

# Effects of *Galium aparine* Extract on the Angiogenic Cytokines and ERK1/2 Proteins in Human Breast Cancer Cells

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## Abstract

Tumor angiogenesis is a multiple step process regulated by a range of pro- and anti-angiogenic factors, cytokines and several signaling cascades, including the ERK1/2 MAP kinase pathway. *Galium aparine* (GA) is a perennial plant of the family Rubiaceae, growing widespread in Anatolia. It has been shown that GA methanol (MeOH) extract has anti-proliferative and apoptotic effects on human breast cancer cells. To investigate the underlying mechanisms that might account for the anti-proliferative effect of GA extract, changes in angiogenic cytokines and ERK1 and ERK2 proteins were investigated in human MCF-7 and MDA-MB-231 breast cancer cells. Viability of breast cancer cells after treatment of GA were determined by Cell Proliferation Kit II. Changes in angiogenic cytokines in breast cancer cells was done by a Human Angiogenesis Antibody Array. Protein levels of phospho ERK1 and ERK2 were evaluated by western blot analysis. GA induced cytotoxicity and apoptosis in a time- and concentration-dependent manner in both cancer cells. Dipeptidyl peptidase-4 (DPP-IV), insulin-like growth factor-binding protein 1 (IGFBP-1) and transforming growth factor beta 1 (TGF- $\beta$ 1) levels were significantly decreased; however, secretion of serpin E1 was significantly increased by GA extract in MCF-7 cells. In MDA-MB-231 cells, the levels of amphiregulin (AR), interleukin 8 (IL-8), insulin-like growth factor-binding protein 3 (IGFBP-3), metalloproteinase-8 (MMP-8), heparin-binding EGF-like growth factor (HB-EGF) and platelet-derived growth factor (PDGF) were decreased by GA extract treatment. The secretion of pro-angiogenic cytokines such as neuregulin-1 (NRG1- $\beta$ 1), vascular endothelial growth factor (VEGF) and tissue factor (TF) were significantly decreased by GA treatment in both breast cancer cells. There was also a significant decrease in protein levels of both phospho ERK1 and ERK2 in MCF-7 and MDA-MB-231 breast cancer cells by GA treatment. These data implies that GA contains potential anti-tumor and anti-angiogenic components for breast cancer cells.

**Keywords** — Angiogenic cytokines, breast cancer cells, ERK1, ERK2, *Galium aparine*.

## 1 Introduction

Breast cancer is one of the most common malignancies and the leading cause of cancer deaths in women [1]. Tumor angiogenesis plays a key role in the pathogenesis and progression of breast cancer [2]. It is a multiple step process regulated by a range of pro- and anti-angiogenic factors, cytokines and several signaling cascades, including the ERK1/2 MAP kinase pathway [2,3].

Despite the improvements in the treatment strategies, the outcome has not changed significantly in breast cancer treatment and the identification of novel agents that are active in this setting remains a challenge.

Plants are the important source of novel pharmacologically active compounds and it is reported that 80% of plant derived drugs were related to their original ethnopharmacological purposes [4]. Various phytochemicals derived from

plants have been investigated for their efficacy in the treatment of breast cancer and today many of the plant-derived products are clinically in use. Vinblastine from Madagascar periwinkle, vincristine from *Catharanthus roseus*, paclitaxel and docetaxel from *Taxus baccata* are well known and effective agents in the treatment of breast cancer [5].

The genus *Galium* L., comprised of approximately 1300 species worldwide and is represented by 101 species in Turkey. It has been used as diuretics, choleric, and against fever, hypertension, epilepsy disease, and cancer in the folk medicine [6-9]. *Galium aparine* (GA) is a perennial plant of the Rubiaceae family, growing widespread in Anatolia. A variety of bioactive substances have been identified in GA such as alkaloids, iridoids, anthraquinones, flavanoids, polyphenolic acids and vitamin C [10,11].

We have previously demonstrated the anti-proliferative and apoptotic effects of GA methanol (MeOH) extract on MCF-7 and MDA-MB-231 human breast cancer cells [11]. Based on these findings, to investigate the underlying mechanisms that might account for the anti-proliferative effect of GA MeOH extract, changes in angiogenic cytokines and phospho ERK1 and ERK2 proteins were investigated in human MCF-7 and MDA-MB-231 breast cancer cells.

