

Research Article

Differential Control of Growth, Apoptotic Activity, and Gene Expression in Human Breast Cancer Cells by Extracts Derived from Medicinal Herbs *Zingiber officinale*

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The present study aimed to examine the antiproliferative potentiality of an extract derived from the medicinal plant ginger (*Zingiber officinale*) on growth of breast cancer cells. Ginger treatment suppressed the proliferation and colony formation in breast cancer cell lines, MCF-7 and MDA-MB-231. Meanwhile, it did not significantly affect viability of nontumorigenic normal mammary epithelial cell line (MCF-10A). Treatment of MCF-7 and MDA-MB-231 with ginger resulted in sequences of events marked by apoptosis, accompanied by loss of cell viability, chromatin condensation, DNA fragmentation, activation of caspase 3, and cleavage of poly(ADP-ribose) polymerase. At the molecular level, the apoptotic cell death mediated by ginger could be attributed in part to upregulation of Bax and downregulation of Bcl-2 proteins. Ginger treatment downregulated expression of prosurvival genes, such as NF- κ B, Bcl-X, Mcl-1, and Survivin, and cell cycle-regulating proteins, including cyclin D1 and cyclin-dependent kinase-4 (CDK-4). On the other hand, it increased expression of CDK inhibitor, p21. It also inhibited the expression of the two prominent molecular targets of cancer, c-Myc and the human telomerase reverse transcriptase (hTERT). These findings suggested that the ginger may be a promising candidate for the treatment of breast carcinomas.

1. Introduction

Despite significant advances toward targeted therapy and screening techniques, breast cancer continues to be a chronic medical problem worldwide, being the most common type of cancer in women and the leading cause of death [1]. Typically, the treatment of breast cancer involves hormonal therapy with tamoxifen or other selective estrogen receptor (ER) modulators [2–4]. However, almost all patients with metastatic disease and approximately 40% of patients that receive tamoxifen experience relapse that ends by death [5]. In addition, the clinical utility of ER antagonists is often limited by side effects [2, 3, 6] and is largely ineffective against ER-negative breast cancer [2, 3]. Furthermore, despite the fact that many tumors initially respond to chemotherapy,

breast cancer cells can subsequently survive and gain resistance to the treatment [7]. Thus, identification of novel agents that are relatively safe but can suppress growth of both ER-positive and ER-negative human breast cancers is highly desirable.

While many factors contribute to the development of cancer, one hallmark is the loss of balance between cell proliferation and cell death, resulting in increased cell replication and the failure of damaged cells to be removed through apoptosis [8]. Apoptosis is an active form of cell suicide controlled by a network of genes and is an essential process during development as well as playing a key role in the pathogenesis of diseases including cancer [9]. Indeed, many studies strongly suggested that the loss of apoptotic control in favor of cell proliferation is responsible for breast

cancer initiation and progression [10]. Tumor cells do not undergo apoptosis easily because they have defects in their ability to activate the death signaling pathway. In turn, one essential strategy for cancer therapy is to activate apoptotic pathways in the tumor cells. Coherent to this notion, many natural products, including plants, vegetables, herbs, and spices, that have been found to be a potential source of novel anticancer drugs over the decades [11] repeatedly reported to exert their antitumor effects by inducing apoptosis [12]. Among natural products, ginger (*Zingiber officinale*), a member of Zingiberaceae family, has attracted the interest of medical scientists. Ginger is cultivated as a spice and for medicinal purposes [13]. The rhizome of this plant has been used as a medicine in Asian, Indian, and Arabic herbal traditions since ancient times [14]. It has been prescribed for the treatment of various conditions, including arthritis, rheumatological conditions, muscular discomfort atherosclerosis, migraine headaches, rheumatoid arthritis, high cholesterol, ulcers, depression, and impotence (cited in [17]). In addition, a significant number of *in vitro* and *in vivo* studies provide substantial evidence that ginger and its organic pungent vallinoid compounds are effective inhibitors of the carcinogenic process. For example, Unnikrishnan and Kuttan [15] found that alcoholic extracts of the ginger were cytotoxic to Dalton's lymphoma ascites tumor cells, human lymphocytes, Chinese hamster ovary cells, and Vero cells in *in vitro*. Rhode and associates [16] proved that treatment of cultured ovarian cancer cells with ginger resulted in profound growth inhibition in all tested cell lines, inhibition of NF- κ B activation, and diminished secretion of angiogenic factors, VEGF and IL-8. Concerning apoptotic activity of ginger, numerous reports confirmed that some compounds present in ginger exert cancer preventive effects by inducing apoptosis in cancerous or transformed cells [17]. For example, exposure of Jurkat human T-cell leukemia cells to various ginger constituents, galanals A and B, resulted in apoptosis through the mitochondrial pathway and accompanied by a downregulation of antiapoptotic protein Bcl-2 and an enhancement of proapoptotic protein Bax expression [18]. β -Elemene, a novel anticancer drug extracted from the ginger plant, triggered apoptosis in non-small-cell lung cancer cells, which was associated with a mitochondrial release of the cytochrome *c*, induction of caspase-3, -7, and -9 activities, decreased Bcl-2 expression, and increased the levels of cleaved poly(ADP-ribose) polymerase [19]. Gingerdione derivative, a component from ginger, induced G1 arrest and apoptosis in human leukemia HL 60 cells through downregulation of G1-associated cyclin D2, cyclin E, and cdc25A and upregulation of p15 and p27 [20].

