytochemicals. The IC₅₀ results presented here suggest that O.V. is more suitable for targeting hormone-positive breast cancer. V.M. is more suitable to targeting MM231. Furthermore, it was found that treated samples had an increased apoptosis rate compared to non-treated samples, for both O.V. and V.M. In breast cancer-related studies, O.V. was shown to decrease cell survival in MCF-7 cells and induce cytotoxic effects against HCT-116 and MDA-MB-231 cell lines [22]. Earlier studies have reported the efficacy of cranberry derivatives on different cancer cell lines [31,59]. Cell viability was decreased for breast cancer in MCF-7 and MDA-MB-435 cell lines [32]. Additionally,

increased apoptosis and cell cycle arrest levels were shown to increase upon exposure to the drug. However, unlike previous studies, our work focuses on the cytotoxic and metabolic response of bone metastatic breast cancer. To this date, studies have yet to investigate the effect of these plant extracts on healthy bone. Our results show that neither O.V. nor V.M. negatively affected the normal bone tissue. After 24 h, viability did not change, even with increasing dosages.

There are several studies in the literature that demonstrate that differences in p53 expression and function between the breast cancer cell lines MCF-7 and MDA-MB-231 [10–12], and thus substantially influence their responses to therapy. MCF-7 cells typically harbor wild-type p53, causing increased apoptosis and cell-cycle arrest under treatments such as doxorubicin, whereas MDA-MB-231 cells express a mutant, stabilized form of p53 that does not respond equivalently to stress, contributes to survival signaling, and shows attenuated p53-mediated apoptotic pathways, resulting in differential sensitivity to chemotherapeutics and altered apoptotic marker profiles between the two lines (e.g., p53 levels rise with treatment in MCF-7 but remain unchanged in MDA-MB-231, and Bax/Bcl-2 ratios and apoptotic responses differ accordingly) [12]. Thus, the protein p53, a tumor suppressor, is mainly implicated in the regulation of the cell cycle and DNA repair [60,61]. In response to DNA damage, apoptosis can be triggered by activation of p53. Previous studies have demonstrated activation of p53 in MDA-MB-231 and MCF7 breast cancer cell lines, from different compounds [62,63]. Bcl-2 is part of the anti-apoptotic family of proteins, which are recognized to be elevated in cancer cells resistant to chemotherapy. Activation of Bcl-2 proteins leads to inhibition or prevention of cellular apoptosis. We observed activation of p53 in all treated samples. Furthermore, 2D cultures experienced higher fold expression compared to 3D BM cultures. The dysregulation of p53 can be linked to chemoresistance in cancer. Additionally, we found 3D BM breast cancer cells had higher fold expression of Bcl-2 compared to 2D cultures. Overall, the presence of the bone microenvironment increased resistance to apoptosis in breast cancer cells. Changes in apoptotic expression were not observed in treated bone tissue. Overall, O.V. and V.M. are potential anti-cancer candidates for bone metastatic breast cancer that are also non-toxic to bone.

Many reports in the literature indicate that Caspase-3/p53 are significant markers of intrinsic apoptosis [64–67]. When apoptosis occurs, cells undergo a reduction in mitochondrial potential and increased ROS production [68]. Intrinsic apoptosis is signified by the stimulation of caspase-9 and effector caspase [69]. They directly affect the mitochondria, thus regulating/initiating ROS production. caspase-9 directly activates caspase-3 [70]. The activation of caspase-3 allows for cell death to be more efficient. We observed that ROS levels decreased post-treatment in bone metastatic cultures, while caspase-9 activations were upregulated. Specifically, our results demonstrate that when 3D cultures were treated with all three extracts, the caspase-9 mRNA expression levels were upregulated. Initiation of apoptosis can be correlated with changes in redox activities. ROS and MtMP were qualitatively measured to understand apoptosis in relation to redox regulation. We observed that the mitochondrial membrane potential was lost in the treated bone metastatic cultures. Indicating that the plant extracts promoted apoptosis through reduction in membrane potential, via stress and bioenergetic loss. Overall, our results suggest that phytochemically-enriched O.V. and V.M. can initiate cell death in both primary site and secondary site bone metastatic breast cancer.