## 2 Materials and Methods

### 2.1 Cell Lines and Extraction of Plant Material

Breast cancer cells (MCF-7 and MDA-MB-231) were obtained from Interlab Cell Line Collection (Genova, Italy). MCF-7 (HER2-/ER+) and MDA-MB-231 (HER2-/ER-) are two well established human breast cancer cells with different molecular subtypes. They differ in their expression profiles and therefore they are usually preferred as in vitro models for breast cancer research studies. Both cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin in 75 cm<sup>2</sup> polystyrene flasks and maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cell-culture supplies were obtained

from Biological Industries (Kibbutz Beit Haemek, Israel).

The plant material (*Galium aparine* L., voucher specimen no:112) was purchased as a dried powder from a pharmaceutical company (Naturin Natural Products Ltd., Izmir, Turkey). The dried plant was powdered to a homogeneous size in a mill, sieved through a 40-mesh sieve. The powder (10 g) was extracted in 100 mL 99.7% (v/v) MeOH and ultrasonically extracted for 30 min, and then cooled at room temperature. 70% ethanol was added to compensate for the lost weight. The final concentration of extract was 0.1 g/mL and stored at 4 °C. The final dilutions were made immediately before use.

### 2.2 Evaluation of Cell Viability via XTT Assay

Viability of breast cancer cells after GA treatment was determined by Cell Proliferation Kit II (Roche Applied Science, Mannheim, Germany). Cells were seeded at 1×10<sup>4</sup> cells/well, in a final volume of 200 µL, in 96-well plates and incubated for 24 h. Then, cells were exposed to the increasing concentrations of GA (100-800 µg/mL) for 24, 48 and 72 h. At the end of incubation, 100 µL of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) was added to each well, and plates were incubated at 37°C for another 4 h. Absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader (DTX 880 Multimode Reader, Beckman Coulter, Miami, FL). Calculation of cell viability was done using the following formula:

$$\text{Cell viability} = \frac{(A_{450} - A_{650}) \text{ of treated cells} \times 100}{(A_{450} - A_{650}) \text{ of control cells}}$$

The mean of triplicate experiments for each concentration was used to calculate the IC<sub>50</sub> values.

### 2.3 Angiogenic Cytokine Profiling by Antibody Array

To determine the changes in angiogenic cytokines by GA extract treatment in MCF-7 and MDA-MB-231 cells, a Human Angiogenesis Antibody Array

(R&D Systems, Inc., Minneapolis, MN, USA) was used according to the manufacturer's instruction manual. The principle of the method involved a membrane that was coated with specific antibodies for each cytokine, forming an array. The cell lysates from untreated controls and the cells that were exposed to GA extract were collected at 72 h. After blocking the membrane, the lysates were added on the membranes and incubated overnight at 4°C. After washing the membranes with wash buffer for three times, membranes were incubated with biotinylated antibody for 2 h and then incubated with horseradish peroxidase-conjugated streptavidin for another 2 h. Chemiluminescence detection of signals was processed by the Kodak® Gel Logic 1500 imaging system.

Analysis of antibody array results were done as previously reported [13]. Briefly, the spots were quantified by a computer assisted system for image analysis (Kodarray 2.6 software); normalized intensities were calculated from each array by first subtracting the local background from each spot and then normalizing by the average intensity of the arrays. The data were then corrected for the cell protein content of each well. The relative expression level of each protein was calculated according to both spot pixel mean  $\pm$  standard deviation (SD) by the Kodarray.

#### 2.4 Western Blot Analysis

The pellets from untreated controls and treated cells were lysed by using MPER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instruction manual. After centrifugation at  $14,000 \times g$  for 15 min, protein concentrations were quantitated by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated on an SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat dry milk prepared in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 1 h. The membrane was then incubated with primary antibody (1:1000 dilutions, dually-phosphorylated

form of MAP ERK-1 and ERK-2, 44 kDa and 42 kDa, respectively, Abcam Ltd., UK) at 4 °C overnight. Following several washes in TBST, membranes were incubated with appropriate secondary antibodies (1:1000 dilutions, Millipore Upstate USA, Charlottesville, VA) at room temperature for 1 h. The protein bands recognized by the antibodies were visualized by the Kodak Gel Logic 1500 Imaging System.

