Apigenin suppresses proliferation and bone metastasis of human breast cancer cells by inducing apoptosis, autophagy and modulation of the MEK/ERK signalling pathway

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Abstract

Introduction: Although several studies have reported the anticancer properties of apigenin, the impact of apigenin on the proliferation and bone metastasis of breast cancer cells has not been examined. This study was therefore undertaken to investigate the anticancer and anti-metastatic effects of apigenin against breast cancer cells.
Material and methods: The breast cancer SK-BR-3 and MB-157 cells were used in the study. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Acridine orange/ethidium bromide (AO/EB) and annexin V/PI assays were performed to detect apoptosis. Electron microscopy was employed for autophagy detection. Bone metastasis was detected in mice xenograft models.
Results: The proliferation assay showed that apigenin causes a remarkable decrease in the proliferation of the SK-BR-3 breast cancer cells and an IC_{50} of 10 µM was observed for apigenin against the SK-BR-3 cells. The inhibition of SK-BR-3 proliferation was found to be due to apoptosis which was accompanied with the upregulation of Bax and downregulation of Bc-2. Apigenin also triggered the activation of autophagy in the SK-BR-3 cells as evident from the upregulated expression of LC3 II and Beclin 1. Furthermore, the transwell assays together with the *in vivo* studies showed that apigenin suppressed the bone metastasis of the human breast cancer cells.
Conclusions: Taken together, the findings of the present study point towards the potential of apigenin in treatment of metastatic breast cancer.

Key words: apoptosis, metastasis, breast cancer, autophagy, proliferation, apoptosis.

Introduction

Together with many other cancer types, the incidence of breast cancer has increased significantly and has become a serious health problem in women [1]. Approximately 1.3 billion breast cancer cases and 0.5 million deaths are reported annually to be due to breast cancer [2]. The varied nature, late diagnosis, unreliable bio-markers and inefficient treatment

strategies hinder the management of breast cancer [3].

A ubiquitously present, vast and diverse group of plant metabolites, the flavonoids exhibit tremendous pharmacological and medicinal properties. They have been found to have cardio-protective, anti-inflammatory and anticancer properties [4, 5]. Approximately 100 mg minimum daily intake of flavonoids is recommended as the constituent of a healthy diet [6]. Both *in vitro* and *in vivo* research studies have proved the anticancer effects of flavonoids [6]. For example, the flavonoids of *Millettia reticulata* have been reported to suppress the proliferation of the hepatocellular carcinoma cells [7]. Similarly, the flavonoid extract of the flower of *Tecoma stans* has exhibited both *in vitro* and *in vivo* anticancer properties [8]. Owing to the health promoting effects of apigenin, it is an essential flavonoid found across the plant kingdom [9]. Apigenin has been shown to suppress the proliferation of pancreatic cancer cells by inducing G2/M cell cycle arrest [10]. Similarly, it has also been shown to inhibit the angiogenesis of lung cancer cells [11]. In yet another study apigenin has been reported to initiate prostate cancer cell death via reactive oxygen species and p53 activation [12]. The effects of apigenin on breast cancer cells have also been examined. For instance, apigenin has been shown to trigger the apoptotic cell death of breast cancer cells via proteasomal degradation [13]. Lee *et al.* reported that apigenin suppresses HGF-promoted invasive growth and metastasis of breast cancer cells via deactivation of the PI3K/Akt pathway [14]. Nonetheless, the effects of apigenin on the bone metastasis of breast cancer have not been reported. This study was therefore designed to examine the impact of apigenin on the growth and the bone metastasis of breast cancer.

Material and methods

Cell lines and culture conditions

The human breast cancer cell line (SK-BR-3) as well as the normal MB-157 cell line was acquired from the ATCC collection centre, USA. The cell lines were cultured and maintained in DMEM (Dulbecco's modified Eagle's medium, Invitrogen Life Technologies, United States). DMEM was supplemented with fetal bovine serum (10%) and 100 U/ml each of streptomycin and penicillin G (Himedia, Pennsylvania, United States of America). Afterwards, medium with procured cells was placed in a CO_2 (5%) humidified incubator at 37°C.

Cell viability assay

The effects of apigenin on cell visibility were estimated through 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) (Roche, United States) assay (a colorimetric assay). MTT is reduced to an insoluble formazan complex in a living cell due to the presence of succinate dehydrogenase in mitochondria. Briefly, the Sk-BR-3 cells at a concentration of 1×10^5 cells/well were precultured in 96-well plates for 24 h in presence of different doses of apigenin in a humidified 5% CO_2 incubator at 37°C. After treatment, each cell line was subjected to PBS washing twice followed by MTT exposure (100 µl) with incubation for 60 min. An ELISA plate reader (ELX 800; Bio-Tek Instruments, USA) was used to record absorbance for estimation of OD (optical density) at 570 nm.

