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Kaempferol and docetaxel diminish side population and down-regulate some cancer stem cell markers in breast cancer cell line MCF-7

Sara SOLTANIAN[1]; Helia RIAHIRAD[1]; Athareh PABARJA[1]; Mohammad REZA KARIMZADEH[2]; Kolsoum SAEIDI[3, 4]

1 Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Kerman, Iran

2 Department of Medical Genetics, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman, Iran

3 Student Research Committee, Kerman University of Medical Sciences, Kerman, Iran

4 Department of Medical Genetics, Kerman University of Medical Sciences, Kerman, Iran

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Abstract: Cancer stem cells (CSCs) are a small subpopulation of cancer cells whose resistance to chemotherapy and radiotherapy plays a pivotal role in treatment failure, tumor recurrence and metastasis. Today, the ability of many phytochemicals in targeting of CSCs has been identified. Kaempferol is a plant phytoestrogen that was recognized as a useful agent for targeting cancer cells by apoptosis induction, cell cycle arrest and inhibition of angiogenesis, migration, and invasion of cancer cells. In this study, we compared the effect of docetaxel as a common breast cancer chemotherapy drug and kaempferol on MCF-7 breast CSCs. Results showed that both docetaxel and kaempferol caused a dose-dependent reduction in cell proliferation. Rhodamin 123 dye staining and flow cytometry analysis revealed that after docetaxel and kaempferol treatment, SP cells proportion of MCF-7 decreased to 11.83±2.2% and 3±0.2 5% respectively relative to proportion of untreated control cells (23±3.2%). Moreover, real time PCR results showed that kaempferol treatment of MCF-7 was more effective than docetaxel in down-regulating the CSC-associated markers (i.e. oct4, nanog, abcb1 and aldh1a1). Taken together, these results indicate that kaempferol is an effective anti-CSCs agent, therefore it may be a therapeutic strategy for eradicating breast cancer through the elimination of CSCs.

Introduction

Breast cancer is the most common cancer in women around the world. Like other types of cancers, one of the main problems in breast cancer treatment is relapse of cancer after radio/chemo therapy. Treatment failure and cancer relapse is due to the presence of a small subpopulation of cancer cells called cancer stem cells (CSCs). CSCs are cells within a tumor with self-renewal capacity and potential of differentiating into different types of tumor cells. Furthermore, CSCs resistance to radio/chemo therapy led them to cause relapse and metastasis after initial treatment (Abdullah *et al.,* 2013; Soltanian *et al.,* 2011). Today CSCs can be distinguished from other cancer cells by distinct properties and specific biomarkers. One of these properties is expression of high levels of ATP-binding cassette (ABC) transporters that include multidrug resistance proteins (MRPs/ABCC), breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (P-gp/ABCB1). These transporters can pump various small molecules such as cytotoxic drugs out of cells leading to drug resistance in CSCs. This characteristic can be determined by capacity of CSCs to expulse some dyes such as Hoechst 33342 and rhodamin 123. This fraction of cells is designed as side population (SP) cells (Deeley *et al.*, 2006). In addition to ABC transporters, aldehyde dehydrogenase (ALDH) as a detoxifying enzyme, pluripotency transcription factors such as OCT4, NANOG and SOX2 and CSC- specific cell surface markers have been used as a tool to distinguish CSCs and evaluate the activities of potential targeting agents on CSCs (Ailles and Weissman 2007; Rizzino 2009; Ucar *et al.*, 2009).

Although docetaxel (Taxotere) and doxorubicin (Adriamycin) have previously shown a significant antitumor activity in breast cancer patients, recurrence of many of them shows their resistance to chemotherapy (Baltali *et al.*, 2001). Therefore, finding new strategies to target breast CSCs can create new hope for treatment. It has been shown that phytoestrogens as natural compounds with molecular structures similar to endogenous steroid hormones can be used in hormone replacement therapy to reduce the risk of