#### 2.5 Statistical Analysis

Graphpad Prism 5.0 software (La Jolla, CA, USA) was used for data analysis and graphs. Experiments were carried out in triplicate and results were presented as the means  $\pm$  S.D. The difference between two groups was analyzed by a two-tailed Student's test, and that between three or more groups was analyzed using one way analysis of variance test (ANOVA) followed by Dunnett's t-test. In these analyses,  $p < 0.05$  (\*) were considered to be statistically significant.

#### 3 Results

To verify the inhibitory effect of GA extract on the viability of breast cancer cells and determine the 50% inhibitory concentration (IC<sub>50</sub>) values for further experiments, XTT analysis was done. GA extract decreased the cell viability of both breast cancer cells in a time- and concentration-dependent manner (Figure 1 and 2). There were 20, 40, and 75% decrease in cell viability of MCF-7 cells exposed to 300, 500, and 800  $\mu\text{g/mL}$  of GA extract, respectively, when compared to untreated controls at 72 h (Figure 1C). In MDA-MB-231 cells, there were 30, 43, and 62% decrease in cell viability by 300, 500, and 800  $\mu\text{g/mL}$  of GA extract treatment, respectively, when compared to untreated controls at 72 h (Figure 1F). The highest cytotoxicity was observed at 72 h and the IC<sub>50</sub> values of GA were found to be  $475 \pm 2.05 \mu\text{g/mL}$  for MCF-7 and  $498 \pm 3.04 \mu\text{g/mL}$  for MDA-MB-453 cells, at 72 h.

Changes in angiogenesis-related cytokine expressions were studied by a human angiogenesis array in MCF-7 and MDA-MB-231 cells. It was found that GA extract treatment resulted in different angiogenic cytokine secretion changes in

MCF-7 (475 µg/mL) and MDA-MB-231 (498 µg/mL) cells.

Figure 1

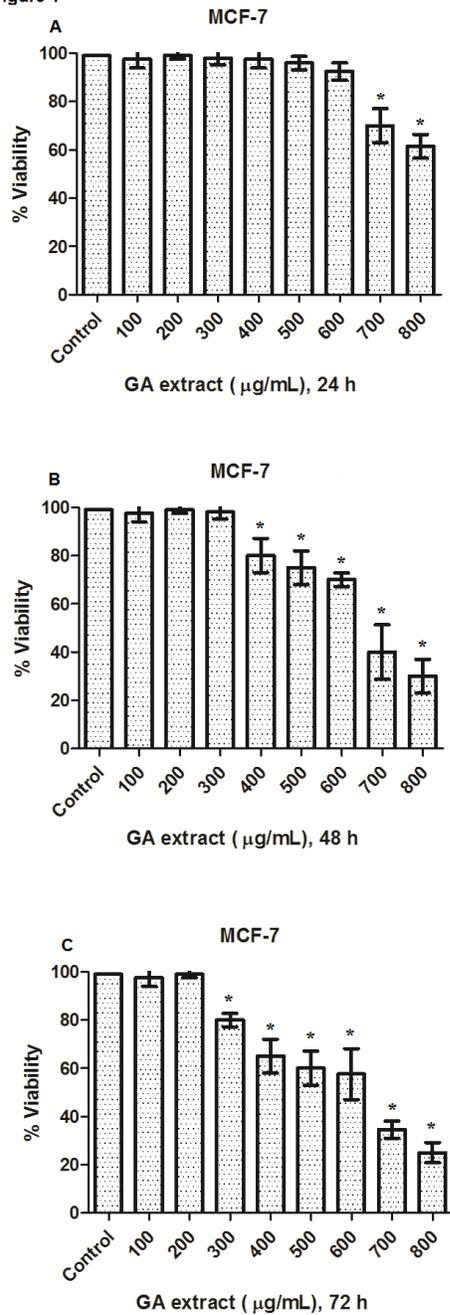


Figure 1 (A-C): Time and concentration dependent inhibition of viability by GA MeOH extract treatment in MCF-7 cells.

