**RESEARCH ARTICLE**

# Suppressive role of *Viola odorata* extract on malignant characters of mammosphere-derived breast cancer stem cells

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## Abstract

**Background** Mammospheres are breast cancer stem cells (BCSCs) that could be yielded through culturing cells in non-adherent and non-differentiating condition. With regard to therapy resistance of cancer stem cells (CSCs), it is essential to discover efficient approaches targeting CSCs. *Viola odorata* extract has been considered as a traditional herbal anti-metastatic drug in several cancer cells. Effect of this drug on BCSCs has not been clearly identified. Current study tries to detect and to compare effect of *Viola odorata* extract on malignant characterization of breast cancer cell lines and BCSCs.

**Materials and methods** MCF7 and SKBR3 and their derived mammospheres as BCSCs were used and the effect of alcoholic extraction of *Viola odorata* on apoptosis and malignant characters of MCF7, SKBR3 and their derived BCSCs were analyzed and compared.

**Results** *Viola odorata* extract induced cell death in MCF7, SKBR3 and their derived mammospheres through apoptosis without any effects on MCF10A. Also, this extract showed anti-migratory, anti-invasion and anti-colony formation activity in MCF7, SKBR3 and their derived mammospheres which was significantly more in MCF7- and SKBR3-derived mammospheres. Also, this extract decreased size and volume of tumors generated by MCF7, SKBR3 and their derived mammospheres in chicken embryo model.

**Conclusion** *Viola odorata* extract exerted anti-cancerous activity on both breast cancer cell lines and their derived BCSCs. Anti-cancerous activity of this extract was significantly more in MCF7-, SKBR3-derived mammospheres in comparison with dedicated cell lines. Data suggest that *Viola odorata* extract mostly targets cancerous cells, not normal cells with exception in high concentration. It acts in a cell-dependent manner.

**Keywords** Breast cancer stem cell (BCSC) · Mammosphere · *Viola odorata* · Invasion · Tumorigenicity

## Abbreviation

| | |
|---|---|
| ALDH | Aldehyde dehydrogenase |
| b-FGF | Basic fibroblast growth factor |
| BCSCs | Breast cancer stem cells |
| CSCs | Cancer stem cells |
| CAM | Chorioallantoic membrane |
| Dox | Doxorubicin |
| DMEM/F12 | Dulbecco's modified Eagle's medium/F12 |
| EGF | Epidermal growth factor |
| EGCG | Epigallocatechin gallate |
| ESA | Epithelial-specific antigen |

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| ER | Estrogen receptor |
|---|---|
| ECM | Extracellular matrix |
| FBS | Fetal bovine serum |
| H&E staining | Hematoxylin and eosin staining |
| HPLC | High-performance liquid chromatography |
| HER2 | Human epidermal growth factor receptor 2 |
| IHC | Immunohistochemistry |
| IC50 | 50% Inhibitory concentration |
| OSCC | Oral squamous cell carcinoma |
| OS | Osteosarcoma |
| PARP | Poly ADP ribose polymerase |
| PR | Progesterone receptor |
| TBMS1 | Tubeimoside-1 |
| TICs | Tumor-initiating cells |

## Introduction

Breast cancer is the most prevalent cancers among women in all over the world. Breast cancer incidence is increasing according to world statistics. In 2017, there were around 40,610 death reports caused by breast cancer in the United States. In 2017, 252,710 new cases of invasive and 63,410 new cases of non-invasive (in situ) breast cancer are estimated to be diagnosed in women in the US (www.breastcancer.org). Breast cancer accounts for 25% of all cancers in women and 15% of all cancer deaths. About 0.8–1% of all breast cancers are male breast carcinoma, but the incidence of breast cancer in men has increased over the past 3 decades. It has been estimated that the prevalence of breast cancer in the world will reach to 2.3 million by 2030 [1, 2]. The most important factors promoting breast cancer are high expression level of estrogen, estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2). On the other hand, mutants in tumor suppressor genes including BRCA1 and BRCA2 are considered as the most important causes of familial breast cancer [3]. There are two main types of breast cancer: ductal and lobular which are divided into invasive and in situ (non-invasive) with several subtypes of breast cancer based on histology. Also, there are several subtypes of breast cancer based on molecular characteristics: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67 expression; luminal A and B (ER + /PR + /HER2 − or ER + /PR −/HER2 −), basal-like triple negative breast cancer (ER −/PR −/HER2 −) and HER2 + with low amount of claudin [2]. There are various types of breast cancer treatment strategy including surgery, chemotherapy, radiotherapy, hormone and targeted therapy [2]. Although these are common treatments of breast cancer, results are short term and may cause long-term side effects. Radiotherapy strengthens the tissues of the breast,

lung and lymph nodes. Using radiotherapy may transform differentiated breast cancer cells into breast cancer stem cells (BCSCs) due to the expression of radiation-induced genes [4]. Other treatments also have several side effects such as osteoporosis and thromboemboli [4–6]. In addition, the above-mentioned strategies have not been fully successful and, in many cases, recurrence has been observed. One of the reasons for recurrence and uncertainty in the treatment of breast cancer is the heterogeneity of tumor cells that complicates the treatment [7]. Over the past decade, a number of cancer cells with stem cell-like properties have been identified called cancer stem cells (CSCs) or tumor-initiating cells (TICs). These cells make cellular heterogeneity in tumors. CSCs play an important role in cancer progression, drug resistance, radiotherapy resistance and relapse [8]. CSCs account for less than 2% of the cancerous mass and have some specific properties including self-renewal, differentiation, metastasis, migration and treatment resistance [8–10]. BCSCs express variety of specific markers in different types of breast cancers such as CD44$^+$/CD24$^−$, epithelial-specific antigen (ESA) or CD326 (EpCAM), additionally, these cells have aldehyde dehydrogenase (ALDH) activity [8]. These minor cell population are capable of developing cancer in mice with NOD /SCID immunity. The CD44 is a surface marker of BCSCs and a receptor of extracellular matrix hyaluronan. It works along with the tyrosine kinase receptor and activates signaling pathways involved in colony formation and proliferation of BCSCs. However, marker of CD24 has been identified as a glycoprotein on the surface of differentiated breast cancer cells [11]. Although new therapeutic approaches such as therapeutic differentiation, nanotechnology and the use of oncolytic viruses have been designed to eliminate these cells, each protocol has some limitations that prevent the development of successful targeted therapies [12]. Also, several drugs such as paclitaxel, nocodazole, 4-oxoretinol and doxorubicin may have concomitant effect on epithelial normal cells as well as cancer cells [13–15]. Considering the recognition of cancer stem cells and their impact on the formation and progression of the disease, and the lack of effective drugs on these cells, it is necessary to find more effective treatments with fewer side effects.

The *Viola odorata* is a mamber of *Violaceae* family plants which has been habituated in Middle East as far as central Asia and large part of Europe. It also is found in North America [16]. *Viola odorata* possesses therapeutic properties including anti-inflammatory [17], anti-rheumatic, asthma and bronchitis [18], anti-hypertension [19], anti-oxidant activity [20] and anti-cancer properties [21, 22]. In traditional medicine, the alcoholic extract of *Viola odorata* leaves show an anti-metastatic effects (www.goldaru-co.com). The active components of *Viola odorata* are saponin, salicylic acid derivatives, glycosides such as vioacercitin, alkaloids such as violins, anthocyanidins and cyclotides

including cycloviolacin [19, 23]. In various studies, the anti-cancer effects of these compounds have also been proven. Recent studies have shown the effects of the cyclotides such as cycloviolacin O2 in different cancers including tongue and throat, breast, lymphoma, stomach, intestine, gastric, rectal, uterine and joints [21]. Mechanism of anti-cancer effects of cyclotides may be degradation of membranes at high concentrations through intercalating with cholesterol-rich and membrane-bound sphingomyelin membranes in membrane lipid rafts [21, 24, 25]. Cyclotides seem to target specific types of cells depending on membrane composition [26]. Some cyclotides also have anti-migratory, anti-metastatic, anti-proliferation effects and apoptotic effects on cancer cells with low concentrations through mediating signaling pathways [27]. Various studies have shown the anti-cancer effects of saponin through activation of apoptosis and autophagy. Saponin increases the sensitivity of cancer cells such as lung adenocarcinoma cells, breast, uterine and colon cancer to radiotherapy and chemotherapy [28–30]. Also, anti-metastatic effects of anthocyanidin have been proven [31]. Anthocyanin, a subset of flavonoids, activates apoptosis pathway through activating Caspase 3, apoptosis induction factor (AIF) and endonuclease G (pro-apoptotic factor). It also inhibits the growth and progression of cancer through cell cycle arrest in G0/G1 and G2/M with increasing p21WAF1 and P27KIP1 expression and decreasing the expression of cyclin A and B [32]. Like other plants, the effective compounds of *Viola odorata* can be dissolved in their alcoholic extract and various fractions from different solvents such as methanol, ethyl acetate, chloroform, n-butanol, hexane and dichloromethane [19, 33–36].