Despite knowledge about the potent anticancer activity of the ginger, the molecular mechanisms underlying this activity are not currently well known in breast cancer. Based on the previously mentioned reported scientific data and considering the fact that in some cases herbal extracts are showing more potency than the purified components [21, 22], the present study was undertaken to investigate the impacts of crude extracts of ginger on growth of breast cancer cell lines.

2. Materials and Methods

2.1. Herbal Material and Extract Preparation. The mature and healthy rhizomes of ginger were purchased from the local market of Jeddah, KSA. They were properly washed and thinly grated and were used to prepare ethanol and aqueous extracts. The dried rhizome was powdered mechanically and extracted by cold percolation with 70% ethanol for 24 h. The extract was recovered and 70% ethanol was further added to the plant material and the extraction continued. The process was repeated three times. The three crude extracts were pooled together, and the combined extract was filtered under suction, evaporated, passed through 0.22 μ m filter, and kept in aliquots at 4°C. To prepare aqueous extract the same procedure was carried out with one modification, extraction processes were carried out using distilled water rather than 70% alcohol. Before use, the stock (ethanol/aqueous) extract was further diluted in tissue culture media to give the final indicated concentrations.

2.2. Cell Culture. Two well-characterized human breast cancer cell lines (MCF-7 and MDA-MB-231) and a non-tumorigenic normal mammary epithelial cell line (MCF-10A) were used as a model. All cells were obtained from King Fahd Center for Medical Research, King Abdulaziz University, Saudi Arabia. MCF-7 and MDA-MB-231 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics in tissue culture flasks under a humidifying atmosphere containing 5%CO₂ and 95% air at 37°C. MCF-10A cells were cultured in Mammary Epithelial Growth Medium (MEGM) supplemented with 100 ng/mL cholera toxin. All cells were subcultured at 3-4 day interval. They were seeded at a density of 5×10^4 cells/mL, then washing the monolayers with phosphate-buffered saline (PBS) followed by a brief incubation with trypsin/EDTA. The washed cells were isolated by centrifugation and resuspended in culture medium for plating or counting. Only cells growing at the exponential phase were used to perform all the experiments.

2.3. Cell Viability Assays. Cell viability assays were done as explained previously [23]. Briefly, the dose-dependent effects of the herb on the viability of MCF-7 and MDA-MB-231 cells were determined by trypan blue dye exclusion assay. Early log phase cells were trypsinized and regrown in 6-well cell culture plates at the concentration of 200×10^3 cells/mL in 3 mL of complete culture medium. Twenty four hours after-treatment, the medium was removed and replaced with fresh medium containing 0.0, 0.025, 0.05, 0.1, 0.15, or 0.2 mg/mL of ethanol, or aqueous, extract of ginger and incubated for 12, 24, 48, or 72 h at 37°C.

At the end of each treatment, always both floating and adherent cells were collected (taking care that none of the floating cells were lost during washes) and pelleted by centrifugation at 700 g for 5 min. The cells were re-suspended in 25 mL phosphate-buffered saline (PBS), mixed with 5 mL of 0.4% trypan blue solution, and counted using a hemocytometer under an inverted microscope. The effect

of herb on growth inhibition was assessed as percent cell viability, where control-treated cells were taken as 100% viable. All experiments were repeated three times.