Autophagy detection

The SK-BR-3 cells were treated with various doses (0, 5, 10 and 20 µM) of apigenin and then trypsinized. The cells were then collected and fixed with the help of glutaraldehyde (2%) in phosphate buffer (0.1 M). Post-fixed in osmium tetroxide (1%), the cells were resin embedded and finally assessed by fluorescence microscopy.

Acridine orange/ethidium bromide staining (AO/EB) assay

To execute the AO/EB staining assay, SK-BR-3 cells were harvested from 6-well plates at a concentration of 0.5×10^5 cells per well. Subsequently, cultured SKBR-3 cells were subjected to various apigenin doses for 24 h. Treated SK-BR-3 cells were then fixed in formaldehyde (5%) Thereafter, glass slides were prepared for loading of treated SK-BR-3 cells for staining with 10 µl of AO/EB solution for 10 min.

Apoptosis assay

The effects of apigenin on cell apoptosis were determined via Annexin V-FITC assay (Sigma-Aldrich). Briefly, human SK-BR-3 cancer cells were cultured in 6-well plates with each well containing 2×10^6 cells. Cells were subjected to incubation for 12 h followed by apigenin treatment at various doses (0, 5, 10 and 20 µM) for 24 h. Apigenin-treated cells were trypsinized and thereafter washed twice with PBS. Afterwards, trypsinized cells were resuspended followed by the addition of binding buffer (250 µl) bearing Annexin V-FITC (20 µl) and propidium iodide (20 µl) to each well. Finally, cells were placed in the dark for further incubation for 30 min and finally apoptosis assessment was performed through flow cytometry (BD Biosciences) [10].

Migration and invasion assay

The impact of apigenin on cell migration and invasion tendency of SK-BR-3 cells was analysed

by transwell chamber assay. Target cells were seeded at 1 × 10^4 cells per ml of density in the upper chambers of a transwell containing cultural media, 10% FBS (fetal bovine serum) and various apigenin doses. Polycarbonate filters with 8 µm pore size were used to grow these cells followed by transfer of chambers to a 24-well plate and incubation for 24 h at 37°C. Afterwards, swabbing was done to eliminate the unmigrated cells and migrated cells were stained with 0.5% crystal violet for about 25 min. Cells were then washed with PBS and finally subjected to microscopic analysis under a light microscope. For cell invasion analysis, a similar procedure was followed except that transwell chambers were coated with Matrigel (Sigma Aldrich, USA).

Western blotting

Using RIPA lysis and extraction buffer, total proteins were isolated from untreated breast cancer cells and cancer cells treated with 0, 5, 10, and 20 µM apigenin for 24 h. The Bradford method was used to quantify the protein concentrations. About 45 µg of total proteins from each sample were separated electrophoretically on 10% SDS-PAGE. The gel was blotted to nitrocellulose membrane which was given the exposure of primary protein antibodies followed by exposure of secondary antibodies. ECL reagent was used for detection of bands corresponding to proteins of interest. The protein expression procedures were normalized with human GAPDH protein.

Xenograft study

The study was approved by the ethical committee of the Fudan University Shanghai Cancer Center, Shanghai, China under approval number FU/335/2018. For the study of the effects of apigenin on the bone metastasis, 10^5 SK-BR-3 cells were put into the left cardiac ventricle post-anaesthesia of female BALB/c mice via an injection. Apigenin at the dose of 20 mg body weight was administered for 4 weeks. However, the normal mice received only the normal saline. The metastasis was examined by bioluminescence imaging (BLI). The Xenogen IVIS system was used for obtaining the bio-luminescent images. Haematoxylin and eosin (H&E) staining was performed to assess the histopathology of the bone tumours.

Statistical analysis

The experiments were performed in triplicate. The values represent the mean ± SD. Student's *t* test and one-way ANOVA were used for statistical analysis. Statistical analysis was considered at $^*p < 0.05$.

Results

Apigenin suppresses the proliferation of breast cancer cells

The effects of apigenin (Figure 1 A) on the proliferation of the breast cancer SK-BR-3 and normal MB-157 cells were evaluated by MTT assay. It was