In our previous study [37], we established MCF7- and SKBR3-derived mammospheres as enrichment breast cancer stem cell models through a variety of in vitro and in vivo experiments. Therefore, considering anti-cancer and anti-metastatic effects of *Viola odorata* on a variety of cancer cells such as breast cancer and the important role of cancer stem cells in the metastasis and progression of the cancers with different profiles of gene expression and characteristics, in comparison with differentiated cancer cells, we proposed different effect of alcoholic extraction of *Viola odorata* on malignant characterization of mammosphere-derived BCSCs in comparison with breast cancer cell lines. Current study is the first study about effect of *Viola odorata* extract on malignant characterization of breast cancer cell line-derived mammospheres as enriched sources of BCSCs. Also, the lowest dose of nominated drug with the highest cell death effects on these cells was compared with the breast cancer cell line and normal epithelial cells. We evaluated and compared effect of alcoholic extraction of *Viola odorata* on apoptosis, migration, colony formation, invasion and tumorigenicity in vivo of breast cancer cell lines and breast cancer cell line-derived BCSCs.

# Method and material

## Culture of cell lines

Two human breast cancer cell lines MCF7 (ER + /PR + / HER2 −) and SKBR3 (ER −/PR −/HER2 +) with different molecular receptors and MCF10A as normal breast cell were purchased from pasture Institute (Iran, Tehran). MCF7 and SKBR3 were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/ streptomycin and 1% non-essential amino acids at 37 °C in humidified atmosphere with 5% CO2. MCF10A was cultured in DMEM/F12 supplemented with 5% horse serum, 1% L-glutamine, 1% penicillin/streptomycin and 1% non-essential amino acids, 10 µg/mL insulin, 20 ng/mL epidermal growth factor (EGF), 100 ng/mL choleratoxin, 0.5 mg/ mL hydrocortisone at 37 °C in humidified atmosphere with 5% CO2 (all components were purchased from sigma, USA).

## Mammosphere culture from cell lines

For mammosphere formation, MCF7 and SKBR3 cells were harvested at 25,000 cells/mL and seeded into agar-coated flask in serum free DMEM/F12 (1:1) supplemented with 10 ng/mL basic fibroblast growth factor (b-FGF), 20 ng/mL EGF and 2% B27 (all purchased from Sigma, USA). Growth factors were supplemented every 2 days. In this condition, cells were grown as non-adherent spheres (termed mammosphere) with a size of > 60 µm. Primary mammospheres were collected every week by centrifugation and dissociated to single cells by trypsin/EDTA 0.05% (v/w). For secondary mammosphere formation, Lombardo et al.'s protocol was used in this study [38]. Mammospheres were passaged during 3 weeks on days 7, 14 and 21 [30, 39, 40].

## Characterization of cancer stem cells derived from breast cancer cell lines

Stemness properties of mammosphere-derived breast cancer cell lines such as expression of CD44 and CD24, expression of stemness genes, *Oct4* and *Nanog*, proliferation rate, migration rate, drug resistance and tumorigenicity of mammospheres in chick embryo model were evaluated and confirmed in our previous study [37].

## Preparation of *Viola odorata* extract

Hydro-alcoholic extraction (EtOH/H2O 70:30) of aerial parts of *Viola odorata* was gifted from Goldaru Company (Iran, Isfahan). *Viola odorata* has been authenticated by Dr. Fariborz Moattar and Dr. Taghi Ghafghazi. Extraction

method is based on Sadeghnia et al. [41]. Protocol Powder of hydro-alcoholic extraction of *Viola odorata* was dissolved in ddH2O.

## HPLC (high-performance liquid chromatography) for hydro-alcoholic extraction of *Viola odorata*

Different solvents extracts yield different phytochemicals of *Viola odorata* such as glycoside, saponins, flavonoids and anthocyanins, alkaloids and cyclotides [42]. Among extracts, hydro-alcoholic extract is a specific solvent for flavonoids and anthocyanin extraction. As several studies showed anti-cancerous effects of flavonoids and anthocyanins, we screened hydro-alcoholic extraction of *Viola odorata* for flavonoids. Qualitative and quantitative HPLC was performed with HPLC system equipped with a pump (1500 series), a column oven, a reversed-phase, pre-packed C18 column ($250 \times 4.6$ mm, 5 µm particle size) and a UV detector (2487). The mobile phase was consisted of acetonitrile/water 1:1 with 1% acetic acid. *Viola odorata* was dissolved in methanol for 24 h at room temperature. 10 µL *Viola odorata* was injected into HPLC and the compounds were detected at 254 nm. Data were recorded with same retention time ($R_t$) values with the standards [43].

## Flow cytometry

MCF7- and SKBR3-derived mammospheres were gathered and enzymatically digested into single cells via 0.05% trypsin/EDTA. Recommended concentration of human CD44-FITC and CD24-PE antibodies (BD Biosciences, San Diego, CA, USA) were added to the cell suspension. Cells were incubated at room temperature in dark for 30 min. Then, the cells were washed with phosphate-buffered saline (PBS) and analyzed by the FACSCalibur flow cytometer (Becton Dickinson, USA).

## MTS assay

The cell viability of MCF7, SKBR3, their derived mammospheres and MCF10A in normal and treatment conditions were measured by MTS assay. In brief, cells were digested into a single cell suspension and plated into 96-well plates with $5 \times 10^3 - 10^4$ cells/100 µL, per well. Cells were cultured in normal condition before treating with different doses of fresh hydro-alcoholic extraction of *Viola odorata* (0–1000 µg/mL) in 37 °C for 24, 48 and 72 h. Twenty microlitre MTS solution (Sigma, USA) was added per well, and incubated at 37 °C for 3 h. Finally, cell viability were measured using plate reader under 492 nm wavelength and the survival rates was calculated [44]. Also, doxorubicin (Dox) (Sigma, USA) with maximal doses of IC50 (50% inhibitory concentration) for each cell line was used as positive control.

## Apoptosis assay

Apoptotic assay was performed according to Annexin V-FITC/PI kit protocol (Sigma, USA). After 48 h treatment with optimized concentration of *Viola odorata* extract, cells enzymatically detached via 0.05% trypsin/EDTA and washed by calcium buffer. Then, 10 µL Annexin V FITC and 10 µL propidium iodide were added to 100 µL cell suspension in calcium buffer and incubated for 20 min at 4 °C in the dark. Flow cytometry analysis was carried out by FACSCalibur flow cytometer (Becton Dickinson, USA) [45].

## Caspase 3/7 and caspase 8 activity assay

Detection of caspase 3/7 activity was performed by CaspaTag caspase 3/7 In Situ assay kit (EMD Millipore, USA). After 48 h treatment with optimized concentration of *Viola odorata* extract, cells enzymatically detached via 0.05% trypsin/EDTA. 10 µL 30X FLICA reagent was added to 300 µL cell suspension. Cells incubated for 1 h at 37 °C under 5% $CO_2$. Then, cells washed by 1X wash buffer and resuspended in 400 µL PBS and 100 µL of cell suspension placed into wells of microtiter plate. Finally, cell absorbance were measured using an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Caspase 8 assay kit (Abcam, USA) was used for detection of caspase 8 activity. After cell harvesting, cells resuspended in 50 µL of ice cold cell lysis buffer. After 10–30 min incubation, supernatant was collected and transferred into wells of microtiter plate. Then, 50 µL of 2X Reaction Buffer/DTT mixture and 5 µL of IETD-*p*NA substrate were added into each reaction well. After incubating plate covered at 37 °C for 1–2 h, output on a microplate reader at OD 400 nm was measured.

## Migration assay (scratch assay)

Scratch assay was used for detection the migration capacity of breast cancer cell lines and their derived mammosphere in control and treatment conditions. Cell line- and mammosphere-derived cells were plated in 6-well dishes at a seeding concentration of $2 \times 10^5$ cells/well. Treatment group was treated with optimized doses of *Viola odorata* extract. After 48 h, to arrest proliferation, 20 µg/mL mitomycin (Sigma, USA) was added to culture dishes with 80–90% confluency for 2 h. Thin scratch/wound was created through scratching with 100 µL pipette tip. Images were taken by Olympus camera (Japan) attached to inverted microscope at $40 \times$ magnifications. Migration rate was detected with modulation of distance between two lines of scratch at 0, 6, 12 and 24 h after scratching. Wound closure (%) was evaluated based on following ratio: % wound closure: $A_{\Delta h}/A_{0h} \times 100\%$. A stands for the size of gap (µm), when the scratch was performed

(0 h), while $A_{\Delta h}$ stands for the difference in the size of gap at the second measurement than the initial size of the gap.