2.4. Colony Formation Assays. Clonogenic survival determination assays were done as explained previously [23]. Briefly, log growth phase of MCF-7 and MDA-MB-231 cells was trypsinized and plated onto 6-well plates at initial cell concentrations of 1×10^3 cells/mL. Twenty-four hours later, the medium was removed and fresh medium was added with the different concentrations (0.0, 0.025, 0.05, 0.1, 0.15, and 0.2 mg/mL) of the herbs for 10 days to allow cells to form colonies. The resulting colonies were fixed and stained with 0.5% trypan blue solution for 30 min. The size and number of stained colonies with >50 cells were counted under a dissection microscope. Colony formation was calculated as a percentage to untreated control cultures. Each condition was repeated three times.

2.5. DNA Fragmentation Assay. The detection of DNA ladder in agarose gel electrophoresis is used as a biochemical marker for the measurement of apoptosis. It is used to determine the presence of internucleosomal DNA cleavage. The MCF-7/MDA-MB-231 cells (10^6 cells/100 mm dish) treated with various concentrations of herbs for 24 h were collected, washed in PBS, and purified using a DNA purification kit, DNeasy Blood, and Tissue Kit, from QIAGEN according to the manufacturer's procedure. The DNA was resolved by electrophoresis on 1% agarose gel at 80~100 V. The gel was stained with ethidium bromide, and DNA was visualized by a UV transilluminator (BIO-RAD).

2.6. Apoptotic Assay. The nuclear morphological changes associated with apoptosis were analyzed using DAPI staining. Cells (2×10^4) were plated on coverslips, allowed to attach overnight, and exposed to vehicle or indicated concentrations of herbal extracts for 24 h. The cells were washed with PBS and fixed with 3.7% paraformaldehyde (Sigma-Aldrich, USA) in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) solution for 10 min at room temperature, then washed twice with PBS. Cells with condensed and fragmented DNA (apoptotic cells) were scored under a fluorescence microscope (Carl Zeiss, Germany) at $\times 40$ objective lens magnification.

2.7. Reverse Transcription PCR. The MCF-7 and MDA-MB-231 cells were plated as explained above. Twenty four hours later, the medium was removed and replaced with fresh medium containing indicated concentrations of ethanol extract of the herb for 24 h. Total RNA was extracted, reverse transcribed, and amplified by PCR using QIAamp RNA Blood Mini Kits (QIAGEN) following instructions of the manufacturer.

Expressions of Bcl-2 and Bax were examined by PCR method using gene-specific primers. The PCR was run as the following 25–30 cycles at 95°C for 5 min, 95°C for 20 sec, 59°C for 40 sec, 72°C for 1 min, and a final extension

at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel and stained with EtBr. Bands on the gel were scanned as digitalized images, and the areas under the peaks were calculated by densitometric analysis using a computerized digital imaging system. The areas were reported in arbitrary units. The relative expression level was calculated by comparing the expression level of the sample with that of the internal standard (HPRT). At least a 2-fold decrease in the relative expression level was considered to be reduced, which was verified by repeat RT-PCR assays. Primer sequences of the NF- κ B heterodimers (p50 and p65), Bcl-2, Bcl-xL, Mcl-1, Survivin (BIRC5), I κ B- α , and HPRT1 were described earlier [24], whereas the primer sequences of the Bax were described in a current study [25].

2.8. Western Blot Analysis. The MCF-7 and MDA-MB-231 cells were plated as explained above. Twenty-four hours later, the medium was removed and replaced with fresh medium containing the indicated concentrations of ethanol extract of the herb for 24 h. The cell pellets were washed three times with PBS and lysed in cold lysis buffer containing 0.05 mmol/L Tris-HCl, and 0.15 mmol/L NaCl, 1 mol/L EGTA, 1 mol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na₃VO₄, 0.5% NP40, 1% Triton X-100, 1 mol/L phenylmethylsulfonyl fluoride (pH 7.4) with freshly added Protease Inhibitor Cocktail Set III (Calbiochem).

