Anti-Breast Cancer Activities of 8-Hydroxydaidzein by Targeting Breast Cancer Stem-Like Cells

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ABSTRACT - Purpose: Cancer stem cells (CSCs) play an important role in various stages of cancer development and therapy refractoriness. 8-Hydroxydaidzein (8-OHD) has revealed anti-cancer activity in different tumors. Accordingly, we aimed to assess the effects of 8-OHD on the suppression of breast cancer stem-like cells (BCSCs). **Methods:** The anti-proliferative and pro-apoptotic properties of 8-OHD were examined by MTS assay and flowcytometry. The expression levels of stemness markers and JAK2/STAT proteins were measured by quantitative real time-PCR (qRT-PCR) and western blotting, respectively. **Results:** 8-OHD significantly decreased three out of six stemness markers and remarkably reduced viability and induced apoptosis of spheroidal and parental cells compared to controls. Further experiments using CD95L, as a death ligand, and ZB4 antibody, as an extrinsic apoptotic pathway blocker, showed that 8-OHD induced apoptosis through the intrinsic pathway, proposing a mechanism by which 8-OHD triggers apoptosis. Results of western blot analysis also revealed a marked decline in the phosphorylation of JAK2 and STAT proteins. **Conclusion:** Our study, for the first time, elucidated an anti-BCSC activity for 8-OHD via decreasing stemness markers, inducing toxicity and stimulating apoptosis in these cells and parental cells. Our results also suggested a novel mechanism by which 8-OHD induces apoptosis in BCSCs.

INTRODUCTION

According to the recent World Health Organization (WHO) report, cancer is the second leading cause of mortality globally, resulting in 8.8 million deaths during 2015 (1). Conventional cancer treatments, such as chemotherapy, surgery, and radiotherapy are not effective in curing cancer and they have many side effects as well (2). Therefore, it is necessary to find new therapeutic approaches that completely treat tumors without any side effect. Cancer stem cells (CSCs) play a pivotal role in tumor development, metastasis, recurrence and they are generally resistant to conventional therapies. Therefore, the removal of the cancer stem cell from a tumor could lead to better clinical outcomes (3, 4). Breast cancer is the most frequently diagnosed cancer among women and the leading cause of cancer mortality worldwide (5). Breast cancer has been reported as the first tumor from which CSCs were identified and isolated (6). Several putative stemness markers such as Wint1, notch1, β-catenin, CXCR4, SOX2, and ALDH3A1 have been reported for CSCs that they determine the stem cell characteristics of these cells (7-9). Breast CSCs (BCSCs) have been evidenced that show relative resistance to radio- and chemotherapy suggesting the need for the new CSC-targeted therapies for this type of cancer (10). To meet this goal, natural products are the best known candidates for use in treating breast tumors by targeting various signaling molecules and pathways in BCSCs (11). Janus kinase (JAK)/transducer and Activator of Transcription 3 (STAT3) pathway has been revealed to have important roles in the regulation of normal and cancer stem cells behaviors and the inhibition of this pathway could cease tumor growth and induces apoptosis in several cancers (12-14). Natural products as rich sources of novel, bioactive compounds with ability to interact with various cellular targets and with limited painful side-effects have been shown to act as anti-cancer agents by inducing cell death in CSCs, causing CSCs to

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differentiate, or sensitizing CSCs to conventional chemotherapy treatments (15). Daidzein (4′,7-dihydroxyisoflavone) or 7-Hydroxy-3-(4-hydroxyphenyl) chromen-4-one is one of the main isoflavonoids found in soybeans that has several pharmacologic properties, such as antioxidant properties and inhibition of tyrosine kinases as well as anticancer and anti-inflammatory properties (16). 8-Hydroxydaidzein (8-OHD) is a hydroxylated derivative of daidzein that can be produced from daidzein, soybean, soybean extract, and soygerm koji by microorganisms isolated from fermented soybean foods or can be converted from daidzein in human and rat liver (17, 18). Antioxidative and anti-inflammatory properties of 8-OHD have been reported in previous studies (19-21). 8-OHD also significantly induces apoptosis and reduces mRNA expression levels of multidrug resistance protein 1 (MDR1), MDR-associated protein (MRP) 1, and MRP2 in human colon adenocarcinoma Caco-2 cells, suggesting that the 8-OHD can be involved in intensifying cytotoxicity and preventing resistance to therapy in cancer cells (22). In the current study, we first attempted to enrich breast cancer stem-like cells (BCS-LCs) in spheroidal structures called mammosphere, and then we tried to examine the effects of 8-OHD on the viability, apoptosis and the eradication of stem cell characteristics of BCS-LCs. Our study, for the first time, established an anti-BCSC effects for 8-OHD, which were exhibited by reducing stemness markers and proliferation as well as the induction of apoptosis in these cells. Also, we suggested a novel mechanism by which 8-OHD stimulates apoptosis in BCSCs.