## Soft agar assay

Soft agar assay was used to evaluate the colony formation or anchorage independent proliferation ability of MCF7, SKBR3, and their derived mammospheres in control and treatment condition. The assay was carried out in six-well plates which pre-coated with 2 mL of 1.2% (w/v) low melting temperature agarose gel (Sigma, USA) in $2\times$ culture medium that solidified in room temperature. Then, cell lines and their derived mammospheres were harvested and adjusted with medium to $10^4$ cells/mL, which then mixed with 1 mL of pre-warm 0.6% (w/v) low melting temperature agarose gel and were placed on top of the present basement gel layer. Complex was solidified before incubation at 37 °C in humid incubator with 5% $CO_2$ atmosphere for 3–4 weeks. During this period, cells were feed with 500 µL/well once a week. At the end of assay, colonies were stained by adding 500 µL 40% MTS for 3 h in incubator. Colonies larger than 150 µm were counted in ten field [46].

## Invasion assay

Cell invasion assay was performed using six-well containing trans-well dishes inserted with 8-µm poly carbonate membranes (Corning, USA). Cells ($3\times10^5$/mL) in 1.5 mL serum free DMEM/F12 medium were plated onto upper chamber (BD BioCoatTM MatrigelTM Invasion Chamber) (Corning, USA), while DMEM/F12 medium with 10% FBS was added to the lower chamber. In both control and treatment groups, after 48 h at 37 °C (5% CO2), non-invading cells were removed from upper chamber with a cotton swab. The invading cells were fixed in 4% paraformaldehyde for 30 min and stained with 0.5% crystal violet solution for 30 min, and then number of invasive cells in ten field was counted microscopically $\times$40 [45].

## Chick chorioallantoic membrane (CAM) tumor formation assay

Chorioallantoic membrane (CAM) in chicken embryo has been identified as an immune-deficient host model for tumor formation in vivo [47–50]. To inoculate treated and control cells onto the CAM on day 3 (starting from the day of hatch chicken egg incubation), 2 mL albumin was withdrawn and a window was opened in the shell with an electric drill equipped with a 0.75 inch wheel tip (Dremel Moto-Tool; Emerson Electric Co., USA). After 9 days of incubation, cells were inoculated carefully onto the CAM, on the top of ectoderm layer at a concentration of $10^6$ cells/100 µL of extracellular matrix (ECM) (Sigma, USA) without touching

CAM. Window was closed with parafilm, and embryo was incubated for up to 5 more days. This experiment was repeated six times. On day 14, the embryos were opened and the CAMs were dissected out to determine the size and volume of formed tumors on the top of CAM. A dissecting microscope was used to determine diameter of tumors [49, 51]. Volume of tumors was calculated according to the following formula: $V = W^2 \times L$, as $W$ is the width and $L$ is the length of the tumor.

## Statistical analysis

Statistical analysis was performed by SPSS for Windows (version 16.0; SPSS Inc., Chicago, IL). The results are presented as mean $\pm$ SEM for at least three independent experiments for each analysis. Student's $t$ test with $p$ values of 0.05 or less was used to indicate statistically significant differences.

# Results

## HPLC of hydro-alcoholic extraction of *Viola odorata*

HPLC was used for detection of flavonoids in hydro-alcoholic extraction of *Viola odorata*. HPLC detected the presence of several flavonoids at retention time 1.97, 2.06, 2.49 and 2.64 for luteolin, quercetin, apigenin and kaempferol, respectively (Table 1).

## Detection of CD24 and CD44 in MCF7 and SKBR3 (2D), and their derived mammospheres (3D)

At the first step, to confirm the stemness potential of mammospheres (3D), we detected CD44 and CD24 markers on the surface of mammosphere-derived cells in all stages of study. Results from flow cytometry revealed that CD44 was not remarkable in MCF7, SKBR3 and their

**Table 1** List of flavonoids detected by HPLC of hydro-alcoholic extraction of *Viola odorata*

| Flavonoids | $R_t$ (min) | mg/kg *Viola odorata* |
|---|---|---|
| Myricetin | 1.79 | nd |
| Luteolin | 1.97 | 180.65 |
| Quercetin | 2.06 | 135.9 |
| Apigenin | 2.49 | 32.03 |
| Kaempferol | 2.64 | 74.76 |
| Isorhamnetin | 2.74 | nd |
| Rhamnetin | 3.75 | nd |

$R_t$ retention time, *nd* not detected

derived mammospheres. Furthermore, CD24 expression in the MCF7 was 63.29%, while in MCF7-derived mammospheres during passages 1, 2 and 3, it was changed to 55.15%, 49.67% and 39.59%, respectively. A similar trend was detected for the expression of CD24 in SKBR3, which was 97.27% and then shifted to 82.42%, 66.74% and 61.66% in SKBR3-derived mammospheres during passages 1, 2 and 3, respectively. Despite the steady-state level of the CD44 marker in MCF7 and SKBR3 mammospheres, the level of CD24 (differentiation marker) was decreased during three passages in MCF7 and SKBR3 mammosphere-derived cells (Supplementary Fig. 1A). Expression of CD24 was significantly decreased during the passage three in MCF7- and SKBR3-derived mammospheres ($p < 0.001$) (Supplementary Fig. 1B).

## Extract of *Viola odorata* induced cell death in MCF7, SKBR3 (2D) and their derived mammospheres (3D)

MTS assay was used to detect cytotoxic potential (IC50) of *Viola odorata* extract on MCF7, SKBR3, their derived mammospheres and also MCF10A. Cell were treated with increasing concentration of the herbal extract (0–1000 µg/mL) for 24, 48 and 72 h. Doxorubicin at optimal concentration of IC50 was used as a positive control (Supplementary Fig. 2). Although cell death was not induced after 24 h treatment with increasing doses of *V. odorata* extract, data showed that cell viability was decreased in a dose- and time-dependent manner after 48 and 72 h period (Fig. 1). Although, MCF7- and SKBR3-derived mammospheres showed a significant drug resistant to increasing concentration of doxorubicin in comparison with corresponding cell lines (Supplementary Fig.2), they did not show resistance to *Viola odorata* extract. IC50 for MCF7 and MCF7-derived mammospheres and SKBR3- and SKBR3-derived mammospheres were 600 and 400 µg/mL, respectively ($p < 0.05$), whereas MCF10A showed a resistance at these concentrations of *Viola odorata* extract. However, *Viola odorata* extract induced cell death in MCF10A at higher concentration, 1000 µg/mL ($p < 0.05$). Therefore, we suggested that *Viola odorata* extract was able to induce cell death in a cell-dependent manner (Fig. 1).

## *Viola odorata* extract induced apoptosis in MCF7, SKBR3 (2D) and their derived mammospheres (3D)

Annexin V kit was used for detection of rate of apoptosis (early and late apoptosis) in MCF7, SKBR3, their derived mammospheres and MCF10A. Results of our study showed that optimized doses of *Viola odorata* extract (600 µg/mL for MCF7- and MCF7-derived mammospheres and 400 µg/mL for SKBR3- and SKBR3-derived mammospheres), which were already notified, induced 8.24%, 31.17% early
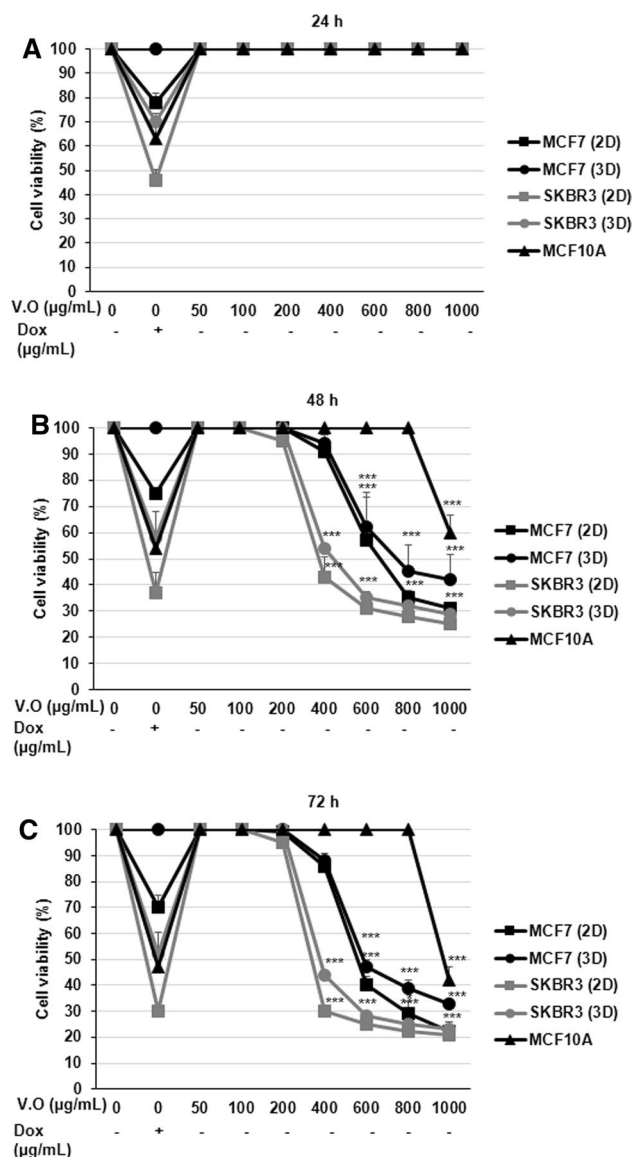