The lysates were collected and cleared by centrifugation, and the supernatants were aliquoted and stored at -80°C . The protein content in the lysates was measured by BCA protein assay (Pierce, Rockford, IL, USA), according to protocol of the manufacturer. Western blot analysis was carried out as previously described [24]. Briefly, aliquots of the lysates containing the same quantity of proteins were boiled for 5 min in sample buffer, electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membranes. After transfer, the membranes were incubated with primary antibody against tested proteins ($\sim 1:1000$), followed by incubation with a secondary horseradish peroxidase—conjugated antibody ($\sim 1:2000$); purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membranes were developed by the enhanced chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ, USA). The membranes were then imaged and autoradiography using X-ray film (Eastman Kodak). Equal loading of proteins was confirmed by stripping the blots and reprobing with β -actin (Sigma).

2.9. Statistical Analyses. All experiments were performed in triplicates and repeated at least five times and the data were presented as mean \pm SD. Statistical analyses were evaluated by Student's *t*-test. Probability values $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of Ginger Extract on MCF-7 and MDA-MB-231 Cell Survival. First, we determined the effect of ginger extract

on cell survival of human breast cancer cell lines, MCF-7 and MDA-MB-231. The MCF-7 cell line is an estrogen receptor positive and estrogen responsive, while the MDA-MB-231 cell line is estrogen receptor negative and estrogen unresponsive [26]. Both cell types were incubated with increasing concentrations (0.0, 0.025, 0.05, 0.1, 0.15 and 0.2 mg/mL) of ethanol, or aqueous, extract of ginger for 12, 24, 48 and 72 h before being harvested and assayed for cell viability by trypan blue dye exclusion assay. The results are summarized in Figure 1. As seen, the ethanol (Panels a and c) or aqueous (Panels b and d) extract of ginger exhibited a dose- and time-dependent anti-proliferative effect on the cell viability of MCF-7 (Panels a and b) and MDA-MB-231 (Panels c and d). Pair-wise comparison between IC_{50} values of the ethanol versus aqueous extract (a versus b and c versus d) shows that the former had a stronger anti-proliferative potentiality, since, generally, the IC_{50} values indicated in Panels a and c were lower than those in Panels b and d. In addition, the maximum effect of the aqueous extract, in the context of both cell lines, was apparently 50% reduction in cell viability, which has been observed after 72 h of treatment and by the highest dose (0.2 mg/mL) (b and d). On the other hand, the maximum effect detected after 72 h by the same dose of the ethanol extract was nearly 15% (MCF-7) and 22% (MDA-MB-231) reduction in cell viability (a and c).

Next, we addressed the question of whether the cytotoxic effect of ethanol/aqueous extract of ginger is selective toward cancer cells. To this end, we utilized a normal mammary epithelial cell line, MCF-10A. This cell line was originally isolated from fibrocystic breast disease and was spontaneously immortalized; it is nontumorigenic in athymic mice and has been used extensively as a representative normal mammary epithelial cell line [26]. The MCF-10A cells have intact cell cycle checkpoints and normal proliferation controls [26]. MCF-10A cells were incubated with increasing concentrations (0.0, 0.025, 0.05, 0.1, 0.15 and 0.2 mg/mL) of ethanol, or aqueous, extract of ginger and their growth was then monitored for 12, 24, 48 and 72 h. The data depicted in Figure 1 (Panels e and f) demonstrate that the MCF-10A cells are significantly more resistant to growth inhibition by ginger compared with MDA-MB-231 or MCF-7 cells. This is because all IC_{50} values shown in Panel e are higher than those in Panel a/c; likewise all IC_{50} values shown in Panel f are higher than those in Panel b/d. Collectively, these results suggest that the ethanol or aqueous extract of ginger inhibited proliferation of MCF-7 and MDA-MB-231 cells and, under the conditions of these experiments, the former extract has obviously the stronger anti-proliferative potentiality. The human breast cancer cells are significantly more sensitive to growth suppression by ginger compared with a normal mammary epithelial cell line. Since the ethanol extract exhibited the stronger anti-proliferative potentiality, we exploited this extract in further mechanistic studies to elucidate the molecular mechanism underlying cytotoxic effect of ginger.