METHODS

Mammosphere Cell Culture and Treatment

The human breast cancer cell line MCF7 was purchased from Pasteur Institute, Tehran, Iran. The cells were cultured in RPMI1640 containing 10% FBS and streptomycin/penicillin (100 U/ml). The cells were incubated at 37°C in an atmosphere of 5% CO2. Mammosphere structure was formed and cultured according to the method that was described by Lombardo, et al. (23). These spheroidal structures were thus dissociated by Accutase™ and seeded into 96-well culture plates at a density of 50 cells per well and were cultured by using serum-free media supplemented by epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) for 7 days. 8-OHD (Sigma–Aldrich, Saint Louis, MO, USA) was dissolved in Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, MO, USA) and the cultured cells were treated with this compound for 48 h. The number of mammospheres in each well was counted under a microscope. The sphere formation rate was determined as the percentage of sphere-forming cells relative to the total number of cells seeded.

Cell Viability and Apoptosis Assay

Cell viability was assayed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit [MTS, 3-(4, 5-dimethylthiazol-2 yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], Promega, according to the manufacturer's instructions. The quantity of formazan product was determined by measuring absorbance at 490 nm using an envision plate reader (PerkinElmer). Flowcytometry was used to determine the percentage of apoptotic cells following treatment with different doses of 8-OHD (0-70μM). The 8-OHD -treated cells were centrifuged (5 min, 1000 × g) at room temperature. Afterward, the cells were resuspended and washed once using 5 ml of phosphate-buffered saline before being stained with annexin-V/PI (apoptosis detection kit; R&D Systems). Flowcytometry was employed for the analysis according to the manufacturer’s protocol.

Real time-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen, Germany), according to the manufacturer's instructions. The synthesis of cDNA was performed by reverse transcriptase-polymerase chain reaction (RT-PCR) method using 1 μg/ml total RNA. RNA concentration was measured by Nanodrop 2000c spectrophotometer (Thermo Scientific, USA), and cDNA was synthesized by cDNA Synthesis Kit (Bio FACT, Daejeon, South Korea). The changes in mRNA expression of Wnt1, Notch1, β-catenin, CXCR4, SOX2, and ALDH3A genes and GAPDH, as internal control, were measured by quantitative reverse transcriptase PCR (qRT-PCR) in a ABI PRISM7900HT (Applied Biosystems) detection system using SYBR GREEN PCR Master Mix. Quantitative RT- PCR was performed using the primers provided in Table.1.

Table 1. Primer sequences used in this study

Gene name	Forward primer	Reverse primer
Wnt1	5'-GAACCTGCTTACAGACTCCAAGAGT-3	5'-CCGG ATTTTGG CGTATCAGA-3
Notch1	5'-CCGCAGTT GTGCTCCTGAA-3	5'-ACCTTGGCGGTCTCGTAGCT-3
B-catenin	5'-CCTTTGTCCCGCAAATCATG-3	5'-ACGTACGGCGCTGGGTATC-3
CXCR4	5'-CAGTGGCCGACCTCCTCTT-3	5'-ACATGGACTGCCTTGCATAGG -3
SOX2	5'-TGCGAGCGCTGCACAT-3	5'-CGGGCAGCGTGTACTTATCC-3
ALDH3A1	5'-TCCAGCAACGACAAGGTGATT-3	5'-GGCAGAGAGTGCAAGGTGATG-3
GAPDH	5'-ACCCACTCCTCCACCTTTGA-3	5'-CT GTTGCTGTAGCCAAATTCGT-3

Western Blot Analysis

To measure the expression of our tested factors at the protein level, the western blotting technique was used. Firstly, the cells were lysed by using RIPA lysis buffer (Thomas Scientific Inc). A total of 40 µg protein was separated using 10% SDS-PAGE, electrotransferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), blocked with 5% non-fat milk for 1 h at room temperature, and incubated with primary antibodies: STAT3, p-STAT3, JAK2, p-JAK2, and anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Thereafter, the blots were incubated with the corresponding horseradish peroxidase conjugated secondary antibody. Immunoblots were then visualized using an enhanced chemiluminescent kit (SuperSignal; Pie Pierce).

Data Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for post hoc comparison by SPSS software version 19. Statistical significance was set at $P<0.05$ and $P<0.01$. All the data that were collected from at least three individual experiments were presented as mean ± SD.

