EFFECT OF NARINGIN, A NATURAL COMPOUND, ON STEM CELL PHENOTYPE OF BREAST CANCER

by

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This thesis is dedicated to my family, my teachers and my friends who have showed me the way when I lost.

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ABSTRACT

THE EFFECT OF NARINGIN, A NATURAL COMPOUND, ON STEM CELL PHENOTYPE OF BREAST CANCER CELLS

Almost all of novel therapies focused to target signaling pathways related to cell growth and survival failed to provide patient survival. These kinds of therapeutic approaches shrink just the tumor bulk and eventually lead to recurrence of cancer. This clinical handicap could be explained by the discovery of cancer stem cell (CSC) which resistant to standard therapies including chemo- and radiotherapy in different cancer types. The residual tumors after conventional therapies are enriched for CSCs and possess epithelial-mesenchymal transition (EMT)-like properties that promote invasion, metastasis. Natural dietary compounds, especially polyphenols have gained increasing attention due to their role in cancer chemoprevention. Recently, various studies showed that many natural compounds may decrease therapy-resistant, slow cycling cancer stem cell proportion and suppress EMT-like features.

In this study, we tested the effect of naringin, a polyphenolic compound derived from grapefruit on the stem cell phenotype of breast cancer. The results showed that naringin treatment lead to decrease the fraction of CSCs and suppress the expression of EMT markers. It was demonstrated that this flavonoid at the concentration that does not cause cell cytotoxicity may affect stem cell properties of breast carcinoma by decreasing number of cells having high ALDH1 activity and CD44+/CD24-phenotype. These data suggest for the first time that naringin could be used to target Breast CSCs and provide over-all survival if used with an agent that eradicates differentiated cells.

ÖZET

BİR DOĞAL BİLEŞEN OLAN NARİNGİNİN MEME KANSERİ HÜCRELERİNİN KÖK HÜCRE FENOTİPİ ÜZERİNDEKİ ETKİSİ

Günümüzde kullanılan ve daha çok hücre büyümesi ve hayatta kalmasıyla ilişkili sinyal yolaklarını hedef alan tedaviler hastanın hayatta kalmasını sağlayamamaktadır. Bu tür tedaviler daha çok tumor kütlesinde artışa neden olan farklılaşmış hücrelerin ölümüne neden olmakta ve sonunda tumorun yeniden oluşmasını engelleyememektedir. Bu kliniğe yönelik sorun, birçok farklı kanserde bulunan, kemo- ve radyoterapiye dirençli olan kanser kök hücrelerin varlığının tespitiyle açıklanabildi. Tedavi sonrası hala ortamda bulunan tumor kütlesinin CSCs açısından zenginleştiği ve aggressifliğiyle yakından ilişki olan epitaliyel-mezenkimal geçiş (EMT) özelliğini gösterdiği tespit edilmiştir. Doğal besin bileşenleri, özelllikle polifenoller son zamanlarda kanseri önleyici özellikleri nedeniyle dikkatleri üzerlerine çektiler. Son zamanlarda bir çok çalışmada bu bileşenlerin, terapiye dirençli ve yavaş bölünen kanser kök hücrelerin oranını azaltabileceği ve EMT özelliğini baskılayabileceği gösterildi.

Bu çalışmada ilk kez, doğal bir bileşen olan ve greyfurt gibi asitli meyvelerden elde edilen naringinin, meme kanseri kök hücreleri üzerindeki etkisini test ettik. Sonuçlar, naringinin kanser kök hücre oranını azaltığı ve EMT ile ilişikili genlerin anlatımında azalmaya sebep olduğu gösterilmiştir. Bu flovanoidin, hücre sitotoksitesine neden olmayan bir dozda meme kanserinin kök hücrelerine özgü bir özellik olan ALDH1 enzim aktivitesinde azalmaya neden olabileceği ve CD44+/CD24- fenotipindeki kanser kök hücrelerin oranı azaltabileceği tespit edildi. Bu veriler, ilk kez naringinin meme kanseri kök hücrelerini yok etmek için kullanılabileceğini gibi ve farklılaşmış hücreleri hedef alan bir ajanla birlikte meme kanseri hastalarında tam bir iyileşmeye neden olabilir.

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5-FU	5-Fluor Urasil
ABCB5	ATP-binding Cassette Sub-family B Member 5
ABCG2	Adenosine triphosphate (ATP)-binding Cassettes Molecule 2
ALDH	Aldehyde Dehydrogenase
BAAA	Bodipy-aminoacetaldehyde
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CSC	Cancer Stem Cell
DAPI	4’,6-diamidino-2-phenylindole
DEAB	Diethyl-aminobenzaldehyde
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGCG	Epigallocatechin Gallate
EGF	Epidermal Growth Factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial Mesenchymal Transition

EpCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
ESA	Epithelial Specific Antigen
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein-iso-thio-cyanate
HER2	Human Epidermal Growth Factor 2
HMLE	Human Mammary Epithelial Cells
KLF4	Kruppel-like factor 4
Lin	Lineage
MMP-2	Matrix Metalloproteinase-2
MMP-9	Matrix Metalloproteinase-9
mRNA	Messenger Ribonucleic Acid
MTS	3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo- phenyl)-2H-tetrazolium
NCBI	The National Center for Biotechnology Information
Ng	Nanogram
Nmoles	Nanomoles
NOD/SCID	Non-obese Diabetic/severe Combined Immunodeficient
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide

PR	Progesterone Receptor
PSA	Penicillin Streptomycin Amphotericin B
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
R1	Region 1
RNA	Ribonucleic Acid
Rpm	Rotation per minute
SD	Standard Deviation
TF	Transcription Factor
V	Volume
VEGF	Vascular Endothelial Growth Factor
W	Weight
α-SMA	α-smooth muscle actin
β –actin	Beta-actin
β –catenin	Beta-catenin
µL	Micro liter
µM	Micromolar

1. INTRODUCTION

1.1. BREAST CANCER

Breast cancer is a heterogeneous disease based on histology, treatment sensitivity and survival. In last decade, the technological advances, such as high-throughput technologies help us to explain this heterogeneity in terms of global gene expression profile and define five different breast cancer intrinsic subtypes which consist of Luminal A, Luminal B, Claudin-low, HER2 positive and Basal-like. [1]. Intrinsic subtypes of breast cancer tumors have revealed critical differences in incidence, survival and response treatment (**Table1. 1**).

Table 1. 1. Molecular classification of breast carcinoma

Classification	Immunoprofile	Other characteristics	Example cell lines
Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	Ki67 low, endocrine responsive, often chemotherapy responsive	MCF-7, T47D, SUM185
Luminal B	ER^+, $PR^{+/-}$, $HER2^+$	Ki67 high, usually endocrine responsive, variable to chemotherapy. $HER2^+$ are trastusumab responsive	BT474, ZR-75
Basal	ER^-, PR^-, $HER2^-$	$EGFR^+$ and/or cytokeratin $5/6^+$, Ki67 high, endocrine nonresponsive, often chemotherapy responsive	MDA-MB-468, SUM190
Claudin-low	ER^-, PR^-, $HER2^-$	Ki67, E-cadherin, claudin-3, claudinin-4 and claudinin-7 low. Intermediate response to chemotherapy	BT549, MDA-MB-231, Hs578T, SUM1315
HER2	ER^-, PR^-, $HER2^+$	Ki67 high, trastuzumab responsive, chemotherapy responsive	SKBR3, MDA-MB-453

EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor [2]

Luminal B subtype is known differentiated and endocrine or trastuzumab responsive breast cancer. However, recent studies showed that this molecular subtype has high ALDH1 enzyme activity which is a marker of Cancer stem cells (CSCs) and HER2 expression in this subgroup drives luminal breast cancer stem cells [3, 4].

1.2. CANCER STEM CELLS

The idea that cancer originated from transformed germ cells or stem cells is proposed 150 years ago. But only recently, accumulating knowledge and technological improvement have allowed us to test this idea and finally it leads to cancer stem cell hypothesis. Cancer stem cells have properties overlap with normal stem cells: the ability to self-renewal and to differentiate. They have unlimited self-renewal potential in addition to differentiation ability into other cells to form tissue or in our case, to form tumor. Cancer stem cells or tumor-initiated cells have been identified firstly in leukemia and until date, the presences of them have been shown in a various tumors. Despite their origin is still in debate, it is known that they are responsible for tumor growth, progression and metastasis. Two models have been suggested for origin of cancer stem cell. One school of thought is that this small subpopulation is originated from tissue-specific stem cell via transformation by the several combinations of mutations. The second one is that they results from other differentiated tissue cells which acquire stem like potential after mutations that cause de-differentiation. Despite their origin, their unlimited proliferation potential and aberrant differentiation into progeny to give tumor heterogeneity has been accepted by both groups [5,6].

The ability of cancer stem cells to self-renew and give rise to progeny which does not have this potential, implicit that this minor population has tumor-initiating capacity. Self-renewal is called a process that stem cells divide asymmetrically to give not just daughter cell but also identical stem cell which is a hallmark of stem cell [7]. Despite progenitor cells derived from asymmetrical division of stem cell are highly proliferative, the stem cells themselves represent slowly dividing cells [8]. Cancer stem cells possess the potential to give rise phenotypic heterogeneity of tumor in immunodeficient mice.

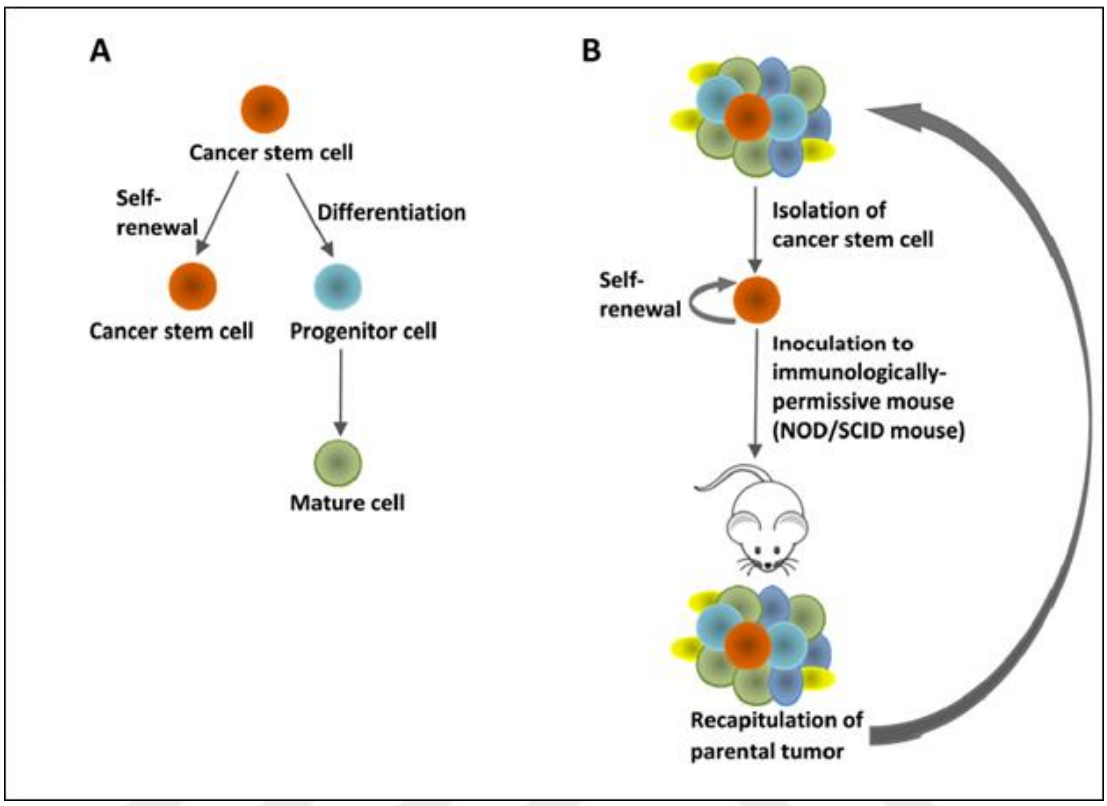

Figure 1.1. Cancer stem cell theory. **(A)** Cancer stem cells are capable of self-renewal and differentiation. **(B)** Isolated cancer stem cells are able to phenotypically recapitulate the parental tumor along serial passaging through multiple recipient mice [9]**.**

In the last decade, a new wave of studies had been carried out to address the cancer stem cell concept. After these studies, to define the existence of CSC population three key observations are used;

- Only a small population of cancer cells within each tumor have usually demonstrated the tumorigenic potential when transplanted into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice in small number.