**Fig. 1** Detection of cytotoxic activity of *Viola odorata* extract on MCF7, SKBR3 (2D) and their derived mammospheres (3D). Various concentrations of *Viola odorata* extract were used for treating the cells in a period of **a** 24 h **b** 48 h **c** 72 h. Results showed that unlike to doxorobicin (Dox), *Viola odorata* extract induced cell death at 48 and 72 h after treatment in both breast cancer cell lines and their derived mammospheres in dose- and time-dependent manner. Efficient concentration of *Viola odorata* extract was 600 µg/mL for MCF7 and MCF7 derived mammospheres and it was 400 µg/mL for SKBR3- and SKBR3-derived mammospheres. Of important that *Viola odorata* extract did not induce cell death in MCF10A, exception in high doses (1000 µg/mL). Stars indicates significant difference between samples and control (0 µg/mL) at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

apoptosis and 33.82%, 9.53% late apoptosis in MCF7- and MCF7-derived mammospheres, respectively ($p < 0.05$), and 32.46%, 9.24% early apoptosis and 12.39%, 35.19% late apoptosis in SKBR3- and SKBR3-derived mammospheres, respectively ($p < 0.05$). Early apoptosis rates of MCF10A

at 400 and 600 μg/mL of *Viola odorata* extract were 5.25% and 4.94%, respectively ($p > 0.05$). Late apoptosis rates of MCF10A at 400 and 600 μg/mL of *Viola odorata* extract were 3.9% and 4.99%, respectively ($p > 0.05$). Data showed that *Viola odorata* extract induced apoptosis in a cell-dependent manner (Fig. 2a–c). Also, some necrosis were observed in both treated and untreated groups (Fig. 2a).

### *Viola odorata* extract induced apoptosis through caspase3/7 and 8 activity in MCF7, SKBR3 (2D) and their derived mammospheres (3D)

The results obtained from the caspase activity assay showed that *Viola odorata* extract induced apoptosis through caspase3/7 activity in MCF7-, MCF7-derived mammospheres, SKBR3- and SKBR3-derived mammospheres, which was a 5.3, 3.7, 3.3 and 2.7-fold increase, as compared to control groups, respectively ($p < 0.05$) (Fig. 2d). Also, *Viola odorata* extract induced activity of caspase 8 in MCF7, MCF7-derived mammospheres, SKBR3- and SKBR3-derived mammospheres, which was a 3.1, 2, 2.9 and 1.9-fold increase, as compared to control groups, respectively ($p < 0.05$) (Fig. 2e).

### *Viola odorata* extract inhibited migration of MCF7, SKBR3 (2D) and their derived mammospheres (3D)

A wound healing assay followed by calculation of gap filling at 0, 6, 12 and 24 h post scratch was performed and the results are presented as migration rate (Fig. 3). Data indicated migration rate was effectively inhibited in herbal extract treated groups 24 h after scratch. Notably, the inhibition was started after 6 h in SKBR3- and SKBR3-derived mammospheres ($p < 0.05$) (Fig. 3). Six hours post scratch, migration rate of herbal extract treated MCF7- and MCF7-derived mammosphere were detected to be 11% and 14%, respectively, approximately 9% decrease in migration compare to their control groups (untreated counter parts) ($p < 0.05$). Gradually, 12 h post scratch, migration rate of treated MCF7- and MCF7-derived mammosphere were 17% and 21%, respectively, showing 8% and 12.5% decrease in comparison with their control groups, respectively ($p < 0.05$). Finally, 24 h after scratch, migration rate of herbal extract-treated MCF7- and MCF7-derived mammosphere was 28% and 30%, respectively, which showed 22% and 59% decrease in compare to their untreated counterparts, respectively ($p < 0.05$) (Fig. 3e). Although migration rate of both treated SKBR3- and SKBR3-derived mammosphere was around 0% at 6, 12 h, these reductions were around 6.5% and 7.2% at 6 h and 8.7% and 9% at 12 h, respectively, in comparison with their control groups ($p < 0.05$). Twenty four later, migration rate of treated SKBR3 and SKBR3-derived mammosphere were around 6% and 10%, respectively, showing 12.5% and 27% reduction in compare to their control

groups, respectively ($p < 0.05$) (Fig. 3f). Taken together, data showed that *Viola odorata* extract significantly inhibited migration of MCF7 and SKBR3 and their derived mammospheres. Furthermore, this inhibition in migration rate of MCF7- and SKBR3-derived mammospheres was more than MCF7 and SKBR3 ($p < 0.05$).

### *Viola odorata* extract inhibited colony formation of MCF7, SKBR3 (2D) and their derived mammospheres (3D)

Soft agar assay was used for detection of colony formation ability of MCF7, SKBR3 and their derived mammospheres in treated and control groups (Fig. 4a). Average counting of colonies in ten field showed that colony formation ability of MCF7- and MCF7-derived mammospheres in control condition were 3.8% and 5.7%, while colony formation ability in treated samples significantly decreased to 1.2% and 2.7%, respectively ($p < 0.05$). Also, colony formation ability of SKBR3- and SKBR3-derived mammospheres in control samples were 2.6% and 6.5%, while colony formation ability in treated counterparts significantly decreased to 0.8% and 3.5%, respectively ($p < 0.05$). Decrease of 2.6% and 3% colonies in treated MCF7- and MCF7-derived mammospheres and 1.8% and 3% colonies in treated SKBR3- and SKBR3-derived mammospheres in comparison with their control groups, showed that *Viola odorata* extract significantly decreased colony formation ability of MCF7 and SKBR3 and their derived mammospheres. Importantly, the reductions were significantly higher in MCF7- and SKBR3-derived mammospheres in comparison with dedicated cell lines ($p < 0.05$) (Fig. 4b).