3.2. Effect of Ginger Extract on Colony Formation in MCF-7 and MDA-MB-231 Cells. Next, the anti-proliferative and

cytotoxic effect of ethanol extract of ginger on MCF-7 and MDA-MB-231 cells were further determined and verified by using anchorage-dependent colony formation assay (also referred to as clonogenicity). This assay measures the ability of tumor cells to grow and form foci in a manner unrestricted by growth contact inhibition as is characteristically found in normal, untransformed cells. As such, clonogenicity provides an indirect assessment of the propensity of tumor cells to undergo neoplastic transformation. As shown in Figure 2, during the 10-day culture period, ginger treatment reduced the number of growing colonies and visibly modulated the size of growing colonies. Clonogenicity of both MCF-7 and MDA-MB-231 cells exhibited 50% inhibition that was clearly evident at 1.0 and 0.2 mg/ml, respectively. Furthermore, more than 95% (MCF-7) and 90% (MDA-MB-231) cells died when cultured with ginger extract at the concentrations of 0.2 mg/ml.

3.3. Apoptotic Effect of Ginger on MCF-7 and MDA-MB-231 Cells.

The above findings demonstrate that ginger has an anti-proliferative impact on MCF-7 and MDA-MB-231 cells, which could be the result of the induction of apoptosis, cell cycle arrest, and/or the inhibition of growth. Since the anti-proliferative effect of many naturally occurring cancer chemopreventive agents is tightly linked to their ability to induce apoptosis [12], we therefore investigated whether ginger could induce apoptosis in breast cancer cells. To characterize the cell death induced by ginger, we examined the morphology of the cells under microscope. The data depicted in Figure 3(a) explain that the treatment of MCF-7 and MDA-MB-231 cells with ginger for 24 h resulted in so obvious cell shrinkage, cellular detachment, and loss of the originally confluent monolayer.

Next, we further ascertained proapoptotic effect of ginger by DAPI assay. As depicted in Figure 3(b), DAPI staining revealed the occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies in MCF-7 and MDA-MB-231 cultures treated with ginger, but not in control cultures. The histogram panels demonstrate that ginger treatment caused a statistically significant increase in apoptotic cells, where the percentage of scored apoptotic cells was increased by ~25- (MCF-7) and 20-folds (MDA-MB-231) upon a 0.1 mg/mL ginger extract treatment and by ~40- (MCF-7) and 30-fold (MDA-MB-231) upon a 0.2 mg/mL of the extract treatment, compared with control-treated cultures.

DNA fragmentation is a hallmark of apoptosis and the detection DNA ladder in agarose gel electrophoresis is commonly used as a biochemical marker for the measurement of apoptosis [27]. Therefore, DNA gel electrophoresis was used to determine the presence of internucleosomal DNA cleavage. The data depicted in Figure 3(c) illustrate that DNA fragmentations were clearly detectable in MCF-7 and MDA-MB-231 cells, at the level of all treated doses of the extract for 24 h.

Currently, there are two known pathways that activate the apoptotic caspase cascade, the intrinsic and extrinsic pathways [9]. Both apoptotic pathways converge downstream