- This tumorigenic cancer cell could be characterized by a distinctive profile of surface markers including CD44, CD24, CD133, CD49F, ESA and can be isolated from non-

tumori- genic ones by means of flow cytometry or other immunoselection procedures reproducibly. The markers used to isolate CSCs in different cancer are shown in **Table 1.2**.

- Tumors grown from highly tumorigenic cells consist of tumorigenic and nontumorigenic cancer cells which lead to the full phenotypic heterogeneity of the parent tumor [10].

Table 1. 2. The markers used to isolate CSCs in different cancer

Cancer	**Cell surface Markers**	**References**
Leukemia	CD34+,CD38+, CD19+	[11]
Breast	CD44+,CD24-,Lin- ALDH1,CD49F+,EpCAM-	[12, 13]
Brain	CD133+	[14]
Melanoma	CD20+, ABCB5	[15, 16]
Lung	CD44+,CD24+ CD133+	[17]
Sarcomas	CD105+, CD44+,Stro1 CD117+	[18]
Liver	CD133+ CD90+,CD44+	[19]
Pancreatic	CD44+,CD24-,ESA+ CD133+	[11]

ESA; Epithelial specific antigen, EpCAM; epithelial cell adhesion molecule, ABCB5; ATP-binding cassette sub-family B member 5, ALDH1; Aldehyde Dehydrogenase.

The discovery of tumor cells exhibiting stem cell properties in breast cancer gives researchers a possible explanation why eradication of cancer is so hard to achieve, as well as suggesting strategies for the targeting of this subpopulation. There is accumulating evidence proved that in numerous cancers not just breast cancer, malignancies are driven by this small population. In addition to driving metastasis and progression of tumor, CSCs is the main reason of resistance to chemo- and radiotherapy [3,20-24].The development of cancer therapeutics based on tumor regression will be helpful to produce the agents which target just the differentiated tumor cells not slowly-proliferating cancer stem cells [25].To be able to cure cancer without observing recurrence after treatment, more effective therapies which require to kill this important cancer stem cell population must be developed. The success of these new approaches depends on the identification, isolation, and characterization of cancer stem cells.

1.2.1. Breast Cancer Stem Cells Markers

1.2.1.1. $CD44^{+/}CD24^{-}$ phenotype

Cancer stem cells express the stem cell markers, such as CD49F, OCT4, KLF4, Nanog as seen in their counterpart, normal stem cells [26–28]. However, the studies showed that they also have different surface makers that will be helpful the isolation of CSC from tissue or cell lines.

The existence of CSCs in breast cancer is firstly shown by Al-Hajj. It was the first direct evidence of CSCs in solid tumors. The results demonstrated that, in most human breast cancers, only a small fraction of the tumor cells, defined as [$CD44^{+}/CD24^{-/low}/linage^{-}$] which represent the 11%–35% of total cancer cells, is capable of tumor growth when transferred into NOD- SCID mice. Despite the 20,000 cells lack this surface markers were unable to form tumor in immmunodeficient mice, only 200 of cells having $CD44^{+}/CD24^{-/low}/linage^{-}$ phenotype form tumor. Tumors grown from these cells were shown to compromises the bulk of tumor and give rise to the phenotypic heterogeneity of the parent tumor, suggesting for the first time the existence of a functional hierarchy of stem cell systems in a solid human epithelial tumor [12]. However

CD44 which is a cell adhesion molecule, takes part in binding of cells to hyaluronic acid, CD24 is a negative regulator of a chemokine receptor called CXCR4. CXCR4 molecule is involved in breast cancer metastasis [29]. The research directed by Fillmore and Kupperwas showed that several breast cancer cell line, including MCF7, MDA-MB-231 and SUM159 have highly tumorigenic cell population that have $CD44^{+}/CD24^{-/low}/ESA^{+}$ and those cell exhibit stem cell like behavior, such as forming mammosphere in suspension culture, self-renewal, differentiation and having slower cell cycle kinetics. Even as few as 100 cells having this phenotype was enough to form tumor in recipient mice. Also previous studies demonstrated that these cells, CSCs were less sensitive to cytotoxic agent, such as paclitaxel and 5-FU [30]. Besides driving carcinogenesis and causing metastasis, the therapeutic resistance of these cells, CD44+/CD24- mammary stem cells has been shown *in vitro* and in mouse models by Philips et al. [31].

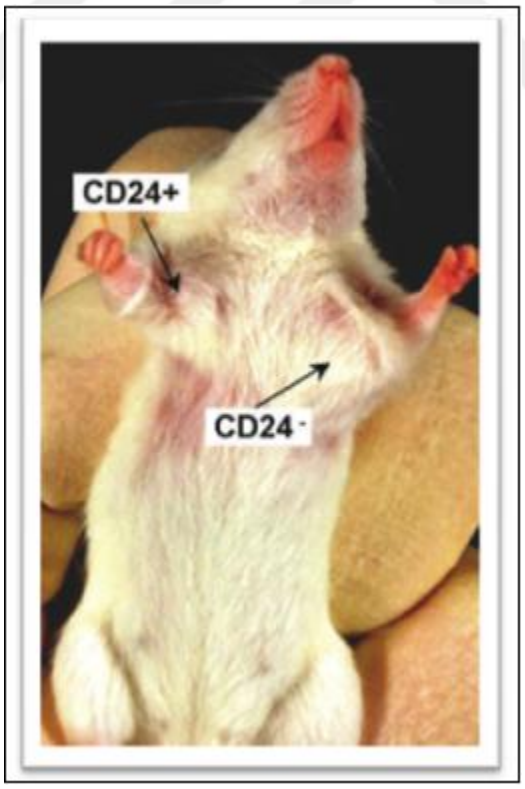

Figure1. 2**.** The tumor growth in a mouse at the $CD44^{+}CD24^{-/low}Lineage^{-}$ injection site, but not at the $CD44^{+}CD24^{+}Lineage^{-}$ injection site [12].

1.2.1.2. Aldehyde Dehydrogenase (ALDH)

The expression of Aldehyde Dehydrogenase which is a detoxifying enzyme responsible for oxidation of intracellular aldehydes, has described by Ginestier et al. as a stem cell marker which could be used to isolate human mammary stem cells [32]. This enzyme is expressed in hematopoietic and neuronal stem as well as progenitor cells. It has a role in early differentiation of stem cells through its role in oxidizing retinol to retinoic acid. The enzymatic function are known to protect stem cells from oxidative injury [33, 34].

Recently an enzymatic assay called ALDEFLUOR Assay is used to detect the expression of ALDH1. In the assay, Aldefluor contains ALDH1 substrate as known bodipy-aminoacetaldehyde (BAAA) that is converted into fluorescent product BODIPY-aminoacetate (BAA)[35] as described in product literature (Stem Cell Technologies). The activity of this charged enzyme is analyzed by flow cytometry. Due to its charge as well as the multidrug-resistance transporters are intentionally inhibited by the ALDEFLUOR reagents, cells that are alive retain BAA. The cells having high levels of ALDH1 activity will display high fluorescence and can be isolated with FACS into two subpopulations; ALDH-hi and ALDH-low [36]

Cancer cells with high ALDH activity have been demonstrated to exhibit stem cell properties in different cancers, including multiple myeloma and leukemia [37]. The cancer stem cell properties of ALDEFLUOR-positive cells but not ALDEFLUOR-negative cells were shown in NOD/SCID mice. Serial passage of the ALDEFLUOR-positive cells was able to generate tumors that demonstrated the phenotypic heterogeneity of the initial tumor.

Interestingly, the population having $CD44^{+}/CD24^{-}/Lin^{-}$ phenotype which is described as cancer stem cell in breast tumors has been shown to have a small overlap with ALDEFLUOR-positive cell population [12, 13]. In the tumors investigated, the overlap represented approximately 1% or less of the total cancer cell population and it is detected that the ALDEFLUOR-positive $CD44^{+}/CD24^{-}/Lin^{-}$ cells is shown to has highly tumorigenic potential. As few as 20 cells from this population were able to generate tumors. Moreover, to identify breast cancer stem cells *in situ,* ALDH1 immunostaining of paraffin-embedded specimens were utilized. Furthermore,

analyses made by Ginestier and Korkaya et al. have shown the importance of *ERBB2* over-expression is related to stem cell phenotype. Ginestier checked 577 human breast carcinomas and showed that the expression of ALDH1 correlates with tumor histological grade, ER and PR negativity, proliferation index as assessed by Ki-67 expression, and *ERBB2* over-expression, suggesting that the expression of stem cell marker is a powerful predictor of poor clinical outcome [32].Korkaya's group found that *ERBB2* over-expression in normal human mammary epithelial cells and mammary carcinomas increases the ratio of stem cells having ALDH1 expression [3]. The clinical relevance of these studies have been shown in a neo-adjuvant breast cancer trial. Tumor regression induced by neo-adjuvant chemotherapy was related to an induction of $CD44^{+}/CD24^{-}$ cancer stem cells in residual tumors. In contrast, breast cancers with *ERBB2* amplification had an increased proportion of $CD44^{+}/CD24^{-}$ cells before treatment that was reduced by administration of the *ERBB2* inhibitor lapatinib, a drug is used to treat *ERBB2*-overexpressed breast cancer subtype [38].The clinical results suggest that *ERBB2* inhibitor lapatinib could be used with new approaches to completely remove this minor and resistant cell population.

The relationship between ALDH1 expression and metastasis has been demonstrated in patients having inflammatory breast cancer (IBC). The study within 109 patient with IBC had been checked showed that expression of ALDH1 is associated with early metastasis and decreased survival [39].

It is obvious that standard therapeutic approaches, such as chemotherapy are not enough to eradicate slowly dividing CSCs. Since ALDH1 is known to metabolize chemotherapeutic agents, such as cyclophosphamide [40], traditional therapies are not a good option to eradicate CSCs.

1.2.2. Isolation and Characterization of Cancer Stem Cells

The techniques could be used for isolation and characterization of cancer stem cells are still under debate. The method which will be selected as an appropriate technique for isolation of these cells must be validated by an assay that can verify the stem cell properties of cell and

differentiation. The first method which is generally used is cell sorting based on stem cells markers. Based on the different CSCs markers in different cancer are known **(Table 1.2.)**, these cells could be isolated from tumor and cancer cell lines via FACS. A well established *in vitro* assays termed tumorosphere or mammosphere for breast cancer, have also been used to isolate for cancer stem cells [41]. The *in* v*itro* culture system is developed by Dontu and her colleagues for human mammary epithelial stem and progenitor cells based on the technique used for neuronal and brain stem cells. This cell culture technique adapted for breast tumor tissue is called mammosphere technique. Cells from human reduction mammoplasty specimens has been demonstrated to be able to survive, grown under low attachment, on non-adherent substrata and form floating cell colonies having spherical structure termed "mammospheres" without differentiated. It has been shown that mammosphere-initiating cells were able to self-renew *in* v*itro* and recapitulate the lobular/alveolar structure of mammary gland, suggesting stem cell properties of these cells. Then this culture system is adapted to grow tumorosphere-initiating cells that have stem cell properties including the ability to survive and grow in suspension under serum-free conditions with addition of some growth factors, including EGF, bFGF [25, 42, 43]. However, more differentiated tumor cells are anchorage-dependent and undergo anoikis in these conditions. Moreover, to be able to find the drugs which target the cancer stem cell populations, the tumorosphere culture has also been used in different studies [44].

The third and last *in vitro* technique can be used to isolate CSCs is side population methods. Side population method is based on the overexpression of transmembrane transporters, such as the adenosine triphosphate (ATP)-binding cassettes molecule *ABCG2/BCRP1* which exclude dyes for instance Hoechst 33342 or Rhodamine 123 in stem cells. This property is not found in differentiated cells that retain the dyes [45, 46]. Steiniger et al. isolated CSCs based on this method from two breast cancer cell line, MCF7 and MDA-MB231 via Hoechst 33342. The author found that these cells are more resistant to cytotoxic agents compared to non-side population cells. Treatment with 5-FU, methotrexate and doxorubicin lead to decrease on cell viability of non-side population to 10-50% whereas SP showed 100% viability. Beside, SP cells have been shown to have stem cell surface markers, such as CD44 and c-Kit [47].

In summary, many *in vitro* techniques have been developed to enrich for and isolate breast cancer stem cells. However, *in vitro* cancer stem cell assays provide an important tool for studies related to pathway and for screening of agents that target CSCs, self-renewal can only be confirmed by serial passage in the most robust model called xenograft model. In the xenograft model, human cancer cells are injected into the humanized as well as cleared fat pad of immunodeficient orthotopically. Whereas the cancer stem cell population initiates and sustains the tumor growth upon serial passage, the tumor cell population, lack of stem cell property's growth only limited-size and cannot maintain tumor growth upon serial dilutions. Due to the disadvantage of this system related to the micro environmental difference found in humans compared to NOD/SCID mice, researcher may encounter potential limitation of these systems [48].