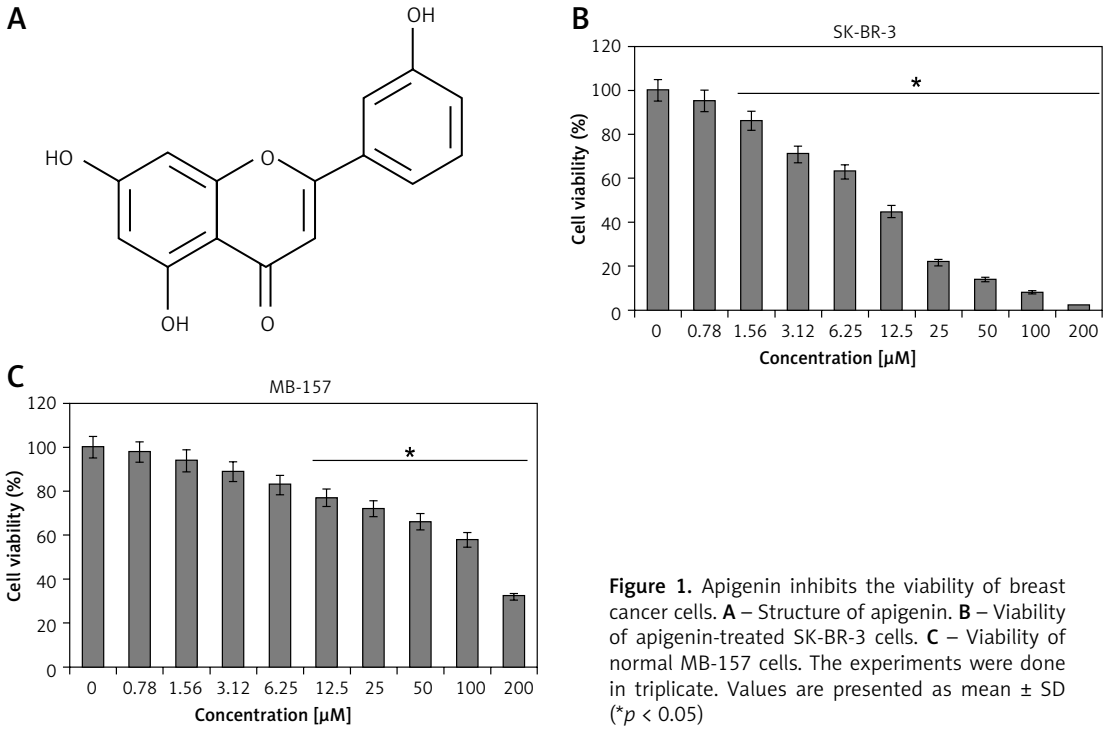

Figure 1. Apigenin inhibits the viability of breast cancer cells. **A** – Structure of apigenin. **B** – Viability of apigenin-treated SK-BR-3 cells. **C** – Viability of normal MB-157 cells. The experiments were done in triplicate. Values are presented as mean ± SD ($^*p < 0.05$)

found that viability of all the SK-BR-3 breast cancer cells declined remarkably with the increasing doses of apigenin (Figure 1 B). The IC_{50} of apigenin against the human SK-BR-3 breast cancer cells was found to be 20 µM (Figure 1 B). However, the effects of apigenin on the normal MB-157 cells was less severe, as evident from the IC_{50} of around 100 µM (Figure 1 C).

Apigenin induces autophagy in the breast cancer cells

The transmission electron microscopic examination revealed that apigenin treatment results in the induction of autophagy in the SK-BR-3 cells as evident from the formation of the autophagic vesicles (Figure 2 A). The western blot analysis also showed that apigenin increased the expression of light chain 3 (LC3) II and Beclin 1 (Figure 2 B).

Apigenin promotes apoptosis in breast cancer cells

The results of fluorescence microscopy of AO/EB stained SK-BR-3 cells showed that the anti-proliferative effects of apigenin on the growth of human breast cancer cells were mainly because of the activation of apoptosis in the SK-BR-3 breast cancer cells as evident from the increase in the nuclear fragmentation of the SK-BR-3 cells (Figure 3 A). The percentage of apoptosis induced by apigenin was determined by annexin V/PI staining. The percentage of apoptosis increase was found to be 0.1%, 10.62%, 27.45% to 36.73% at the dosage of 0 µM, 5 µM, 10 µM and 20 µM apigenin (Figure 3 B). The expression of Bax was increased while the proteins level of Bcl-2 showed a dose-dependent decrease (Figure 3 C).

