Silibinin affects tumor cell growth because of reduction of stemness properties and induction of apoptosis in 2D and 3D models of MDA-MB-468
Pegah Abdollahia, Marzieh Ebrahimib, Nasrin Motameda and Fazel S. Samanib

Silibinin, with a strong antioxidant activity and a weak cytotoxic property, is considered a candidate for cancer prevention. As there is no information on its effect on breast cancer tumor-initiating cells (cancer stem cells (CSCs)) in a 3D culture model, which more closely mimic natural tissues, we carried out this study to determine whether silibinin can target breast CSCs in MDA-MB-468 cells cultured under 3D and 2D conditions. Silibinin was added to culture medium of MDA-MB-468 at a half maximal inhibitory concentration (IC50) dose in 2D and 3D models. Then, stemness properties were assessed using colony and sphere-formation tests. Flow cytometry and real-time PCR were used to determine the different expression levels of stem cell-related marker at protein and mRNA levels under both culture conditions. Our results showed that silibinin inhibits cell growth in a dose-dependent manner by induction of apoptosis, alteration of the cell cycle, reduction of stemness properties and function, and induction of tumoral differentiation. The mechanism of silibinin action and also the response of tumor cells differed when cells were cultured in a 3D model compared with a 2D model. Silibinin may potentially target breast CSCs. Moreover, tumor-initiating cells are more sensitive to silibinin in a 3D culture than in a 2D culture. Anti-Cancer Drugs 00:000–000 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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Keywords: 2D culture, 3D culture, apoptosis, breast cancer stem cell, silibinin

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Background
Breast cancer as a heterogeneous tumor model consists of a distinct subtype of cells with different proliferative, differentiative, and tumorigenic properties [1]. It is the most common cause of cancer-related death among women in Iran [2] and developed countries [3,4]. Growing data showed that a subset of cells called breast cancer stem cells (CSCs) are present in cancers with capacities for self-renewal, multilineage differentiation, tumorigenicity, chemotherapy, and radiotherapy resistance [5,6], whereas they cause cancer establishment and progression as well as resistance to current treatments [1,7]. These types of cells express putative CSC markers such as CD44 and CD133, enhance ALDH activity, over-express stemness-related genes, and functionally initiate tumors in vitro (by enhancing colony-forming and spheroid-forming ability) and in vivo (when the cells are transplanted to NOD-SCID mice) [8–10].

Most traditional anticancer therapies have strong cellular cytotoxicity properties and severe side effects [11] on both cancerous and noncancerous cells, but cannot target CSCs to reduce tumor relapse. Moreover, about 90% of promising preclinical drugs fail to result in effective treatments for humans in all therapeutic classes, which leads to considerable drain in time and money and delays in the discovery of successful interventions [22]. Use of a 2D culture model and expansion of cells on polystyrene plastic dishes are the main problems in the field of pharmaceutical studies of cancer. In fact, a 2D culture model lacks the real complexity of a cell microenvironment and is an unnatural environment [23]. However, the use of animal models in tumor biology is expensive, time consuming, and frequently fails [24,25]. Therefore, a 3D culture model may be useful to resolve the existing in-vitro defects in drug discovery, screening, and safety assessment.

To date, dietary supplements and other phytotherapeutic agents could be used as an alternative source in cancer treatment because their synergistic efficacy properties minimize the systemic toxicity of chemotherapeutic agents. Silymarin and its major constituent, silibinin, are extracts from the medicinal plant *Silybum marianum* (milk thistle) and have traditionally been used for the treatment of liver cirrhosis. However, it has been reported that its orally active component exerts anticancer effects with no adverse effects on human and rodent cells at doses as high as 2 g/kg of body weight [12–14]. Its effects were mainly exerted by inhibition of cell proliferation,
inhibition of the Wnt/β-catenin pathway, and associated transcriptional activity [15–21].

As there are no data on the effect of silibinin on stemness properties in breast cancer, in this study, we first investigated the tumor-suppressing effect of this drug on cell growth by assessing cell viability, apoptosis, cell cycle, and stemness-related function in a 2D culture of MDA-MB-468 breast cancer cells and then compared the results with 3D cultures as an in-vitro model for cytotoxicity.

Materials and methods
Ethical standards
All experiments were conducted in compliance with the current laws of the Islamic Republic of Iran. The Ethics Committee of the Royan Institute approved the study.