* Address correspondence to: Kolsoum Saeidi,
kolsoum.saeidi@gmail.com

diverse human cancers such as breast cancer (Bilal *et al.,* 2014). Antioxidant activity and therapeutic potential of kaempferol as a phytoestrogen for cancer prevention has been shown in some studies (Kim *et al.,* 2013). Furthermore, the effect of kaempferol in suppression of epithelial-mesenchymal transition (EMT) and migration of cancer cells has also been shown (Jo *et al.,* 2015; Liang *et al.,* 2015). EMT is an important process that is often activated during development and in the initiation of metastasis in cancers. Those cells undergoing EMT express stem cell markers and show features similar to CSCs such as increase in drug efflux pumps and anti-apoptotic effects. Therefore, there is a direct link between the EMT and gain of CSC properties (Kim *et al.* 2014). Due to the suppressive effect of kaempferol on EMT and cell migration, it is likely that kaempferol has an inhibitory effect on CSCs (Chen *et al.,* 2013; Liang *et al.,* 2015). The aim of this study was to compare the effects of kaempferol with that of docetaxel (a commonly used drug in breast cancer chemotherapy) on human breast cancer stem-like cells. For this purpose, expression of some CSC markers such as *oct4, nanog, abcb1* and *aldh1a1* were measured before and after treatment of MCF-7 with docetaxel and kaempferol. Moreover, proportions of SP cells and cancer cells positive for some breast CSC markers including CD44 and CD133 were evaluated in kaempferol and docetaxel-treated MCF-7 cells compared with parental (untreated control) cells.

Material and methods

Cell culture

Human breast cancer cell lines MCF-7 were obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Biowest) supplemented with 10% fetal bovine serum (FBS; Biowest, France), 100 U/mL penicillin, and 100 mg/mL streptomycin (Biowest, France) and maintained at 37°C in humidified air with 5% CO_2.

Measurement of cell viability

First, 5×10^3 MCF-7 cells were seeded in triplicate into 96-well plates. For cytotoxic analysis, cells were treated for 48h with docetaxel (Sanofi Winthrop, France) and kaempferol (Cat. No. K013; Sigma-Aldrich) at different concentration of 1.25-160 µM and 5-320 µM, respectively. For MTT assay, 20 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Atocel, Austria) (5 mg/ml) was added to each well. After incubation for 3h, the remaining MTT solution was removed and 100 µl DMSO was added to each well to dissolve the formazan crystals. Finally, the absorbance was measured at 490 nm using a microplate reader (BioTek-ELx800, USA). The percentage of cell viability was calculated by dividing the mean absorbance of each treatment to the mean absorbance of its controls multiplied by 100. All tests were performed in triplicates.

Treatment with docetaxel and kaempferol

MCF-7 breast cancer cell line was exposed to media containing 80 µM of docetaxel or 700 µM of kaempferol. Expression of CSC markers were compared between docetaxel- or kaempferol-treated and untreated control MCF-7 cells by flow cytometry and real-time polymerase chain reaction (PCR).

Analysis of side population (SP) cells

For detection of the SP, cells were collected from sub confluent culture by trypsinization and resuspenion at 10^6 cells/ml in PBS containing 2% fetal bovine serum (FBS). Then, cells were incubated with 0.1 µg/mL rhodamin123 (Cat. No. R8004; Sigma-Aldrich) at 37°C for 30 min. After two times washing with ice-cold PBS, cells were incubated at 37°C for 40 min to allow them to efflux rhodamin123 dye. The cells were then washed in cold PBS and were labeled with 1µg/ml propidium iodide (PI) (Cat. No. P4170; Sigma-Aldrich) to distinguish live from dead cells before analysis. Rhodamine123 staining was detected using a flow cytometer (Partec FloMax) exciting at 488 nm and detecting rhodamine123 with a 530/30 broad pass (BP) filter and propidium iodide with a 585/42 BP filter. In parallel experiments, rhodamin123 and 100 µM verapamil (Cat. No. V4629; Sigma-Aldrich) were added together to cells to confirm that the SP population was sensitive to verapamil, an inhibitor of ABC transporters.