In summary, to this date, the cytotoxic effects of O.V. and V.M. extracts against bone metastasis and healthy bone tissue have yet to be investigated. Here, we report the significant cytotoxicity of both phytochemical-enriched plant extracts against the bone metastasis of breast cancer. Utilizing a 3D bone metastatic testbed, we tested these extracts against breast cancer that has metastasized to bone. Our results indicate that the initiation of

apoptosis is activated intrinsically and is possibly redox-regulated. In addition, these plant extracts induce no apparent toxicity to healthy bone cells, while the observed potentially healing effect will need to be evaluated further. Future studies are needed to study the redox and enzymatic activity of BM breast cancer cells, before and after treatment. Overall, we present the tremendous potential of phytochemically-enriched plant extracts (O.V. and V.M) for therapies for bone metastasis of breast cancer, and they should be considered for clinical trials.

4. Materials and Methods

4.1. Phytochemically-Enriched Plant Extract Solution Preparation

V.M. extract was purchased from Decas Cranberry Products Inc. (Carver, MA, USA). O.V. extract was purchased from Barrington Nutritionals (Harrison, NY, USA). Initially, a stock solution (1000 ppm) of O.V. and V.M. was prepared by dissolving 0.1 g of each herbal extract (dry powder) in 100 mL of 10% ethanol solution. Next, the phytochemically-enriched therapeutic solution was sterilized with a 0.22 μ m filter. Further, the stock solutions were diluted in serial dilutions using serum-free DMEM (ATCC, Manassas, VA, USA). Molecular-grade water (ATCC, Manassas, VA, USA) was used to prepare the 10% ethanol solution and plant-extract solutions.

4.2. Preparation of Nanocomposite PCL/In Situ HAPclay 3D Scaffolds

We followed the methods reported earlier for preparation of PCL/in situ HAPclay scaffolds [51,71]. In order to enhance the d-spacing between clay sheets, 5-aminovaleic acid is first used to alter Na-MMT clay. The University of Missouri: Clay Minerals Respiratory supplied the Na-MMT clay. After that, hydroxyapatite (HAP) is biomineralized inside the intercalated nano-clay galleries to create in situ HAP clay. We bought sodium phosphate (Na_2HPO_4), calcium chloride (CaCl_2), polycaprolactone (PCL), 5-aminovaleic acid, and 1,4-dioxane from Sigma Aldrich in St. Louis, MI, USA. 1,4-dioxane is combined with 10% in situ HAPclay and PCL. After stirring, the mixture was transferred into cylindrical molds. Scaffolds of PCL/in situ HAP clay were created by the freeze-drying technique. The scaffolds were employed for the studies after being cut to 3 mm thick, with a 12 mm diameter.

4.3. Cell Culture and Cell Seeding

Human breast cancer cell lines (MDA-MB-231 and MCF-7) were sourced from the American Type Culture Collection (ATCC, Manassas, VA, USA), and hMSCs were purchased from Lonza (Rockville, MD, USA). The authentication of breast cancer cell lines was defined using short tandem repeat (STR) profiling, which had been conducted within the past three years at ATCC. hMSCs were authenticated using HLA-A, HLA-B, and HLA-C testing. All experiments were performed using mycoplasma, virus-free cells.

The media used for MDA-MB 231 (MM231) cells consisted of 10% FBS, 89% Dulbeccos Modified Essential Medium (DMEM), and 1% P/S. 89% Eagle's Minimum Essential Medium (EMEM), 10% FBS, 0.01 mg/mL human recombinant insulin, and 1% P/S were used to cultivate MCF-7 cells (ATCC, Manassas, VA, USA). An MSCGM bullet kit media (Lonza, Rockville, MD, USA) was used to sustain human mesenchymal stem cells. Every culture was maintained in a humidified incubator at 37 °C and 5% CO_2 . 5×10^4 breast cancer cells were cultivated on 2D tissue culture polystyrene (TCPS), whereas breast cancer cell lines were employed at passages 4–8, and hMSCs from passages 2–5. Scaffold sterilization begins with 70% ethanol immersion for 24 h, followed by 45 min of UV irradiation. After that, they had two 12-h PBS washes before being kept in a CO_2 incubator submerged in a culture medium. Then, before adding culture medium, 1×10^5 hMSCs per scaffold were sown and incubated for 4 h. To develop a bone niche, scaffolds seeded with cells were

grown for 23 days. Then, on a bone-mimetic testbed, 1×10^5 breast cancer cells were sown per scaffold and cultivated for 10 days (Figure 7).

