



Acacetin Inhibits Cell Proliferation, Survival, and Migration in Human Breast Cancer Cells

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Received: 4 Oct 2018 / Accepted: 12 Nov 2018 / Published online: 1 Jan 2019

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Abstract

Acacetin is an O-methylated flavone which is extracted from various edible and medicinal plants such as *Turnera diffusa* (Damiana), *Robinia pseudoacacia* (black locust) and honey. Acacetin has been studied to possess anti-malarial, anti-inflammatory and anti-tumor efficacy. Although, its potential as an anti-tumor agent against breast cancer has not been explored in detail. In this present study, we have studied the effect of acacetin on breast cancer cells MCF-7 and MDA-MB-468. Following treatment with acacetin, the viability of breast cancer cells was measured by MTT assay which showed 41-76% decrease. The strong growth inhibitory effect of acacetin was confirmed by trypan blue assay. The inhibitory effects of acacetin were observed on migratory potential of breast cancer cells using wound-healing assay, and western blotting showed downregulation of vimentin and N-cadherin. Cell cycle arrest was also observed using flow cytometric analysis. Acacetin inhibited ERK1/2 and AKT signaling and modulated the expression of cell cycle regulators. Overall, the results suggested that acacetin strongly decreases the survival and growth of breast cancer cells and causes cell cycle arrest along with inhibiting the migratory potential and EMT in breast cancer cells.

Keywords

Acacetin, breast cancer, chemoprevention, EMT, cell cycle arrest, migration.

1. INTRODUCTION

Breast cancer is still the most diagnosed form of malignancy in women worldwide. According to GLOBOCAN 2018, there will be 2.1 million new breast cancer cases diagnosed in women, and this corresponds to approximately 25% of all cancers diagnosed in females. The prevalence of breast cancer in Asian countries has also surged rapidly in the past few years [1]. Occurrence of breast cancer has been associated with various factors such as diet, obesity, hereditary factors, and level of estrogen

hormone. These factors, in turn, contribute to the development of multiple mutations, cell cycle aberrations, loss or gain of apoptosis and survival related markers and alterations in various signaling molecules [2]. Understanding of biology of cancer, technological advances, and development of tools and techniques have helped immensely in the diagnosis and therapeutics of breast cancer although traditional methods such as surgery, chemotherapy, and radiation have proved very beneficial in treating various cancers. However, it comes with severe side

effects that have a massive impact on the quality of life of a patient. Also, most of the anti-tumor drugs can inhibit the cancer growth in the initial stages but after cancer has metastasized very few anticancer agents are proved to be effective. Several studies have demonstrated that metastasis is accountable for approximately 90% of cancer deaths [3]. Therefore, it is necessary to find novel natural compounds with low toxicity towards normal cells and strong selectivity against cancer cells in initial as well as advanced cancer stages.

Natural products such as flavonoids have garnered a lot of attention in the past few years for their potent and selective anticancer efficacy against various types of cancers and also due to their relative non-toxicity towards normal cells [4]. Studies have concluded that the long-term consumption of a diet rich in flavonoids has an inverse relationship with the development of various cancers [5]. Acacetin (Fig. 1A) is a bioflavonoid found in several dietary items and plants including *Robinia pseudoacacia*, *Turnera diffusa*, and *Betula pendula*. Acacetin is known to possess anti-inflammatory, antiplasmodial, anti-angiogenic, anti-oxidant, and anti-cancer properties [6-8]. Several studies with acacetin have shown that it can modulate various signaling pathways to inhibit cancer growth [7,9,10]. It also has anti-angiogenic and anti-migratory properties which indicate that it can also inhibit the EMT, angiogenesis and metastatic cascade in cancer cells [6,11]. Although acacetin is not explored on breast cancer much and also it is not much known about its anti-cancer and anti-migratory potential against breast cancer cells. Herein, we studied the effect of acacetin on MCF-7 and MDA-MB-468 breast cancer cells. We investigated the anti-proliferative and anti-migratory activity of acacetin along with its cell death-inducing potential on human breast cancer cells.

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

Acacetin, MTT, Trypan blue, sodium phosphate dibasic (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), sodium chloride, acetic acid, ethylenediaminetetraacetic acid (EDTA), hydrochloric acid, sodium hydroxide, Trizma Base, calcium chloride, sodium dodecyl sulphate (SDS), and β -mercaptoethanol (BME), DMSO (molecular grade) was purchased from Merck (Darmstadt, Germany). PVDF membrane was bought from Amersham (USA) and Millipore (USA). Triton-X 100 and NP-40 were procured from Merck (Darmstadt, Germany). RPMI 1640 and DMEM high glucose cell culture medium, fetal bovine serum (FBS), and antibiotic/antimycotic

solution (100X) were from Gibco Life Technologies, Invitrogen (Grand Island, NY).