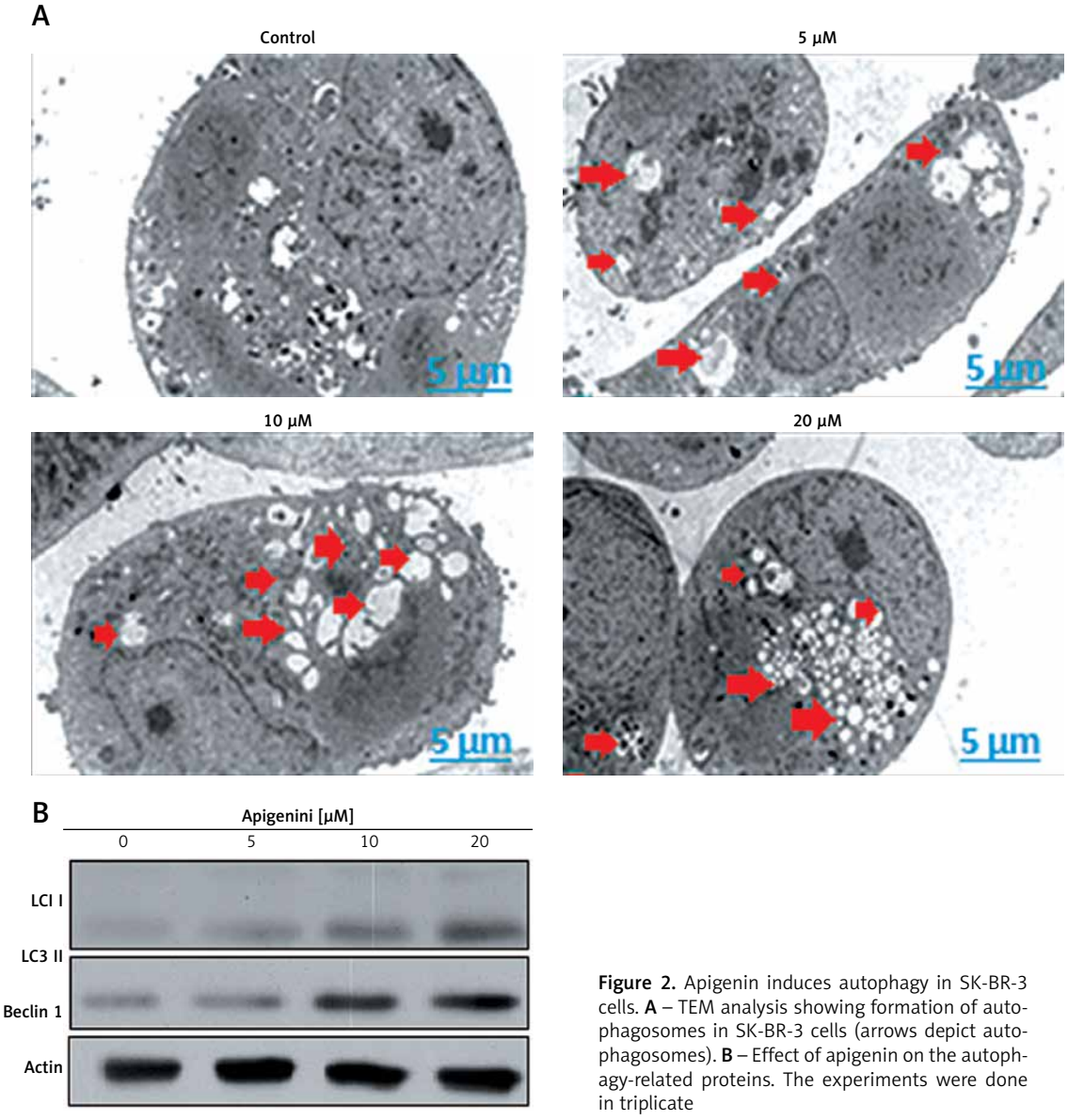

Figure 2. Apigenin induces autophagy in SK-BR-3 cells. **A** – TEM analysis showing formation of autophagosomes in SK-BR-3 cells (arrows depict autophagosomes). **B** – Effect of apigenin on the autophagy-related proteins. The experiments were done in triplicate