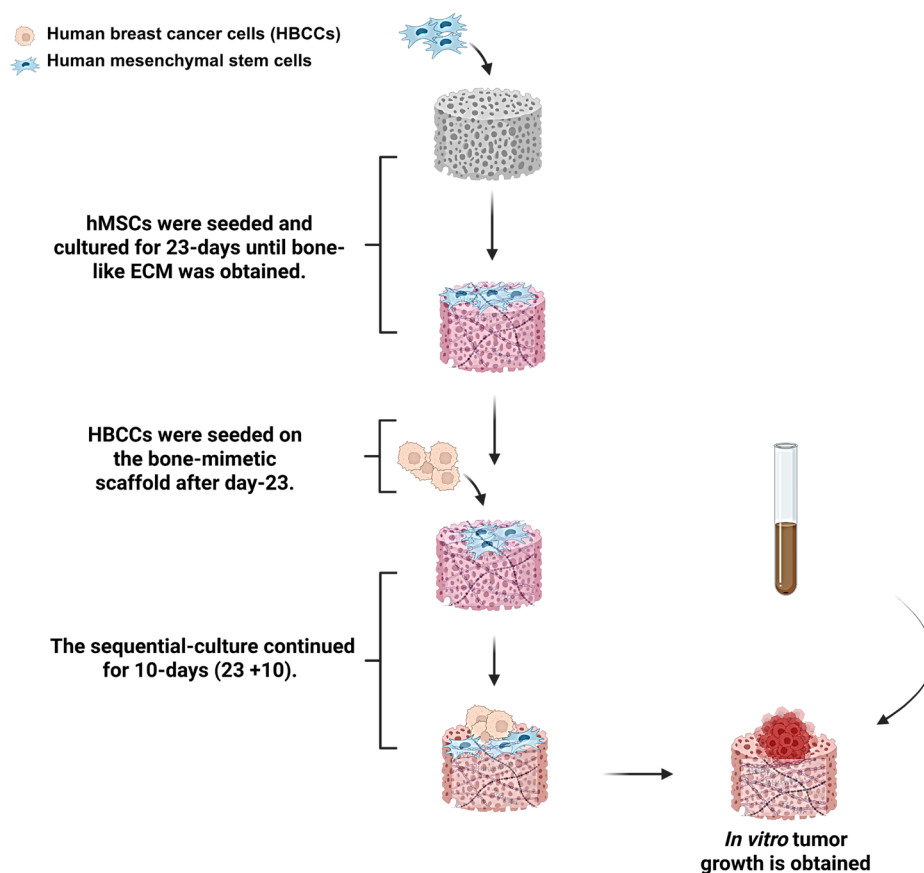


Figure 7. Schematic illustrating the sequential culture steps in creating the bone niche and then recapitulating the bone metastasis of breast cancer. Created in Biorender. Preetham Ravi. (2024). <https://app.biorender.com/illustrations/6361a8453a1b5ae370700561?slideId=e4d01940-f7bd-4e87-a2bd-cacdad76a4f5> (accessed on 27 February 2024).

4.4. Cell Viability Assay

Breast cancer and bone cultures were made serum-free for 24 h before treatment. Afterwards, all cultures were incubated with various concentrations (0, 100, 200, 400, 800 ppm) of O.V. and V.M. solutions for 24 h. Alamar blue assay was executed to assess the dose-dependent viability of treated and untreated (control) samples, per the guidance of the manufacturer's protocol (Invitrogen, Cambridge, MA, USA). Briefly, after 24 h, cells were rinsed with PBS (phosphate-buffered saline). Next, Alamar blue stock solution was diluted 1:10 in phenol red-free DMEM and added to the cultures for 4 h. After, fluorescence was measured at 570 nm. Determination of half-maximal inhibitory concentrations (IC_{50}) for all samples was calculated using GraphPad Prism (v7.04).

4.5. Live/Dead Assay

Live/dead assay was used to determine the viability of MM231 and MCF-7 bone metastatic cultures and normal bone cultures. Samples imaged were untreated and treated with IC_{50} concentrations with O.V. and V.M. Cells seeded on the different scaffolds were stained with a live/deadTM Cell Imaging Kit (Thermofisher Scientific, Dreieich, Germany) according to the manufacturer's protocol. Live/dead solution was prepared by mixing the Calcein AM solution and BOBO-3 iodide and diluting it to a working concentration with PBS. Next, treated and untreated samples were incubated with Calcein AM and BOBO-3 iodide. Lastly, scaffolds were kept at room temperature for 15 min. Imaging was performed

using the Olympus–Bruker Bio-AFM confocal system and FITC/TRITC filters. (Olympus, Tokyo, Japan and Bruker, Billerica, MA, USA).