1.2.3. Cancer Stem Cells and Metastasis

Due to metastasis is responsible 90 per cent of cancer related to mortality, the prevention and targeting metastasis has the big attention for clinical success of treatment [49]. On a biological level, metastasis consists of series distinct steps that compromise the invasion-metastasis cascade. First step requires that, cancer cells in primary tumor area have the potential to invade the surrounding tissue by breaching the basement membrane. Then, these tumor cells must enter into lymphatic and blood vessels which is called intravasation and survive through these vessels. The next step is extravasation, exit from the vasculatory system. The final step requires disseminated tumor cells to have the properties to adapt and proliferate in a foreign microenvironment as well as form macroscopic metastasis [50]. To accomplish the initial steps of metastatic cascade, carcinoma cell must acquire a number of traits which orchestrated by a small numbers of centrally acting transcription factors (TFs).

TFs choreograph the Epithelial Mesenchymal Transition (EMT**)** which confers the mesenchymal properties on epithelial cells and related to acquisition of aggressive character of tumor cells. EMT-associated traits enable the translocation of primary tumor cells to a distant location. (Figure 1.3).

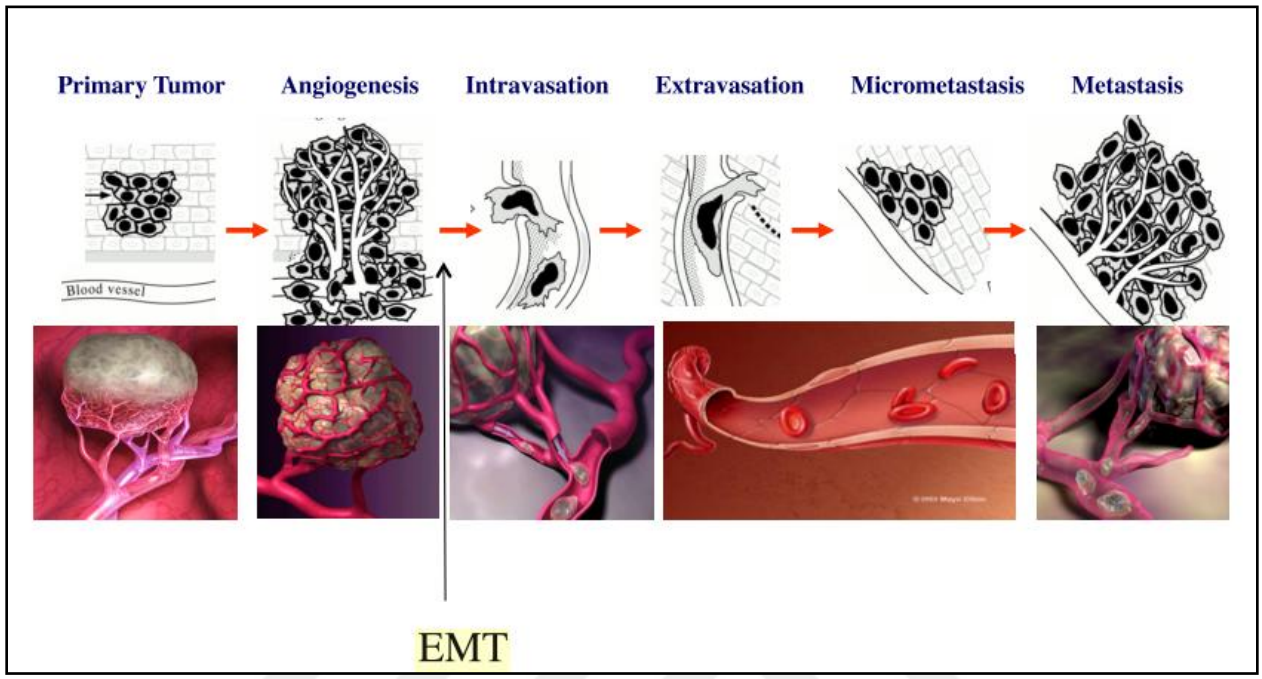

Figure 1. 3. The metastatic cascade; EMT programs are involved in early steps of the metastatic cascade, where they enable the invasion into the stroma and translocation of carcinoma cells to distant parenchyma. [51]

EMT can generate cellular behavior related to malignancy, motility, invasiveness and resistance to apoptosis which lead to seeding the large colonies of cancer cells that form macroscopic metastases [52]. In the early stage of EMT, most transcription factors act as transcription repressors, such as Snail, Slug, Zeb1, Twist directly repress mediator of epithelial adhesion. Also Snail, Slug has been demonstrated to have a direct role in the repression claudins which involve in the assembly of between neighbor cells [50, 53]. Moreover, the mechanism lead to the acquisition of mesenchymal character involve the up-regulation of N-cadherin, the mesenchymal intermediary filament vimentin and extracellular matrix (ECM) components such as fibronectin. These changes lead to gain of ability to invade surrounding tissue and migration of primary tumor cells. Furthermore, accumulating evidence proposes that EMT generates cells that have the properties of CSCs in a great number of malignancies, including breast [51,54], hepatocellular [55], pancreatic carcinoma[56].

Mani et al. showed the direct relationship between EMT and CSC phenotype. The induction of an EMT in immortalized human mammary epithelial cells (HMLEs) by ectopic expression of either Snail or Twist results in the acquisition of mesenchymal traits and increase the expression of stem cell markers. Moreover, the most of these cells acquired a $CD44^{high}/CD24^{low}$ phenotype, CSCs pattern and exhibited increased ability to form mammospheres [51]. The connection between EMT programs and metastatic CSCs should indicate the necessity to develop new strategies that interfere with gaining CSCs phenotype through EMT program.

Due to the hypothesis that cancer stem cells is the major reason for resistant to standard therapeutic approaches, including chemotherapy and radiotherapy, new therapies should be modulated to eradicate this subpopulation [23, 40, 57, 58]. Since the results have provided enough evidence for their anti-tumor effect on a variety of cancers in the last decade, natural dietary compounds have received attention to target cancer stem cells.

1.3. Natural Compounds as Cancer Chemopreventive Agents

Botanical and nutritional compounds have been used for either the treatment of cancer and prevention of cancer. Due to that high consumption of vegetable reduces the risk of cancer; a great deal of studies has been focused on the natural dietary compounds as chemoprevention [59]. Cancer chemoprevention is defined as pharmacological intervention with synthetic or naturally occurring compounds. In addition to prevent, inhibit, or reverse carcinogenesis, chemoprevention has potential to prevent the development of invasive cancer which is described as a major field of scientific investigation [60]. Till date, substantial investigation in epidemiological and animal studies besides *in vitro* studies have illustrated that dietary compounds, specially polyphenolic compounds and carotenoids have protective role against the cancer [58, 59, 61–63]

The family of natural polyphenolic compounds called Flavonoids, are composed of several classes including flavones, flavonols, flavanols , flavonones, iso-flavonoids and antho-cyanidins. The anti-cancer and chemopreventive property of these phenolic compounds have been researched by different groups in numerous epidemiological studies [62, 64–66] and broad range

of effects of flavonoids at the cellular and molecular levels have been demonstrated. The modulation of cellular processes, including cell cycle and apoptosis promote their anti-proliferative effects [67].

Given that these polyphenol compounds are usually multi-targeted, they may mediate other cellular events, such as stimulation of CSC differentiation and making CSCs more sensitive to chemotherapeutic agents. However; the limited reports showed inhibition of CSCs by dietary compounds, many of them have been demonstrated to be involved in repressing the self-renewal pathways of CSCs [15, 68–76]. Table1. 3 shows the natural compounds could be used to target the self-renewal of CSCs and their structure.

Table 1.3. Natural compounds could be used to target the self-renewal of CSCs and the pathways they affected

Compounds	**Biochemical Class**	**Pathway Related to Self-renewal**
Curcumin	Polyphenol	Notch, Wnt/β-catenin, Hedgehog, Akt
Resveratrol	Polyphenol	Notch, Wnt/β-catenin, Akt
Sulforaphane	Isothiocyanate	Hedgehog, Wnt/β-catenin,
Genistein	Flavone	Notch, Wnt/β-catenin, Akt
Epigallocatechin gallate (EGCG)	Polyphenol	Hedgehog, Akt
Quercetin	Flavone	Wnt/β-catenin

Besides self-renewal, treatment with curcumin or sulforaphane has been showed to decreased the ratio of cells expressing ALDH-1A1 and the number of tumorsphere [77, 78]. EGCG and resveratrol inhibit the metastasis by decreasing the expression of VEGF matrix metalloproteinase including MMP-2 and MMP-9, and modulating EMT, respectively [79, 80].

In addition to compounds discussed above, several compounds known to have anti-cancer effect have been waiting to be searched for their possible effect on CSCs.

1.3.1. Naringin

Naringin is a citrus flavonoid derived from grapefruit and related citrus species. It provides citrus species its characteristic bitter flavor. A serious line of investigations has been shown that it display strong biological activity against cancer. Its structure contains aglycone moiety called narigenin that links to a dioside,the neohesperidoside. In the body, naringin is converted to naringenin and its conjugates. Those metabolites are eliminated by renal excretion [81]. It has been reported that naringin or its metabolite naringenin exert its biological and pharmacological properties on different pathways related to anticarcinogenic [82], lipid-lowering [83], superoxide scavenging [84], anti-apoptotic [85], anti-atherogenic, metal chelating [82] and antioxidant activities [84]. Recently, the effect of naringin on apoptosis and cell cycle arrest has been show related to Wnt/B-catenin pathway which related to self-renewal of cancer stem cell [86] Figure 1.4. shows the molecular structure of naringin. Based on this study, it is concluded that naringin may have an impact on stem cell phenotype.

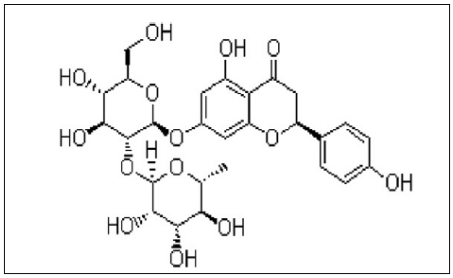

Figure 1.4. Molecular structure of Naringin. Naringin [7-(2-O-(6-deoxy-alpha-L-mannopyranosyl)- beta-D-glucopyranosyloxy)-2,3-dihydro-40,5,7-trihydroxyflavone].

AIM OF THE STUDY

In this study, it was aimed to examine the effect of naringin, a natural compound on stem cell phenotypes of breast cancer. To show that breast cancer cell line treated with different concentration of this flavonoid. Then, ALDH1+ or CD44+/CD24- stem cell fraction were counted by flow cytometry. To evaluate the effects of naringin on the aggressiveness of breast carcinoma, EMT and stem cell signature were checked at the transcriptional level and invasion assay was carried out.

2. MATERIAL and METHODS

2.1. MATERIALS

2.1.1. Cell Lines

The human breast cancer luminal subtype cell line BT474 was gifted by Dr. Korkaya (cancer center, Georgia Regents University, Augusta, GA, USA) and the human breast cancer triple negative breast cancer cell line MDA-MB231 was obtained commercially from ATCC – American Type Culture Collection, Manassas, VA, USA.

2.1.2. Cell Culture

Dulbecco‟s Modified Eagle Medium, DMEM (Invitrogen, Gibco, UK, cat # 31885), DMEM /F12 (Invitrogen, Gibco, UK, cat # 11320-074),Fetal bovine serum, FBS (Invitrogen, Gibco, UK, cat # 10270-106), Penicilin/Streptomycin/ Amphotericin, PSA (Invitrogen,Gibco, UK, cat # 15240-062), 0.25 per cent (w/v) Tyrpsin-EDTA solution (Invitrogen, Gibco, UK, cat # 25200), T75 Cell Culture Flasks (Biofil, TCP, Switzerland cat # 90075), 6-well culture plate (Biofil, TCP, Switzerland, cat # 92006), 96-well culture plate (Jetbiofil, cat # TCP011096), Serological pipets; 5 ml, 10 ml, 25 ml (LP ITALIANA SPA), Centrifuge tubes, 15 ml (Isolab), Incubator (Nuare, US, model no; NU5841E).