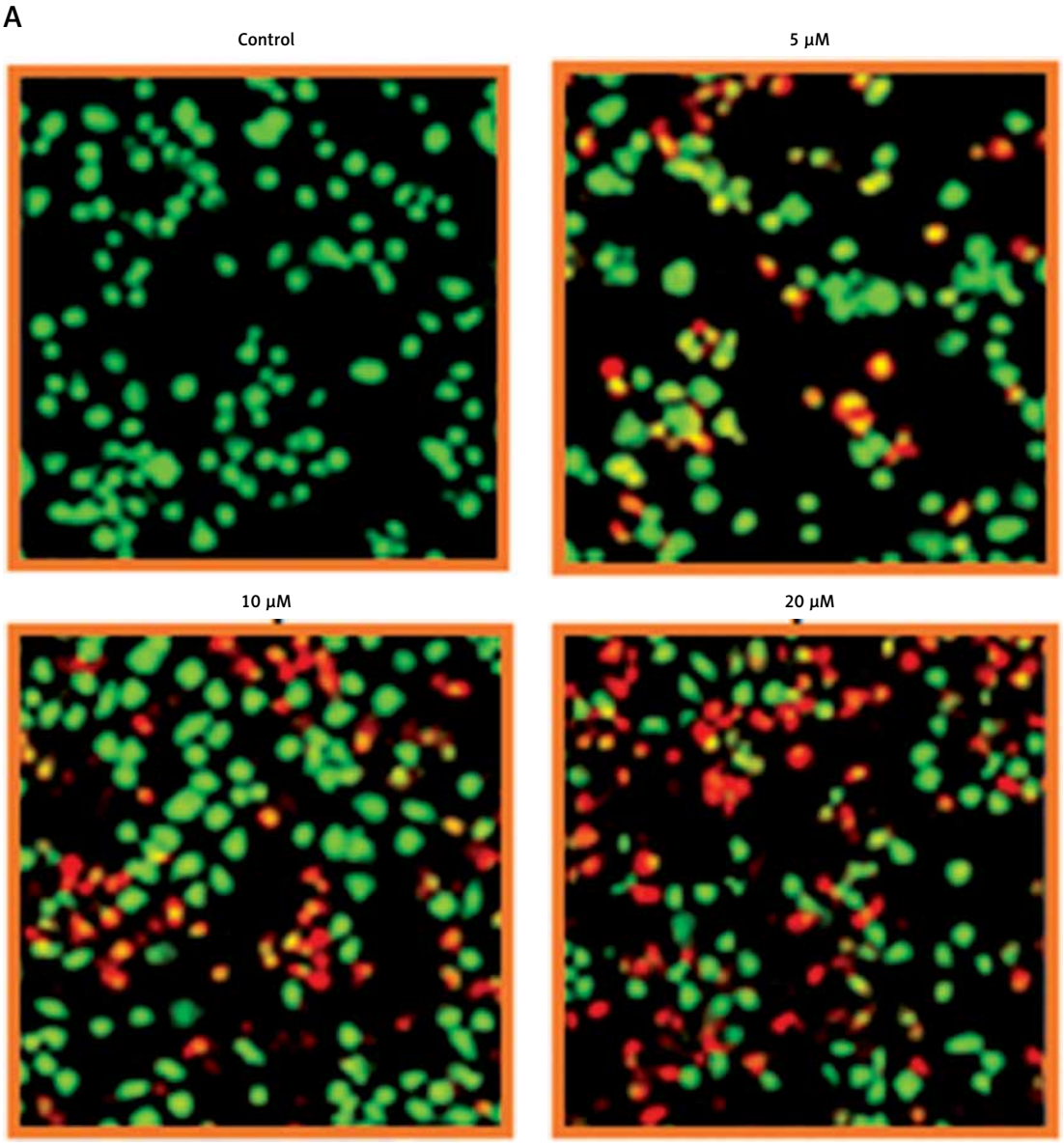

Figure 3. Apigenin induces apoptosis in SK-BR-3 cells. **A** – AO/EB staining of the apigenin-treated SK-BR-3 cells

Apigenin blocks MEK/ERK signalling

The effects of apigenin on the MEK/ERK signalling pathway were also evaluated. It was found that the protein levels of p-MEK1/2 and p-ERK declined significantly and concentration dependently (Figure 4). However, there was no apparent effect on the total MEK1/2 and ERK.

Apigenin suppresses the bone metastasis of SK-BR-3 cells

Next, the effects of apigenin on the bone metastasis of the human SK-BR-3 breast cancer cells was determined. The results revealed that apigenin suppressed the migration and invasion of the SK-BR-3 cells (Figure 5). The migration was found to be decreased by 62% and the invasion was 68% relative to untreated SK-BR-3 control cells (Figure 5). The effects of apigenin on the bone metastasis were also examined in the xenograft mice model at one dosage of 20 mg/kg body weight. The bioluminescent imaging showed a remarkable increase in bone metastasis of control mice. The mice also developed osteolytic bone lesions as revealed by the X-ray and H&E staining. However, mice administered apigenin showed relatively low metastasis and osteolytic bone lesions (Figure 6). These results suggest that apigenin suppresses the bone metastasis of breast cancer cells and indicates that investigation of effects of multiple doses of apigenin in future experiments may provide more insights.