2.2 Cell Culture and Treatment

MCF-7 and MDA-MB-468 (human breast cancer cells) were acquired from ATCC (Manassas, VA). MCF-7 cells were grown in RPMI-1640 medium, and MDA-MB-468 cells were maintained in DMEM high glucose medium. Media for both the cell lines were supplemented with 10% FBS and 1% PSA (penicillin, streptomycin and amphotericin B) solution. The cells were cultured as an adherent monolayer at 37°C in humidified 5% CO_2 incubator and regularly passaged when the confluence reached ~70-80%. A stock of 100 mM acacetin was prepared in DMSO and 20-80 μM final concentrations in culture medium were used to treat cells. DMSO was not more than 0.1% v/v in every treatment.

2.3 Cell Viability Assay

Approximately 8×10^3 cells/well were plated in a 96-well plate in triplicates and cultured for 24 h. The cells were then subjected to acacetin treatment (20-80 μM) for 24 h and 48 h. After each treatment time was over media was aspirated and 50 μl of MTT (5 mg/ml) per well was added and kept in a CO_2 incubator at 37°C for 4 h. After incubation period was over MTT solution was aspirated out and followed by gentle washing with 1X PBS. 100 μl of DMSO was added and incubated for 5 minutes to dissolve the formazan crystals that yielded purple color. Optical density was read at 570 nm using a BioTek microplate reader (USA) [4].

2.4 Trypan Blue Assay for Cell Growth and Death

MCF-7 and MDA-MB-468 cells were grown in 24-well plates (3×10^4 cells/well), and at ~70% confluency was treated with acacetin (20-80 μM) for 24 and 48 h as previously reported [8]. After the treatment time was over cells were trypsinized and subjected to centrifugation for 5 minutes at 1500 rpm. Cells were resuspended in 1X ice-cold PBS and stained with trypan blue dye. Blue stained dead cells and unstained live cells were counted on Neubauer hemocytometer.

2.5 Cell Cycle Analysis by Flow Cytometry

1×10^5 cells/well were seeded in a 6-well plate and treated as mentioned above. After each treatment, cells were trypsinized and processed as reported earlier [12]. Cells were resuspended in 500 μl of saponin/PI solution containing 25 mg/ml PI (w/v), 0.3% saponin (w/v) with 0.1 mM EDTA and 10 mg/ml RNase A (w/v) in PBS. The cells were then incubated overnight at 4°C in dark. DNA content analysis was done using a flow cytometer (BD Biosciences, USA).

2.6 JC-1 Assay for Mitochondrial Membrane Variation

The effect of acacetin treatment on mitochondrial membrane potential was explored using the fluorescent dye JC-1 as described previously [4]. Approximately 5×10^4 cells/well were plated in a 12-well plate in triplicates. Briefly, after 24 h of acacetin treatment (0-80 μ M) cells were harvested as in trypan blue assay and 2 μ l JC-1 dye (5 mg/ml) was added to each sample and kept in an incubator for 15 minutes in the dark at 37°C. This was followed by washing cells thrice with ice cold 1X PBS. Further, cells were immediately analyzed using flow cytometry.

2.7 Wound Healing Assay

MDA-MB-468 cells were cultured till confluent monolayer was achieved in a 12-well plate and serum starved for 24 h to inhibit proliferation. A scratch was created with a sterile 200 μ l pipette tip. Three washes of 1X PBS to remove the detached cells was followed by treatment with acacetin. Images were taken with a digital camera attached to an inverted microscope (Carl Zeiss, Germany). The wound gap widths were measured using Microsoft powerpoint software.

2.8 Lysate Preparation and Western Blotting

After the treatment time was over, total cellular protein was extracted using non-denaturing lysis buffer as reported earlier and quantified using Bradford reagent [13]. Next, 30-55 μ g of the protein samples were mixed in 5X reducing sample buffer and kept in boiling water for 8 minutes. The samples were then loaded and separated on SDS-PAGE gels and transferred to PVDF membrane using electroblotting. The membranes were blocked in 5% skimmed milk/0.05% Tween/PBS solution for 1 hour at room temperature, followed by overnight incubation with the specific primary antibodies. Appropriate HRP labeled secondary antibodies were used and protein bands were visualized by dipping the membrane in a chemiluminescence substrate followed by exposure to X-ray films.

2.9 Statistical analysis

Statistical analysis of the data was done by GraphPad Prism version 6 software. Experiments were repeated to ensure reproducibility. Student's t-test was done to evaluate the statistical significance between groups. Data was considered significant when the P value was less than 0.05.