2.1.3. Natural compound

Naringin,, HPLC graded, 95 per cent (Sigma Aldrich, cat # 71162)

2.1.4. Cell Viability Assay

MTS-assay (3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2- (4-sulfo-phenyl)-2H-tetrazolium) (MTS Reagent Powder, Promega, UK, cat # G1111; PMS (Phenazine Methasulfate), Sigma Aldrich, cat # P9625), 96-well culture plate (Jetbiofil, cat # TCP011096), ELISA (Biotek, model no: EL800).

2.1.5. Apoptosis Analysis

Annexin V-FITC Apoptosis Detection Kit (eBiosciences, UK, cat# BMS500FI)

2.1.6. Cell cycle Analysis

Triton-X 100 (Bio Basic Inc, cat# 9002-93-01), Paraformaldehyde (Sigma-Aldich, cat# P6148), PI (Sigma Aldrich, cat# P 4170), RNase (Invitrogen, UK, cat #12091-039), Flowcytometry (BD Biosciences FACS Calibur, Cell Quest Pro software)

2.1.7. ALDEFLUOR Assay

ALDEFLUOR™ Kit (Stem Cell Technologies, Grenoble, France), Flowcytometry (BD Biosciences FACS Calibur, Cell Quest Pro software).), DMEM /F12 (Invitrogen, Gibco, UK, cat # 11320-074), T75 Ultra low attachment Flask (Corning, cat# CLS3814), B-27® Serum-Free Supplement (Invitrogen, Gibco, UK, cat # 17504044), BSA (Invitrogen, Gibco, UK, CAT # 17504-044), EGF (Sigma-Aldich, cat# E9644), Human Basic Fibroblast Growth Factor (Sigma-Aldich, cat# F0291)

2.1.8. Flow Cytometry (FACs) Analysis

Conjugated anti-bodies against CD44-FITC (clone G44-26 ,cat # BD555478, BD Biosciences, San Francisco, CA, USA), CD24-PE (clone ML5 ,cat # BD555428, BD Biosciences, San Francisco, CA, USA), Flowcytometry (BD Biosciences FACS Calibur, Cell Quest Pro software).

2.1.9. Real Time PCR

RNA easy plus mini Isolation Kit (Qiagen, cat # 174136), Revert-aid First Strand cDNA Synthesis Kit (Fermentas, cat# K1622), SYBR® qPCR Master Mix (Fermentas, cat# K0251), Primers (Macrogen, South Korea), BioRad RT-PCR C1000 Touch Thermal Cycle CFX96 detection system (Bio-Rad, Hercules, CA, USA, Bio-Rad CFX Manager Module).

2.1.10. Matrigel Invasion Assay

Growth Factor Reduced BD Matrigel Invasion Chamber (BD Biosciences; CA, USA, cat # 354483), Paraformaldehyde (Sigma-Aldich, cat# P6148), Incubator (Nuave, US, model no: NU5841E). Giemsa stain (Sigma Aldrich,USA cat#G4507)

2.2. METHODS

2.2.1. Cell culture

Human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM low glucose medium (Invitrogen Life Technologies, Inc., CA, USA) supplemented with 10 per cent v/v fetal bovine serum (FBS) and 1 per cent Penicillin/Streptomycin (Invitrogen Life Technologies, Inc., CA, USA) at 5 % CO2, 37 ℃. Human luminal A subtype breast cancer cell line,BT-474 was gifted by Dr. Hasan Korkaya, was cultured in DMEM/F12 medium supplemented with 10 % v/v fetal bovine serum (FBS) and 1 per cent Penicillin/Streptomycin (Invitrogen Life Technologies, Inc., CA, USA) at 5 % CO2, 37 ℃.

2.2.2. Preparation of Naringin

A stock solution of 20 per cent (w/v) was prepared in DMSO (Sigma, USA). The solution was stored in aliquots at -20 ^{0}C. Further dilutions were made in DMEM or DMEM/F12 to adjust the required concentrations from 0.05 to 0.15 per cent (w/v) for the treatment of breast cancer cells.

2.2.3. Cell Viability Assay (MTS)

MDA-MB-231 and BT-474 were seeded in a 96-well flat bottom plate at a density of 5 x 10^3 cells/well or 10 x 10^3 cells/well respectively. After 24 hr, naringin (Sigma) dissolved in DMSO at a final concentration of 0.05, 0.10, 0.15 per cent w/v and vehicle (0.2 per cent v/v DMSO) were added and incubated for 1,2,3 days at 5 % CO2, 37 ℃. Cell viability was assayed using MTS kit purchased from Promega (Uppsala, Sweden) and it was performed according to the kit manual.

2.2.4. Apoptosis Analysis with Annexin V Staining

BT474 cells were seeded in a 6-well plate at a density of 1.5 x 10^5 cells/ well. After 48 hours of naringin treatment at a concentration of 0.05, 0.10 or 0.15 per cent w/v, cells were trypsinized. Then, the Annexin-5 kit (eBiosciences) was used to analyze the apoptosis with using a FACS Calibur (BD Biosciences), according to the manufacturer's instructions. Firstly, cells were washed twice with cold PBS and 1 x 10^6 cells were resuspended in 400 ml 1X binding buffer. Then, 100 µl of cell suspension was transferred to a 5 ml culture tubes and for each group four tubes prepared. Three of them were prepared as control and adjustment of Flowcytometry. The last one is used for measuring apoptosis level. **Table 2.1.** explains the preparation of cells for analyses.

Table 2.1. Preparation sample of tubes for apoptosis analyses

Tubes	**Material**	**Amount**	**Incubation Time**
1	- (Negative Cotrol)	-	-
2	Annexin V antibody	5µl	15 min
3	PI (10 µg/ml)	5µl	5 min
4	Annexin V	5µl	15 min
	PI (10 µg/ml)	5µl	5 min

After the incubation time at RT in the dark, the cells were washed with 1X binding buffer. Following washing, 400 µl of 1X binding buffer was added to each tube to analyses with flow cytometry within 1 hour.

2.2.5. Analysis of Cell Cycle Progression

Cells were seeded in 6 well plates at a density 25 x 104 cells/well. After cells reached 80 per cent confluence, interested concentration of naringin or vehicle was added to the wells and incubated

for 2 days. Cells were trypsinized, harvested, and fixed in 1 ml 70 per cent cold ethanol in eppendorf and waited at -20^{o}C for overnight. After incubation, cells were centrifuged at 1,500 rpm for 5 min and following washing with phosphate-buffered saline, cells were permebiliazed with 0.1 per cent Triton X-100 for 20 minutes at room temperature. Then, the cell pellets were resuspended in 500 µl RNase A (0,3mg/ml) and incubated at 37^{o}C for 30 minutes. Cell cycle distribution was calculated from 10,000 cells with Cell Quest software (Becton Dickinson, CA, USA) using FACS Calibur (Becton Dickinson, CA, USA) following PI addition.

2.2.6. ALDEFLUOR assay

For ALDH1 enzyme activity, 25000 cells/ ml were seeded in T75 flask containing serum-free media with naringin or vehicle as a control under ultra low attachment conditions. The ALDEFLUOR kit (Stem Cell Technologies, Grenoble, France) was used to analyze the cell population with high ALDH enzymatic activity, using a FACS Calibur (BD Biosciences), according to the manufacturer's instructions. Briefly, cells were incubated in ALDEFLUOR assay buffer containing ALDH substrate (1 mmol/ per 1.10^6 cells). In each experiment, a sample of cells was incubated, under identical conditions, with 50 mmol/l of diethylaminobenzaldehyde, a specific ALDH inhibitor, as a negative control. After 30 minutes, cells were washed with ALDEFLUOR assay buffer and analyzed via FACS Calibur. The experiment was repeated twice.

2.2.7. Flow cytometry

Cells seeded in ultra low T75 flask at 25.10^3 cells/m in serum-free condition which supplemented with EGF, basic FGF, BSA, B27 and cultured for 3 days. After incubation in CO_2 incubator, cells were washed once with phosphate-buffered saline (PBS) and then harvested with 0.05 per cent trypsin/0.025 per cent EDTA. Single cells were washed with PBS containing 5 per cent FBS and 1 per cent penicillin/streptomycin (wash buffer), and resuspended in the wash buffer ($5*10^5$ cells/100 µl). After suspension cells were fixed with 2 per cent paraformaldehyde for 30 minutes, followed by washing with wash buffer. Combinations of fluorochrome-conjugated

monoclonal antibodies obtained from BD Biosciences against human CD44-FITC and CD24-PE were added to the cell suspension at concentrations recommended by the manufacturer and incubated at 4°C in the dark for overnight. The labeled cells were washed in the wash buffer, then BD FACSCalibur™ flow cytometer is used to determine cell ratio.

2.2.8. Primer Designing and RT-PCR Analysis

Primers of α-SMA, CD49F, Nestin, Beta-actin genes were designed by using Primer BLAST online software of The National Center for Biotechnology (NCBI) and ordered from Macrogen company to be synthesized at 100 nmoles. The other primers (OCT4, KLF4, Vimentin, Fibronectin, and Snail1) sequences were used as previously described in the literature. **Table 2.2.1.** shows the primers that were used in this study.

Table 2.2. Primers that were used in this study

Primers	**5'- 3', sense**	**5'- 3', antisense**
OCT4	GCATTCAAACTGAGGCACCA	AGCTTCTTTCCCCATCCCA
∝ SMA	CCGACCGAATGCAGAAGGA	ACAGAGTATTTGCGCTCCGAA
CD49F	ATGGAGGAAACCCTGTGGCT	ACGAGAGCTTGGTCTTGGA
Vimentin	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC
B-actin	GCTTCCTGTAGGTGGCAATC	TGATCCACATCTGCTGGAAGGT
Nestin	CTGCGGGCTACTGAAAAGTT	AGGCTGAGGGACATCTTGAG
Snail1	AAGATGCACATCCGAAGCC	CGCAGGTTGGAGCGGTCAGC
Fibronectin	CAGTGGGAGACCTCGAGAAG	CAGTGGGAGACCTCGAGAAG
KLF4	GATTACGCGGGCTGCAAAACCTACACA	TTGTAGTGCTTTCTGGCTGGGCTCC

BT474 cells were seeded into 6-well plates at a concentration of 150.000cells/well. The cells treated with the desired concentration of naringin or vehicle. Total RNA from treated cells were isolated using Qiagen mini RNA isolation kit according to the manufacturer's instructions after 48 hours of the treatment. cDNA was synthesized using Revert-aid First Strand cDNA synthesis kit. SYBR green real time PCR method was used to detect the gene levels. cDNAs were mixed with primers and SYBR® qPCR Master Mix in a final volume of 10µL. Beta actin (*β-actin*) gene was used as the housekeeping gene for normalization of the data. All RT-PCR experiments were done using Bio-rad CFX RT-PCR manager system. Microsoft Excel is used to analyze the results.

2.2.9. Matrigel invasion assay

The invasion assay was performed with the Growth Factor Reduced BD Matrigel Invasion Chamber in 24 well plate (BD Biosciences; CA, USA). Matrigel Invasion Chambers are hydrated for at least 2 h in the tissue culture incubator with 500 µl serum free DMEM in the bottom of the well and 500 µl in the top of the chamber. After hydration, 25,000 cells in 200 µl of serum-free media with different concentration of naringin or vehicle were placed on the top insert which containing matrigel. 700 µl media with 10 per cent serum were placed in the bottom well. After incubation at 37^0C for 22 in CO_2 incubator, cells were fixed with 3.7 per cent paraformaldehyde. Following fixation, 100 per cent methanol is applied for permeabilization. Then, invaded cells are stained with giemsa stain. Cotton tips were used to remove the cells that remained on the matrigel or attached to the upper side of the filter. The number of cells that invaded through the matrigel was counted under a phase-contrast microscope. For each group, five different areas were counted and average of this five area is used to determine invaded cell ratio.

2.2.10. Statistical Analysis

All data are shown as the means ± standard errors. Graphics were drawn using GraphPad Prism 5 software and Microsoft Office Excel program. The results of real time PCR data were normalized to the mRNA level of β-actin. The statistical analysis of the results were performed one-way ANOVA followed by the multiple-comparison Tukey's test using GraphPad Prism 5 software. Statistical significance was determined at $P < 0.05$.

3. RESULTS

3.1. EFFECT OF NARINGIN ON CELL VIABILITY OF BREAST CANCER CELL LINE

We firstly examine the cytotoxicity of naringin on human breast cancer cell line. Based on the literature, the toxicity assay was performed at three different concentration of naringin for 3 days. According to MTS assay results, on the first day naringin did not change the viability at the concentration of 0.05 and 0.10, whereas it decreased the viability at the concentration of 0.15. On the second day, the viability of cells on the concentration of 0.10 per cent slightly increased and cell viability of cells treated with 0.15 per cent were decreased, however 0.15 per cent of naringin did not exhibit significant effect. On the third day, for all concentration, cell viability was increased. The MTS results have been shown on Figure 3.1.