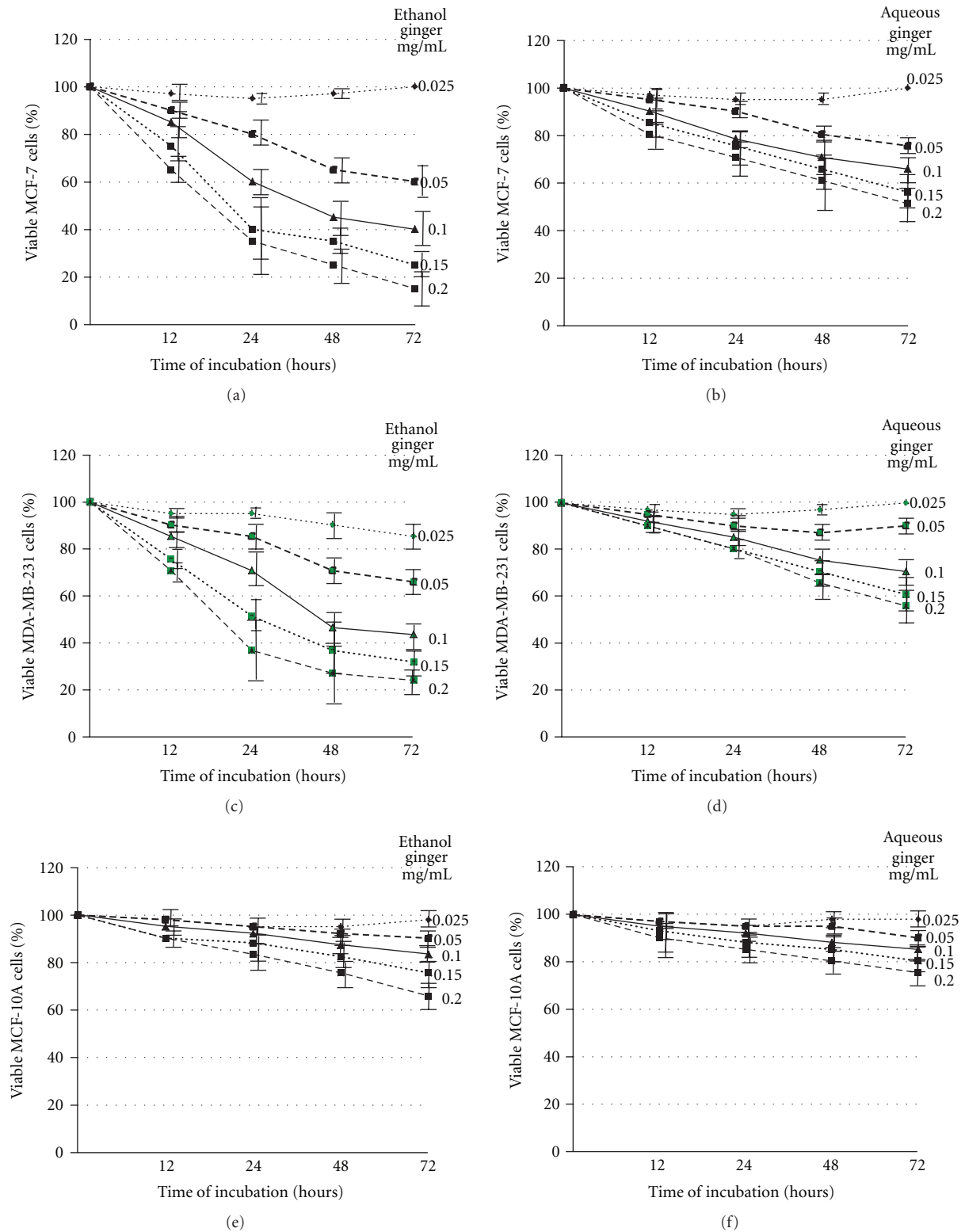


FIGURE 1: Ethanol and aqueous extracts of ginger inhibit proliferation of MCF-7 and MDA-MB-231 cells. MCF-7 (a and b), MDA-MB-231 (c and d), and MCF-10A (e and f) cells were incubated with the indicated concentrations of ethanol (a, c, and e) or aqueous (b, d, and f) extract of ginger for displayed time intervals. The cell viability was measured by trypan blue dye exclusion assay, as described in Materials and Methods. The experiments were repeated five times in triplicates, and cell viabilities at each dose of ginger extracts were expressed in terms of percent of control and reported as the mean \pm SD.