### *Viola odorata* extract inhibited invasion of MCF7, SKBR3 (2D) and their derived mammospheres (3D)

Cell invasion assay through six-well trans-well cell culture inserts was performed for detection of invasion ability of MCF7, SKBR3 and their derived mammospheres in control and treated samples (Fig. 5a). Average invading cells in ten field showed that the invading cells of MCF7- and MCF7-derived mammospheres in control condition were 0.38% and 1.02%, while the cells in treatment condition significantly decreased to 0.23% and 0.42%, respectively ($p < 0.05$). Also, the invading cells of SKBR3- and SKBR3-derived mammospheres in control condition were 0.6% and 1.37%, while the invading cells in treated condition significantly decreased to 0.23% and 0.44%, respectively ($p < 0.05$). Decrease of 0.15% and 0.6% invading cells in treated MCF7- and MCF7-derived mammospheres and decrease of 0.37% and 0.93% invading cells in treated SKBR3- and SKBR3-derived mammospheres, respectively, compared with their control counterparts showed that *Viola odorata* extract significantly
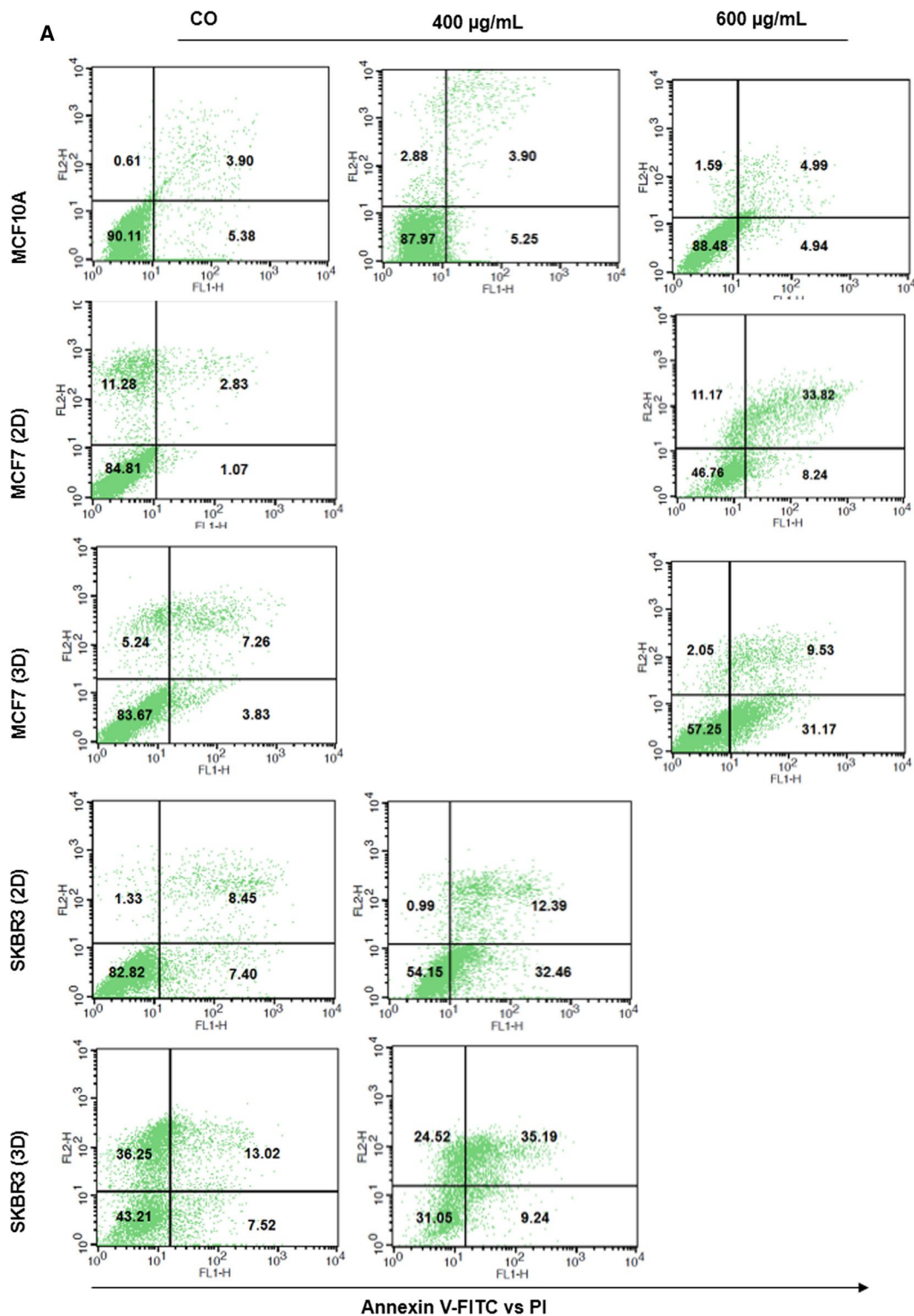
**Fig. 2** *Viola odorata* extract induced apoptosis through Caspase 3/7 and 8 activity in MCF7, SKBR3 (2D) and their derived mammospheres (3D). **a** Annexin V cytometry analysis for detection of rate of apoptosis in MCF7, SKBR3, their derived mammospheres and MCF10A at optimized concentration, **b**, **c** data showed that *Viola odorata* extract induced early and late apoptosis in MCF7, SKBR3 (2D) and their derived mammospheres (3D). *Viola odorata* extract didn't induce apoptosis in MCF10A at both 400 and 600 µg/mL concentration. **d**, **e** Data from caspase 3/7 and 8 assay showed that *Viola odorata* extract induced apoptosis through increased caspase 3/7 and caspase 8 activity in MCF7, SKBR3 (2D) and their derived mammospheres (3D). Star indicates significant difference between samples and control (CO: 0 µg/mL of *Viola odorata* extract) at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$
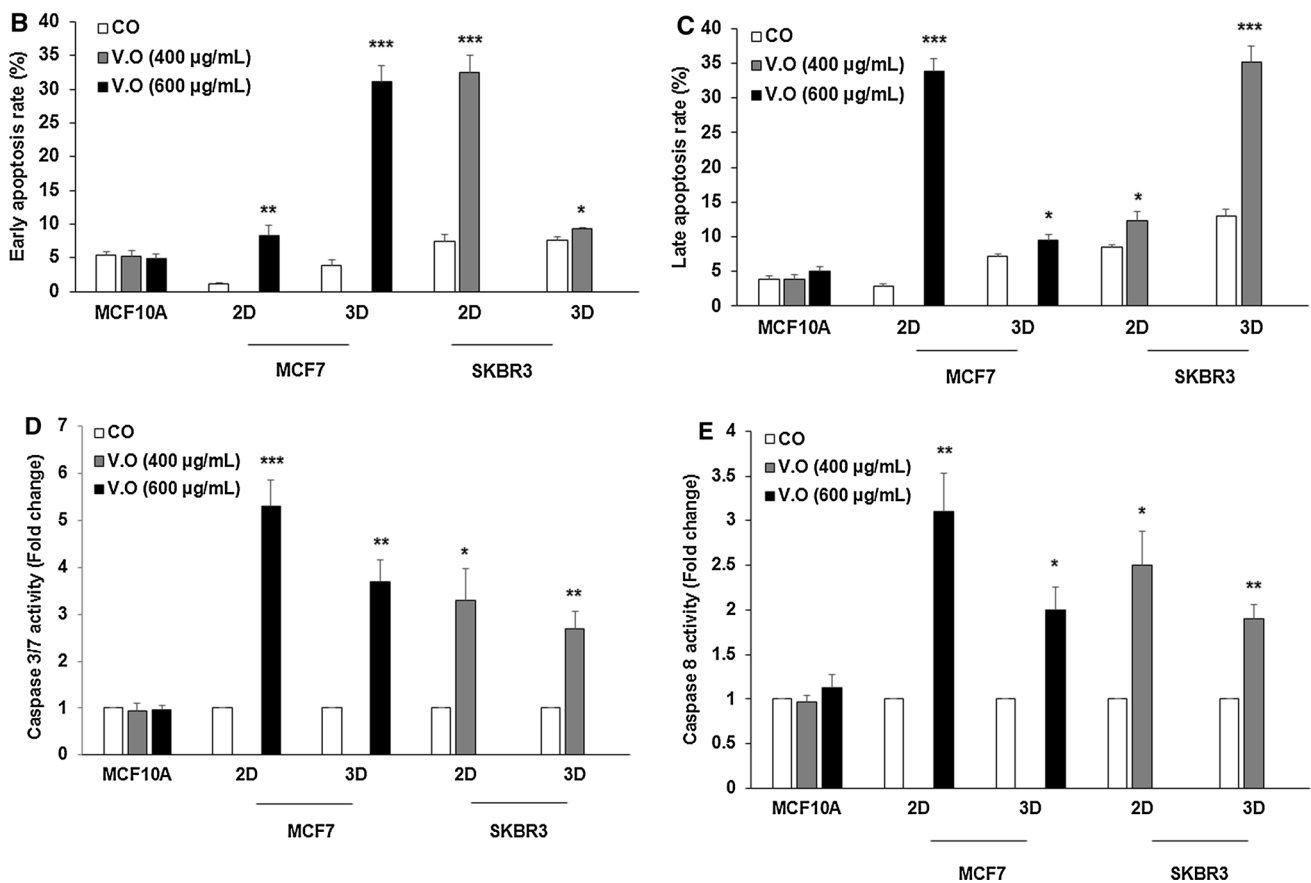
**Fig. 2** (continued)

decreased invasion ability of MCF7 and SKBR3 and their derived mammospheres and the reductions were significantly higher in MCF7- and SKBR3-derived mammospheres ($p < 0.05$) (Fig. 5b).

