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Original Research

Naringenin inhibits human breast cancer cells (MDA-MB-231) by inducing programmed cell death, caspase stimulation, G2/M phase cell cycle arrest and suppresses cancer metastasis

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Abstract: The current study was designed to unveil the anticancer effects of naringenin against breast cancer MDA-MB-231 cells. Cytotoxic effects were estimated via MTT viability assay. Clonogenic assay was performed to assess clonogenic potential of MDA-MB-231 cells. Apoptosis was examined via AO/EB staining, quantified via annexin V/PI staining and western blotting was performed to monitor apoptosis allied protein expressions. Cell cycle was analyzed through flow cytometric analysis. Transwell chambers assay was executed for determination of cell migration and cell invasion tendency of MDA-MB-231 breast cancer cells. Results indicated significant anticancer potential of naringenin drug against MDA-MB-231 cells. On evaluation of cell proliferation rate of breast cancer cells by MTT assay, it was observed that naringenin inhibited proliferation rate in dose as well as time dependent manner. AO/EB staining assay revealed potential morphological changes indicating apoptotic cell death. Annexin V/PI staining assay revealed increased apoptotic cell percentage with increased drug doses. The apoptosis inducing potential of naringenin drug was observed to be mediated via caspase activation. Flow cytometric analysis predicted cell cycle arrest at G2/M phase of cell cycle. Further cell migration as well as cell invasion tendency of MDA-MB-231 cells was reduced to minimum upon application of naringenin drug.

Key words: Breast cancer; Naringenin; Apoptosis; Cell cycle; Metastasis.

Introduction

Flavonoids are a major class of natural products with several subclasses including flavones (1). Flavonoids are of great medicinal as well as biological reputation and are found in a variety of Chinese herbs and edible plants. Recent studies have revealed that flavonoids show anti-tumor, anti-oxidant, anti-viral, anti-allergic, and anti-inflammatory behavior (2-9). The anti-tumor effects of flavonoids have been discovered of their apoptosis inducing potency in cancer cells (10,11). Flavones are found in vegetables and fruits like flavone, luteolin and apigenin, are regarded as important dietary phytochemicals with significant cancer chemopreventive potency (12,13). Flavones have been reported to induce their anticancer effects via effecting several signaling pathways and mechanisms against different model cancer cells. Breast cancer ranks among most dangerous cancers prevailing in women globally. Breast cancer incidences are amplifying at an alarming rate, mostly in western countries. From past one decade, novel and effective cancer chemotherapeutics have been recognized. Still breast cancer related deaths and incidences are multiplying day by day with over one million cases yearly (14). The study of breast cancer from last two or three decades have evolved a number of risk factors regarding breast cancer etiology like age, gender, genetic, smoking, alcoholics and obesity (15-18). Several cellular changes that result in the development of breast cancer include suppressed cellular apoptosis, enhanced cell division as well as cancer cell metastasis. Pivotal signal transduction pathways are often dysregulated by these cancer initiating changes that result in the activation of several transcription factors. These factors get activated by the extracellular signals that ultimately induce genetic modifications. It has been reported that breast cancer involves enhanced expressions of AKT/PKB (protein kinase B) which enhance cellular resistance as well (19,20). Breast cancer patients are often treated with radiation therapy, chemotherapy, and surgical resection. Still, breast cancer lethality is enhanced due to distant disease metastasis, lack of effective chemo-preventives and lower overall survival rate. Which results in the emergence of desperate need of effective and novel treatment strategies. Naringenin molecule belongs to flavanone subclass of flavonoids and is prevalent in large amounts among Citrus fruits, tomatoes and bergamot. This phytochemical has been accounted with different pharmacological applications including antiviral, anti-inflammatory, anti-bacterial, anti-cancer, antioxidant, cardioprotective and antiadipogenic (21-24). The current investigation was designed to unveil the

anticancer effects of naringenin against drug-resistant human breast cancer cells (MDA-MB-231). Tendency of inducing cellular apoptosis, caspase activation, G2/M phase cell cycle arrest and suppressing cancer metastasis by naringenin were assessed as well in this study.