Immuno-flow cytometric analysis

To examine the expression of CD44 and CD133, dissociated cells were washed with cold PBS and then one million cells resuspended in PBS/2% FBS at concentration of 10^6 cell/ml. For analysis of CD44, cell suspension was incubated with appropriate dilution (1:5) of FITC- CD44 Clone G44-26 (also known as C26) antibody (Cat. No. 560977; B.D Bioscience) for 45 min in dark at room temperature. After washing and 5 min before flow cytometry analysis, cells were incubated with PI at 1 µg/ml concentration to exclude dead cells. For analysis of CD133/2 expressions, 100 µl of cell suspension was incubated with suggested dilution (1:10) of PE-CD133 antibody (Cat. No. 293C3; Miltenyi Biotecclon) and 5 µl 7-amino-actinomycin D (7-AAD) (Cat. No. 559763; B.D Bioscience) to stain dead cells for 30 min in dark at room temperature. In the end of the procedure, unbounded antibodies were removed by washing and cells were analyzed by flow cytometer (Partec FloMax, Münster, Germany) using fl1 and fl2 filters for detection of FITC-CD44 and PI and fl2 and fl3 filters for detection of PE-CD133 and 7-AAD. FITC mouse IgG1, isotype control clone MOPC-21 (Cat. No. 555748; BD Bioscience) and PE mouse IgG1, isotype control clone MOPC-21 (Cat. No. 554680; BD Bioscience) served as control in analysis of flow cytometry results.

Isolation of RNA and quantitative PCR analysis

Total RNA was extracted from untreated and treated cells using total RNA isolation kit (DENAzist Asia, Mashhad, Iran) according to the manufacturer's instructions and then via a nanodrop and agarose gel electrophoresis, optimal quantity and quality of RNA were confirmed. The total RNA was treated with DNase I (Cat. No. EN0521; Thermo Fisher Scientific) to remove contaminating genomic DNA. For reverse transcription–polymerase chain reaction, 1 µg of RNA was reverse-transcribed in presence of 0.5 µg oligo $(dt)_{18}$ (Cat. No. MAN0013109; Thermo Scientific), 1 mM dNTPs (Cat. No. R0192; Thermo Scientific), and 200 U of M-MuLV reverse transcriptase (Cat. No. EP0441; Thermo Scientific) in a final reaction volume of 20 µl. First, the reaction was incubated at 42°C for 60 min and then was followed by 10 min at 72°C.

To quantify the level of transcripts for *oct4, nanog, abcb1* and *aldh1a*, RT reactions were subjected to quantitative PCR amplification. One microliters of complementary DNA products were amplified with SYBR Green real-time PCR Master Mix (Cat. No. PB20.11-01; Biosystems, Barcelona, Spain) in Analytik Jena Real-Time PCR System. PCR primers were used as follows: *oct4* forward: 5′-CCGAAAGAGAAAGCGAACCAGTAT-3′, reverse: 5′-CCACACTCGGACCACATCCTTC-3′; *nanog* forward: 5′-TGAGATCCTGAGCCTTTGG-3′, reverse: 5′-CTGCGTCACACCATTGCTATTCT-3′; *abcb1* forward: 5′-CACCACTGGAGCATTGACTR-3′, reverse: 5′-CAGTGTTAGTTGCCAACCAT-3′; *aldh1a1* forward: 5′-TCAGCAGGAGTGTTTACCAA-3′, reverse: 5′-CTTACCACGCCATAGCAA-3′; *β2M* forward: 5′-CTCCGTGGCCTTAGCTGTG-3′, reverse: 5′-TTTGGAGTACGCTGGATAGCCT-3′. Amplification conditions for *oct4, nanog* and *β2M* were: 95°C for 4 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30s, and 72°C for 30 s. Plate read step to collect fluorescence for mentioned genes was at 72°C. The same program was used for *abcb1, aldh1a1* except that annealing temperature was 62°C. At the end of the PCR runs, to derive melting curves, temperature was increased in steps of 1°C for 10 s from 61°C to 95°C. The specificity of products in reactions was validated by analyzing the melting curve of the products and gel electrophoresis. PCR readings were performed in triplicate. Serial dilutions (1:10) of cDNA samples were amplified by real-time PCR in the presence of SYBR Green to acquire standard curves. Slope of standard curve was used to calculate PCR efficiency according to the following equation: $E=2^{(-\Delta\Delta CT)}$. Quantification of target genes was normalized using the β2M gene as a reference and subsequently expressed as relative to untreated control cells.