## *Viola odorata* extract inhibited tumorigenicity of MCF7, SKBR3 (2D) and their derived mammospheres (3D) in CAM of chicken embryo

To evaluate tumorigenicity potential of MCF7, SKBR3 (2D) and their derived mammospheres (3D) in control and treated cells in vivo, cancer cells were inoculated in CAM of chicken embryo, on the ectoderm layer. After 5 days, visible tumor masses were produced in all testing groups. Average size and volume of tumors were measured (Fig. 6a). Average size of tumors from MCF7 and MCF7 derived mammospheres were calculated as were 1.5 and 3.3 mm, respectively, whereas average size of tumors from treated MCF7 and MCF7 derived mammospheres were 1 and 2.5 mm, respectively. Also, average size of SKBR3- and SKBR3-derived mammospheres (3D) were measured 3.16 and 3.37 mm, whereas average size of tumors from treated SKBR3- and SKBR3-derived mammospheres were 2 and 2.2 mm. Data showed that *Viola odorata* extract significantly decreased

tumor size of MCF7, SKBR3 and their derived mammospheres ($p < 0.05$) (Fig. 6b). Average volume/depth of tumors produced by MCF7 and MCF7 derived mammosphere was detected as 4.5 and 20.5 mm$^3$, respectively, whereas average volume of tumors produced by treated MCF7- and MCF7-derived mammosphere were 1 and 2.5 mm$^3$, respectively (Fig. 6c). Average volume of tumors produced by SKBR3- and SKBR3-derived mammospheres (3D) were detected as 12 and 23.6 mm$^3$, respectively, whereas average volume/depth of tumors produced by treated SKBR3- and SKBR3-derived mammospheres were 0.7 and 0.4 mm$^3$, respectively. Data showed that *Viola odorata* extract significantly decreased size and volume/depth of MCF7, SKBR3 and their derived mammospheres ($p < 0.05$) (Fig. 6c). These reductions were not significant between treated MCF7- and MCF7-derived mammospheres as well as treated SKBR3- and SKBR3-derived mammospheres ($p > 0.05$).

## Discussion

As breast cancer incidence is increasing in all over the world, efforts to discover effective drugs and treatment are also increasing. BCSCs, a minor cell population, are the
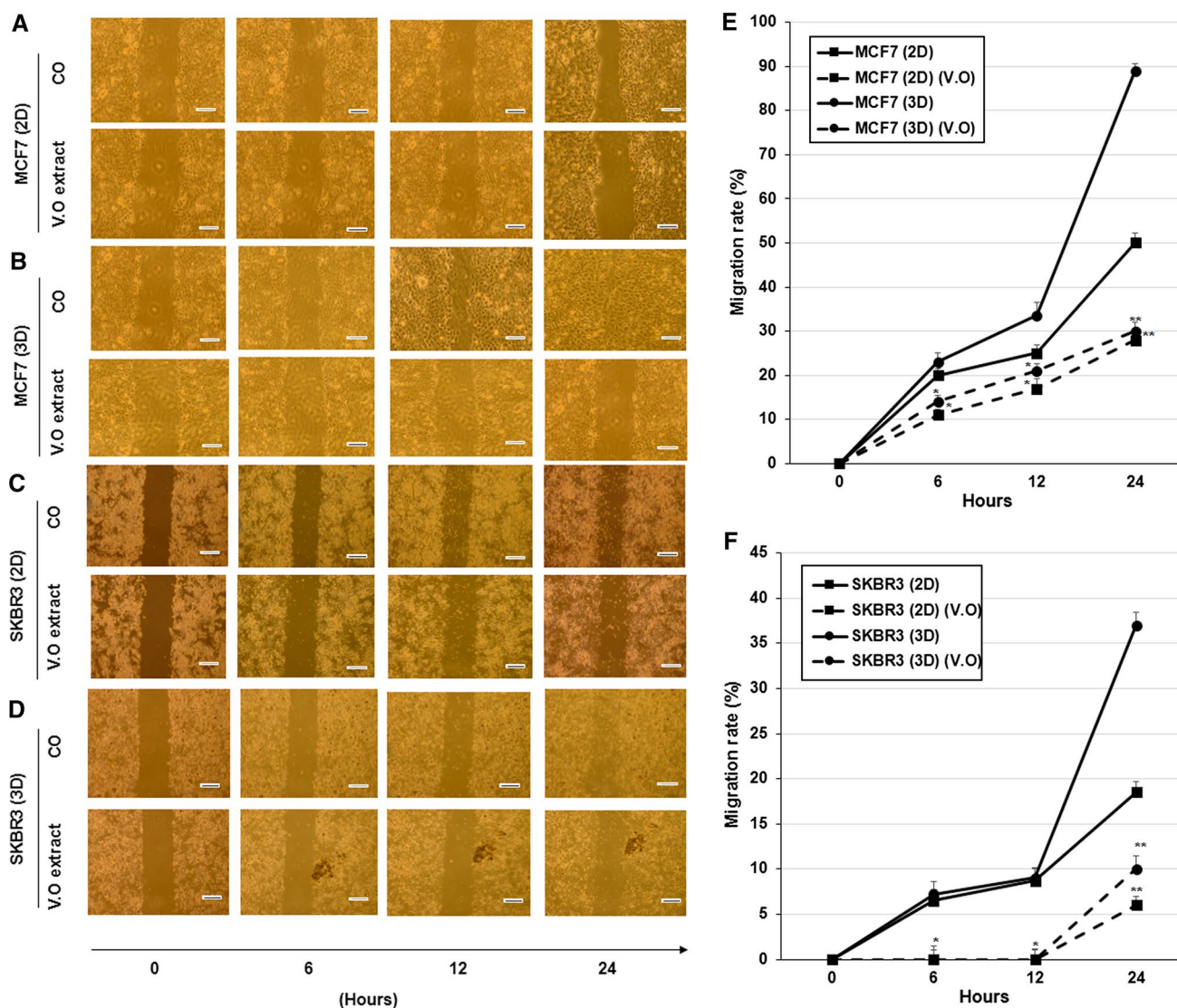
**Fig. 3** Detection of migration ability of MCF7, SKBR3 (2D) and their derived mammospheres (3D) treated by *Viola odorata* extract. A series images of scratch assay from MCF7, SKBR3 and derived mammospheres was taken at 0, 6, 12 and 24 h post scratch in both control and treated samples. Cell lines were **a** MCF7 (2D) **b** MCF7 (3D) **c** SKBR3 (2D) **d** SKBR3 (3D). Bar is 200 μm. **e, f** Quantification of migration rate of MCF7, SKBR3 and their derived mammospheres monolayers in both control and treated samples. As shown migration rate of treated groups in all types of cells was significantly lower than their control counterparts at 6, 12 and 24 h ($p < 0.05$). Results showed that *Viola odorata* extract significantly inhibited migration of MCF7 and SKBR3 and their derived mammospheres. Represented value bars are the mean of triplicate independent experiments ± SEM. Star indicates significant difference between samples and controls (V.O untreated) at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

most highly malignant cells making tumors more aggressive. In previous study, we introduced MCF7 and SKBR3 mammospheres as an enrichment system for BCSCs through some in vitro and in vivo confirmation assays [37]. Also, significant decrease in CD24 expression level as a differentiation marker in cell line-derived mammospheres was confirmed during all stages of the study.

Common cancer treatment strategies such as chemotherapy, radiotherapy and hormone therapy usually have many side effects including drug resistance [4–6]. Also, chemical drugs may show toxicity in normal epithelial cells [13–15]. Recently, natural components have been pinpointed among scientists for their possible therapeutics application. Many studies have reported a variety of plant extracts such as *Salvia*, *Lakoochin A*, *Clinacanthus nutans*, *Gefitinib*, *Krukovine*, *Euterpe oleracea, Strobilanthes crispa* Blume hexane extract, and *viola tricolor* as herbal medicine which inhibit development of cancer cells such as breast cancer, hepatocellular carcinoma, melanoma, lymphoma, colorectal cancer, lung cancer and breast cancer [41, 52–58]. Among them,