RESULTS

Enrichment of Breast Cancer Stem-Like Cells by Mammosphere Culture

Previous studies have evidenced that BCSCs can be propagated in serum-free media supplemented with EGF and bFGF (24, 25). Therefore, we employed this cell culture model to enrich the BCSC populations from the MCF-7cell line. $CD44^+/CD24^-$ have been previously reported as specific markers for identifying BCSCs (26). After developing mammospheres from MCF-7 cell culture, flowcytometry analysis was performed to sort the breast cancer cell populations based on the expression of CD44 and CD24 in these cells. As depicted in figures 1A and 1B, the expression of $CD44^+/CD24^-$ cell populations were significantly increased in mammosphere cells. The results also showed that the spheroid culture could be a suitable method to expand the BCSC populations from the MCF-7 cell line. To characterize and confirm the mammosphere cells, the expression levels of stemness markers were quantitatively measured. The results revealed a significant increase in the expression of BCSC stemness markers such as Wnt1, Notch1, β-catenin, CXCR4, SOX2, and ALDH3A1 in mammosphere culture compared to parental cells (Fig. 1C).

Effects of 8-Hydroxydaidzein on Stemness Markers and Viability of Breast Stem-Like Cells

According to previous reports and results of our study, breast cancer cells-derived mammospheres are highly enriched with cells that have BCSC phenotype and generally show increased expression of the BCSC markers (27). In our study, we also examined the effect of 8-OHD (70 µM) on stemness markers such as Wnt1, Notch1, β-catenin, CXCR4, SOX2, and ALDH3A1 in mammosphere cell culture. As shown in Fig.2, 8-OHD treatment significantly reduced the expression of CXCR4, SOX2, and ALDH3A1 in BCSCs- enriched mammospheres. To assess the effect of 8-OHD on the proliferation and viability of cells, mammospheres and parental cells (MCF-7) were treated with varying concentrations of 8-OHD (0-70µM) for 48 h. Cell viability was detected by Methyl Tetrazolium salt (MTS) assay. The MTS assay data demonstrated that 8-OHD significantly inhibited the proliferation of mammospheres and parental cells in a dose and time-dependent manner (Fig. 3). According to our obtained data, parental cells were more sensitive to 8-OHD in comparison to the mammosphere cells.