Statistical analysis

Data are expressed as mean±SD of at least three independent experiments. Statistical analyses were performed using SPSS version 16. Statistical significance was determined using Student's *t*-test for comparisons between treated versus control cells and $p<0.05$ was defined as the level of statistical significance.

Results

Cytotoxic effects of docetaxel and kaempferol on MCF-7 cell line

To identify the IC50 value of docetaxel on MCF-7 breast cancer cell lines, cells were treated with various concentration of docetaxel (1.25-160 μM) for 48 h. Analysis of cell survival by MTT assay showed that IC50 value of docetaxel on MCF-7 cells was 22.5 μM after 48h of its administration (Fig. 1-A). To compare this cytotoxic efficiency with the effects of kaempferol, MCF-7 cells were also treated with different concentration of kaempferol (5-320 μM) for the same period of time. The IC50 value of kaempferol was identified as 175 μM (Fig. 1-B).

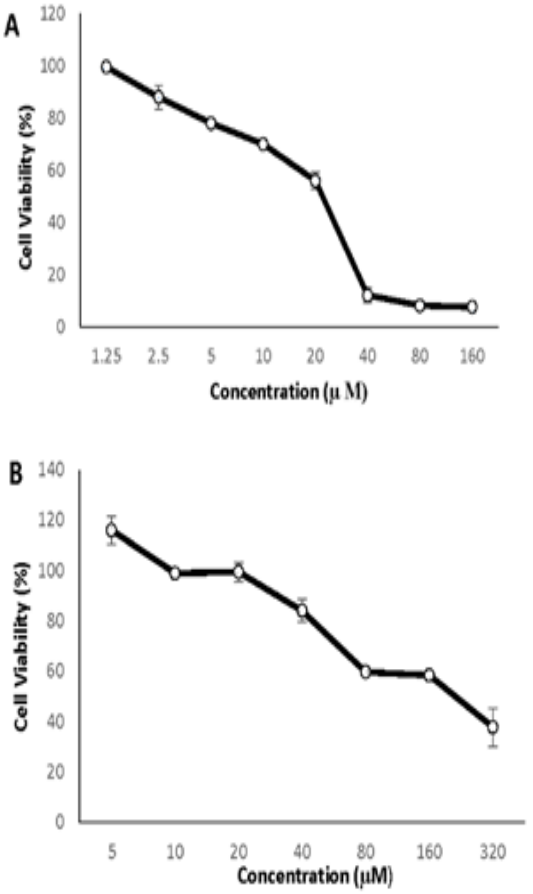

FIGURE 1. Effect of Docetaxel (A) and kaempferol (B) on growth of MCF-7 breast cancer cells. MCF-7 cells were treated with increasing concentrations of docetaxel and kaempferol for 48 h and cell viability was determined by MTT assay. All the points represent results from three independent experiments performed in triplicate. Data are expressed as mean±SD.

Effects of docetaxel and kaempferol on breast cancer stem-like cells

To examine the effects of docetaxel and kaempferol on breast CSCs, MCF-7 cells were treated with 22.5 μM docetaxel or 175 μM kaempferol for 48h and then the SP cells were analyzed by flow cytometry. Untreated control MCF-7 cells showed 23±3.2% SP cells (Fig. 2-A). It has been shown that rhodamin dye exclusion is dependent on ABCB1 transporter (Ford and Hait 1990). Here we observed that incubation of cells with verapamil, that blocks function of ABCB1 transporter leads to loss of the SP cells (Fig. 2-B). This confirms that rhodamin dye exclusion in MCF-7 cells is dependent on ABCB1 transporter. Docetaxel and kaempferol treatments decreased the proportion of SP cells to 11.83±2.2% (Fig. 2-C) and 3±0.2% (Fig. 2-D), respectively.