Cell cultures
The human breast cancer cell line MDA-MB-468 was obtained from the Pasteur Institute (Tehran, Iran). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mmol/l glutamine, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 10% fetal bovine serum in 5% CO2 humidified air at 37°C. A trypsinized single-cell suspension was seeded into culture dishes for the monolayer cultures. DMEM, penicillin, streptomycin, fetal bovine serum, and trypsin/EDTA were purchased from Gibco (Invitrogen, Grand Island, New York, USA).

Spheroids were cultured using a liquid overlay method. Briefly, round-bottom 96-well plates and T25 flasks were coated with 12 mg/ml of poly (2-hydroxyethyl methacrylate) (Poly-HEMA; Sigma, St Louis, Missouri, USA) in 95% ethanol and washed once with phosphate-buffered saline before cell seeding. The mean diameter (d) of the spheroids was determined using the following equation: $d = (a \times b)^{1/2}$, where a and b are the orthogonal diameters of the spheroid [26].

Cell proliferation assay
First, 13 000 cells/cm² and 16 000 cells/cm² were seeded on 3D poly-HEMA-coated plates and 2D plastic surfaces, respectively, on the initial day of culture. Cells were then exposed to silibinin for 24, 48, and 72 h on day 1 for the monolayer cultures and on day 4 for the spheroid cultures.

Cytotoxicity assay
Cells were seeded on day 0 and exposed to silibinin for 48 h on day 1 for the monolayer culture and on day 4 for the spheroid culture. The number of viable cells was determined before and after exposure to the anticancer agent to estimate cytotoxic parameters following a general assay protocol using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Fitchburg, Wisconsin, USA) in a 2D culture and propidium iodide (PI) assay in a 3D culture.

Apoptosis detection
MDA-MB-468 cells were treated with silibinin at an half maximal inhibitory concentration (IC50) value in a 2D (500 µmol/l) and a 3D (1500 µmol/l) model, respectively, for 48 h. The apoptotic effects of silibinin on cells were measured using the Annexin-V/PI Staining Kit (Sigma) according to the manufacturer’s instruction. Binding of annexin-V to phosphatidylserine suggests early apoptosis, whereas binding of annexin-V to PI indicates late apoptosis.

Cell cycle analysis
MDA-MB-468 cells were treated with silibinin at the same concentration and incubation time used for apoptosis detection (500 and 1500 µmol/l in 2D and 3D culture, respectively, for 48 h). Cells in control (untreated) and test (treated with silibinin) groups were stained with a solution containing PI (100 mg/ml) and ribonuclease (50 mg/ml) for about 20 min. Stained cells were analyzed using the BD FACSCalibur Flow Cytometry System (BD Biosciences, San Jose, California, USA).

Colony-formation assay
Two hundred cells of MDA-MB-468 from both control and test groups were plated in each well of six-well plates and allowed to grow for 9 days. Then, colonies were counted under light microscopy.

Spheroid-formation assay
MDA-MB-468 cells untreated and treated with silibinin were plated at a density of 10 000 cell/ml in six-well ultra-low attachment plates in serum-free DMEM supplemented with 20 ng/ml epithelial growth factor (Royan Institute, Tehran, Iran) and 20 ng/ml basic fibroblast growth factor (Royan Institute). After 7 days, all spheres greater than 100 µm and up to 400 in diameter were counted in each well. The cutoff can reflect 3D cell–cell interactions and is appropriate when attempting to establish pathophysiological conditions with hypoxic areas in the spheroid center.

Flow cytometry analysis
Fluorescence-activated cell sorting analysis was carried out using the BD FACSCalibur Flow Cytometry System with the following antibodies: (i) fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated CD44 (BD Biosciences) and (ii) PE-conjugated CD133 (MACS, Auburn, California, USA). Cells stained with isotype antibodies, including PE Mouse IgG2b and FITC Mouse IgG2b (Dako, Carpinteria, California, USA), served as respective controls.