4.6. Flow Cytometry–Apoptosis Assay

We followed the methods of Kar, Sumanta et al. 2023 [51]. Cells were dosed with corresponding IC50 values for 12 h in order to perform flow cytometric analysis. After that, both treated and untreated cells were taken out and given three rounds of washing in cold PBS. Cells were labeled with propidium Iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V, after they had been remixed in cold annexin binding buffer, with a specific concentration of 1×10^6 cells/mL. The BD Accuri C6 Flow Cytometer was used to run the samples, and FlowJo v10 software (BD Biosciences, Franklin Lakes, NJ, USA) was used for processing.

4.7. Reactive Oxygen Species (ROS) Assay

Live bone-metastatic cultures of MM231 and MCF-7 were stained following the manufacturer's protocols (Abcam, Waltham, MA, USA). Briefly, samples were treated with O.V. and V.M. for 12 h, then rinsed with PBS before adding DCFDA solution. The DCFDA solution was diluted and added to the samples. Then, samples were stored in the dark, at 37 °C, for 45 min. After that, samples were rinsed with $1 \times$ buffer and then imaged. Live cell imaging was performed using an Olympus—Bruker bio-AFM confocal system with a FITC filter (Olympus, Tokyo, Japan and Bruker, Billerica, MA, USA).

4.8. Mitochondrial Membrane Potential (MtMP) Assay

Manufacturer's protocols were followed for live cell imaging (Cell Signaling Technology, Danvers, MA, USA). Bone metastatic cultures of MM231 and MCF-7 were treated with O.V. and V.M. for 12 h. After, the samples were washed with PBS. Next, 200 nM of TMRE labeling solution was added to all samples. Samples were placed in a CO₂ incubator for 20 min. Samples were imaged using an Olympus bio-AFM confocal system, using the orange-red filter.

4.9. Gene Expression Studies

Before seeding breast cancer cells, hMSCs were cell cycle-arrested with ten µg/mL of Mitomycin C. Both 2D, 3D-BM, and bone cultures were treated with IC50 drug concentration. After 24 h of treatment, RNA was separated using the Direct-zol RNA isolation kit (Zymo Research, Irvine, CA, USA). To produce cDNA, extracted RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA), arbitrary primers, and a heat cycler (Applied Biosystems, Carlsbad, CA, USA). Lastly, Real-Time Polymerase Chain Reaction (RT-PCR) was conducted using thermal treatments consisting of a holding stage with 2 min at 50 °C and 10 min at 95 °C, followed by a cycling stage of 40 cycles of 15 s at 95 °C, and 60 s at 60 °C using a 7500 Fast Real-Time System (Applied Biosystems, Carlsbad, CA, USA). Table S1 presented in the supplementary document contains primer sequences of genes analyzed in RT-PCR experiments. Differences in p53, Bcl-2, and caspase-9 mRNA expression between treated and untreated cultures were measured. Gene expressions were normalized to β-actin. Target gene expressions were calculated using the comparative C_t method ($2^{-\Delta\Delta C_t}$).

4.10. Statistical Analysis

It was determined that the data were displayed as mean ± standard deviation (n = 3). The post hoc Tukey test was used after the one-way ANOVA to determine the statistical significance/p-values among numerous comparisons. Using GraphPad Prism v7.04 and an

unpaired Student's *t*-test, statistical differences between the treated and untreated groups were deemed significant at a 95% probability level ($p < 0.05$).

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052355/s1>.

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Conflicts of Interest: The authors K.K., D.K., K.S., D.S. have applied for a patent related to phytochemicals as anticancer therapy. The authors K.K., D.K. and K.S. have a startup company, 3D Phytochemicals for Cancer Therapeutics, LLC.

Abbreviations

The following abbreviations are used in this manuscript:

BAX	Bcl-2-associated X protein
Bak	Bcl-2 homologous antagonist/killer
Bcl-2	B-cell lymphoma
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
ER	Estrogen Receptor
EMEM	Eagle's Minimal Essential Medium
FBS	Fetal Bovine Serum
HAP	Hydroxyapatite
HER2	Human epidermal growth factor receptor 2
hMSCs	Human mesenchymal stem cells
IC-50	Half-maximal inhibitory concentration
MCF-7	Michigan Cancer Foundation 7
MDA-MB-231	Monroe Dunaway Anderson-Mammary Luminal B-231
MtMP	Mitochondrial Membrane Potential
O.V.	<i>Origanum Vulgare</i>
p53	Tumor suppressor protein p53
PCL	Polycaprolactone
ROS	Reactive Oxygen Species
V.M.	<i>Vaccinium Macrocarpon</i>
FITC	Fluorescein isothiocyanate
PBS	Phosphate-buffered saline
Caspase	Cysteine-aspartic acid protease

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