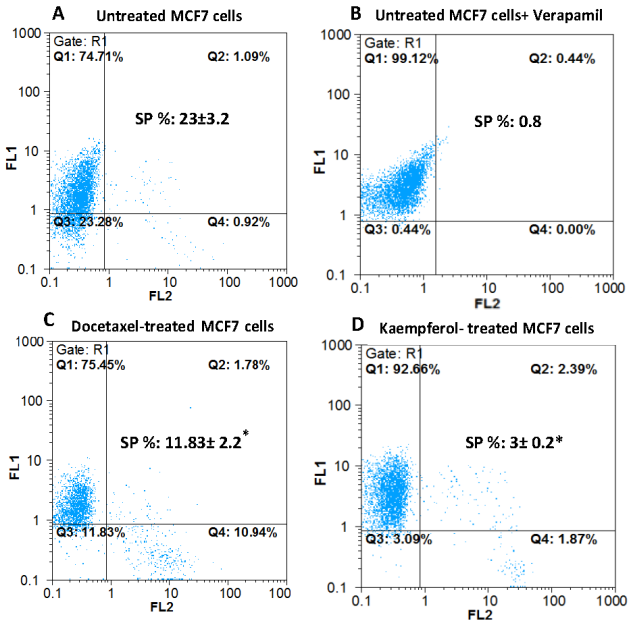

FIGURE 2. Docetaxel and kaempferol diminish side population in MCF-7 cells. Untreated MCF cells in absence (A) and presence of verapamil (B), docetaxel (80 μM) treated MCF7 cells (C) and kaempferol (700 μM) treated MCF7 cells (D) were stained with Rhodamine 123 and propidium iodide dyes and analyzed using flow cytometry as described in materials and methods. Cell within Rhodamin 123-+Pi- compartment (Q3) were identified as side-population cells. Results are the mean ± SD of 3 independent experiments. **P*<0.01 compared to the control as tested by the Student's *t*-test.

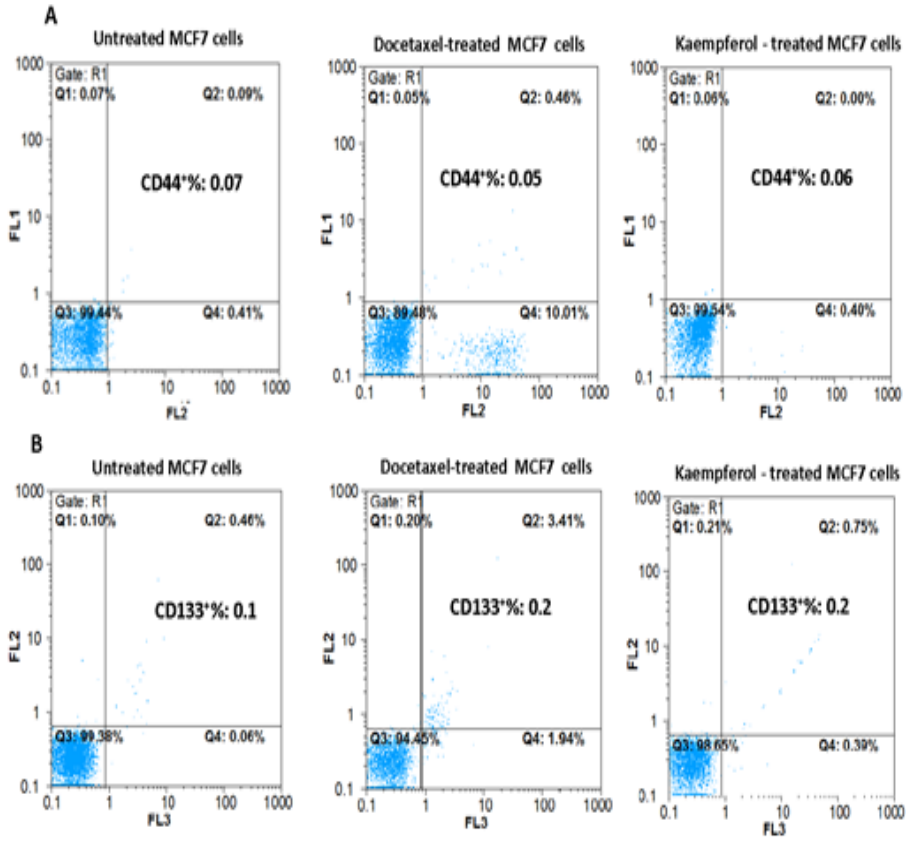

FIGURE 3. FACS analysis on the expressions of CD133 and CD44. Untreated MCF7 cells, docetaxel-treated MCF7 cells and kaempferol treated MCF7 cells were immunostained in separate test with a FITC anti- human CD44 Antibody+ Pi (A) and PE-conjugated antibody against human CD133+7-AAD (B) and then analyzed using flow cytometry. The results represent three independent experiments.