3. RESULTS

3.1 Acacetin Inhibits Proliferation and Viability Human Breast Cancer Cells

The cytotoxic efficacy of acacetin treatment on MCF-7 and MDA-MB-468 cells was studied by MTT assay (Figure 1A & 1B) for 24 and 48 hours. The data indicated that with increasing concentrations of acacetin (20, 40 and 80 μ M), viability of cells decreased in a time- and dose-dependent manner as compared with the control group. The percentage inhibition with acacetin (20-80 μ M) treatment in MCF-7 cells were 7-71% at 24 h and 24-76% at 48 h ($P < 0.05-0.001$) (Fig. 1B) and in MDA-MB-468 cells were 7-32% at 24 h and 21-41% at 48 h ($P < 0.05-0.001$) (Fig. 1C). These results indicate that acacetin significantly inhibits the survival of human breast cancer cells.

3.2 Acacetin Reduces Cell Growth of Breast Cancer Cells

Growth inhibitory effect of acacetin was further analyzed using trypan blue assay. Acacetin treatment inhibited the live and total cells and increased the percentage of dead cells in both the cell lines in a dose- and time-dependent manner. Cells treated with acacetin (20-80 μ M) for 48 h reduced total cell number by 32-67% ($P < 0.05-0.001$) in MCF-7 cells (Fig. 2A) and by 10-22% ($P < 0.05-0.001$) in MDA-MB-468 cells (Fig 2C). Further acacetin treatment also elevated the dead cells by 1.5 to 3-fold in both the cell lines (Fig. 2A and 2C). Acacetin also caused a substantial reduction in cell growth and increased dead cells in a time-dependent manner (12-72 h) at 40 μ M dose in MCF-7 cells (Fig. 2B). These observations suggest that 20-80 μ M doses of acacetin is potent in inhibiting cell proliferation and inducing cell death in breast cancer cells.

3.3 Acacetin Inhibits Cell Cycle Progression in Human Breast Cancer Cells

To gain insight into the growth inhibiting effect of acacetin on breast cancer cells and to explore the mechanisms involved, we analyzed the influence of acacetin on cell cycle progression. The data obtained using flow cytometry demonstrated that the percentage of cells arrested by acacetin treatment in various phases at different time points are different. Cells were arrested in sub-G1 and G1 phase at 24 h and S & G2/M phase in 48 h (Fig. 3A). This data is in correlation with the previous study published in which acacetin was shown to increase sub-G1 population at 24 h in MCF-7 cells [10].

3.4 Acacetin Disrupts Mitochondrial Membrane Potential in MCF-7 Breast Cancer Cells

JC-1 assay was done on MCF-7 cells to analyze the efficacy of acacetin to induce mitochondrial membrane potential disruption in breast cancer cells. The data suggested that acacetin was able to

disrupt the mitochondrial membrane potential in MCF-7 cells at 24 h time point (Fig. 3B). As mitochondrial membrane disruption is an early sign of mitochondria-mediated apoptosis, the data suggested that acacetin may cause mitochondrial-mediated apoptosis in breast cancer cells.