**Fig. 4** Detection of colony formation ability of MCF7, SKBR3 (2D) and their derived mammospheres (3D) treated by *Viola odorata* extract. **a** Colonies of MCF7, SKBR3 (2D) and their derived mammospheres (3D) in control and treatment conditions in soft agar. Bar is 50 μm. **b** Colony formation ability of MCF7, SKBR3 (2D) and their derived mammospheres (3D) in control and treatment conditions in soft agar. *Viola odorata* extract decreased the colonies formation ability of MCF7, SKBR3 (2D) and their derived mammospheres (3D). Star indicates significant difference between samples and related controls (V.O untreated) at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$
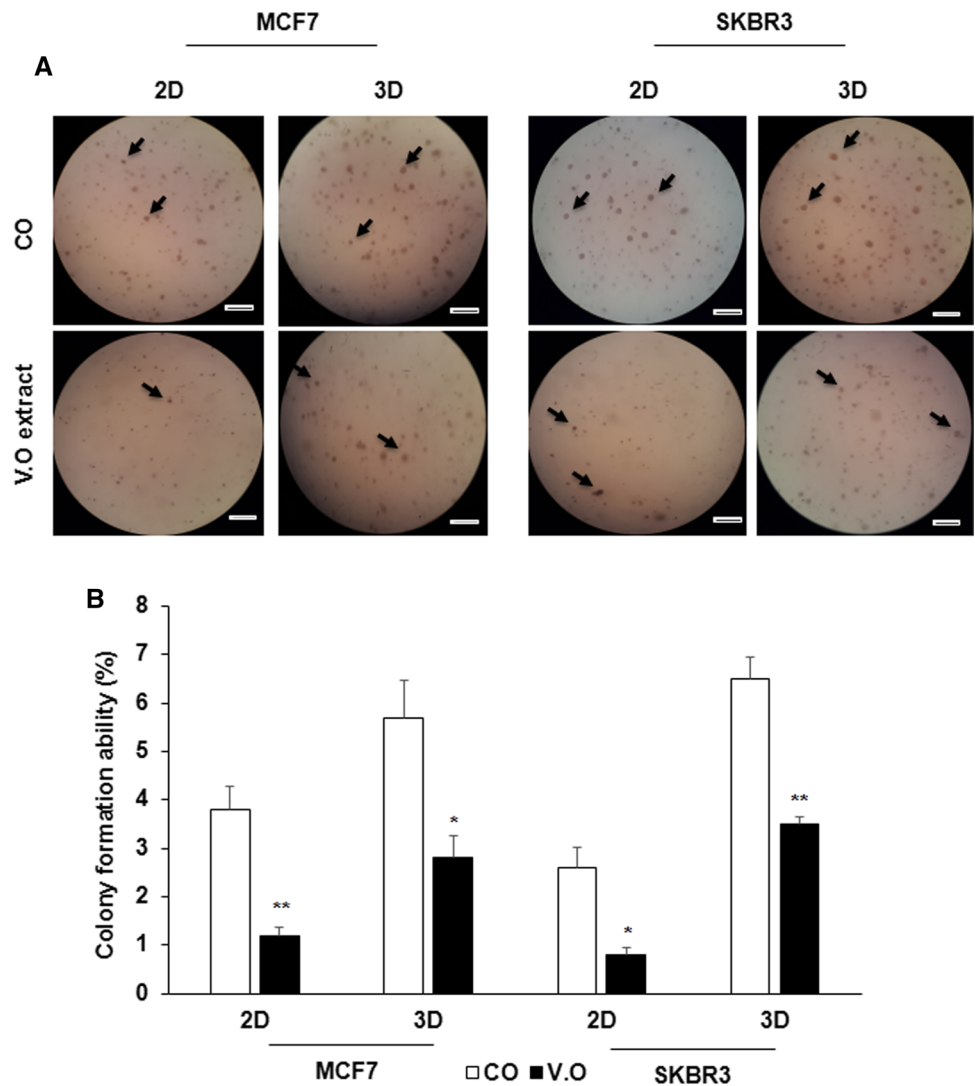


*Viola odorata* has been identified as a source of bioactive components and herbal medicine with anti-cancer effects [21, 22]. The active components of *Viola odorata* with anti-cancerous effects are cyclotides including cycloviolacin, saponin, salicylic acid derivatives, glycosides such as vioacercitin, alkaloids such as violins, anthocyanidins and flavonoids. Data from HPLC detected several flavonoids such as luteolin, quercetin, Apigenin and Kaempferol in *Viola odorata* extract. Several studies have confirmed anti-cancer effects of flavonoids [59–61]. We showed that whole alcoholic extract of *Viola odorata* induce cell death in both cell lines and cell line-derived mammospheres without toxicity effects on normal breast cells representing that *Viola odorata* extract may induce cell death through cell-dependent manner based on membrane composition [26]. It induces cell death in MCF10A at high concentration as 1000 μg/mL. Along with our study, several studies showed that components of microalgae extract inhibit proliferation of cancer cells such as breast cancer, lung carcinoma, melanoma and prostate cancer without any effects on normal cells [62, 63]. Data from annexin V showed that *Viola odorata* extract induced cell death at optimal concentration through early and late apoptosis in MCF7, SKBR3 and their derived mammospheres. Also, *Viola odorata* extract induced apoptosis through increased activity of caspase 3/7 and 8. Necrosis observed in both groups (treated and untreated) may be as a programmed necrosis in cancer cells encountering with nutrient deprivation and insufficient supply to tumor-promoting activity [64]. Also, necrosis has been confirmed as a result of late apoptosis [65]. Yi et al. showed that Kaempferol, a natural flavonoid induced apoptosis through decreased expression of Bcl2 and poly ADP ribose polymerase (PARP) and increased expression of Bax and cleaved PARP in breast cancer cells [59]. Also, Quercetin, a flavonoid detected in *Viola odorata* extract induced cell cycle and apoptosis in breast cancer cells through modification of Foxo3a signaling [60].

**Fig. 5** Detection of invasion ability of MCF7, SKBR3 (2D) and their derived mammospheres (3D) treated by *Viola odorata* extract. **a** Invasive cells of MCF7, SKBR3 (2D) and their derived mammospheres (3D) in control and treated samples. Bar is 50 μm. **b** Invasive rate of MCF7, SKBR3 (2D) and their derived mammospheres (3D) in control and treated samples of trans-well membrane. *Viola odorata* extract decreased the invasion rate of MCF7, SKBR3 (2D) and their derived mammospheres (3D). Star indicates significant difference between samples and their control counterparts (V.O untreated) at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$
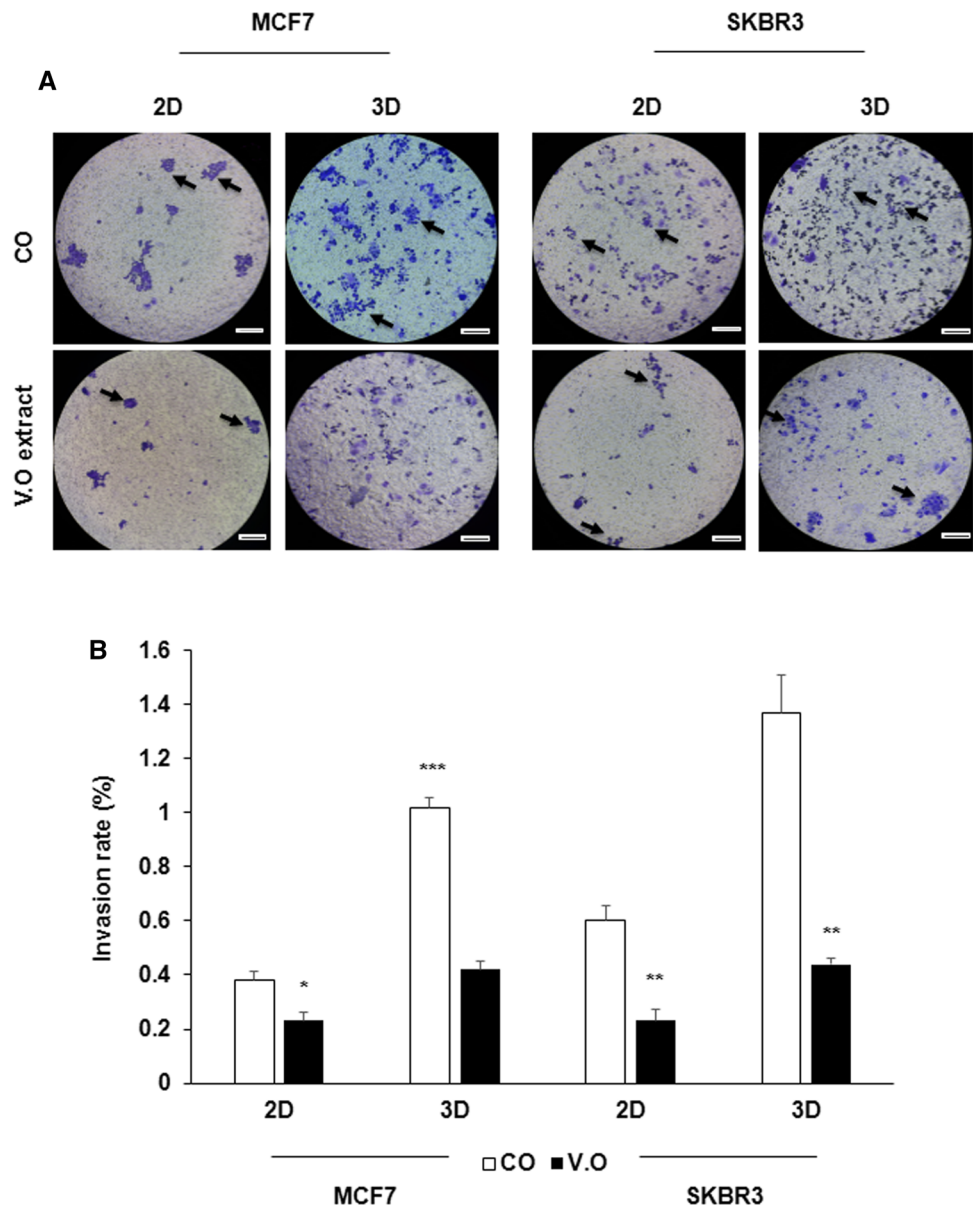