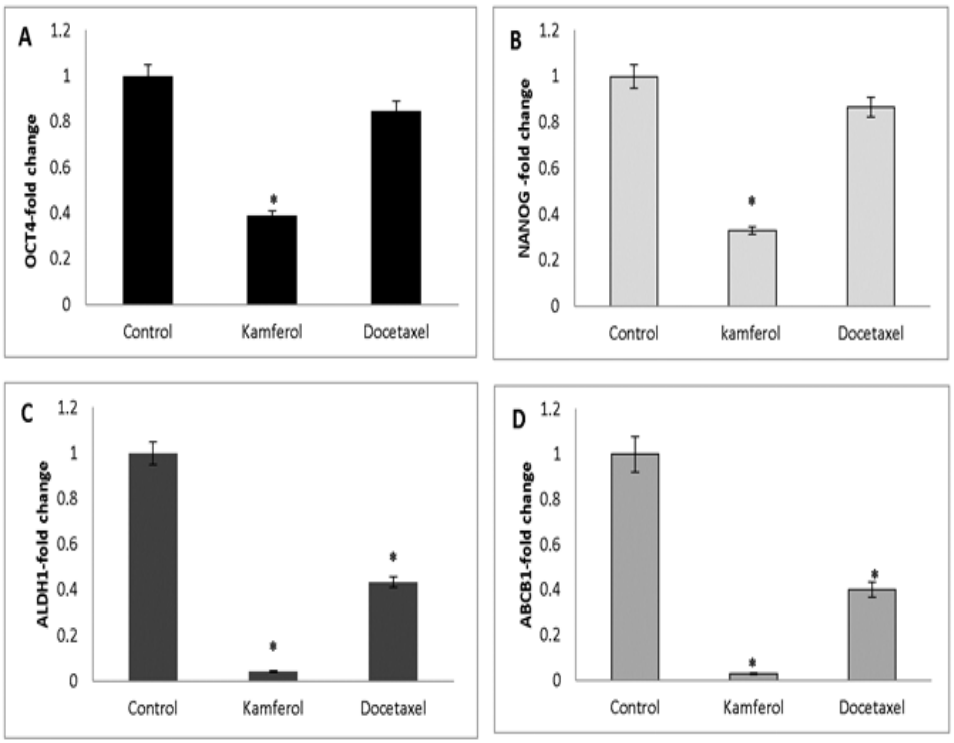

FIGURE 4. Quantitative RT-PCR analysis of OCT4 (A) and NANOG (B) gene expression after treatment of MCF-7 with kaempferol or with docetaxel. MCF7 cells were treated with kaempferol (700 μM) or with Docetaxel (80 μM) for 48h and expression of OCT4, NANOG, ALDH1A1 and ABCB1 were measured and compared to untreated cells as control by real time PCR. Fold change in gene expression was normalized using B2M as a reference. The graph represents the mean data±SD (error bar) of at least three independent experiments. *p<0.05 against control.

These results showed that docetaxel and kaempferol not only inhibits the overall growth of MCF-7 cells, but also has the ability to kill MCF-7 cancer stem-like cells. Moreover, kaempferol reduced the proportion of SP cells more than docetaxel indicating that kaemperol might be a more effective targeting agent for breast CSCs than docetaxel.

In order to examine effect of docetaxel and kaempferol on other CSC markers, flow cytometry was carried out with specific antibodies for surface markers including $CD44^+$ and $CD133^+$. CD44 and CD133 expression was examined in untreated control and docetaxel- or kaempferol-treated MCF-7 cells. As presented in Fig. 3, untreated MCF-7 did not show expression of CD44 and CD133 markers and treatment of cells also did not induce expression of these two markers.