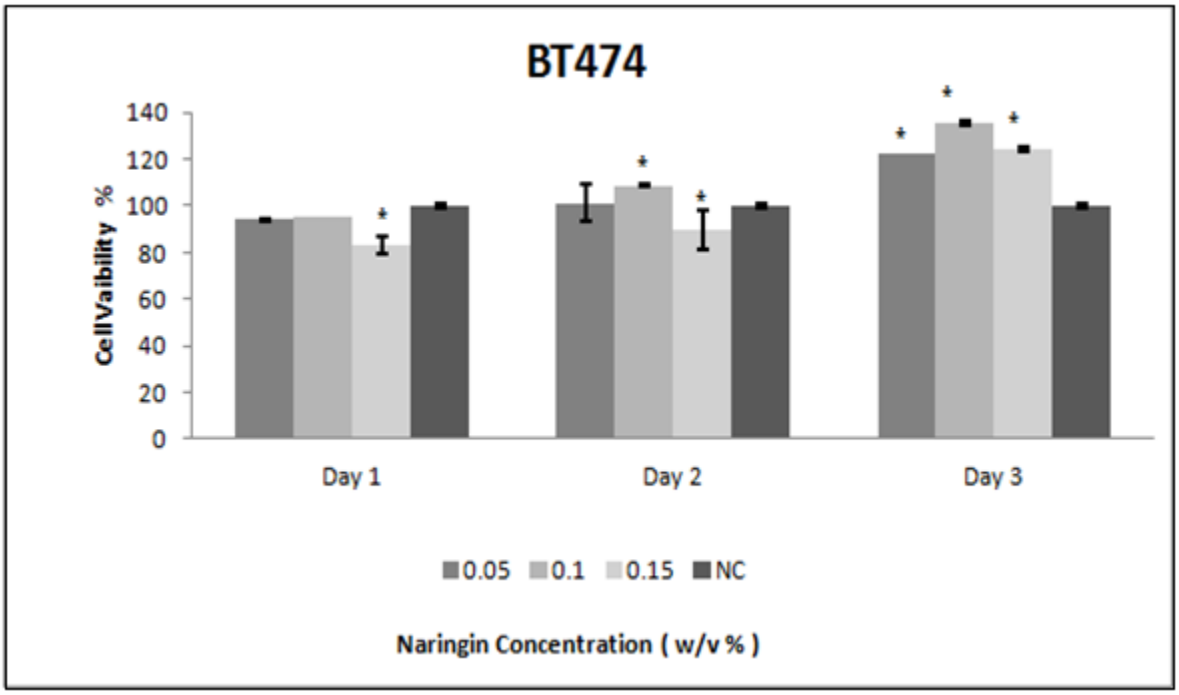

Figure3.1**.** Cell viability of BT474 cells at three different concentration of naringin. *P < 0.05 versus the control (vehicle).

3.1. EFFECT OF NARINGIN ON APOPTOSİS OF BREAST CANCER

After 48 h treatment with different concentration of naringin or vehicle (0.2 per cent DMS0), the cell that went to the apoptosis was detected by using Anexin-5 kit. The apoptotic and necrotic cells were counted by Flow cytometry in the presence of PI and Annexın-5-FITC. The percentage of apoptotic cell was indicated and apoptosis graphic was drawn in Microsoft Excel. The experiment repeated twice. The results have been observed in similar fashion with MTS assay. At the concentration of 0.15, naringin caused to more apoptosis compared to other concentrations but this effect was not statistically significant.

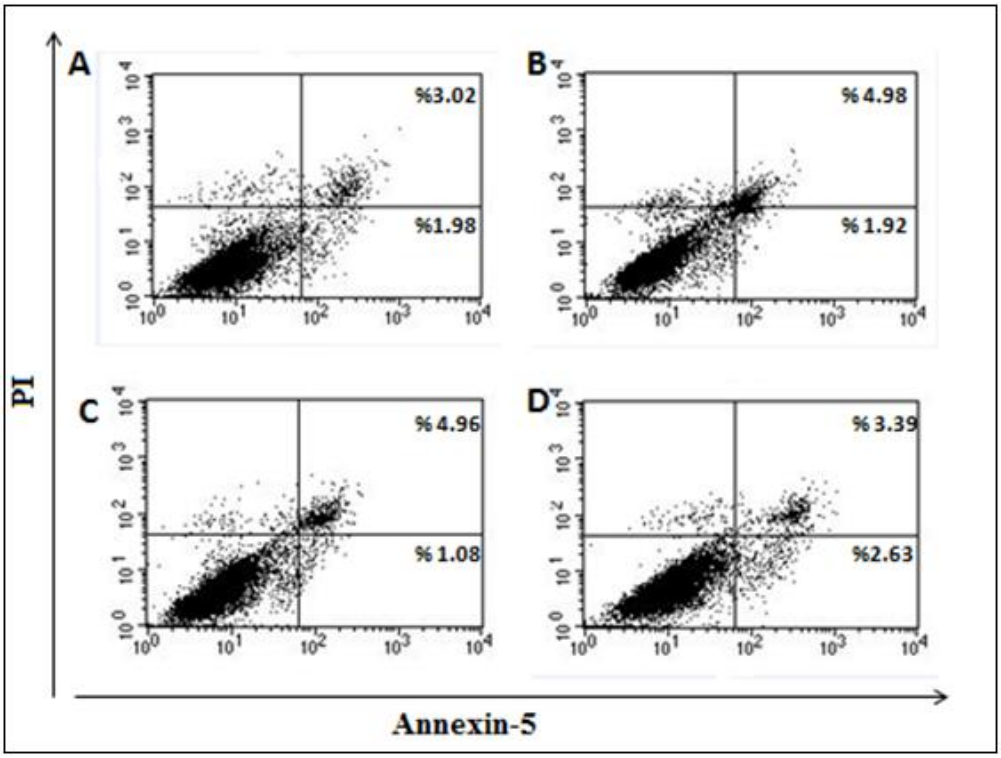

Figure 3.2**.** BT474 cells stained with Anexin-5 after 48h treatment. Dot-blot view of cells shows in the quadrant. Upper left indicates necrotic cells stained with PI, upper right shows cells in late apoptosis phase stained with Annexin-5-FITC and PI. On the other hand, lower left part represent unstained and healthy cells and lower left shows cells in early-apoptotic phase. A represents the vehicle, B, C and D stands for cells treated with naringin at the concentration of 0.05, 0.10 and 0.15, respectively.

As shown in Figure 3.3, naringin treatment did not cause significant increase of the apoptotic cell ratio.

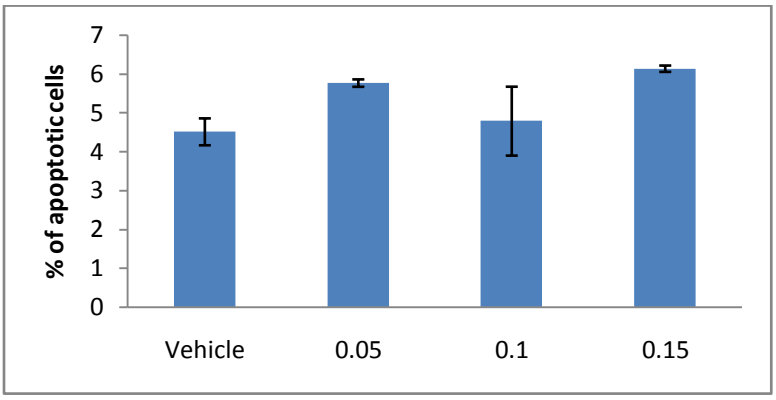

Figure 3.3. The percentage of apoptotic cells after 48h treatment with different concentration naringin or vehicle.

3.3. ANALYSIS OF CELL CYCLE PROGRESSION

BT474 cancer cells were treated with naringin or vehicle for 2 days. Cells were harvested after treatment, and then fixed with 70 per cent ethanol for overnight. Following staining with PI, cells were analyzed for DNA content. The distributions of cells in G0/G1, S, G2 /M phase of cell cycle are indicated in Figure 3.4. According to flow cytometry results, cell ratio in G2/M phase increased on the basis of naringin concentration which is shown in Figure 3.5.

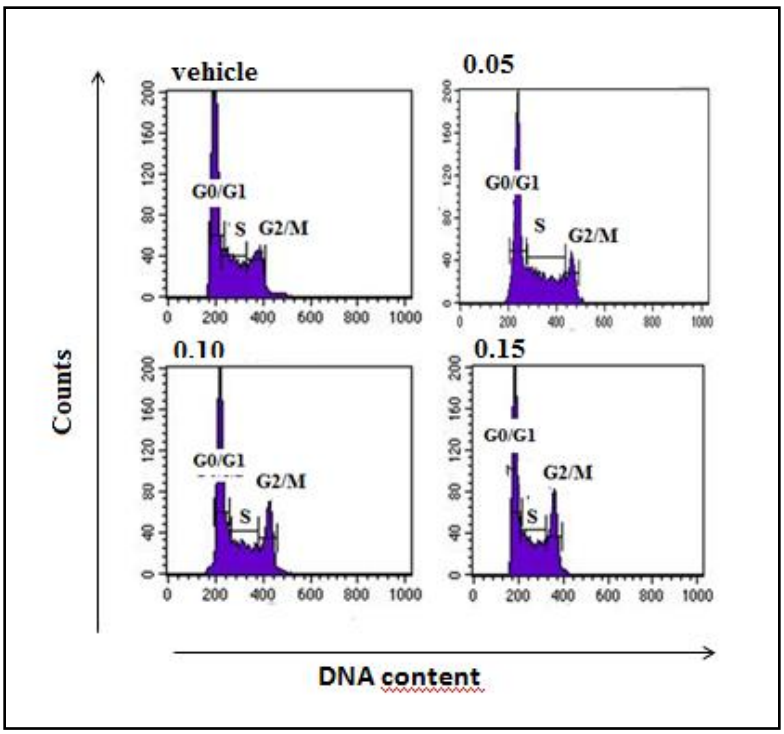

Figure 3.4. The analysis of cell cycle progression. The distribution of cells in G0/G1 (first peak), S (between two peaks), G2 /M (second peak) phase of cell cycle are indicated. X axis indicates the DNA content. Cells in G0/G1 phase gives peak at 200 and in G2/M locates in 400 because of the DNA content they have.

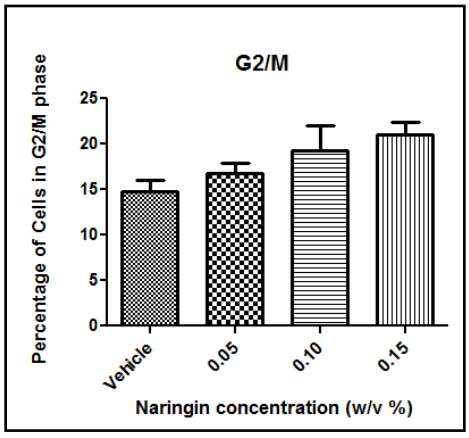

Figure 3.5. The percentages of cells in G2/M phase. Y axis stands for percentage of cells. The cell ratio in G2/M phase was increased when the concentration of naringin increase.

3.4. NARINGIN REDUCE THE PERCENTAGE OF CELLSEXPRESSIN STEM CELL MARKERS

It is known that, the cells with high aldehyde dehydrogenase1 (ALDH1) activity in breast carcinoma can be assessed by the Aldefluor assay that has been demonstrated to enrich tumorigenic stem/progenitor cells [32]. This cell population has potential for self-renewal and to generate tumors resembling the parental tumor. Since BT474 has a relatively high percentage of ALDH-positive cells, we selected this cell line to examine whether naringin inhibits the tumor-initiating ALDH-positive cells *in vitro* in the first place. To provide evidence that Naringin is able to reduce the stem cell populations, we examined the effects of these compounds on the proportion of breast cells expressing the stem cell marker ALDH1 by flow cytometry. As shown in Figure 3.6. and Figure 3.7. naringin treatment reduced the proportion of ALDH-1 expressing at the three concentrations significantly. However, the more effective concentration was 0.10.