ALDH activity was measured using the Aldeflour Kit (Stemcell Technologies, Vancouver, Canada) according
The effect of silibinin on the morphology, proliferation, cellular viability, and cell cycle of MDA-MB-468 in 3D and 2D models. (a) Phase-contrast microscopy showed that living cells transformed into a typical apoptotic morphology after treatment in 2D and 3D models. (magnification ×20). (b) Silibinin reduced the cell number dose and time dependently in 2D and 3D models. (c) The cell viability decreased in both culture models 48 h after treatment when tested by PI and MTS assays. The viability of control was considered 100%. DMSO as a solvent of silibinin exerted no effect on the viability of MDA-MB-468 cells (right panel). (d, e) Cell cycle analysis by flow cytometry showed that silibinin arrested the cells in the S phase in the 2D model and had no effect on 3D cultured cells. Data are expressed as the mean ± SD of three separate biological experiments. *P < 0.05. DMSO, dimethyl sulfoxide; PI, propidium iodide.
to the manufacturer’s instruction. The ALDH inhibitor diethyl-aminobenzaldehyde served as a negative control.

Quantitative real-time reverse transcription-PCR
Total mRNA was isolated from MDA-MB-468 cells cultured in 2D (treated by 500 μmol/l) and 3D (treated by 1500 μmol/l) using TRIzol Reagent (Invitrogen) according to the manufacturer’s instruction. cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, Massachusetts, USA). SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara, Berkeley, California, USA) was utilized to perform quantitative real-time reverse transcription-PCR (RT-PCR) using Rotor-Gene 6000 (Corbett). The GAPDH gene transcript was measured as a normalizer to determine the other gene relative transcripts (ΔΔCt).

Statistical analysis
Student’s t-test was used to determine the statistical significance in the differences between the silibinin-treated and the control groups as well as the difference between 2D and 3D cultures. All experiments were conducted in triplicate. The data are presented as means±SD or mean±SEM. The silibinin-induced changes were considered significant at P value less than 0.05.

Effect of silibinin on induction of apoptosis in MDA-MB-468 cells. (a) MDA-MB468 cells stained with annexin-V (early apoptosis) and PI (late apoptosis) following silibinin treatment (500 μmol/l in 2D and 1500 μmol/l in 3D culture) for 48 h. (b) Graphic representation of cell percent apoptosis in response to silibinin in 2D and 3D models. (c) Mean values of fold change after silibinin treatment for two proapoptotic genes (BAX, Caspase 3) and two antiapoptotic genes (BCL2, survivin) relative to the control (no treatment) after 48 h in 2D and 3D culture. Normalization relative to GAPDH was performed. All experiments were conducted as three different replicates. Results were mean±SEM. *P < 0.05; **P < 0.007, compared with untreated cells. FITC, fluorescein isothiocyanate; PI, propidium iodide.
Results and discussion

Different effects of silibinin on cell viability in 2D and 3D cultures

Morphologically, MDA-MB-468 seeded in 2D culture plates appeared like epithelial cells with oval and/or polygonal shapes, whereas those on 3D-poly-HEMA-coated-culture plates formed grape-like spheres with poor cell-cell contacts and a diameter greater than 200 μm (Fig. 1a). After incubation with silibinin for 48 h, the typical morphology of apoptotic cells, including loss of adherence, condensed cytoplasm, and formation of apoptotic bodies, was observed under both culture conditions using light microscopy. Moreover, silibinin induced the formation of disintegrated spheroids under the 3D condition (Fig. 1a). The maximum changes in morphology were observed at an IC50 dose of silibinin.

To assess the effect of silibinin on cellular proliferation, a preliminary screening was performed at different concentrations of silibinin for 24, 48, and 72 h in cells cultured in 2D and 3D models. Silibinin significantly inhibited cell proliferation in a dose-dependent and time-dependent manner (Fig. 1b). However, multicellular structures of those cells showed higher growth than their monolayer-grown counterparts (Fig. 1b), which was resistant to silibinin at a lower dose. Then, we determined whether