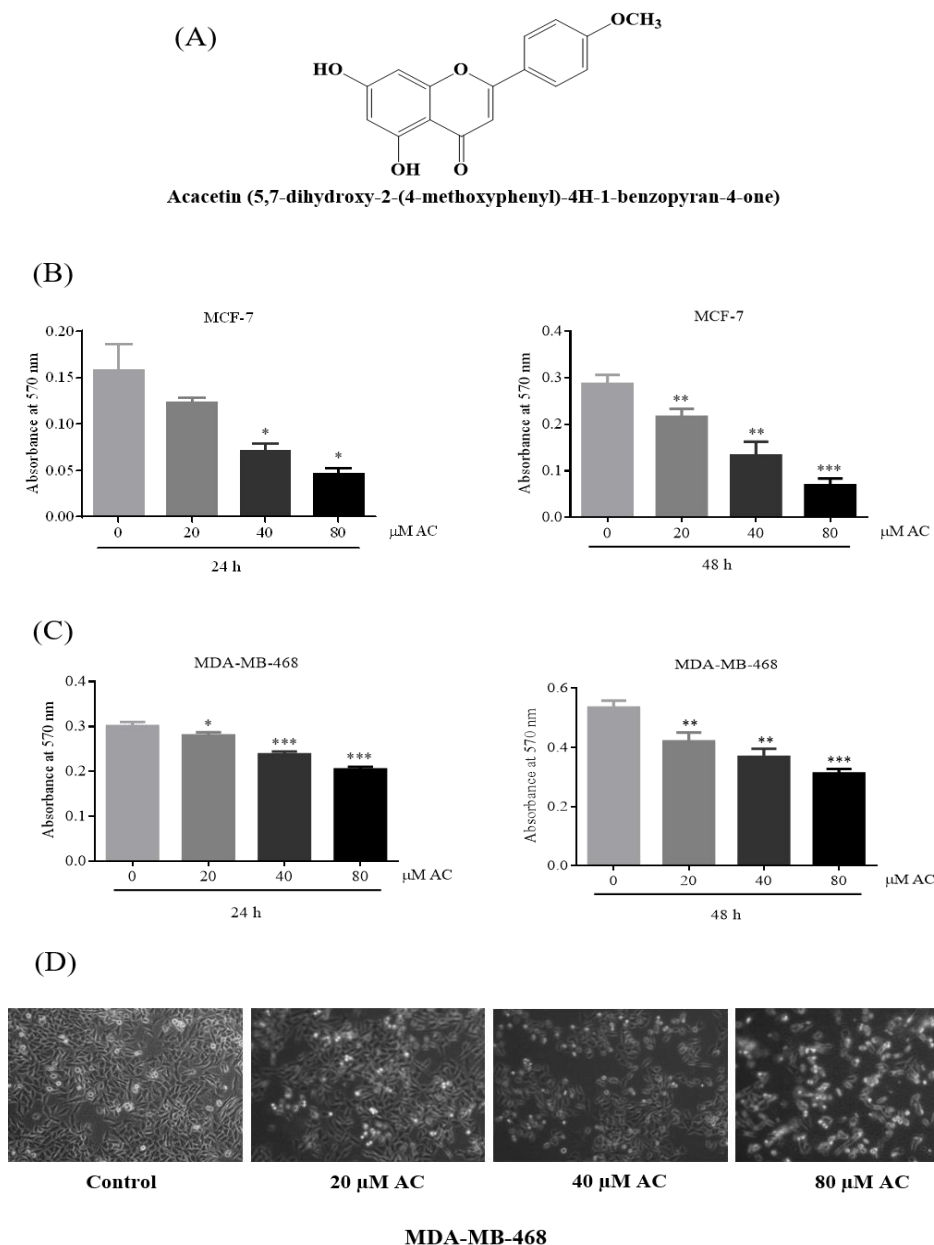
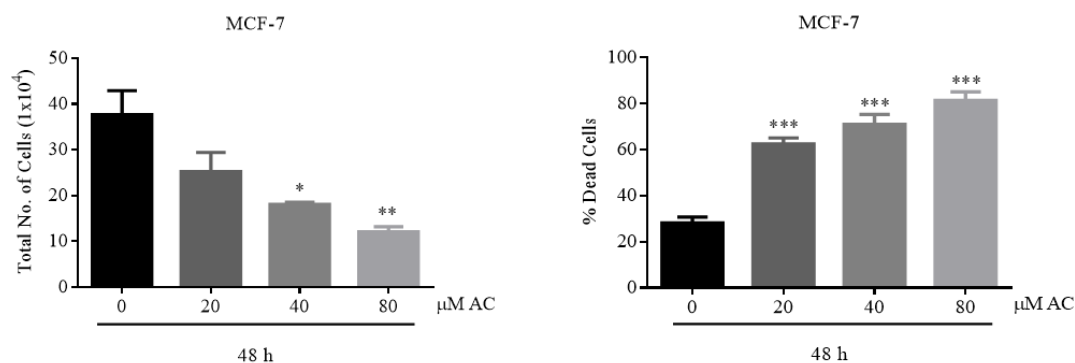
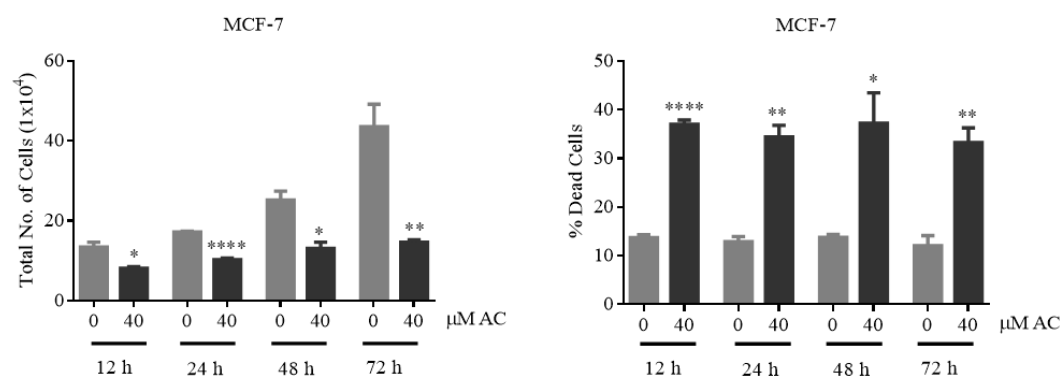