Analysis of the expression oct4, nanog, abcb1 and aldh1

To study the effect of docetaxel and kaempferol on CSCs population, expression of *oct4, nanog, abcb1* and *aldh1a1* as CSC markers were quantitated in treated cells relative to untreated MCF-7 cell line. Treated cells showed lower expression of each of these CSC markers than untreated MCF-7 cells. After docetaxel and kaempferol treatment, expression of *oct4* decreased to 15% and 61% of that of untreated MCF-7 cells, respectively (Fig. 4-A). The *nanog* expression in docetaxel and kaempferol-treated cells showed decrease by 13% and 67%, respectively (Fig. 4-B). Similarly, transcription of *aldh1a1* and *abcb1* showed 56% and 59% drop after treatment of cells with docetaxel, whereas in kaempferol-treated cells, aldh1a1 and abcb1 were more down-regulated and showed 95% and 97% drop, respectively (Figs. 4-C-D). Altogether, real time PCR assay also showed that the effect of kaempferol on reduction of CSC markers was stronger than that of docetaxel.

Discussion

The fact that CSCs are responsible for treatment resistance, cancer recurrence and metastasis makes them important candidates for cancer therapy. This intrinsic therapy resistance of CSCs to various chemotherapy drugs has been shown in many reports. For instance, chemotherapy with 5-fluorouracil (5-FU) plus oxaliplatin (FOLFOX), which is a backbone of colorectal cancer therapy, resulted in enrichment of CSCs (chemo-surviving cells) that may lead to cancer relapse (Patel *et al.*, 2010). Although cisplatin-based chemotherapy regimen has been used for treatment of numerous human cancers including bladder, head and neck, lung, ovarian, and testicular, enrichment of CSCs after cisplatin treatment have been seen in many cancers that lead to relapse and decreased overall survival of patients (Shafee *et al.,* 2008; Wang *et al.,* 2017). As other examples, however, combination of docetaxel and doxorubicin, as well as tamoxifen, are widely used as first-line chemotherapy in treatment of breast cancers, but the percentage of breast CSCs was not decreased after treatment with these common chemotherapy drugs (Baltali *et al.,* 2001). In addition to resistance of cancer cells to chemotherapy drugs, toxicity of these agents to normal tissues and their side effects have motivated researcher to look for new therapeutic approaches. To this end, it has been shown that plant-derived compounds in combination with chemotherapy drugs may improve the outcome of cancer therapy with less toxic side

effects (Park *et al.,* 2014; Patel *et al.,* 2008; Shankar *et al.,* 2011). Thus, identification of new phytochemical agents targeting CSCs is very promising. Phytoestrogens are plant-derived xenoestrogens consumed by eating phytoestrogenic plants. Since these relevant plant-derived compounds have structural similarities to estradiol, they can be used instead of classical hormone replacement therapies in cancers (Dixon 2004). Kaempferol is a phytoestrogens and a flavonoid found in a variety of plants including apple, grape, tomato, green tea, pine, broccoli and ginkgo leaf. Antineoplastic activities of kaempferol via apoptosis induction, cell cycle arrest and inhibition of angiogenesis, migration, and invasion of various cancer cells have been shown. In breast cancer, kaempferol interacts with the estrogen receptor pathway and slows down the growth and induces apoptosis in cancer cells (Kim *et al.,* 2013). The present study confirmed that kaempferol decreases not only the whole cell viability but also the proportion of SP cells in MCF-7. Expression of CSC markers was also down-regulated after treatment with kaempferol. Moreover, we found that kaempferol was more effective than docetaxel, a standard drug for breast cancer treatment, against breast CSCs.