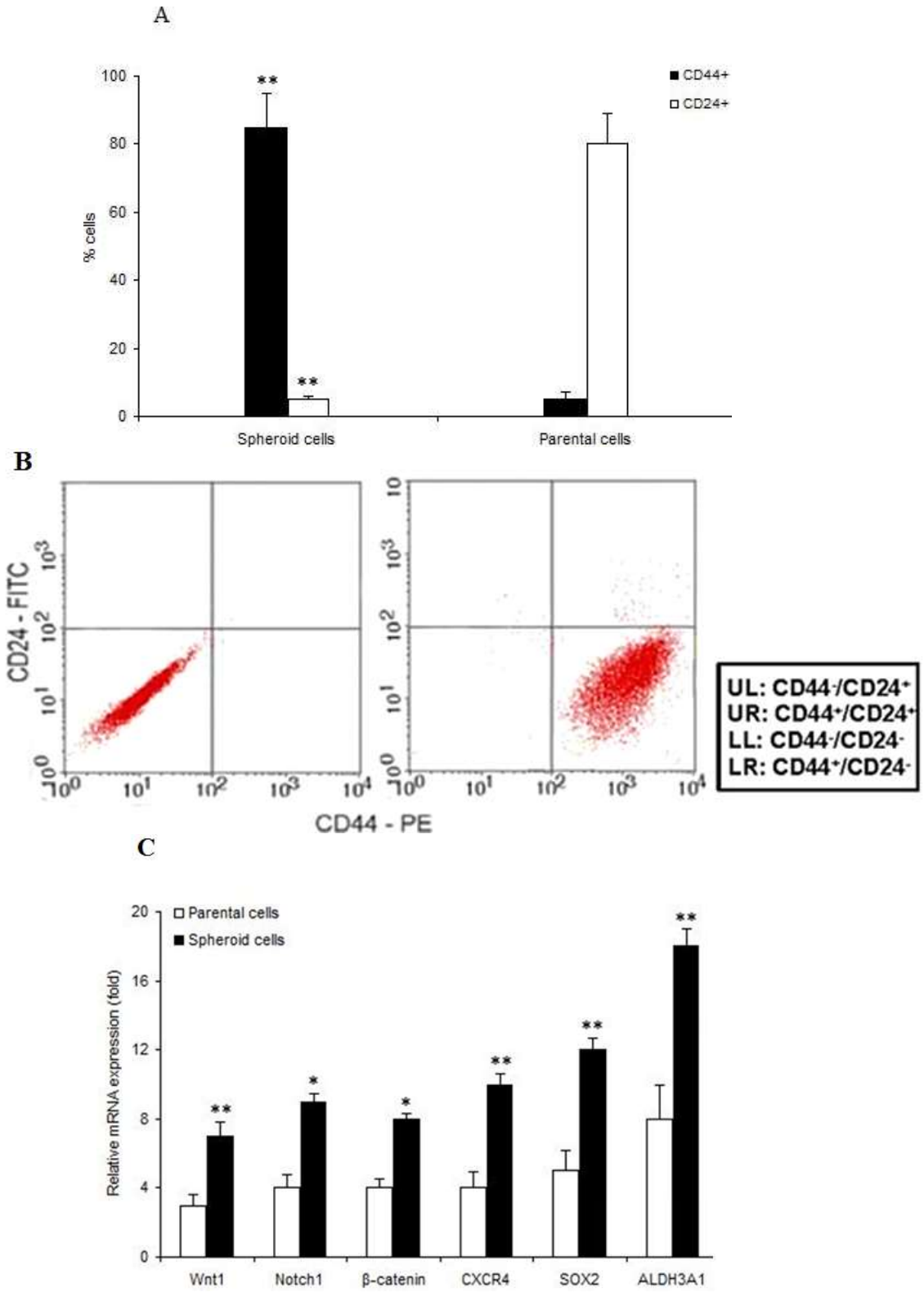


Figure 1. (A) Estimating the percentage of the CD44+/CD24-/low population in MCF-7 cells. Cells were stained with PE-CD24 and FITC-CD44. The results are representative of three separate experiments. Significant differences ($P<0.05$, Student's t-test) were found between mammosphere cells and parental cells. (B) Flowcytometry analysis data. The fluorescent intensity of CD 24 expression is on the Y axis and of CD44 expression on the X axis. (C) Real-time PCR analysis of the gene expression of Wnt1, Notch1, β-catenin, CXCR4, SOX2, and ALDH3A1. The data are presented as means ± SD for the three separate experiments (* $P<0.05$, ** $P<0.01$, in comparison with the parental cells).

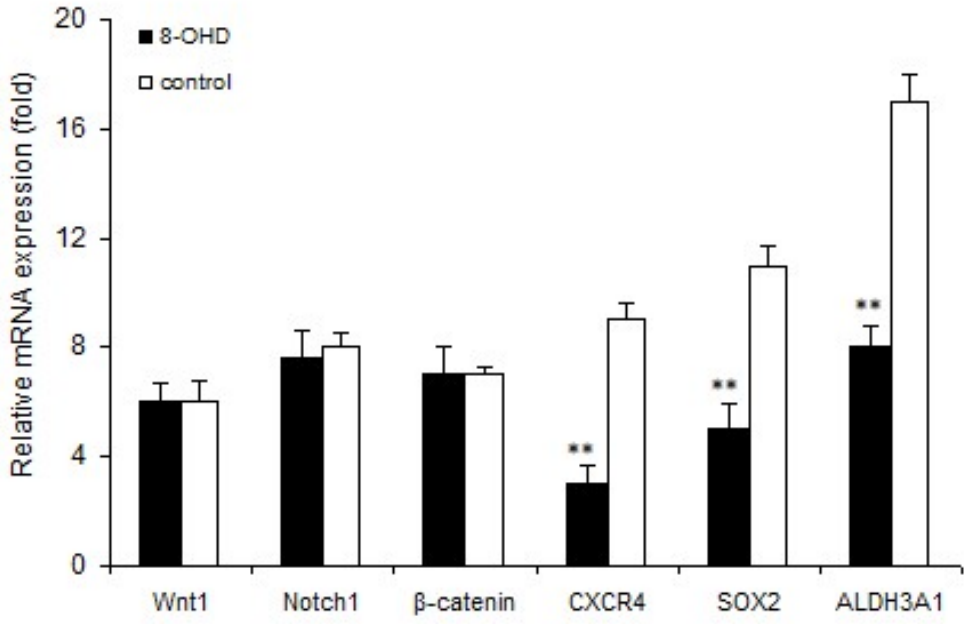


Figure 2. Analysis of the expression levels of stemness markers such as Wnt1, Notch1, β-catenin, CXCR4, SOX2, and ALDH3A1 in mammosphere cell culture following exposure to 70 µM 8-hydroxydaidzein (8-OHD) for 48h. The results are representative of at least three separate experiments. All results were significant at ** P<0.01.