Figure 1. Acacetin suppresses human breast cancer cell viability: Breast cancer cells MCF-7 & MDA-MB-468 were treated with DMSO or different concentrations of Acacetin (20–80 μM) for 24 & 48 h. At the end of each treatment time, cell proliferation and toxicity were analyzed using MTT analysis. (A) Structure of Acacetin. (B) MTT assay on MCF-7 cells and (C) MTT assay on MDA-MB-468 cells. Columns are representative of three independent experiments. Data points are the means ± S.E.M. of three experiments. $P < 0.001$ (***), $P < 0.01$ (**), $P < 0.05$ (*). The P-value is determined by comparing each treatment with the control group.

(A)



(B)



(C)

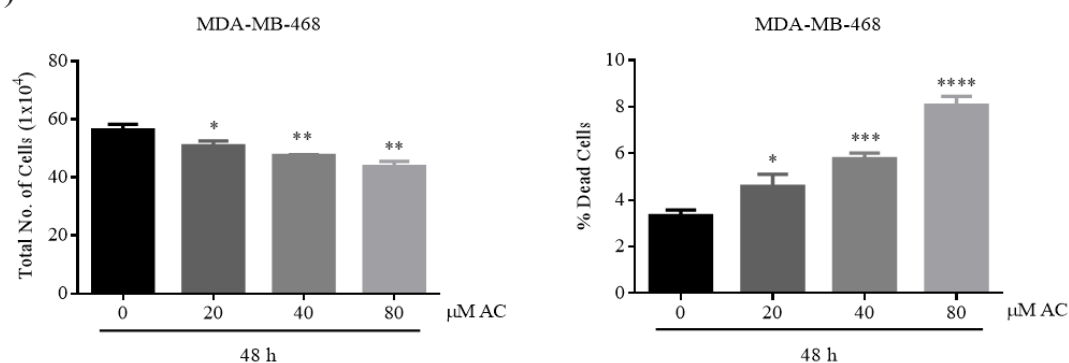


Figure 2. Effect of acacetin on cell proliferation and death in human breast cancer cells: Breast cancer cells MCF-7 & MDA-MB-468 cells were treated with DMSO or different concentrations of Acacetin (20–80 μ M) for 48 h. At the end of each treatment time, cells were collected and processed for determination of total cell number and dead cells as mentioned in Materials and Methods. (A) A total number of cells and dead cells of MCF-7. (B) Total number of cells and dead cells of MDA-MB-468. Columns are representative of three independent experiments. Data points are the means $\pm$ S.E.M. of three experiments. $P < 0.001$ (***), $P < 0.01$ (**), $P < 0.05$ (*). The P-value is determined by comparing each treatment with the control group.