Although due to self-renewal ability and capacity to differentiate, gold standard assay for identifying CSCs was used in serial transplantation of tumor cells into immunocompromised mice, but there are several in vitro assays to isolate and identify CSCs. High expression and activity of membrane transporters belonging to ABC transporter family are common features of CSCs that makes them multidrug-resistant. SP cells, which are a population with overexpression of ABC transports have many characteristics of CSCs (Guo *et al.,* 2016; Litman *et al*., 2000). Hence, SP analysis has also been used here to study the effect of docetaxel and kaempferol on breast CSCs. We found that docetaxel and kaempferol have inhibitory effects on cell proliferation of MCF-7 cells and kaempferol was more effective in reducing size of the SP population. The human *ABCB1 (MDR1*) is one of many ABC transporter genes that give rise to a protein named P- glycoprotein which is able to pump out various anticancer drugs and is elevated in drug-resistant tumors. It was shown that rhodamin 123 dye exclusion is partly mediated by the ABCB1/P-gp protein and therefore expression of P-glycoprotein is inversely related with rhodamin 123 staining of cancer cells (Gillet *et al.,* 2010). Expression analysis of *ABCB1* by real time PCR showed that *ABCB1* is more down-regulated by kaempferol than docetaxel treatment which confirm results obtained by flow cytometry.

Another widely used method for identifying CSCs is based on specific CSC surface markers. Some markers such as CD133, CD24 and CD44 are among the prevalent solid CSC markers (Ailles and Weissman 2007; Clarke *et al.*, 2006). According to many studies in breast cancers, CSCs are characterized and accepted as $CD44^{+}CD24^{-}$ cells. In addition, CD133-expressing breast tumor cells also show CSC properties such as drug-resistance, self-renewal, differentiation ability, high proliferation and ability to form tumors in xenografts. Therefore, breast tumors contain distinct $CD44^{+}/CD24^{-}$ and $CD133^{+}$ cells with CSC characteristics (Wright *et al.,* 2008).

In the present study, CD44 and CD133 markers were analyzed to investigate the potential inhibitory effects of docetaxel and kampferol on breast CSCs. Flow cytometry results showed that MCF-7 cells lack a CD44+ and CD133+-subpopulation and treatment by docetaxel and kaempferol did not induce expression of these two markers. Similarly, it was shown that MCF7 cells grown as monolayers were strongly positive for CD24 with undetectable CD44 (Liu *et al.,* 2014) and it was also reported that MCF-7 express low levels of CD133 (Park *et al.,* 2015). On the contrary, in other studies $CD44^{+}/CD24^{-}$ cells and CD133 + which showed characteristic of CSCs were isolated from the MCF-7 cultures (Nadal *et al.,* 2013; Sun *et al.,* 2013). It is likely that the use of various antibodies gives rise to different results for detection of CD44 and CD133 markers on MCF-7 cell line.

ALDH1 confer resistance to specific chemotherapeutics by oxidizing aldehydes in to carboxylic acids (Hellsten *et al.,* 2011; Ikawa *et al.,* 1983; Jimeno *et al.,* 2009; Ma *et al.,* 2008; Nakahata *et al.,* 2015; Ucar *et al.,* 2009). ALDH1 high activity is characteristic of CSCs in various cancer including pancreatic cancer, breast cancer, rhabdomyosarcoma, human multiple myeloma, and acute myeloid leukemia. Therefore, ALDH1 expression or activity can be used as a common marker for CSC population (Ginestier *et al.,* 2007). We identified that expression of this enzyme is reduced following docetaxel and kaempferol treatment, however *aldh1a1* expression levels were higher in docetaxel-treated cells than kaempferol-treated cells. OCT4, NANOG and SOX2 are proteins involved in self renewal and maintaining pluripotency of normal and cancer stem cells, and their expression have been analyzed for targeting of pancreatic, hepatoma, and glioblastima CSCs by different chemotherapy drugs and plant-derived components (Chambers *et al.,* 2003; Fong *et al.,* 2008; Takeda *et al.,* 1992; Yao *et al.,* 2012). Using quantitative real-time PCR, we showed that kaempferol is more effective in the down-regulation of *oct4* and *nanog* than docetaxel.

In conclusion, a dramatic decrease in the SP population and downregulation of CSC markers such as *oct4, nanog, abcb1* and *aldh1a1* after docetaxel and kaempferol treatment indicates that these two agents are either leading to reduce expression of CSC markers or to loss of cells expressing these markers from population. Furthermore, stronger effects of kaempferol than docetaxel on SP cells and CSC markers suggest that kaempferol is an effective plant-derived anticancer agent targeting breast CSCs.

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