Figure 2

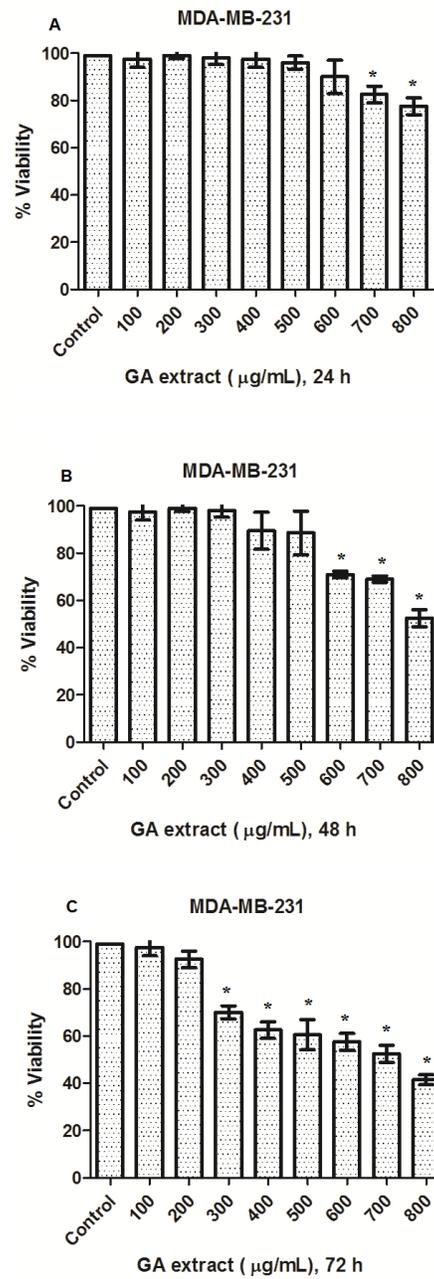


Figure 2 (A-C): Time and concentration dependent inhibition of viability by GA MeOH extract treatment in MDA-MB-231 cells.

Table 1 shows the fold changes in angiogenic cytokine secretion of MCF-7 and MDA-MB-231 breast cancer cells after GA treatment. The results from repeated experiments showed that the secretion of pro-angiogenic cytokines such as neuregulin-1 (NRG1-β1), vascular endothelial growth factor (VEGF) and tissue factor (TF) were significantly decreased by GA treatment in both breast cancer cells (p<0.05). Moreover, there was a 2.8-, 2.4- and 3.2-fold decrease in dipeptidyl peptidase-4 (DPPIV), insulin-like growth factor-binding protein 1 (IBP-1) and transforming growth factor beta 1 (TGF-β1) levels in MCF-7 cells, respectively (p<0.05). However, secretion of serpin E1, an anti-angiogenic cytokine, was increased by 4.4-fold in MCF-7 cells by GA extract (p<0.05).

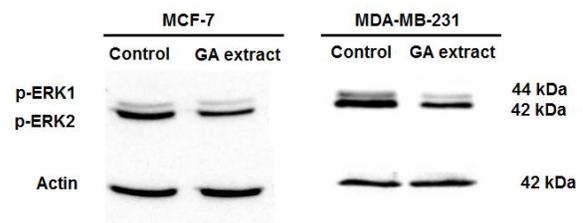
**Table 1:** Fold in the secretion of angiogenic cytokines by GA extract in breast cancer cells (p<0.05) (ns: non-significant as compared to untreated controls).

| Abbreviation | Cytokine name                                | Fold change |            |
|--------------|--|-------------|------------|
|              |  | MCF-7       | MDA-MB-231 |
| VEGF         | Vascular endothelial growth factor           | -3.6 ± 0.8  | -4.0 ± 0.8 |
| NRG1-β1      | Neuregulin 1                                 | -2.1 ± 1.0  | -2.5 ± 0.4 |
| TF           | Tissue factor                                | -3.6 ± 0.6  | -2.2 ± 1.4 |
| DPPIV        | Dipeptidyl peptidase-4                       | -2.8 ± 0.8  | ns         |
| IBP-1        | Insulin-like growth factor-binding protein 1 | -2.4 ± 1.4  | ns         |
| TGF-β1       | Transforming growth factor beta 1            | -3.2 ± 0.8  | ns         |
| Serpin E1    | Plasminogen activator inhibitor              | +4.4 ± 0.8  | ns         |
| AR           | Amphiregulin                                 | ns          | -2.6 ± 1.2 |
| IL-8         | Interleukin 8                                | ns          | -2.8 ± 0.6 |
| IBP-3        | Insulin-like growth factor-binding protein 3 | ns          | -2.7 ± 1.2 |
| MMP-8        | Metalloproteinase-8                          | ns          | -3.1 ± 1.4 |
| HB-EGF       | Heparin-binding EGF-like growth factor       | ns          | -3.4 ± 2.0 |
| PDGF         | Platelet-derived growth factor               | ns          | -3.4 ± 0.4 |