Materials and Methods

Cell cytotoxicity assay

Cellular viability of Breast MDA-MB-231cancer cells was testified by MTT cell cytotoxicity assay. Briefly, a preliminary concentration of 2×10^5 cells each well were seeded onto a 96-well plate and incubated in a 5% CO_2 incubator at 37°C for 24h. Subsequently, cells were exposed to changing naringenin doses viz 0, 20, 40, 80 and 160 µM for a time period of 48 h and 24 h, followed by incubation. Next, MTT standard solution (5mg/ml) was supplemented to each well substituting cultural media followed by additional incubation for 4 h. To each well 150 µL of DMSO was added and centrifuged for about 10 min. Finally, ELISA reader was utilized to monitor absorbance at 620 nm taking 490nm as reference wavelength.

Clonogenic assay

Clonogenic assay was implemented to assess the influence of naringenin on colony forming potency of breast MDA-MB-231 cancer cells. Cancerous cells were seeded in 6-well plates and then treated with different naringenin doses viz 0, 20, 80, and 160 µM. Cells were washed thoroughly with PBS three times. Fixing of cells was performed with a mixture of methanol and acetic acid. After accomplishment of culturing of cell colonies, staining of cells was done by crystal violet staining (0.1%). Those colonies which contained more than 50 cells were considered for counting. Finally the cells were viewed under microscope.

AO/EB staining assay

The morphology of apoptotic cells was observed by AO/EB staining assay through fluorescence microscopy. MDA-MB-231 cells were harvested at logarithmic phase of growth followed by trypsinisation with trypsin (0.25%). Cells were launched into 96-well plate containing 10% FCS (fetal calf serum) with an initial density of 2×10^4 cells/ml. These well plates were then incubated and exposed to varying naringenin doses of 0, 20, 80, and 160 µM. Additionally, cells were supplemented with 20 µl trypsin and loaded over glass slides. AO/EB staining solution (100 µg/ml each) was placed over slides and covered with coverslips. Fluorescent microscope (OLYMPUS, Japan) was used for detection of apoptotic cell morphology.

Annexin V/PI assay

For annexin V-FITC/PI assay, Annexin V Apoptosis Detection Kit (BD, USA) was used for about 15 min strictly following manufacturer's guideline at room temperature. Finally, apoptosis cell percentage was determined by flow cytometric analysis.

Assessment of different cell cycle phases

For cell cycles phase distribution assessment, MDA-MB-231 cells were collected at 80% of growth confluence. Afterwards, cells were treated with naringenin drug doses viz 0, 20, 80, and 160 µM followed by washing with PBS (phosphate buffer saline) twice. Washed cells were fixed overnight in cold ethanol at 4°C and then filtered with a 0.05 mm cell strainer. Afterwards, cells were incubated with PBS comprising of 0.2% (v/v) Triton X-100, 100 µg/mL RNase A and 50 µg/mL propidium iodide (PI). Finally, different MDA-MB-231 cells with different cell cycle phases were observed flow cytometrically (C6, BD, NJ, United States).

Cell migration and invasion determination

Cell migration and invasion potency of MDA-MB-231 cells was checked by transwell assay. Upper transwell chambers were filled with cancer cells with an initial concentration of 1×10^6 cells along with cultural media and 10% FCS (fetal calf serum). Upper chambers were also supplemented with different naringenin doses viz 0, 20, 80, and 160 µM. Lower chambers of transwell were filled with FCS and 200 ml of media only. Afterwards, transwell chambers were incubated for 24h followed by fixation in methanol and crystal violet staining. Finally, the cells were analysed and counted under an inverted microscope with 200X of magnification. For cell migration simple transwell chambers were used while in case of cell invasion chambers were coated with Matrigel.