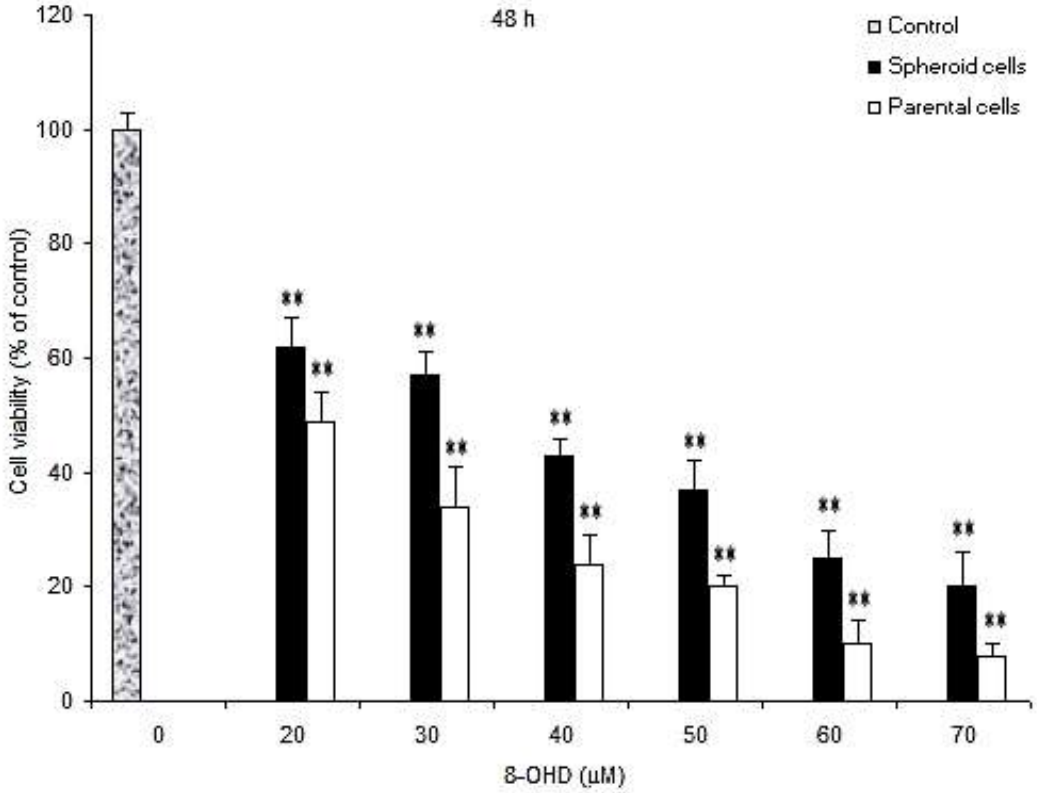


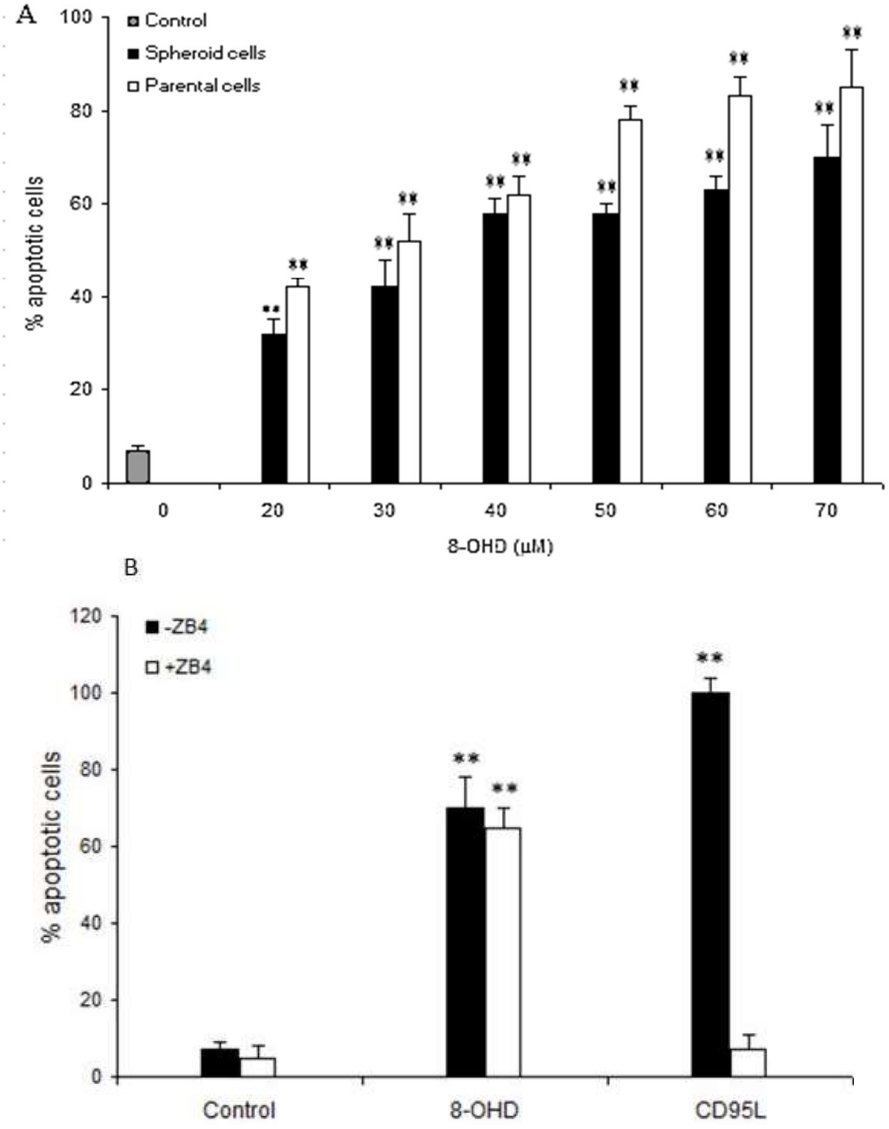
Figure 3. Cytotoxic effects of 8-OHD on MCF-7 spheroid and parental cells. Cells were treated with 8-OHD (0-70µM) for 48 hours. Cell viability was examined using MTS assay. The data are expressed as mean ± SD of separate experiments. Significant differences ** P<0.01) were found between the treatment and control groups.