In MDA-MB-231 cells, there was a 2.6-, 2.8-, 2.7-, 3.1-, 3.4- and 3.4-fold decrease in the levels of amphiregulin (AR), interleukin 8 (IL-8), insulin-like growth factor-binding protein 3 (IBP-3) metalloproteinase-8 (MMP-8), heparin-binding EGF-like growth factor (HB-EGF) and platelet-

derived growth factor (PDGF) by GA extract treatment, respectively (p<0.05).

To investigate the possible effect of GA extract treatment on ERK signaling, we examined the expressions of active, dually-phosphorylated form of ERK-1 and ERK-2 protein levels by western blot analysis. There was a significant decrease in protein levels of both proteins in MCF-7 and MDA-MB-231 cells exposed to IC50 values of GA extract for 72 h (Figure 3).



**Figure 3:** The expression levels of phosphor ERK-1 and ERK-2 by GA extract treatment in MCF-7 and MDA-MB-231 cells.

#### 4 Discussion

Most of the plant-derived compounds inhibit cell viability via targeting multiple cellular signaling pathways including growth factors, cytokines, protein kinases, and angiogenesis which are frequently deregulated in cancer cells. Angiogenesis is one of the key processes in breast cancer development and metastasis which is modulated by a range of pro- and anti-angiogenic factors [2,3]. In addition to their effects on angiogenesis, the levels of these factors have been correlated with tumor stage, survival, and malignancy, rendering them potential diagnostic markers and therapeutic targets [13].

Galium specieses are traditionally used in Europe and Northern America for treatment of cancerous ulcers or breast cancer [9]. Several studies have reported the anti-proliferative, antioxidant and apoptotic effects of Galium specieses in vitro. However, limited knowledge is available concerning its effect on angiogenic cytokines and related pathways. The effect of Galium verum

aqueous extract (33.3 µl/mL) on extracellular matrix substrate invasion was investigated in drug sensitive and resistant laryngeal carcinoma cell lines and it was revealed that it has remarkable anti-invasive properties in these cell lines [14].

Previously, we have demonstrated that MeOH extract of Galium aparine was cytotoxic in both breast cancer cell lines impairing normal breast epithelial cells and induced both apoptotic and non-apoptotic cell death in breast cancer cells. To investigate the underlying mechanisms that might account for the anti-proliferative effect of GA extract, changes in angiogenic cytokines by GA treatment were investigated in MCF-7 and MDA-MB-231 human breast cancer cells. This is the first study investigating the effect of GA extract on angiogenic cytokines in breast cancer cells. There were differences among the breast cancer cell lines in regards to the levels of expression of angiogenic cytokines by GA treatment. It could be explained by the genetic differences between human MCF-7 and MDA-MB-231 breast cancer cells.

DPPIV is one of the significantly decreased pro-angiogenic cytokine by GA extract in MCF-7 cells. It is ubiquitously expressed peptidase between different cancers and interacts with the components of ECM, thereby involving in the control of malignant transformation, cell proliferation, migration and invasion [15, 16]. Serum DPPIV levels were found to be higher in breast cancer patients as compared to the healthy control group indicating its role in breast tumor development [17].

IBP-1 is another angiogenic cytokine decreased by GA extract treatment in MCF-7 cells. It is a member of insulin-like growth factor binding proteins (IGFBPs). IBP-1 binds with high affinity to insulin-like growth factor type 1 and 2 which are important mitogens for transformed cells and tissues, including breast cancer [18].

Among pro-angiogenic cytokines, TGF-β1 was significantly decreased in MCF-7 cells by GA extract treatment. TGF-β1, a multifunctional growth factor, functions as a potent inducer of angiogenesis and stimulator of vascular maturation

[19]. High levels of TGF-β1 in breast cancer patients found to be associated with worse survival, and plasma TGF-β1 levels might be predictive of distant metastasis [20].