Colony and sphere-formation ability tested before and after treatment in 2D and 3D models of MDMB-468. (a) Colony number showed a significant reduction under 2D and 3D culture conditions at an IC50 dose (500 μmol/l in the 2D model and 1500 μmol/l in the 3D model for 48 h) and (b) Spheroid-formation efficiency was reduced after silibinin treatment in the 2D culture, but not in the 3D culture. (c) Silibinin induced morphological changes in colonies and (d) Spheroids in 2D and 3D cultures (magnification ×20). (e) Spheroid sizes were reduced before and after silibinin treatment in 2D and 3D cultures *P<0.05, compared with untreated cells. IC50, half maximal inhibitory concentration.
growth inhibition was because of induction of apoptosis-related cell death, reduction of cell viability, and/or alteration in cell cycle. Our results showed that silibinin decreased cell viability in a dose-dependent manner in both 3D and 2D cultures (Fig. 1c). However, the IC50 dose in the 3D culture was three-fold higher than the related value in the 2D culture (1500 vs. 500 µmol/l), which may be related to an increase in cellular resistance or drug inaccessibility in the 3D culture model. Meanwhile, dimethyl sulfoxide as a silibinin solvent did not exert any effect on viability (Fig. 1c). The results of cell cycle analysis and apoptosis induction showed that low cell growth in 2D culture may be related to a significant reduction in the G0/G1 phase population in the 2D culture [39.49 ± 6.76% (treated group) vs. 62.29 ± 3.48% (control group); P = 0.05] (Fig. 1d and e), a cell cycle arrest at the S phase, an

![Fig. 4](a) Mean values of fold change for stemness-related genes (CD133, ALDH, C-MYC, KLF4, OCT4, NANOG, SOX2) and differentiation genes (BRCA1, GATA3) after culturing cells under the 3D condition relative to the control (2D culture condition). GAPDH mRNA was utilized to normalize gene expression data. Results were mean ± SEM. *P < 0.05; **P < 0.007, compared with 2D cultured cells. (b) Graph showing the mean values of fold change after treating cells with an IC50 dose of silibinin for stemness-related genes (CD133, ALDH, C-MYC, KLF4, OCT4, NANOG, SOX2) and differentiation genes (BRCA1, GATA3) relative to the control (no treatment) for 48 h in 2D and 3D cultures. GAPDH mRNA was utilized to normalize gene expression. Results were mean ± SEM. *P < 0.05; **P < 0.007, compared with untreated cells. IC50, half maximal inhibitory concentration.
increase in the percentage of early apoptosis (Fig. 2a and b), and downregulation of BCL2 (of antiapoptotic genes; \( P = 0.003 \)) expression (Fig. 2c). However, the reduction in the number of cells in 3D culture might be because of an increase in the percentage of late apoptotic cells (Fig. 2a and b), and downregulation of BCL2 and SURVIVIN (of IAP family; \( P = 0.00 \)) (Fig. 2c). Silibinin exerted no effect on the cell cycle in 3D cells (Fig. 1d and e).

The apoptotic cells with fragmented nuclei that appeared as cells with hypodiploid DNA content could be detected at the sub-G0 peak. Thus, the cell cycle result indicated that the silibinin-treated samples showed a significant increase in the sub-G0 phase compared with the untreated sample under both culture conditions, which also confirmed the apoptosis-inducing activity (Fig. 1d and e).

As mentioned above, silibinin reduces tumor cell growth through induction of apoptosis. In 3D culture, cells entered into late apoptosis by downregulating BCL2 and SURVIVIN, but in 2D, most of the cells entered into early apoptosis by downregulating BCL2. However, cells in 2D showed resistance to apoptosis by upregulating Caspase 3, which is a proapoptotic gene that plays a central role in the execution phase of cell apoptosis; no alteration was observed in 3D cultured cells [27]. Another reason may be the increased expression of SOX2 and OCT4 in 2D culture after silibinin treatment, showing its ability to promote antiapoptosis induction [28].

The second mechanism of silibinin action is the change in cell cycle. We showed that in contrast to 2D, which led to the arrest of cell cycle in the S phase, in the 3D culture, silibinin could not alter the cell cycle, which may indicate how the cells will respond to this drug in real models. In general, the progression of the cell cycle in eukaryotes is a complex process involving a resting G0 phase, and cell growth involving G1, S, and G2/M phases in a step-wise manner. Silibinin caused cell cycle arrest in different phases in different cancer cells [29–31].

**Alteration of stemness properties after treatment with silibinin at IC\textsubscript{50} doses in 2D and 3D models**

Because of the accumulating evidence, CSCs play important roles in tumor growth, metastasis, and relapse after chemotherapy or radiotherapy; therefore, a number of studies have attempted to identify drugs that especially kill CSCs [32–34]. As an alternative strategy for cancer therapy focusing on CSCs, in this study, we assessed the influence of silibinin in inducing differentiation in CSCs. For this purpose, we evaluated sphere-forming and colony-forming capacities, which are considered properties of CSCs.