Western blotting assay

Cultured MDA-MB-231 cells were exposed to variant naringenin doses viz 0, 20, 80, and 160 µM prior to lysing with lysis buffer. 10 µg of total protein quantified by performing BCA assay was subjected to 8% SDS-PAGE gel. Resolving of the protein content was accomplished electrophoretically. Afterwards, proteins were loaded to PVDF membranes followed by primary antibody treatment (antibodies against Caspase-3, Caspase-8, Caspase-9, BAX and BCL-2). Thereafter, membranes were subjected to secondary antibodies overnight at 4°C. Finally, the protein signals were detected and visualized via ECL (enhanced chemiluminescence.

Statistical analysis

Data for each study group was imitated from execution of individual triplicate experiments. Comparison of values for statistical analysis was performed with Chi-Square or Student's t-test. $P<0.05$ indicated considered as statistically significant and results were presented as the mean ± SD.

Results

Naringenin inhibits cell proliferation and colony formation tendency of MDA-MB-231 cells

Proliferation rate of breast MDA-MB-231 cancer cells was evaluated by execution of MTT viability assay. Control cells were taken as 100% viable cells. On application of naringenin (Figure 1) (20 µM), viability reduced dramatically from 100% to near about 90% after 24h of treatment. On higher concentrations (160 µM) the viability was restricted to about 35%. When time of exposure was increased to 48h, the viability reduced to about 15% (Figure 2). Therefore, outcomes of MTT assay revealed both time as well as dose dependent inhibi-

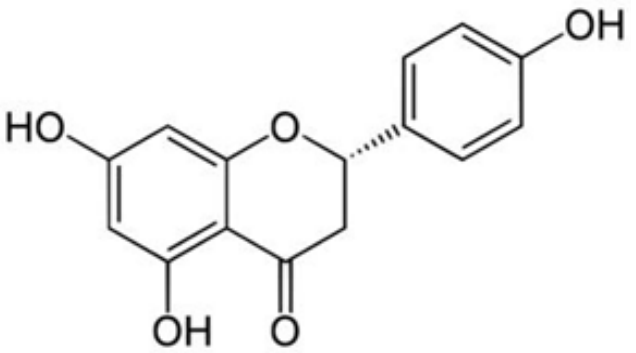

Figure 1. Chemical structure of naringenin molecule.

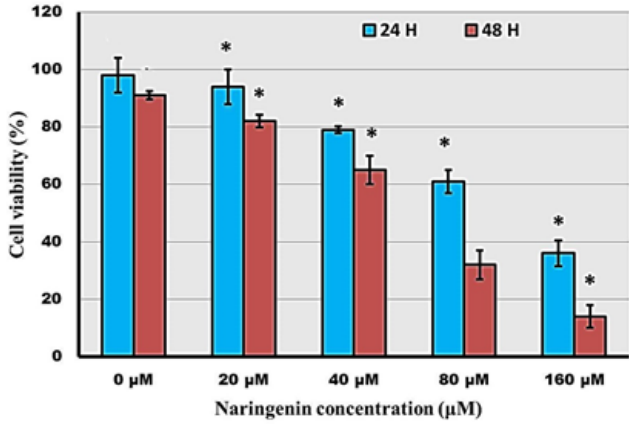

Figure 2. MTT viability assay for determination of cellular viability of MDA-MB-231 cells after exposure to varying naringenin viz 0, 20, 40, 80 and 160 µM. Cells were exposed for 24h and 24h with target drug. Results indicated suppression of viability both time as well as dose dependently. Data is shown as means ± standard deviation with replications n=3. P< 0.05.

tion of MDA-MB-231 cancer cell viability. Application of naringenin on MDA-MB-231 cell colonies predicted that it potentially inhibited their growth. It was observed that the number of cell colonies declined from 350 to about 130 on varying doses from 0-160 µM (Figure 3).