Serpin E1 is a serine protease inhibitor which is involved in mammary gland development. It is shown to regulate cell adhesion, motility, apoptosis, and angiogenesis. The inhibitory action of serpin E1 induces tumor progression by preventing excessive degradation of the ECM consequently promoting tumor angiogenesis [21]. GA extract treatment significantly increased serpin E1 levels in MCF-7 cells.

Several growth stimulatory factors such as EGF, TGF, IGF and PDGF have important roles in the growth of breast cancer. PDGF-AB is a homo/heterodimeric growth factor composed of an A and/or a B chain, was significantly reduced in MDA-MB-231 cells. It stimulates proliferation, migration and angiogenesis in cancer cells [22, 23]. Over expression of PDGF is accepted as a negative survival marker for many types of human cancers [24].

HB-EGF is the other growth factor belonging to the EGF family, significantly reduced by the extract treatment in MDA-MB-231 cells. HB-EGF plays important roles in tumorigenesis, metastasis, and drug resistance in breast cancer and thus accepted as a therapeutic target for breast cancer treatment [25].

IGFBP-3 is the most abundant binding protein among the IGFBP family proteins has critical role in cell growth, apoptosis, migration, and attachment [26, 27]. Many studies revealed that more aggressive ER- cells secrete higher levels of IGFBP-3 as compared to ER+ cells [28]. IGFBP-3 levels were significantly reduced in ER- MDA-MB-231 cell line by the GA extract treatment.

MMP-8 is a protease that cleaves fibrillar collagens, extracellular matrix proteins, as well as other proteases, cell adhesion proteins, protease inhibitors, growth factors, and chemokines [29]. MMP-8 proteolytically activates IL-8 and, thereby, regulates neutrophil chemotaxis in vivo [30]. IL-8 is a pro-inflammatory cytokine, belongs to the CXC

chemokine family and potently induces migration, adhesion, and invasion IL-8 by activating G protein-coupled receptors (CXCR1 and CXCR2) in multiple intracellular signaling pathways [31, 32]. Upregulated IL-8 levels have been detected in several types of human cancer cells, including metastatic breast cancer [33]. GA extract treatment significantly reduced both MMP-8 and IL-8 levels in MDA-MB-231 cells.

There are some pro-angiogenic cytokines which were significantly reduced by GA extract in both MCF-7 and MDA-MB-231 breast cancer cells such as NRG1- $\beta$ 1, TF and VEGF. NRG1- $\beta$ 1 is a ligand for the HER3 receptor which has no tyrosine kinase activity. NRG1 binding activates HER3 receptor heterodimerisation and induces expression of proteins that are involved in invasion and metastasis of breast cancer cells [34-36]. TF has shown to be involved in intracellular signaling pathways regulating cell proliferation, migration, gene expression and mature microvessel formation. Elevated TF levels were found to be associated with increased angiogenesis and accelerated tumor growth [37]. The pro-angiogenic signaling molecule VEGF is a key player in the process of tumor angiogenesis [38]. VEGF is expressed by breast tumors and content of VEGF in tumor cells correlates with the prognosis of patients. Moreover, the presence of VEGF receptors on both endothelial and tumor cells indicates that VEGF is of vital importance in autocrine-dependent tumor cell proliferation and invasion [39]. Therefore it has been an important focus for anti-cancer drug development.

Besides the modulation of pro- and anti-angiogenic factors, activation of the ERK1/2 signaling pathway is required for angiogenesis in endothelial cells. Thus, this pathway was considered as the target for anti-angiogenic agents. GA extract treatment significantly decreased both the levels of active p-ERK1 and 2 protein levels in both MCF-7 and MDA-MB-231 cells.

A hallmark of tumor cells is metastasis characterized by increased cell migration, tissue invasion, and organ colonization. Current data suggest that tumor metastasis is regulated by

soluble factors secreted from the tumor cells [40]. Thus, in this study, changes in various angiogenic cytokines investigated by antibody array after GA treatment in breast cancer cells and obtained results suggest that GA extract might exert its anti-proliferative effect by modulating various angiogenesis-related cytokines and ERK1/2 pathway. The development of drugs that target angiogenic cytokines simultaneously could result in efficacious and selective killing of breast cancer cells.

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