As shown in Fig. 3a, cells in both 2D and 3D models could form colonies and spheres. However, there were no significant differences in their ability for colony and sphere formation (\( P = 0.05 \)). Interestingly, the number and also the morphology of colonies were significantly altered in both culture models after treatment with silibinin (at IC\textsubscript{50} for 48 h). The numbers of colonies were 40.66 ± 2.02 and 31.67 ± 5.96 before silibinin treatment in 2D and 3D cultures, respectively; these values reduced to 0.66 ± 0.57 and 2 ± 0.5 after silibinin treatment, respectively (\( P = 0.00 \)) (Fig. 3a). However, the reduction was significantly higher in the 2D culture relative to the 3D culture (\( P = 0.03 \)). Moreover, cells treated with silibinin were much larger in size while establishing spindle-shaped cells, suggesting that silibinin can induce differentiation in MDA-MB-468 (Fig. 3c).

Although silibinin reduced the number and morphology of colonies in both culture models, it caused a reduction in sphere-forming abilities only in the 3D culture model (6.14 ± 0.43 before vs. 1.55 ± 0.07% after treatment) (Fig. 3b). Meanwhile, spheroid sizes were significantly decreased after incubation with silibinin in both models (Fig. 3e).

Because of reduction of sphere-formation and colony-formation ability after treatment with silibinin in the 3D model, we investigated whether there were any changes in the expression of presumptive markers of breast cancer stem and differentiated cells; therefore, we evaluated the expression of CD133, ALDH, C-MYC, KLF4, OCT4, NANOG, and SOX2 of stemness-related genes and BRCA1 and GATA3 of breast differentiation markers using RT-PCR. As expected, KLF4 was significantly upregulated (2.4 ± 0.2) in cells cultured in the 3D model, whereas the expression levels of other stemness-related genes including CD133, ALDH, C-MYC, NANOG, and SOX2 were downregulated in cells cultured in 3D by 0.42 ± 0.19, -0.22 ± 0.01, -0.51 ± 0.04, -0.09 ± 0.02, and 0.22 ± 0.03-fold, respectively, compared with the related values of cells cultured in the 2D model (Fig. 4a). Also, 3D culture did not induce a significant alteration in the expression of OCT4 compared with the related value of the 2D culture. In addition, differentiation genes, GATA3 and BRCA1, were downregulated to 0.66 ± 0.02-fold and upregulated to 1.85 ± 0.04-fold in the 3D model, respectively. Then, the expression levels of stem/progenitors and differentiation markers were measured after treating cells by 500 µmol/l silibinin in a 2D culture and 1500 µmol/l in a 3D culture for 48 h. According to our findings, cells cultured in 3D could induce differentiation by downregulation of all stemness-related genes and by upregulation of the differentiated marker (GATA3) following silibinin treatment (Fig. 4b). Cells cultured in the 2D model showed downregulation of CD133, ALDH, C-MYC, and GATA3 relative to untreated cells; however, OCT4 and SOX2 from stemness genes and BRCA1 from differentiation markers were significantly upregulated after silibinin treatment (Fig. 4b).
The percentage of positive cells for ALDH/CD133, CD44/CD133, and ALDH/CD44 in 2D and 3D models of MDA-MB-468 before and after treatment with silibinin. (a) Flow cytometry plots for MDA-MB-468 cells stained with ALDH, CD133, and CD44 following silibinin treatment in 2D and 3D cultures. Control analyses were carried out using an isotype-matched antibody. The percentages in the right top quadrant refer to ALDH+/CD133+, CD44+/CD133+, and ALDH+/CD44+ populations and (b) graphic representation of cell percent ALDH+/CD133+, CD44+/CD133+, and ALDH+/CD44+ in silibinin-treated and untreated cells in 2D and 3D cultures. Data are expressed as the mean ± SD of three separate experiments. *P < 0.05; **P < 0.007, compared with untreated cells. FITC, fluorescein isothiocyanate.
Moreover, we used flow cytometry to assess protein expression of CSC-related markers including ALDH/CD133, CD44/CD133, and ALDH/CD44 in 2D and 3D cultures. Statistically, 3D culture induced a significant reduction in ALDH+/CD133+ (P = 0.04) and CD133+/CD44+ (P = 0.01) expression levels relative to the 2D model. Also, the proportion of cells with phenotypes of ALDH+/CD133+, CD44+/CD133+, and ALDH+/CD44+ decreased under two culture conditions after treatment with an IC50 dose of silibinin (P < 0.05) (Fig. 5a and b). However, a significant reduction in the CD133+/CD44+ phenotype was observed in the 3D culture compared with the 2D culture.