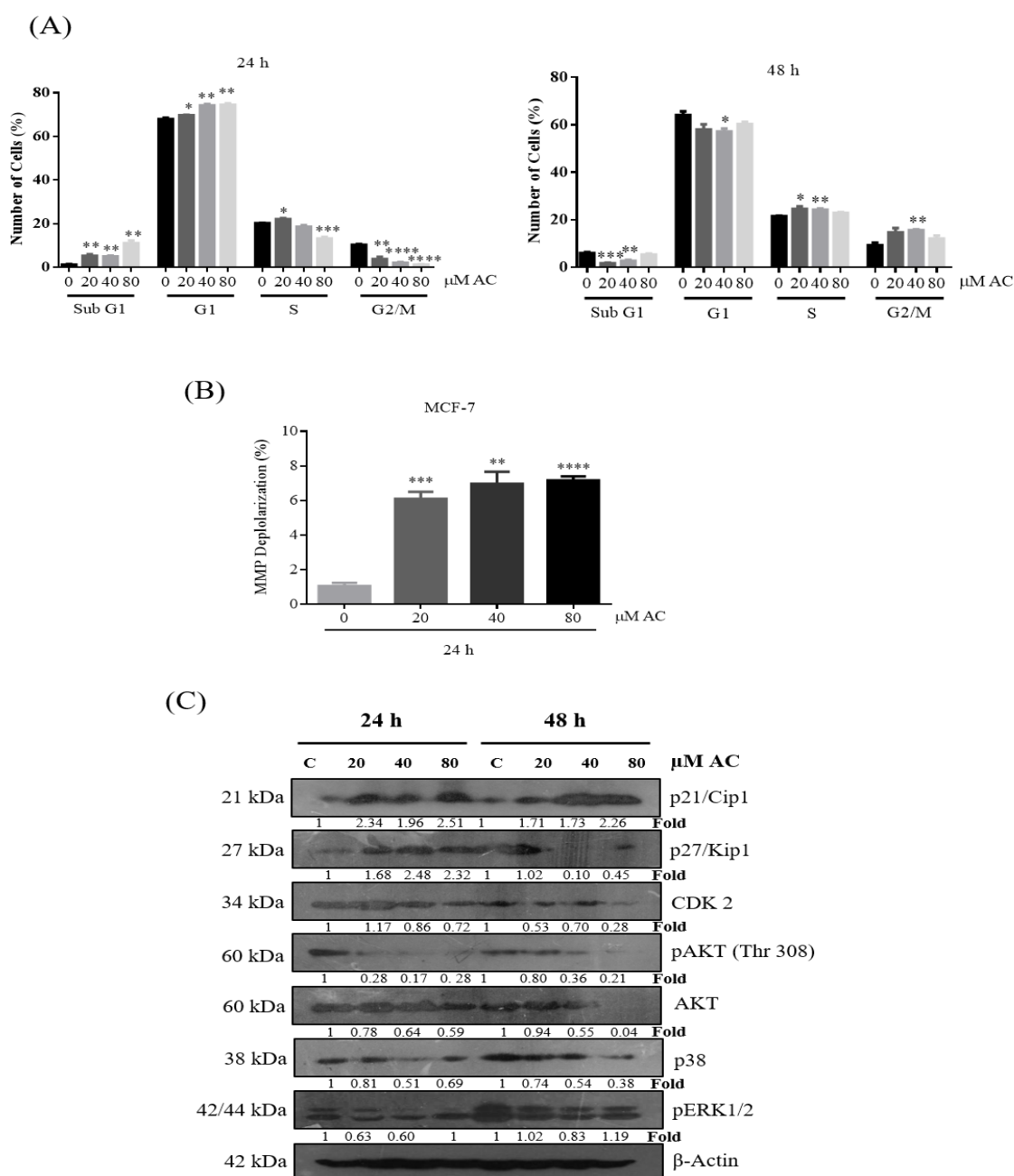


Figure 3. Effect of acacetin on cell cycle progression and survival of human breast cancer cells: The cells were treated with DMSO or 20-80 μ M of acacetin for 24 and 48 h. After indicated treatment times, cells were collected, washed with PBS, digested with RNase A, and then cellular DNA stained with propidium iodide. PI-stained cells were analyzed for cell cycle phase distribution as detailed in materials and methods. (A) Cell cycle distribution in MCF-7 cell line in presence of different treatment groups. (B) JC-1 assay was carried out to investigate the role of mitochondria in acacetin induced apoptosis in breast cancer cells. MCF-7 cells were treated with indicated doses of acacetin for 24 h and analyzed using flow cytometry. (C) Western blotting was done for cell survival (ERK1/2, p38, and AKT) and cell cycle markers (p21, p27 and CDK2) in MDA-MB-468 cells. Membranes were stripped and reprobed with anti β -Actin antibody to ensure equal protein loading. Numbers below the bands represent fold changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for β -Actin loading control.