Effect of 8-Hydroxydaidzein on Apoptosis in Breast Stem-Like Cells

As previously has been described, during the early stages of the apoptosis process, phosphatidylserine (PS) translocates to the outer side of the cell membrane. Therefore, we tried to explore the induction of apoptosis by 8-OHD using annexin V-FITC/PI staining (28). Accordingly, mammospheres and parental cells were exposed to the different concentrations of 8-OHD (0-70 μM) for 48 hours. The results indicated that 8-OHD markedly induced apoptosis in both mammospheres and parental cells (Fig. 4A). To unravel the involvement of intrinsic or extrinsic pathways in the induction of apoptosis by 8-OHD, we tended to design additional experiments. According to the literature, fas ligand (FasL or CD95L) plays an important role as death ligand, which mediates apoptosis induction by binding to its receptor, CD95 (29). Besides, anti-Fas (human, neutralizing, clone ZB4) antibody has been identified to block the extrinsic pathway of apoptosis (30). Accordingly, we used CD95L (100 ng/ml) and ZB4 (1μg/ml, 1 h) to further elucidate which pathway of apoptosis may be activated by 8-OHD. Flowcytometry analysis of the spheroid and parental cells with either CD95 receptor blocked by pre-incubation with anti-CD95 antibody ZB4 (1μg/ml, 1 h) or unblocked CD95 receptor and subsequent addition of 8-OHD (70μM) or soluble CD95L (100 ng/ml) as the positive control for 48 h. The results show that there is a significant difference between the percentages of apoptotic cells in the presence and the absence of ZB4 (Fig. 4B and C). These data confirmed that 8-OHD induced apoptosis through the intrinsic pathway, suggesting the mechanism of the induction of apoptosis in mammosphere cells. As described in the literature, JAK/STAT signaling pathway has an important role in regulating normal and cancer stem cells behaviors allowing to provoke tumor development (12), and hence the blocking of JAK/STAT signaling pathway leads to the suppression of tumor growth and induction of apoptosis in cancers cells (14). Therefore, we were interested in examining the expression levels of these two proteins in the mammospheres by western blot analysis. The mammospheres were treated with 8-OHD (70μM) and in combination with interleukin 6 (IL-6) for 48h. IL-6 was used to activate cytokine receptors to induce the phosphorylation of STAT3 and JAK2. Treating spheroid cells with IL-6 for 4 hours demonstrated an elevation in the phosphorylation of JAK2 and STAT3 compared to the control group as shown in figure 5. However, 8-OHD inhibited the activation of JAK2 and STAT3 in breast stem-like cells by blocking the phosphorylation of these two proteins in comparison to the IL-6 treated group. This compound also apparently prohibited the stimulatory effects of IL-6 by decreasing the phosphorylation of JAK2 and STAT3 following exposure of the cells to the combination of IL-6 and 8-OHD.