Tubeimoside-1 (TBMS1), a triterpenoid saponin extracted from tubeimoside induced 13–14% apoptosis through reduction in PARP, P-ERK1/2, Bcl2, caspase 3, 7 and 8 expression as well as increased PARP and cleaved caspases in oral squamous cell carcinoma (OSCC) [66]. Kang et al. showed that a major component of Delphinidin, anthocyanidin induced 33 and 57.8% apoptosis through mitochondria dependent pathway in osteosarcoma (OS) [67].

Cell migration and invasion are the specific processes in the metastasis of cancer stem cells. We showed that *Viola odorata* extract decreased migration and invasion of MCF7, SKBR3 and their derived mammospheres. Difference rate between control and treated groups was higher in MCF7- and SKBR3-derived mammospheres in comparison with MCF7 and SKBR3 ($p < 0.05$). Anthocyanidin may decrease

migration and invasion of cancer cells through decrease in MAPK signaling factors such as p-ERK, p38 [67]. Also, anthocyanidin inhibits HER + breast cancer metastasis by suppressing FAK signaling, phosphorylation of FAK, c-src and p130 as well as decrease in mesenchymal factors, fibronectin, vimentin [68]. Also, Kaempferol suppresses migration and invasion breast cancer cells through down-regulation of RhoA and Rac1 [69]. Furthermore, saponin inhibits migration and invasion of cancer cells through decreased expression of c-myc and MMP7 [66]. Current study showed that *Viola odorata* extract decreases anchorage independent proliferation of breast cancer cells and BCSCs through colony formation in soft agar. Also, difference rate between control and treated groups was higher in MCF7- and SKBR3-derived mammospheres in comparison with

**Fig. 6** Detection of tumo-
rigenicity ability of MCF7,
SKBR3 (2D) and their derived
mammospheres (3D) treated
by *Viola odorata* extract in
chicken embryo through CAM
assay. **a** Tumors generated by
MCF7, SKBR3 (2D) and their
derived mammospheres (3D) in
control and treatment condi-
tions. Bar is 1 mm. **b** Size of
tumors generated in control
and treatment conditions. *Viola
odorata* extract decrease the
size of tumors generated by
MCF7, SKBR3 (2D) and their
derived mammospheres (3D).
**c** Volume/depth of tumors
generated in control and treat-
ment conditions. *Viola odorata*
extract decrease the volume/
depth of tumors generated by
MCF7, SKBR3 (2D) and their
derived mammospheres (3D).
Star indicates significant dif-
ference between V.O treated
samples and untreated controls
at *$p < 0.05$, **$p < 0.01$,
***$p < 0.001$
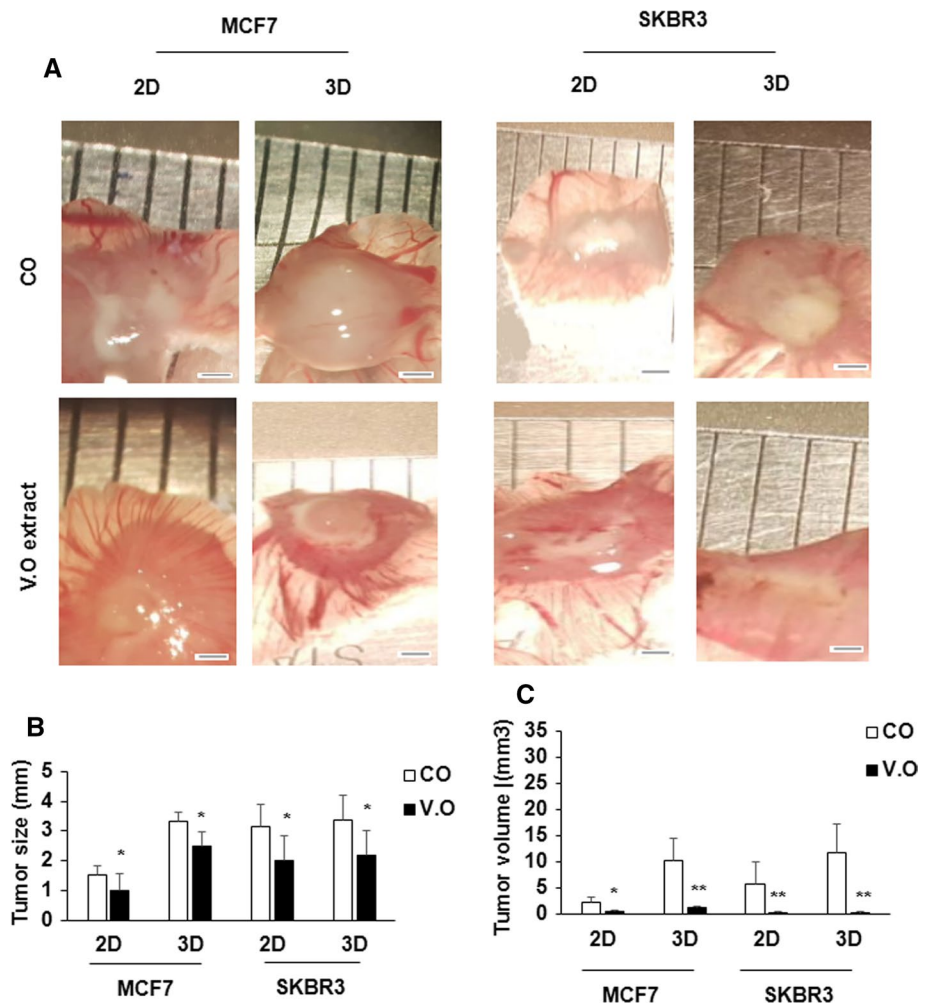


MCF7 and SKBR3. Quercetin, anthocyanidin and saponin in herbal medicine were shown to decrease colony forma-tion [67, 70–72]. Anti-cancerous activity of Luteolin and Apigenin which were detected in *Viola odorata* had been confirmed by several studies [44, 61]. Also, Apigenin has been shown to suppress stemness phenotypes of breast cancer cells by inhibiting YAP/TAZ activity [55]. Here, we showed the suppressive effects of *Viola odorata* extract on tumorigenicity capability of MCF7, SKBR3 and their derived mammospheres through decreased size and volume/depth of generated tumors in CAM of chicken embryo.

Results showed that although *Viola odorata* extract induces cell death in a similar doses for both breast cancer cell line and its derived mammospheres, it inhibits migra-tion, colony formation and invasion of breast cancer cells derived mammospheres more than corresponded breast can-cer cell lines. Along with our study, several studies targeted BCSCs and signaling pathways by photochemicals, anti-diabetic drug metformin and some natural compounds, such as quercetin, genistein, capsaicin and green tea polyphenol, epigallocatechin gallate (EGCG), polyphenols, Chestnut leaf

extract, *Strobilanthes crispa* Blume hexane extract, *Caesal-pinia spinosa* extract [73–79].

## Conclusion

Finally, we suggested *Viola odorata* extract as an anti-meta-static drug through significant decrease of migration, colony formation and invasion of BCSCs compare with the breast cancer cell lines. Also, we showed that it may act in a cell-dependent manner. At optimal concentration, *Viola odorata* extract may target breast cancer and BCSCs not normal epi-thelial cells. Although, further studies are needed to prove the exact route of the drug's effect, our study results strength the traditional medicine beliefs that *Viola odorata* can be a relative inhibitor of metastatic breast cancer.

## Compliance with ethical standards

**Conflict of interest** No conflict of interest.

**Research involving human participants and/or animals** The permission to use chicken embryo based on the standard protocols was obtained by Ethics Committee of Royan Institute (The permission to use chick embryo based on the standard protocols was obtained by Ethics Committee of Royan Institute (Project no. IR ACECR ROYAN REC IR ACECR ROYAN REC).

**Informed consent** Not applicable.

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