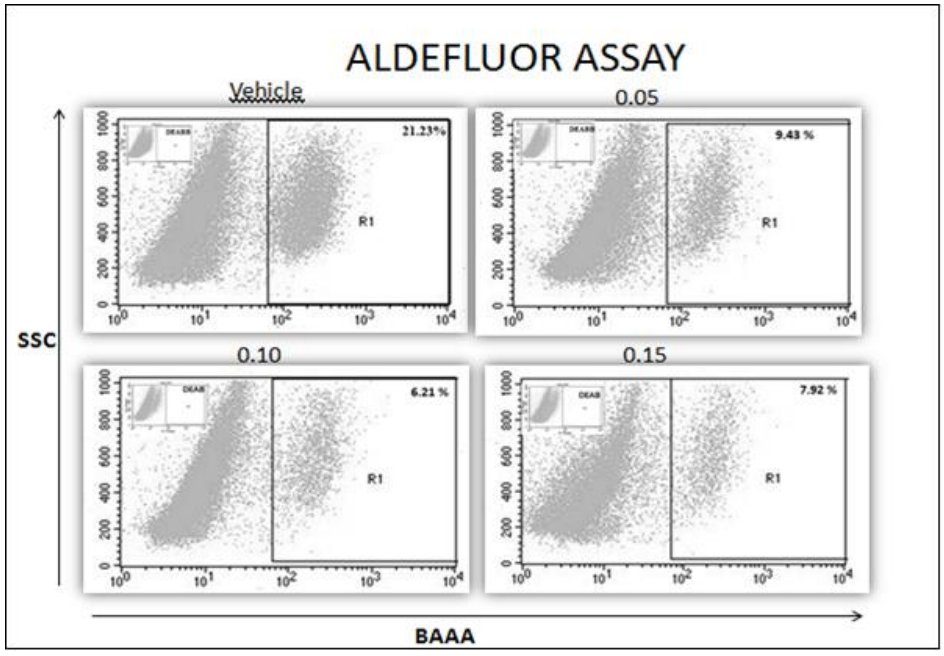

Figure 3.6. Representative dot plots of flow cytometry results for naringin effect on stem cell population of breast carcinoma. The inhibitor of ALDH, diethyl-aminobenzaldehyde, (DEAB) were used to establish the baseline fluorescence of these cells (upper right window in each graph) and to define the ALDEFLUOR-positive region (R1).

Quantification of ALDH-positive cells in each treated group is shown (Figure 3.7). As shown in Figure 3.7(B) naringin reduced the proportion of ALDH-1 expressing cells significantly from 22 per cent to 7-10 per cent. The best results were seen in the cells treated with 0.10 per cent naringin which decreased stem cell ratio to almost 6%. The treated cells form mammosphere under serum–free condition. The mammospheres treated with vehicle was bigger comparatively to naringin treated groups and more spherical in compact shape were observed in control groups as seen in Figure 3.7. (A).

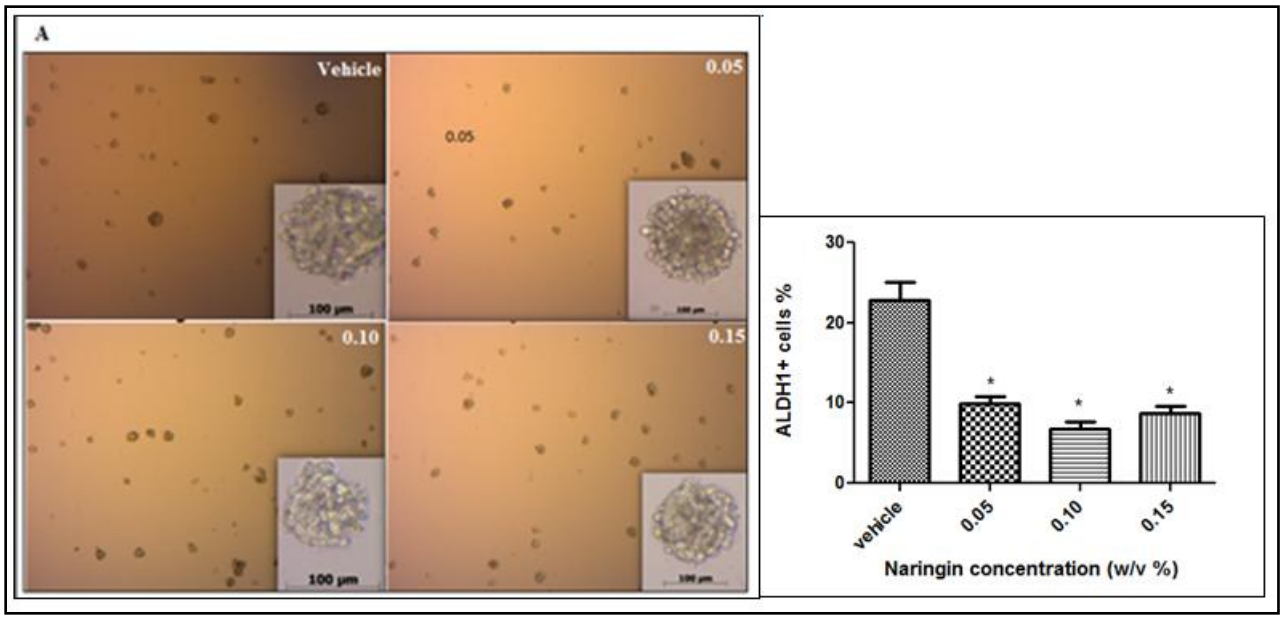

Figure 3.7. A; Primary mammosphere formed from human breast stem/early progenitor cells in suspension culture with dietary polyphenol naringin or vehicle treatment**.** The picture on left side show the sphere with 20 x magnification and big picture represent spheres with 4 x magnifications. **B;** The proportion of ALDH-1 expressing cells growth mammosphere condition and treated with naringin or vehicle. *P < 0.05 versus the negative control.

3.6. THE EFFECT OF NARNGIN on CD44+CD24- PHENOTYPE of MDA-MB231

Due to not having CD44+CD24- phenotype of BT474, we checked the the effect of naringin on MDA-MB231 which has high portion of these tumorigenic cells. After treatment with naringin for 3 day under low attachment, serum-free conditions, it was found that naringin decrease CD44+CD24- cancer stem cell ratio significantly in triple negative breast cancer cell line in a dose-dependent manner. As shown Figure 3.8. and Figure 3.9., naringin treatment impeded the CSCs with $CD44^{+}$/ $CD24^{-}$ ratio from 90 per cent to 57-22 per cent.

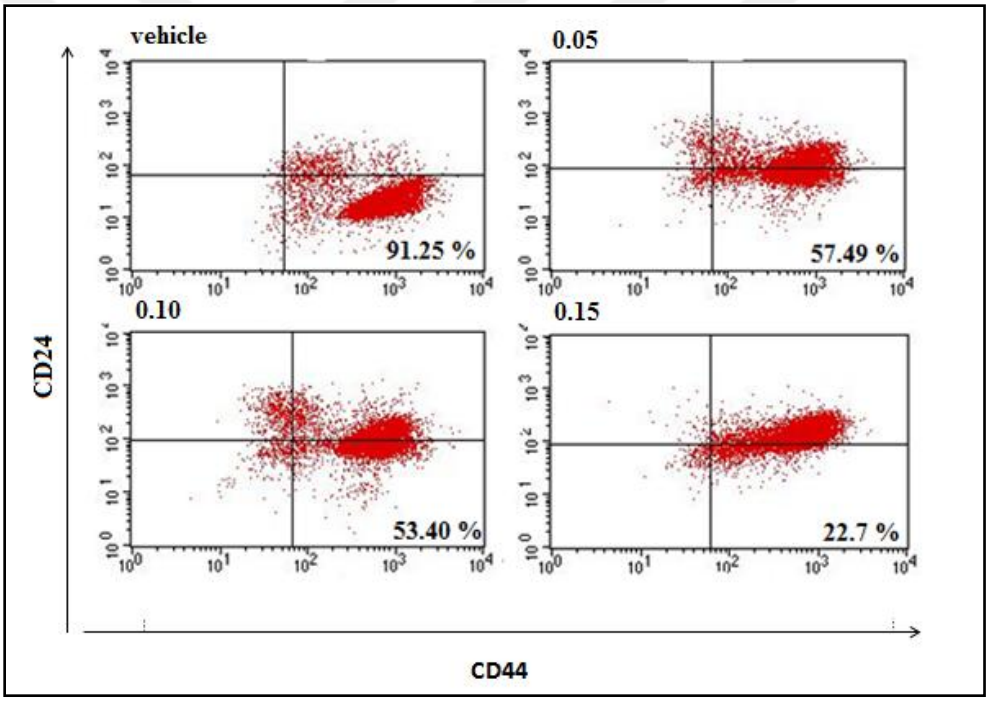

Figure 3.8. Representative of the dot-blot of naringin treated MDA-MB231 cells for CD44 and CD24 staining. The percentages of CD44+/ CD24- cells have been shown in the quadrant lower right side.

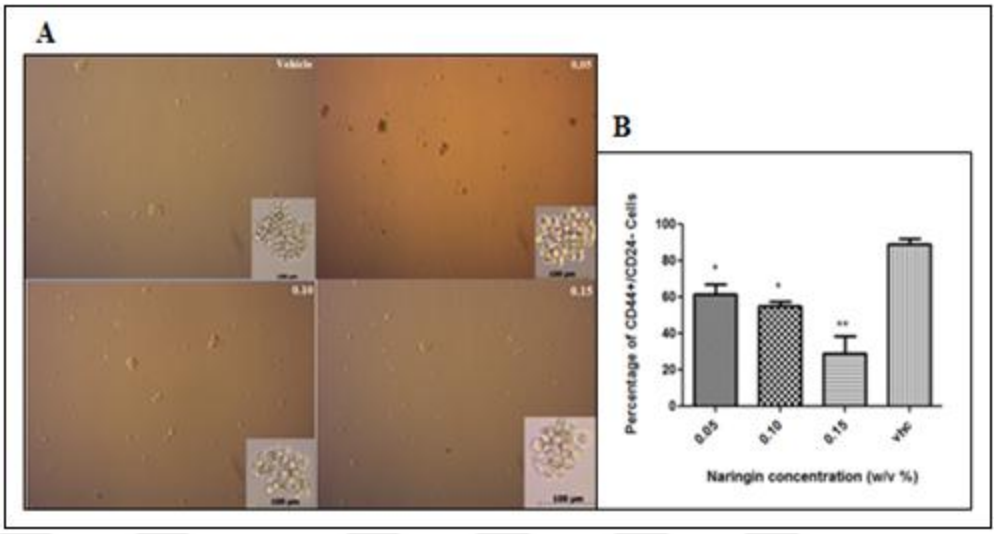

Figure 3.9. Naringin treatment decreased the cancer stem cell ratio in MDA-MB231. A; mammosphere formation under treatment of naringin (vehicle: upper left, 0.05: upper right,0.10: lower left, 0.15: lower right), B; represent the percentage of CD44+/CD24- cells after treatment with naringin for 3 day under serum-free, low attachment conditions.*$p < 0.05$/**$p < 0.01$/***$p < 0.005$ versus vehicle.

3.6. REAL TIME PCR RESULTS

3.6.1. Naringin Decreased the Stem Cell Marker Expression on Transcriptional Level

CD49F is an integrin which homodimerize with other integrins and binds laminin to lead to attachment of epithelial cells to the extracellular matrix as well as communication between the cell and the extracellular matrix by co-operating with receptor tyrosine kinases bidirectionally. It is known that in breast cancer, elevated CD49f expression is associated with reduced survival [87]. Since the increased expression of stem cells markers , including CD49F, OCT4, KLF4 have been seen in breast cancer stem cells and related aggressive phenotype of breast carcinoma, we checked the expression of these markers after naringin treatment for 2 days. The qRT-PCR results showed that naringin treatment decrease the mRNA expression of these markers compared to control group, significant. Naringin treatment at the concentration of 0.10 cause twofold decrease of each stem cell marker. The *CD49F* expression was decreased 1.5 fold by this phenolic

compound at the concentration of 0.05 and 0.15. The expression of *KLF4* was decreased almost 2 fold for each concentration, whereas *OCT4* expression was decreased for 0.05 significantly but 0.15 did not change the expression of *OCT4*, significantly (Figure 3.10). β-actin was used as a housekeeping gene to normalize the results.