DISCUSSION

BCSCs production has been found to play a crucial role in therapy resistance of breast tumors with the ability to regenerate tumor (31). Therefore, targeting these cell populations may aid in improving therapeutic approaches and causes the suppression of tumor growth and progression. Mammosphere culture is one of the efficient ways to isolate and enrichment of tumorigenic breast cancer stem cells (32). Accordingly, we cultured the mammosphere from the MCF-7 cell line to explore the effects of 8-OHD on different aspects of this BCSC-bearing model. To establish the formation of mammospheres enriched with BCSCs, the gene expression levels of a group of stemness markers such as Wnt1, Notch1, β-catenin, CXCR4, SOX2, and ALDH3A1 as well as CD44/CD24, as BCSC markers, were analyzed. Corresponding data identified high expression levels of all six genes and showed $CD44^{+}/CD24^{-}$ phenotype in mammosphere cells as compared with the parental cells, proposing the formation and enrichment of BCSCs in mammosphere structure. As mentioned earlier, cancer-preventive properties of 8-OHD have been reported in several previous investigations (16, 22), However, the precise molecular mechanisms of its anticancer effects, especially against BCSCs, are not yet clear. To meet this purpose, we first examined the effect of this compound on stemness markers in BSCS-enriched mammospheres. Interestingly, we found that 8-OHD significantly reduced the expressions of CXCR4, SOX2, and ALDH3A1 in mammospheres; however, it had no considerable effects on the other three stemness markers in comparison to control cells. To the best of our knowledge, there is no previous data investigating stem cell marker expressions following exposure to daidzein or its derivatives in BCSCs.


A
Control
Spheroid cells
Parental cells
% apoptotic cells
8-OHD (µM)
B
-ZB4
+ZB4
% apoptotic cells
Control
8-OHD
CD95L

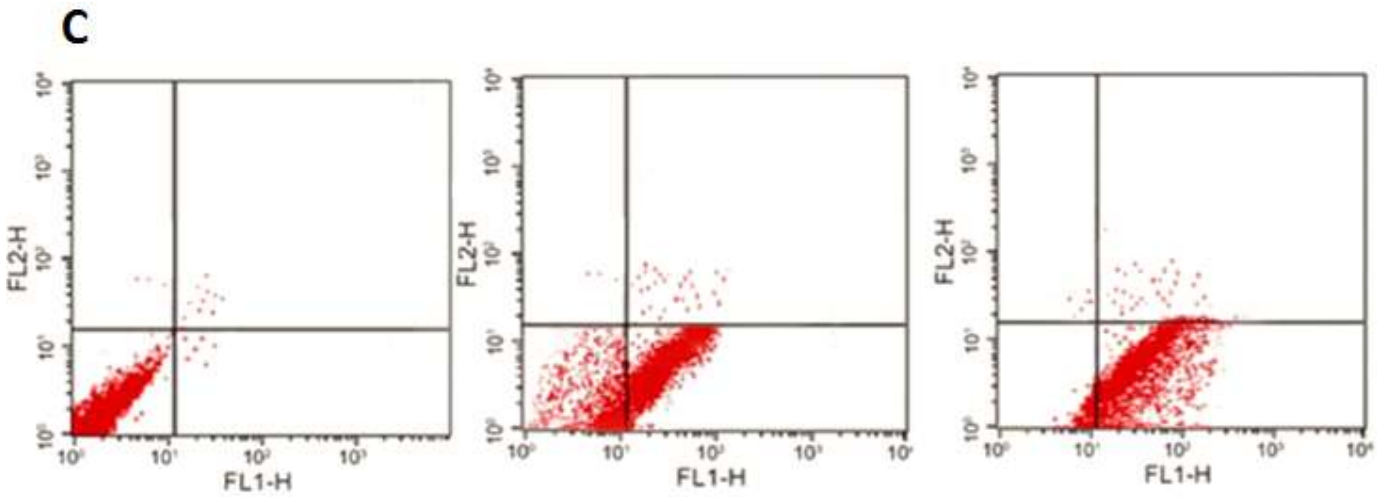


Figure 4. (A) Flowcytometry analysis of the MCF-7 cells treated with 8-Hydroxydaidzein (8-OHD, 0-70 μM) for 48 h. (B) 8-OHD-induced apoptosis occurred independent of CD95L binding to the CD95 receptor (**P< 0.01). (C) Flowcytometry analysis data.