Apoptosis assessment after naringenin treatment

Apoptosis is one of the vital mechanisms by which multicellular organisms maintain their health and homeostasis. Apoptosis is naturally occurring programed cell death and targeting apoptosis in a cancer cell serves as a breakthrough in chemotherapy. Herein, naringenin inhibited cell proliferation rate of MDA-MB-231 cells and an attempt was made to unveil its underlying mechanism. Apoptosis was first analyzed by AO/EB staining assay. Results predicted that naringenin treatment induced specific modifications in the cell morphology of MDA-MB-231 cells that indicated apoptotic cell death. It showed damaged DNA, membrane blebbing, nucleus condensation and membrane rupture (Figure 4). Apoptosis was further quantified annexin V/PI staining assay. The results indicated that the numbers of apoptotic cells at controls were minimum and on application of naringenin drug the percentage of apoptotic cells increased remarkably (Figure 5). Further, western blotting assay was performed to check the Caspase mediation of apoptosis. Outcomes from western blotting indicated increased caspase-3, -8, -9 and BAX activities while as BCL-2 activity was observed to be declining with increased naringenin doses (Figure 6). Thus, the results from AO/EB staining, annexin V/PI staining and western blotting assay indicated that naringenin induced antiproliferative effects mediated via induction of Caspase-dependent apoptosis.

Naringenin inhibited G2/M phase cell cycle arrest

Different cell cycle phases of MDA-MB-231 cells were analyzed through flowcytometry. Frequent cell division results in the development of tumor growth and targeting cell division remains a target in cancer chemotherapy. The G2/M-phase cells were observed to be increase with increasing drug doses as compared to S-phase and G0/G1-phase cells. This indicated that the cell cycle stopped at G2/M-phase (Figure 7). Hence, the application of naringenin induced cell cycle arrest in MDA-MB-231 cells in a dose-reliant manner.

Naringenin treatment inhibited MDA-MB-231 cancer cell migration and invasion

Cell migration and invasion are the two major mechanisms in breast cancer metastasis. Therefore, target-

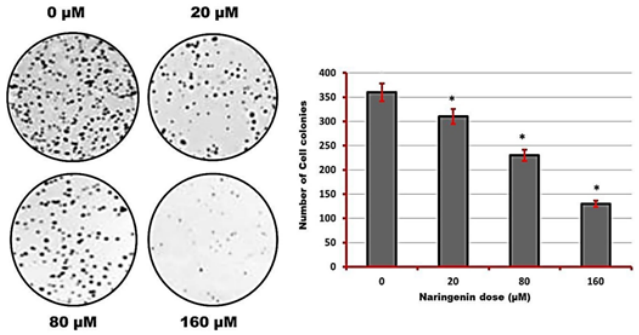

Figure 3. Clonogenic analysis of MDA-MB-231 cells after naringenin exposure at indicated doses. Graphical representation of reduced cell colonies after drug exposure is also presented. Data is shown as means ± standard deviation with replications n=3. P< 0.05.