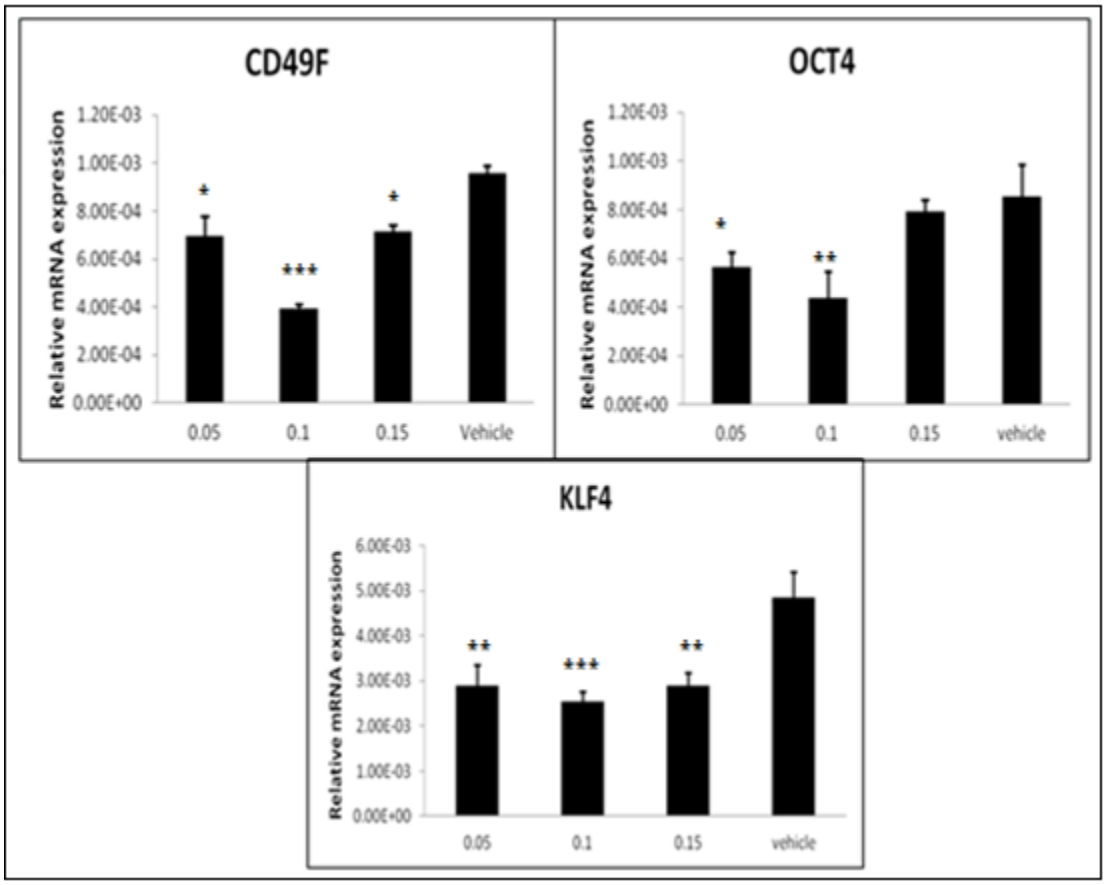

Figure 3.10. The relative mRNA expression of stem cell markers; KLF4, OCT4, CD49F. **p* < 0.05/***p* < 0.01/****p* < 0.005 compared to vehicle.

3.6.2. Naringin Decrease the EMT-Related Markers Expression on Transcriptional Level

Epithelial Mesenchymal Transition (EMT**)** which confers the mesenchymal properties on epithelial cells and related to acquisition of aggressive character of tumor cells. In the earlier stage of EMT, transcription factors, such as Snail1 acts as repressor on epithelial behavior whereas the mechanism lead to gaining mesenchymal character requires the expression of mesenchymal filament and extracellular matrix metalloproteinase, including vimentin and fibronectin. Real Time PCR results showed that in the cells treated with naringin for two days, the expression of EMT related markers decreased for all interested concentration compare to control (Figure 3.11). Naringin decrease the mRNA level of vimentin and Snail1 in a dose-dependent manner, whereas 0.10 % of naringin exhibit more inhibiting effect on fibronectin**.** β-actin was used as a housekeeping gene to normalize the results.

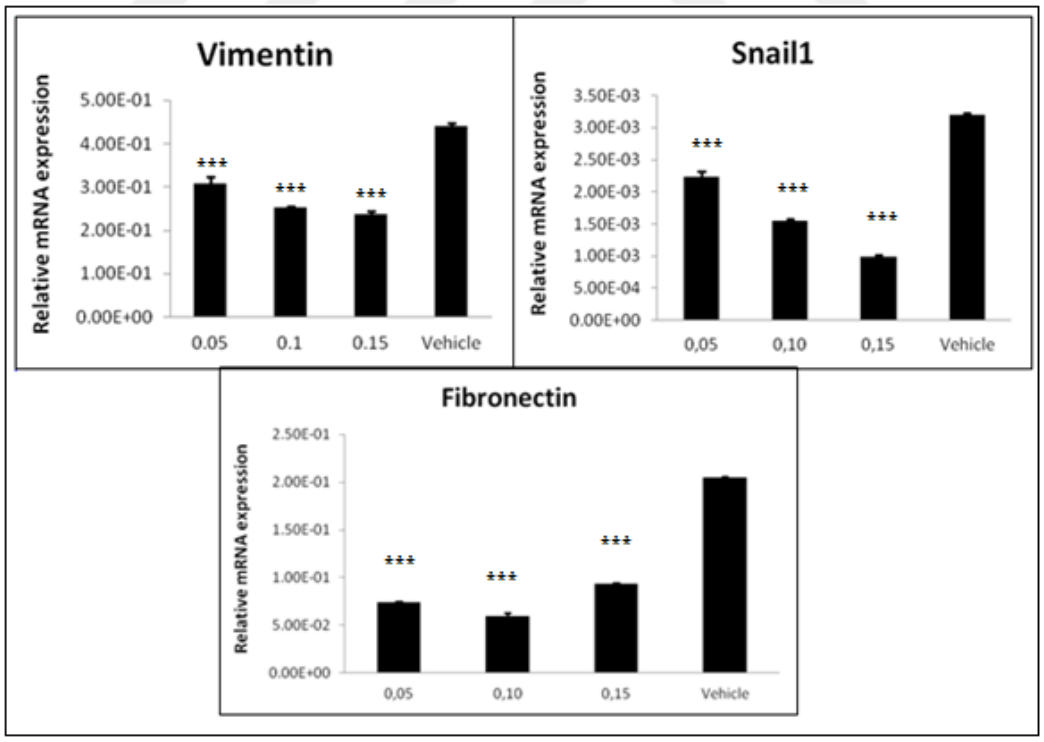

Figure 3.11. The effect of naringin on mRNA expression of genes related to EMT phenotype of CSCs. $*p < 0.05$/$*p < 0.01$/$***p < 0.005$ compared to vehicle.

3.6.3. Naringin Decrease the Differentiation Markers Expression on Transcriptional Level

After observing that naringin exerts its effect on the stem cell phenotype of CSC by decreasing ALDH1+ stem cell and leading to decreased EMT-related markers, it was suspected whether naringin may cause the differentiation of this subgroup. In order to see it, the markers expressed on progenitor cells, Nestin and differentiated breast cancer cells, α-smooth muscle actin (α – SMA) was checked by qRT-PCR. It demonstrated that naringin decrease the *Nestin* expression; however the treatment lead to increase on expression of (*α –SMA*) (Figure 3.12.) β-actin was used as a housekeeping gene to normalize results.

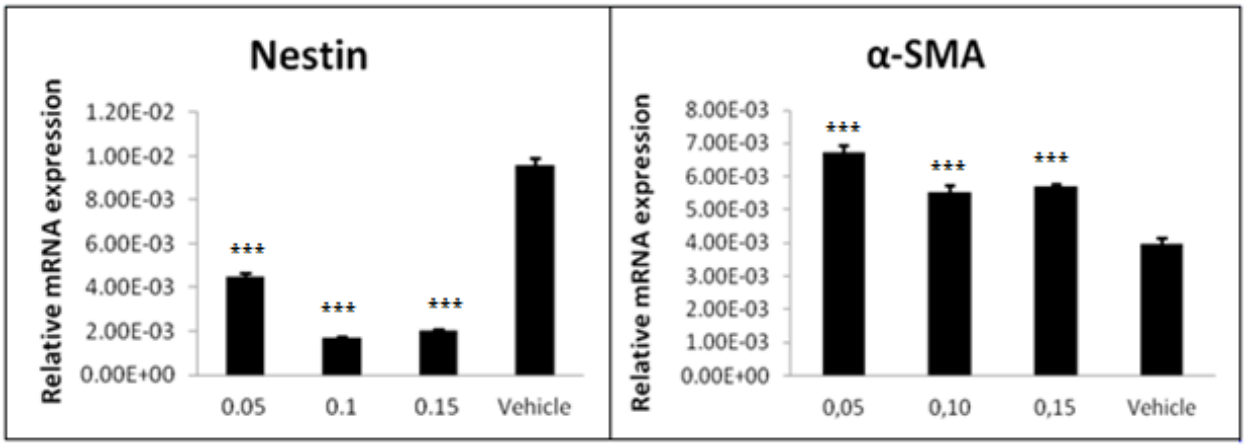

Figure 3.12. The effect of naringin on mRNA expression of Nestin and α –SMA. *$p < 0.05$/*$p < 0.01$/***$p < 0.005$ compared to vehicle.

3.7. THE EFFECT OF NARINGIN ON INVASIVE PROPERTIES OF CANCER CELLS

One of the features of cancer stem cell is being invasive. To check the invasive potential of cells treated with naringin, the invasion assay was carried out. Cells were seeded on matrigel treated with naringin or vehicle. After 22h, five areas randomly selected were counted for cells invaded the matrigel under phase-contrast microscopy following the staining with Giemsa stain. However the treatment decrease the portion of cells invade matrigel, this effect was not significant.

Figure 3.13. shows the microscopic image of the cells invaded matrigel (A) and graphic represented the average of treated cells that invade the matrigel.

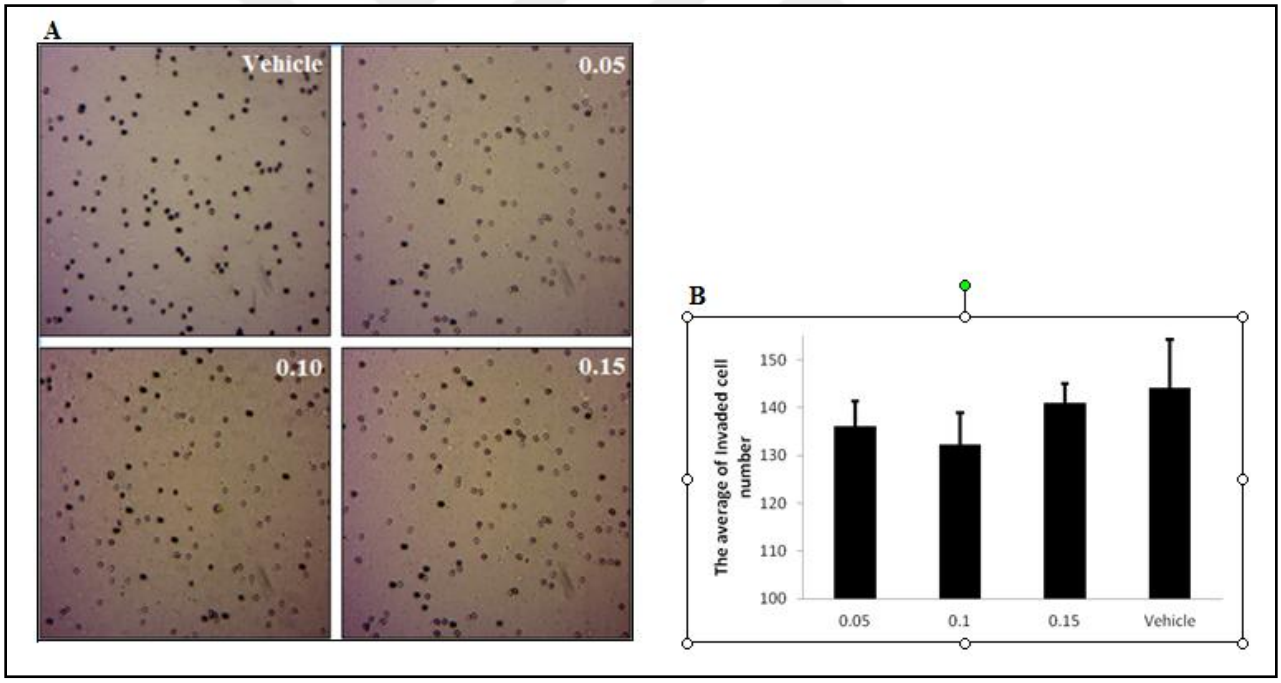

Figure 3.13. Matrigel invasion assay for comparing invasiveness of treated breast cancer cells**.** Invasion assay was carried out using BD matrigel-coated 24-well transwell. Then randomly chosen fields were photographed (×20) **(A),** and the number of cells migrated to the lower surface was calculated as a percentage of invasion. **(B);** Data are shown as the mean ± SD of three independent experiments by analysis of one way- Anova and Tukey's Multiple Comparison Test.