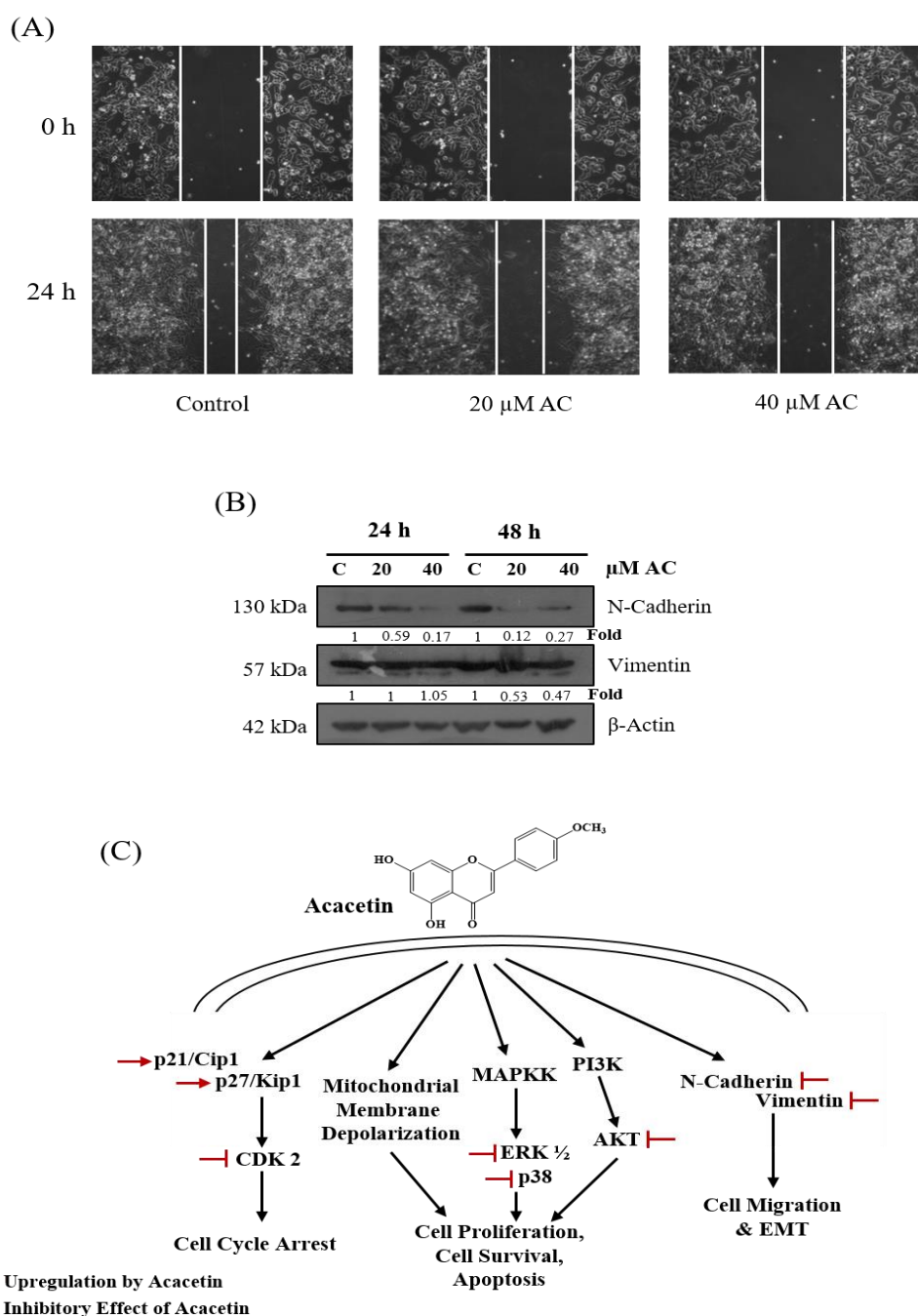


Figure 4. Effect of acacetin on cell migration and EMT in MDA-MB-468 breast cancer cells: The cells were treated with DMSO or 20-40 μ M of acacetin for 48 h. After treatment time, wounds were created using a pipette tip and were photographed at 0 and 24 h time points as described in materials and methods. (A) Representative figures for wound healing assay in MDA-MB-468 cells. (B) Immunoblotting was performed for the expression level of N-cadherin and vimentin as described in materials and methods. The membrane was stripped and re-probed with anti- β -Actin for loading control. Densitometric data shown below each band are fold-change of the protein levels from control and normalized to β -actin. (C) Proposed mechanism of acacetin induced cell cycle arrest, cell death and inhibition of EMT in human breast cancer cells.

3.5 Acacetin Modulates Cell Cycle and Cell Survival Molecules

Modulatory effect of acacetin on the expression levels of cell cycle related markers and cell survival molecules was assessed using western blotting. Whole-cell lysates of MDA-MB-468 cells were extracted at 24 and 48 h and subjected to immunoblotting. It was observed that acacetin significantly increased the expression levels of cyclin-dependent kinase inhibitors (CDKI) such as p21 and p27 in a dose-dependent manner, although the expression of p27 decreased in acacetin treated groups at 48 h time point. The decrease in the expression of p27 at higher doses and time point may be because Cip1 and Kip1 are upregulated at several transcriptional and post-transcriptional events. These proteins are said to have very short half-life, therefore it may be a likely reason for the decrease in p27 level at higher time points [14]. Acacetin treatment also decreased the expression levels of phosphorylated and total forms of ERK1/2 and AKT, and a decrease in the expression level of p38 was also seen at both time points suggesting that acacetin could inhibit the cell proliferation and survival *via* downregulating ERK/AKT signaling pathways (Fig. 3C).