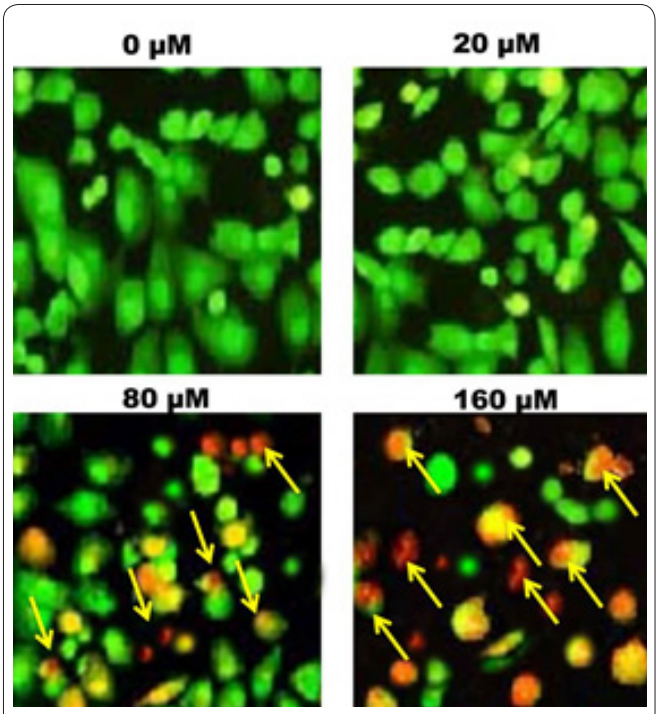

Figure 4. Cellular morphology detection with AO/EB staining assay. The arrows represented apoptotic cell morphology like membrane blebbing and nuclear condensation. n=3.

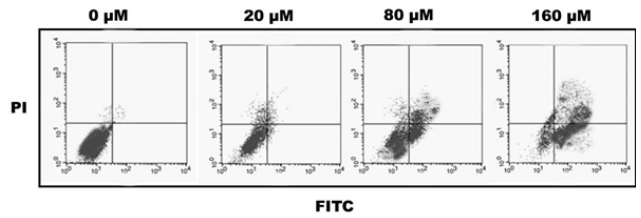

Figure 5. Calculation of the number of apoptotic cells via annexin V-FITC/PI dual staining assay after naringenin exposure at different doses. Experiments were performed in triplicates.

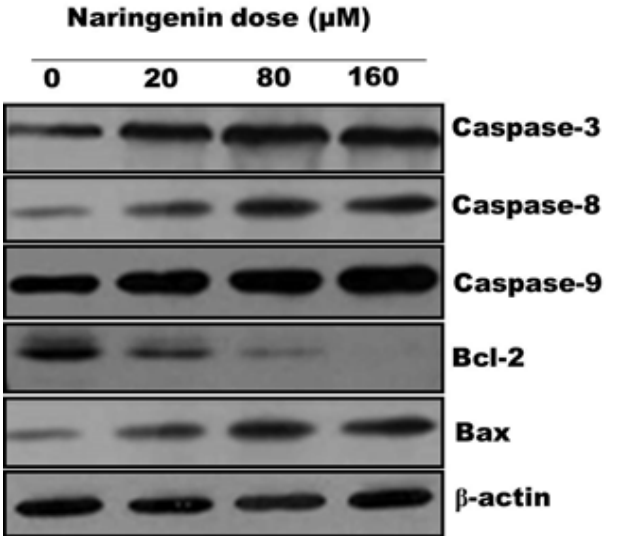

Figure 6. Western blotting assay for determination of activity of apoptosis allied proteins and its caspase dependence. Experiments were performed in triplicates.

ing cell migration and invasion inhibits breast cancer metastasis. Transwell chambers assay was performed to assess cell migration and invasion potency of breast cancer cells. Results from transwell chambers migration assay indicated dose reliant inhibition of cell migration. The number of migrated cells was reduced to minimum by naringenin exposure (Figure 8). The transwell chambers assay for cell invasion also indicated minimization in the number of invaded cells after drug exposure (Figure 9). Thus, it was established by performing transwell chambers assay that naringenin inhibited breast cancer metastasis.

Discussion

Besides recent developments in the field of medicinal chemistry, acquired and intrinsic resistance towards chemotherapy and higher probability of disease relapse poses strong drawbacks in breast cancer treatment (25). Therefore, the research has been diverted to natural products which poses very few/or no side effects with higher efficacy. Natural products have come up with novel drug candidates with anticancer potential and different therapeutic targets (26,27). Quercetin and emodin drugs are of natural origins which were reported with apoptosis inducing potential and cell proliferation inhibitive effects against different human cancer cell lines (28-29). Apoptosis is a natural phenomenon of cell death with both intrinsic as well extrinsic nature. To discard damaged, malfunctioning and old cells, apoptosis plays a vital role in multicellular organisms. Targeting and bringing apoptosis into play in cancer cells by application of a potential drug serves as an important strategy in cancer management (30). Another important aspect of cancer lethality is distant metastasis. Can-