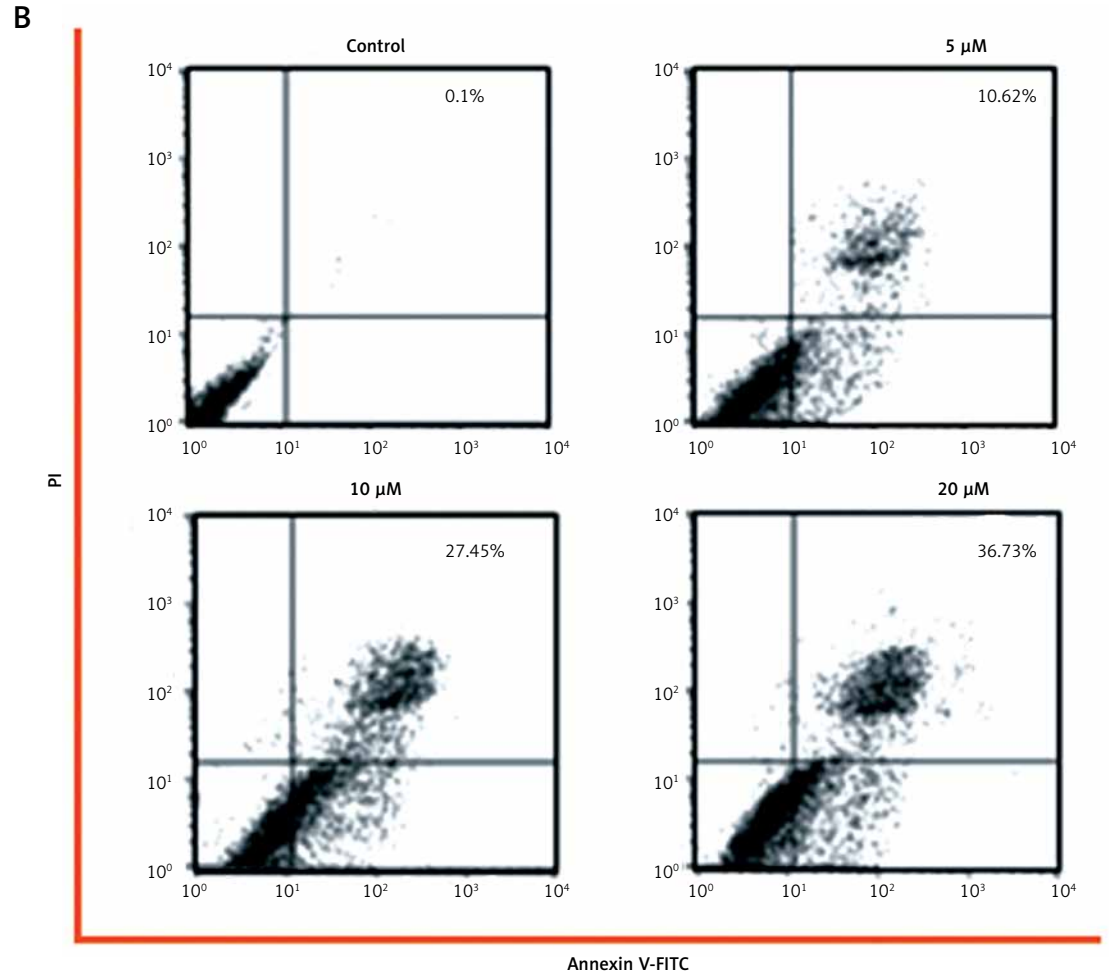

Figure 3. Cont. **B** – Annexin V/PI staining showing apoptosis in SK-BR-3 cells. **C** – Expression of Bax and Bcl-2 in apigenin-treated SK-BR-3 cells. The experiments were performed in triplicate

Discussion

One of the most frequently detected cancers, breast cancer is responsible for approximately 23% of all the cancers and 14% of the deaths caused by cancer [15]. Recent developments in cancer treatment and diagnostics have caused a decrease in the breast cancer-related mortality [16]. However, the adverse effects of the chemotherapeutic agents and their lower efficacy limit its treatment. This study investigated the anticancer properties of one of the ubiquitously present plant flavonoids, apigenin. The results showed that apigenin causes a remarkable decrease in the proliferation of the SK-BR-3 cancer cells with relatively low cytotoxic effects on normal cells. One of the main characteristics of cancer cells is the dysregulated apoptosis. It is therefore believed that natural compounds that can induce apoptosis in cancer cells may prove beneficial in the development of cancer chemotherapy [16]. A vast number of drugs used for cancer treatment are extracted from plants and these molecules inhibit the proliferation of the cancer cells via different signalling cascades [17]. This study revealed that apigenin promoted apoptotic cell death of the SK-BR-3 cells via upregulation of Bax and depletion of Bcl-2. Autophagy is another vital process that helps to get rid of harmful and

damaged cells or cell organelles [18]. This study revealed that apigenin also activated autophagy in the SK-BR-3 cancer cells via enhancement of LC3 II and Beclin 1 expression. Previous studies have shown that some plant-derived molecules induce apoptosis and autophagy in cancer cells [19]. Undoubtedly, the MEK/ERK signalling pathway contributes a core effect in regulating cell proliferation, differentiation and survival in the signalling networks [20]. Due to this, it has been studied and discussed to determine the pathogenesis of several types of human cancers [21]. The MEK/ERK signalling cascade has been shown to be remarkably and aberrantly activated in cancer cells [22]. Activation of the MEK/ERK pathway promotes the proliferation and development of cancer [21]. Hence it is believed that the MEK/ERK signalling pathway could act as a therapeutic target for the treatment of different cancers. Therefore, we also examined the effects of apigenin on this pathway. Interestingly, it was found that apigenin blocks this signalling pathway in a dose-dependent manner. Additionally, the transwell assays showed that apigenin suppressed the migration and invasion of cancer cells. Under *in vivo* conditions, apigenin suppressed the bone metastasis of the breast cancer cells. All considered, apigenin may prove to be an essential anti-proliferative and anti-metastatic agent and