**Discussion**

The tumor microenvironment plays an important role in response to chemotherapy, but monolayer cultures are the bases of most in-vitro screening approaches. It is, therefore, very important to develop more predictive pharmacological models for the assessment of new therapeutic strategies. Tumor spheroids present a level of intermediate complexity and incorporate the concept of the tumor microenvironment [35–45]. Because of the presence of some potential side effects of chemical compounds, we investigated the effects of a natural compound named silibinin [30] on cell growth, cell viability, cell cycle, apoptosis, and modulating CSCs. The results of this study showed that silibinin could inhibit tumor cell growth by different mechanisms. Also, we found that the responses of tumor cells differ when cells are cultured under 3D rather than 2D conditions. The first and main mechanism of silibinin is induction of apoptosis, which was also reported previously by other studies [19,39,40]. Interestingly, cells in the 3D model entered into late apoptosis, but cells in the 2D model entered into early apoptosis after treatment with silibinin at an IC50 concentration. The second mechanism was alterations in the cell cycle. However, 2D cells and not cells in the 3D culture arrested on S phase by silibinin. The third mechanism that silibinin used for cell growth regulation was reduction of stemness properties and induction of differentiation. In fact, here, we report the first evidence of the effect of silibinin on breast CSC regulation. However, the effect of silibinin on both the number and the size of colonies has been reported previously in colon cancer cell lines [41]. Indeed, reduction in colony-forming capacity and an alteration in the morphology of colonies to spindle shape were observed, suggesting differentiation of human breast carcinoma MDA-MB-468 cells. This differentiation was further confirmed by upregulation of the differentiation marker (BRCA1) in 2D and (GATA3) in 3D cultures, by downregulation of stem cell markers (OCT4, NANOG, SOX2, CD133, ALDH, KLF4, and C-MYC) in 3D and (CD133, ALDH, KLF4, and C-MYC) in 2D models, as well as by a reduction in the expression of ALDH+/CD133+, CD44+/CD133+, and ALDH+/CD44+ in both 2D and 3D models, which are considered as profiles of breast CSC markers. On the basis of the results summarized in Table 1, it seems that the structural architecture in a 3D tissue model regulates differentiated cell functions through changes in cell shape, as well as increased cell–cell and cell–matrix interactions affecting the response to external agents [42,43].

Moreover, the data presented here showed promising cytotoxicity of silibinin against CSCs in MDA-MB-468 cells because it most likely means that treated CSCs were promoted to a more differentiated state. However, comparison of the results of the effect of silibinin on CSCs under two culture conditions showed that its IC50 dose effect (Fig. 6) was more significant in the 3D culture than in the 2D culture. It seems that transformation of the phenotype in the 3D culture and also into the CSC phenotype could make these cells more sensitive to silibinin [41], thus supporting the fact that its multiple treatments were more effective in reducing both the number of stem cells and the size of the colonies or spheres. Another possible mechanism is a shift in the symmetrical division that is responsible for self-renewal

| Table 1 Summarized results of silibinin effects in 2D and 3D models |

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↓ reduction or downregulation; ↑, enhancement or upregulation; ↑↑, without significant changes after silibinin treatment in 2D and 3D cultures; ↓↓, high reduction; ↑↑, high increase.
into the asymmetric type [41]. In addition, modulation of Wnt/β-catenin/FGF/Notch family proteins that play a critical role in the growth and survival of CSC [41–45] can inhibit tumor growth.

Conclusion
Our data provide evidence that silibinin exerts a dual effect against both MDA-MB-468 bulk cells and their CSCs, wherein it interferes with CSC pool expansion and function. Also, the results in 3D culture strongly suggest the importance of conducting further investigations, especially using in-vivo models, to promote the use of silibinin as a chemopreventive agent against breast CSCs, which is the key component of chemoresistance, recurrence, and metastasis.

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P.A. conceived the idea, performed the literature search, experiments, and drafted the manuscript; F.S. conducted flow cytometry experiments and contributed toward drafting the manuscript; M.E. and N.E. supervised the project, made substantial contributions to the concept and design of manuscript, and drafted the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest
There are no conflicts of interest.

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