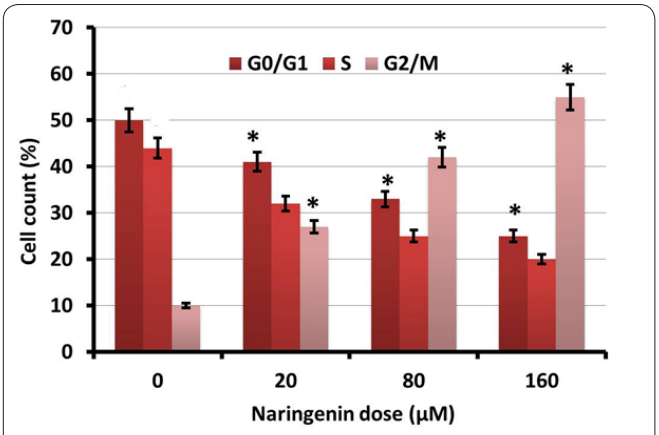

Figure 7. Flow cytometric analysis of different cell cycle phases presenting increased number of G2/M-phase cells after naringenin exposure at indicated doses. Data is shown as means ± standard deviation with replications n=3. P< 0.05.

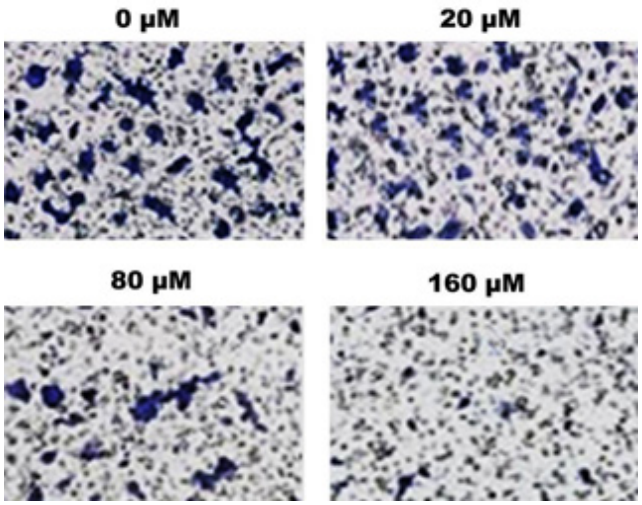

Figure 8. Transwell chambers assay representing migrated cell populations of MDA-MB-231 cells after exposure to naringenin drug at indicated doses. Experiments were performed in triplicates.

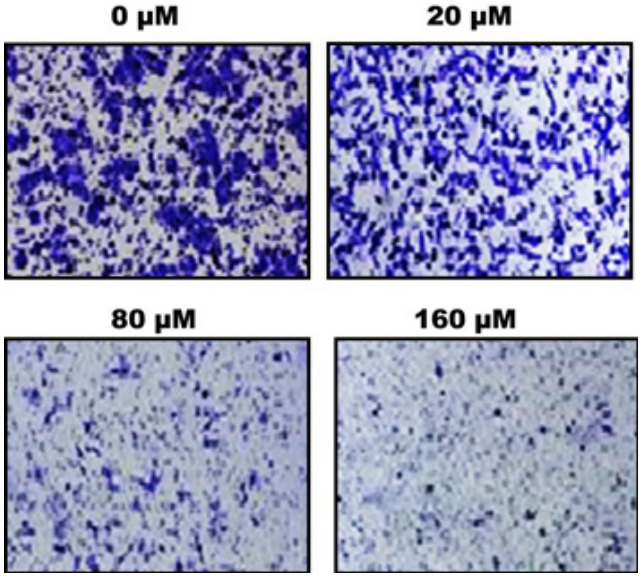

Figure 9. Transwell chambers assay representing invaded cell populations of MDA-MB-231 cells after exposure to naringenin drug at indicated doses. Experiments were performed in triplicates.