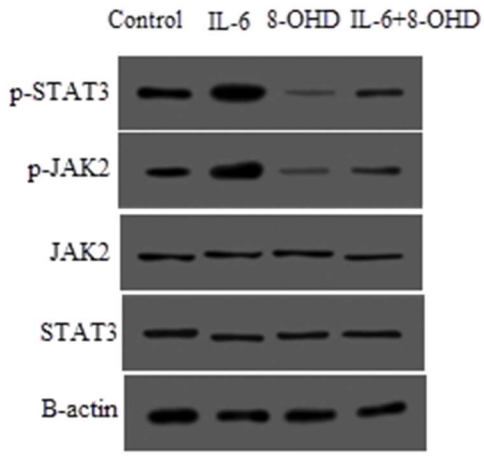


Figure 5. The effects of 8-Hydroxydaidzein (8-OHD, 70 μM) treatment alone and in combination with interleukin 6 (IL-6, 50 ng/mL) on the expressions and phosphorylation of the JAK2 and STAT3 proteins in the spheroid cells. IL-6 was utilized to activate the pathway, leading to the JAK2 and STAT3 phosphorylation. As is illustrated in the figure, 8-OHD inhibited STAT3 and JAK2 phosphorylation induced by IL-6. There are no remarkable differences in the expressions of unphosphorylated proteins between the groups.

However, a study by Kwon, et al. revealed that daidzein had no apparent effect on the expression of stem cell marker, Oct4, in porcine induced Pluripotent Stem Cells (33). They also showed that daidzein didn't affect the apoptosis and proliferation of these stem cells. Another study identified daidzein as a potent ALDH2 inhibitor, the enzyme that has protective effects on the development and differentiation of osteoblastic stem cells (34). The results of that study suggest an inhibitory property for daidzein against this stemness marker, which is in agreement with our observation in the case of ALDH3A1 marker expression. The other study confirmed that daidzein treatment inhibits the formation of ovarian sphere cells, ovospheres, proposing the prohibitory effects of daidzein on stem-like cell properties of these cancer cells (35). The data obtained from that study also identified reduced proliferation, migration, invasion, and induced cell cycle arrest and apoptosis in ovarian cancer cells that seem to occur through regulation of FAK and PI3K/AKT pathway by daidzein, as a phytoestrogen, binding to estrogen receptors. Similarly, our findings showed that the

treatment of spheroid and parental cells with 8-OHD led to a dose-dependent loss of cell viability, and induction of apoptosis in these cells as compared with the control cells. More interestingly, parental cells were more sensitive to 8-OHD in comparison to mammosphere cells. This observation supports the hypothesis that the CSCs are more resistant to the therapeutic agents than the cancer cell itself. Several lines of previous evidence affirmed that daidzein or its derivatives affect cancer cell viability by stimulating the apoptotic process in different types of cancer (36-39). In a second step, we attempted to conduct additional experiments to uncover the mechanism (s) by which 8-OHD may induce cell death. Accordingly, we used CD95L and ZB4 for inducing apoptosis and blocking the extrinsic pathway, respectively. Interestingly, we revealed that 8-OHD stimulates apoptosis via the intrinsic pathway. Some previously published data have also declared that daidzein induces apoptosis of cancer cell lines by modulating the intrinsic pathway (40, 41). Additionally, we further examined the effects of 8-OHD on JAK/STAT signaling pathway to find a possible molecular mechanism for the intrinsic apoptotic pathway induction. As previously has been unraveled, the inhibition of the JAK2/STAT3 pathway could induce apoptosis via the mitochondrial pathway (42). Therefore, we used IL-6 to stimulate the JAK2/STAT pathway and then treated the mammospheres with the combination of IL-6 and 8-OHD as well as the 8-OHD alone. IL-6 considerably increased the levels of phosphorylated JAK2 and STAT3 proteins. However, the 8-OHD treatment decreased the phosphorylation of these proteins either alone or in combination with IL-6 compared with the control and IL-6 treated groups. We proposed that the inhibitory effect of 8-OHD on the phosphorylation of JAK2 and STAT3 may cause the blockage of JAK2/STAT3 signaling and triggers apoptosis of mammosphere cells.

CONCLUSION

According to our data, we implied that 8-OHD exhibits strong anticancer properties by reducing CSCs characteristics, decreasing cell viability and inducing apoptosis in CSCs and cancer cells. Our study, for the first time, elucidated an anti-BCSC activity for 8-OHD. Moreover, our data suggested a novel mechanism by which 8-OHD may stimulate apoptosis in BCSCs. Taken together; it seems that this daidzein derivative has the potential to be a promising anticancer agent for treating breast cancer. However, we are still very much in the early steps of considering 8-OHD as a potent anti-breast cancer compound, and more detailed experiments are required to prove precise anti-breast cancer mechanisms of this natural product.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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