3.6 Acacetin Inhibits Cell Migration and EMT in Breast Cancer Cells

Effect of acacetin treatment on the migratory abilities and the epithelial-mesenchymal transition was assessed using wound healing assay and western blotting. Acacetin (20 & 40 μ M) decreased the migratory potential of MDA-MB-468 cells which was evident in wound healing assay as the width of the wounds in treated groups was more in comparison to control at 24 h time point (Fig. 4A). Treatment of acacetin for 48 h on breast cancer cells was also able to significantly downregulate the expression levels of classical mesenchymal markers such as N-cadherin and vimentin (Fig. 4B). Several studies have shown that N-cadherin and vimentin play a vital role in the epithelial to mesenchymal transition of cells that are responsible for metastasis. This data suggests that acacetin is effective in inhibiting migration and EMT in human breast cancer cells.

4. DISCUSSION

Chemoprevention may be defined as the use of natural, synthetic or biological agents for the inhibition, delay, or reversal of carcinogenesis or cancer development. In recent years, several studies have shifted their focus on the use of natural dietary phytochemicals for cancer chemoprevention as they have proven to be a safe and effective option than

the synthetic chemotherapeutic agents that are often accompanied by toxic side effects [15-17]. The purpose of this study was to explore the antitumor efficacy of acacetin against breast cancer. MCF-7 representing ER+ & PR+ breast cancer and MDA-MB-468 representing triple-negative breast cancer (TNBC) was used in this study.

We were able to illustrate that acacetin effectively inhibits cell proliferation in breast cancer cells. The inhibitory effect of acacetin was observed more on MCF-7 cells in comparison to MDA-MB-468 cells as was seen in MTT assay and this was further confirmed with trypan blue assay in which acacetin significantly increased the percentage of dead cells in MCF-7 breast cancer cells. Acacetin also increased the number of dead cells in MDA-MB-468 cells, but it was not as effective as it was for MCF-7 cells.

The cell cycle is a critical biological event which is necessary for the growth of cells, and in cancer cells, many cell cycle checkpoint regulators are expressed aberrantly thus disturb the cell cycle regulation. Therefore, for the regulation of uncontrolled cell proliferation *via* control of cell cycle regulatory molecules, the small molecules could be an alternative to cancer chemotherapeutics, and it can also have the potential for prevention as shown in the past few decades [7,18,19]. CDK's and CDKI's like p21 and p27 are the critical cell cycle regulatory molecules. Acacetin treatment restricted MCF-7 cells early in sub-G1 and G1 phase and later in S & G2/M phases. In MDA-MB-468 breast cancer cells, acacetin was able to downregulate CDK-2 which is essential for G1 to S phase transition. Acacetin treatment was also able to increase the expression levels of p21 and p27 in MDA-MB-468 human breast cancer cells. These findings suggest that acacetin treatment causes cell cycle arrest *via* up-regulation of p21 and p27, which is followed by the down-regulation of CDK expression.

Mitochondrial dysfunction is a key and early event which indicates that cells are undergoing apoptosis [20]. Acacetin treatment induces mitochondrial membrane depolarization in human breast cancer MCF-7 cells at 24 h time point. This data indicates that acacetin-induced apoptosis involves the mitochondrial pathway. ERK and AKT are the pivotal survival markers that are upregulated in several cancers including breast cancer. Alteration in ERK and AKT cell signaling pathways leads to dysregulated cell proliferation, survival, invasion, and metastases. Thus, ERK and AKT can be potential targets and of clinical relevance to all three major subtypes of breast cancer [21]. Acacetin treatment also caused a downregulation in the expression

levels of an activated form of ERK1/2, p38, and AKT in breast cancer cells. This data further confirms the cell proliferation inhibition by acacetin in breast cancer cells and also indicates that acacetin treatment may drive the cells towards apoptosis *via* inhibition of ERK/AKT signaling pathways.

Metastasis is responsible for the majority of the cancer patient deaths from solid tumors. Initiation of metastatic cascade starts from epithelial to mesenchymal transition and cellular migration and invasion. Therefore, EMT and cell migration play a pivotal role in metastatic cascade [22]. We were also able to demonstrate that acacetin treatment diminished the migratory abilities of MDA-MB-468 cells. Acacetin treatment also downregulated the expression levels of classical mesenchymal markers such as N-cadherin and vimentin.

Collectively, the data suggest that acacetin can prove to be a potential anticancer agent against breast cancer cells. Treatment of acacetin not only inhibits cellular proliferation and causes cell cycle arrest but also restrains cell migration and EMT in breast cancer cells (Fig. 4C). Further, *in vitro* and *in vivo* mechanistic studies may establish acacetin as a preclinical anticancer candidate agent for breast cancer treatment.

5. Acknowledgments

The work was supported by funds from the Central University of Gujarat, Gandhinagar, India. KK acknowledges University Grant Commission, New Delhi, India for financial support in the form of fellowship. CIF facility of the Central University of Gujarat is highly acknowledged.

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