cerous cell migrates to distant places where it invades and starts growing through rapid proliferation. Thus targeting cell migration and invasion also serves as a therapeutic target in stopping distant cancer metastasis (31). Another characteristic feature of cancer cell is to rapidly proliferate (rapid cell division). Targeting cell cycle also reduces the proliferation of malignant tumor. In the current study, naringenin was investigated for inhibition of drug-resistant human breast cancer cells (MDA-MB-231) growth by inducing cellular apoptosis, caspase activation, G2/M phase cell cycle arrest and metastasis.

Several studies have mentioned antiproliferative effects of naringenin against different human cancer cells like Chang et al. reported that naringenin blocks proliferation of A549 (lung cancer) cells (32). A similar study demonstrates antiproliferative and anti-viability activity of naringenin against MCF-7 (breast cancer) cells via reduction in the expressions of ERK1/2 and AKT proteins (33). Herein, we assessed cell proliferation rate by MTT assay which illustrated that naringenin is a potential cell proliferation inhibitor of breast cancer MDA-MB-231 cells. Therefore, efforts were made towards finding of its underlying mechanism.

Additionally, several studies have mentioned the pro-apoptotic nature and potency of flavanones (34). Our results are concordant to those results demonstrating pro-apoptotic behavior of flavanones. Herein, we found that naringenin suppressed proliferation of MDA-MB-231 cells via promotion of apoptosis. Apoptosis and survival signaling pathways determine the fate of cells either to survive or die. The balance between these two is maintained by several genes and molecules including Bcl-2, BAX, caspases, Bad, Bcl-XL. These proteins are key signalling regulatory proteins in stimulation and progress of intrinsic apoptosis. Naringenin has been previously shown to induce apoptosis in gastric cancer cells via blocking of AKT pathway and imbalance between proapoptotic and antiapoptotic proteins (35). Similarly, we found that naringenin induced apoptosis in breast cancer cells via upregulation of caspase-3, -8, -9 and BAX and downregulation of Bcl-2.

Uncontrolled proliferation of cancer cells is a result of multiple cell divisions accruing simultaneously. The dysregulation of cell cycle mechanism is a key component in carcinogenesis and tumor growth (36). Naringenin has been previously reported of cell cycle inhibitory effects against hepatocellular carcinoma cells. Herein, flow cytometric analysis demonstrated that naringenin blocked the progression of cell cycle at G2M-phase of cell cycle. Therefore, it was also established that antiproliferative effects of naringenin are also mediated via cell cycle arrest. Distant metastasis of malignant disease is one of the lethal features of cancer. Cell migration and invasion play crucial role in cancer metastasis. Naringenin and other flavanones have been shown to exhibit great potency to inhibit cell migration and invasion of different cancer cells (32). Similarly, we found that naringenin suppressed both migration and invasion of MDA-MB-231 cells in a dose reliant manner, which predicted that naringenin in a potential metastasis inhibitor.

Various studies performed using *in vitro* and *in vivo* experimental models strongly indicate the potential of naringenin molecule in treating various diseases. Several clinical studies have also been done on this molecule focusing on its cardioprotective action as well as bioavailability. Naringenin has also been shown to improve endothelial function. In brief, naringenin can prove to be a promising clinical drug provided further studies on its pharmacokinetic and pharmacodynamic profiles are performed.

Taking together, all the results of current investigation revealed that naringenin inhibits drug-resistant human breast cancer cells (MDA-MB-231) growth by activation of caspase dependent cellular apoptosis, G2/M phase cell cycle arrest and halts cancer metastasis, dose-reliantly.

Conflict of interest

The authors declare that there is no conflict of interest to indicate.

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