4. DISCUSSION

Breast cancer is a significant public health problem despite advances in the understanding of the molecular and cellular events that underlie the disease. However, the progress in medical technology; metastasis, tumor relapse and resistance to therapy lead to death in breast cancer patients. The heterogeneity and molecular complexity of breast cancer are the reasons which lead to fail the development of effective strategies to prevent and cure this disease. This hypothesis termed this minor population as cancer stem cell or tumor-initiating cells [9].

Cancer stem cells are a small fraction of tumor cells that possess the capacity of self-renewal and unlimited slow proliferation. They are currently known to be the locomotive engine of tumor, which contribute to resistance to therapy, tumor recurrence, and distant metastasis. This small population driving the tumorigenesis is highly resistant to chemo-radiation therapy compare to its differentiated daughter cell and this explains why tumor regression may not correlate with patient survival [56, 88, 89].

The presence of CSC in breast cancer has been showed by Al-Hajj. In this study, breast cancer stem cells have been shown to have $CD44^+/CD24^-/Lin^-$ phenotype [12]. The studies to indicate markers for breast CSCs provided that these small fraction may have different surface markers, including $CD49F^+$/ $EpCAM^-$, $CD44^+/CD24^-/ESA^+$ or high enzyme activity such as ALDH1 [32, 90]. Moreover, it has been found that cells has high ALDH1 activity and $CD44^+/CD24^-$ phenotype is highly aggressive in breast cancer [91].

The development of effective therapies which target CSCs is necessary since this small fraction has important implications for early detection, prevention and treatment of breast cancer. Due to being resistant to standard all-care therapies, including radiation, new therapeutic strategies must be developed. Recently, natural dietary compounds, especially phenolic compounds have been demonstrated to have impact on stem cell population of tumors through self-renewal pathway or

EMT. For example, curcumin or sulforaphane treatments decrease ADLH1+ stem cells whereas ECGC effect the stem cell through EMT process [77, 92, 93].

Naringin, a citrus flavanoid with physiological properties could be one of the potential natural compounds will be used against the cancer stem cells. It has a broad range of effects at cellular and molecular level that contribute to its chemo-preventive and anti-carcinogenic properties. The modulation of cellular processes such as cell cycle and apoptosis promote anti-proliferative effects of this dietary compound [94]. In recent study, the inhibitory effect of naringin on breast cancer cells have been proposed to be associated with inhibition of Wnt/B-catenin pathway which is one of the pathways related to self-renewal of stem cell. There was no report showing impact of naringin on stem cell, however our group showed that naringin treatment increases the condrogenic differentiation of human stem cell (unpublished data). Therefore, this study will be designed to investigate the effects of phenolic compound, naringin on stem cell phenotype of breast cancer.

In this study, the inhibitory effect of naringin on the stem cell phenotype of breast cancer cell line has been shown. The cytotoxic effect of interested concentration of naringin on breast cancer cell line, BT474 was demonstrated by MTS assay. The results showed that the concentrations of naringin used for this study were not toxic to the cells. In the first day, cell viability was decreased by 0.15 per cent of naringin slightly and at day 2; only in group supplemented with 0.10 per cent showed slightly increase and 0.15 per cent of naringin slightly decrease the viability. However, at day 3, cell viability was increased in treated groups. Since it is know that cytotoxicity may increase stem cell fraction [15], we choose to treat cell for two days. This will let us to see three different effect; 0.05 which did not affect viability, 0.10 that lead to increase in viability and 0.15 which cause to decrease in the viability of cell. Then, we checked how the treatment of naringin affects the apoptosis of breast cancer. The Annexin-5, an apoptosis assay, results showed that treatment with naringin at these concentrations did not cause any significant differences in apoptosis compared to vehicle after two days treatment. Then, the signatures related to stem cell phenotype that are cell cycle progression, ALDH1 activity, $CD44^{+}/CD24^{-}$ cell fraction and EMT signature as well as stem cell markers have been investigated.

The transverse of cell in the cell-cycle phases is well-controlled. Cells commit to enter the cell-cycle in G1 phase and prepare to duplicate their DNA in S phase. Following S phase, cells enter the G2 phase, in which repair might be seen along with preparation for mitosis in M phase. In the M phase, the separations of chromatids and daughter cells have been seen. The cells can enter G1 or G0, a quiescent phase after M phase [95]. Stem cells are slowly dividing cells that for extended period of times largely stay in G0 phase of cell cycle. These make them resistant to cell cycle active chemotherapeutic agents. In this study, it has been observed that naringin treatment decrease the number of breast cell in G0/G1 phase and lead to arrest in G2/M phase of cell cycle. The G2/M arrest by naringin is consistent with literature. Ramesh et al. has also shown that naringin arrest SiHa cells, cervical cancer cell line at G2/M phase of cell cycle [85]. Treatment with this flavanoid will make stem cell population or differentiation cells more sensitive to chemotherapeutic agents. In other words, naringin could be used with other drugs to target both stem cells and tumor bulk.

For the first time , the effect of naringin treatment on cells having high enzyme activity of Aldehyde dehydrogenase1 (ALDH1) which is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes was shown by us[40]. Aldehyde dehydrogenase, a stem cell marker that can be utilized to isolate human mammary stem cells. Ginestier et al. demonstrated that ALDEFLUOR-positive cells isolated from human breast cancer display properties of cancer stem cells and may generate tumors in immunodeficient mice [32].Due to, it is known that Luminal B subtype breast cancer cell line, BT474 has high level of ALDH1 activity, it was used in this study to examine the effect of naringin on ALDH1+ cells, which is defined as cancer stem cells. Our results showed that naringin supplementation to serum free, mammosphere forming culture decrease the ALDH1+ breast cancer stem fraction from 22 per cent to 7-10 per cent. Moreover, this flavonoid caused the less number of mammosphere formation with smaller size. Luminal B BT474 cells are known to form tightly cohesive structures displaying robust cell–cell adhesions in ultra low attachment, serum-free culture condition [2].

Another stem cell property of breast cancer stem cell is having CD44+/CD24- phenotype. Luminal subtype of breast cancer cell lines contain these cells less than 1 per cent, however,

claudin-low subtype breast cancer cell line, such as MDA-MB231 has a high fraction of this cell. Because of that, we chose this cell line to demonstrate the effect of naringin treatment on breast cancer stem cell having CD44+/CD24- phenotype The findings proved that naringin application decrease the proportion of this subgroup in a dose dependent manner. Treatments cause the reduction CD44+/CD24- cells from 91.5 per cent to 57-22 per cent. The best results were observed in the group treated by 0.15 per cent naringin. Given to the impact of naringin on ALDH1+ and CD44+/CD24- cell fraction in two different breast cancer cell line, naringin may be an alternative to treat cancer with other agents.

After observing the effect of this naturally-occurring dietary compound on stem cell population, the impact of naringin on stem cell was investigated at the molecular level in this study. The data suggested that naringin treatment down-regulate the stem cell and EMT markers. Moreover, supplementation of flavone lead to decrease the expression of *Nestin* that is marker expressed in breast cancer progenitor cells and induction *alpha-smooth muscle actin (α-SMA*) which is a marker for differentiated stem cells [96].

The ectopic expression of stem cell markers, including OCT4, KLF4 in differentiated cells has been shown to induce gaining of stem cell like phenotype [97–100]. All these studies prove that these factors has important role for stem cell behavior. Recently, two paper provided that these two factors are also important for cancer stem cells. However, earlier reports have shown that 70% of breast carcinomas have increased KLF4 expression and that increased nuclear staining for KLF4 is associated with a more aggressive phenotype (Foster et al., 2000; Pandya et al., 2004), the importance of KLF for CSC and eventually in the initiation of tumor has been shown by Yu et al. He and his colleagues showed that knockdown of KLF4 inhibits migration and invasion of breast cancer cells as well as suppression of self-renewal of cancer stem cell. Moreover, inhibition of KLF4 suppressed mammary tumor development in a xenograft tumor model. These results suggested that KLF4 may act as an oncogenic protein and it is essential for maintaining cancer stem cells as well as for promoting migration and invasion [101]. Also, it has been founded that he ectopic expression of OCT4 in mouse breast cancer cell lead to more aggressive phenotype by

increasing number of tumor-sphere and up-regulating the expression of cancer stem cell markers [27].

Given to the importance of KLF4 and OCT4 for tumor migration and metastasis, we checked the effect of naringin on the expression of these markers. The results showed that this natural phenolic compound decreased the expression of *KLF4* for each concentration in transcriptional level and the most effective down-regulation was seen in group treated with 0.10 per cent naringin. However, down regulation of *OCT4* expression was seen in groups treated with 0.05 per cent and 0.10 per cent naringin, there was no significant reduction in 0.10 per cent naringin treated group. Moreover, the impact of this flavonoid on CD49F which is a stem cell marker for breast cancer has been showed that it is effective to target stem cell population. The high expression of CD49F is associated with CSCs profile [87, 102]. Taken to the effect of naringin on stem cell markers expression in transcriptional level, naringin could be used to target cancer stem cells. Especially, at the concentration of 0.10 per cent, naringin inhibits the breast cancer aggressiveness.

When a cell detaches from his microenvironment, it tend to die by anoikis. However, tumor cells escape this fate by entering a process called the epithelial mesenchymal transition (EMT) that let them to acquire a mesenchymal-like phenotype and become invasive. During EMT, the expression of the proteins Fibronectin, Vimentin, several metalloproteinases and transcription factors like Snail1 (Snail), Snail2 (Slug), Zeb 1 increases [49]. EMT has an important role in the induction of cancer cell invasion, migration and phenotype and function of CSCs. Also, cancer stem cell has fundamental clinical implication because of its involvement in resistance to treatment, invasion and metastasis that lead to tumor aggressiveness. Large number of evidence proved that EMT process generate cells with self-renewal ability and drug resistance, CSCs [52, 54, 56, 103]. Additionally, CSC in tumor tissue undergo EMT process to have a mesenchymal phenotype that let CSC to migrate to distant side to form new tumors [56].

In our study, it was also demonstrated that naringin treatment resulted in down regulation of Vimentin, mesenchymal intermediary filament and Snail1 which is transcription factors that

orchestrates the EMT in a dose–dependent manner. The transduction of primary mammary cancer cells with Snail in mice showed that Snail contributes the tumor recurrence [104]. The inhibition of Snail1 by naringin may prohibit the relapse. Moreover, treatment with this phenolic compound has led to decrease Fibronectin, one of extracellular matrix components, significantly. This data indicated that naringin could be used to inhibit EMT process and eventually gaining and promoting CSC phenotype.

Since naringin treatment result in down regulation of stem cell related markers, it was also suspected whether it might induce the differentiation of these cells. Then, the expression of *Nestin*, marker for progenitor cells and *(α-SMA*), markers expressed in differentiated myoepithelial breast cancer cells. The results demonstrated that treatment caused the suppression of *Nestin* in transcriptional level and induction of the expression of differentiation marker, *α-SMA*. It may be concluded that naringin decrease the stem cell fraction by stimulating differentiation of these stem cells in breast cancer.

It has been believed that tumor invasion and metastasis may be mediated by the CSC population [21, 105]. To determine whether the effect of naringin on EMT was a direct effect on tumor invasion or was due to decrease in CSCs, *in vitro* matrigel assay was used. The results showed that naringin lead to decrease invaded cell ratio in group 0.10, however this effect was not significant. This results may be related to that, Snail1 and other EMT protein expressed in BT474 may not be stable [104] and not allow these cell to have invasive character. Taken into account that Luminal B cell lines are not so invasive, to examine the effect of naringin on invasion more aggressive cell lines, for instance MDA-MB231 must be checked.

In conclusion, the presence of CSCs in breast cancer is the driving factor for tumorigenesis. They are the main reason of recurrence, metastasis and resistance standard therapies. Due to resistance to chemo- radiation therapy, new strategies must be developed to target the locomotive of carcinogenesis. Based on their role in chemoprevention and being less or not toxic compare to chemotherapeutic, natural compounds could be alternative to eradicate this minor and resistant,

slow cycling group. However, the effect of some dietary compounds on stem cell has been demonstrated, the impact of naringin on the stem cell phenotype of cancer has been shown for the first time in this study. Our data showed that naringin, especially at the concentration of 0.10 per cent could decrease the fraction of breast cancer stem cells. Since it is known that this concentration has no cytotoxic effect on differentiated cells, which may acquire cancer stem cell phenotype [106], naringin could be used with other agents to target differentiated cells. This will lead to eradication of both CSCs and tumor bulk and eventually prevent the recurrence of tumor.

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