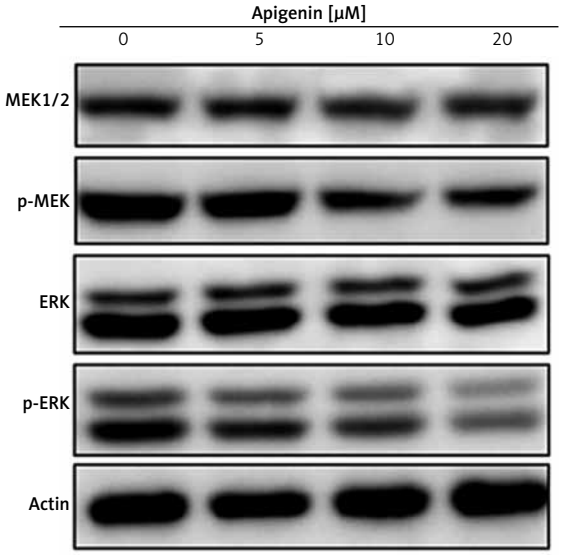

Figure 4. Apigenin block MEK/ERK signalling. Western blot analysis showing the effects of apigenin on the phosphorylation of MEK and ERK. The experiments were performed in triplicate

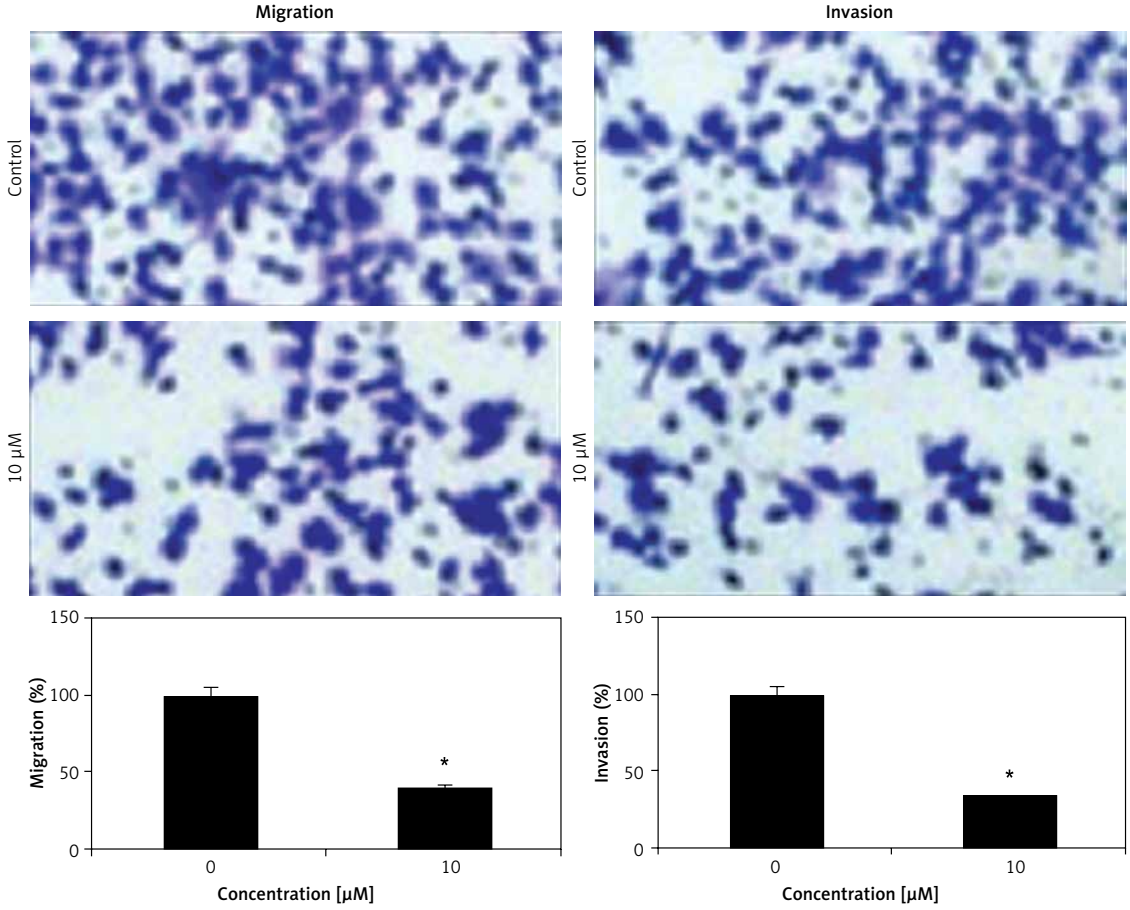

Figure 5. Apigenin inhibits migration and invasion of the SK-BR-3 cells. The transwell assay of the apigenin-treated SK-BR-3 cells showing inhibition of migration and invasion. The experiments were performed in triplicate

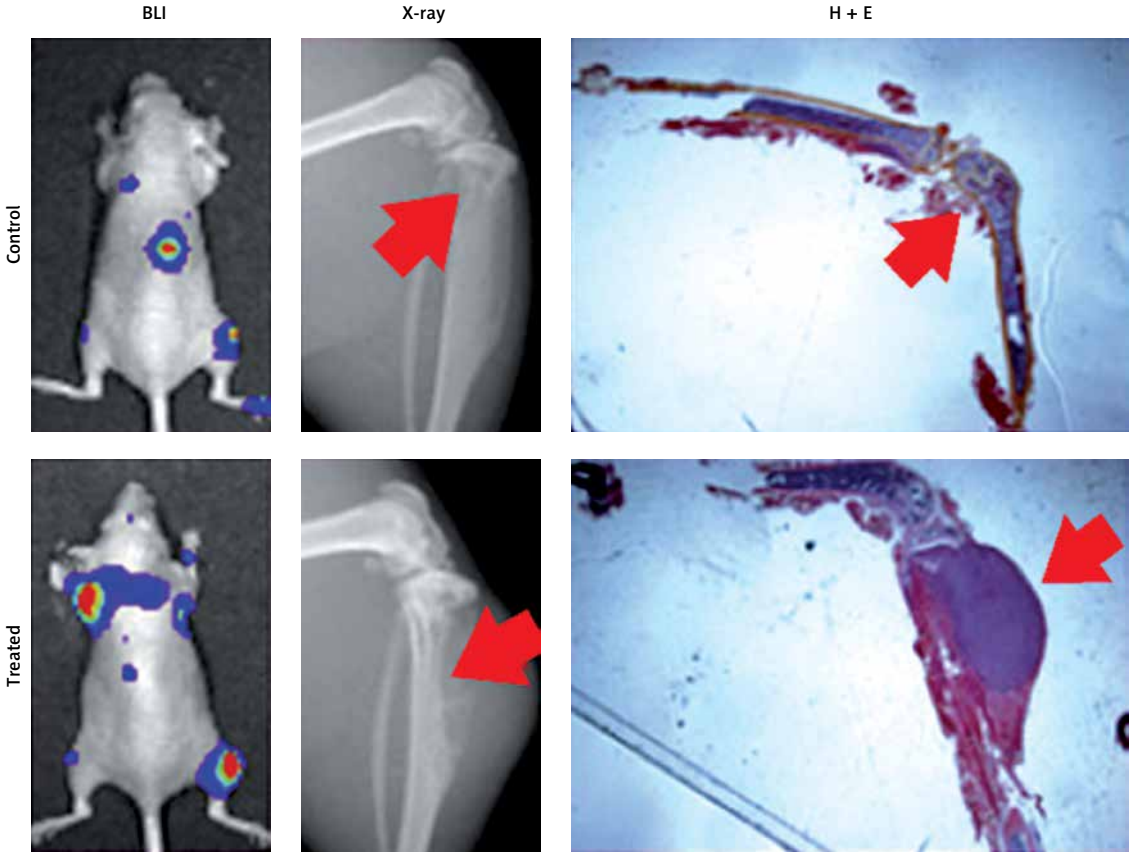

Figure 6. Apigenin inhibits bone metastasis of bone cancer cells. *In vivo* xenografted mice model showing the BLI, X-ray and H + E staining of control and apigenin-administered mice showing inhibition of bone metastasis. The experiments were performed in triplicate

may be used as a lead molecule for the development of breast cancer chemotherapy.

In conclusion, the study revealed the remarkable antiproliferative and anti-metastatic effects of apigenin against human breast cancer cells. These effects were found to be due to the ability of apigenin to induce apoptosis and autophagy via deactivation of the MERK/ERK signalling pathway. Taken together, these observations indicate that apigenin may prove to be an essential lead molecule for the management of breast cancer.

Acknpowledgments

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Conflict of interest

The authors declare no conflict of interest.

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