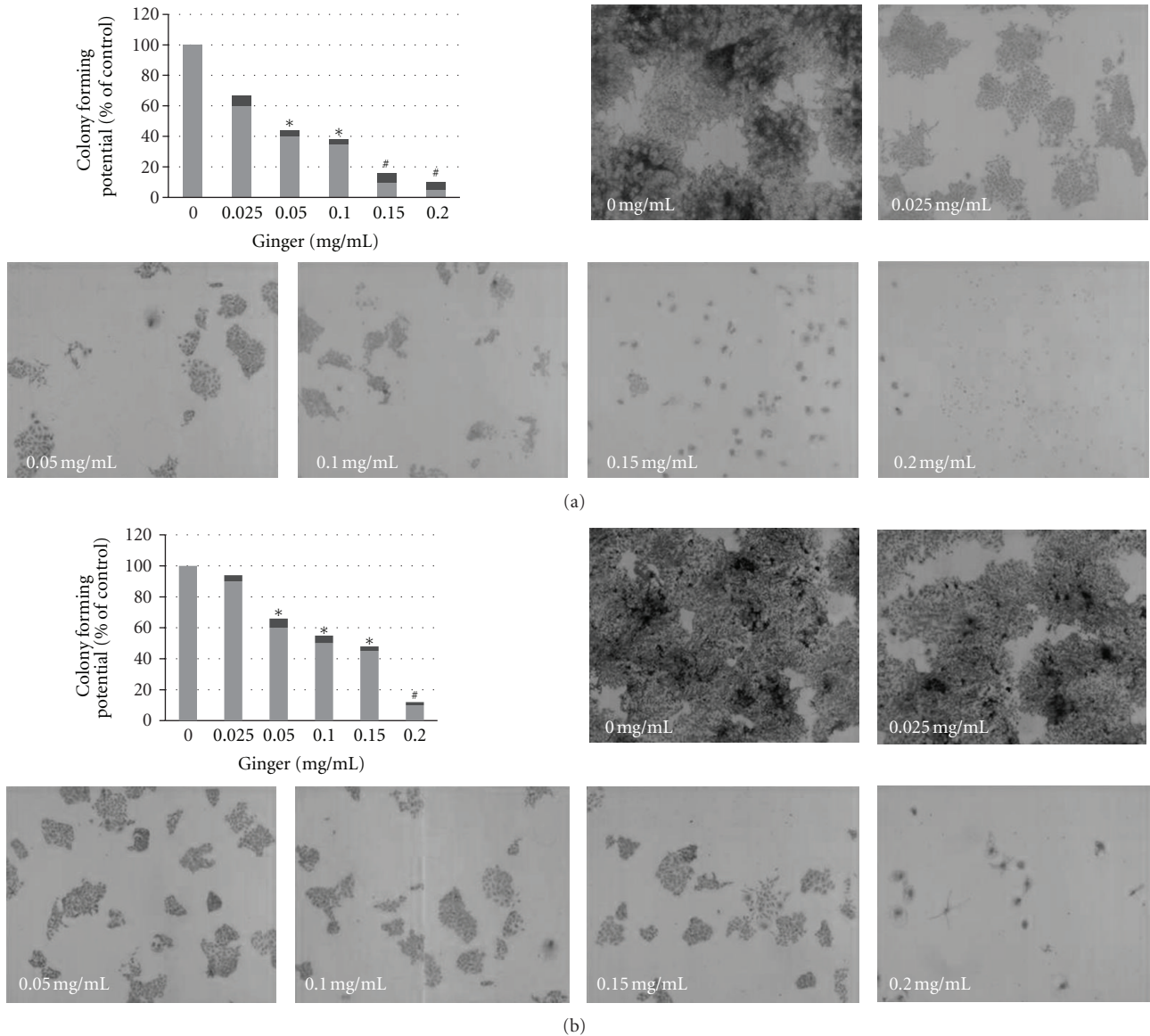


FIGURE 2: Ginger extract inhibits clonogenicity of MCF-7 and MDA-MB-231 cells. Cells were seeded onto a 6-well plate at 300/well and treated with indicated concentrations of the ginger extract of ginger for 10 days. A survival of 100% corresponds to the number of colonies obtained with cells that were not treated with ginger extract (control culture). Dose-dependent colony formation inhibition was clearly evident. The experiment was repeated five times in triplicates, and the colony-forming potential of the cells at each dose of ginger is expressed in terms of percent of control and is reported as the mean \pm SD. $P < 0.05$ (*); $P < 0.001$ (#).

with the activation of caspase-3 to irreversibly commit cells to apoptosis. Therefore, a key event in apoptotic cascades is activation of caspase-3, which normally exists as a 32-kDa inactive precursor. The activated caspase-3 (17-kDa fragment) proteolytically cleaves and activates other downstream caspases and other relevant target molecules in the cytoplasm or nucleus [28]. To this end, to determine whether caspase-3 is involved in apoptosis induced by ginger, the cleavage of procaspase-3 was examined by Western blot analysis. Treatment of MCF-7 and MDA-MB-231 cells with increasing doses of ginger extract caused a dose-dependent proteolytic cleavage of procaspase-3, which was apparent within all applied doses of the ginger extract (Figure 3, Panel

d, Top Gel). Once activated, caspase-3 proteolytically cleaves its endogenous substrate, the 116-kDa PARP (poly(ADP-ribose) polymerase) protein, into an 85-kDa fragment, which is considered to be a biochemical characteristic of apoptosis [29]. Therefore, the Western blot was reprobed with antibody raised against PARP protein. The results depicted in Panel d (Middle Gel) demonstrate that ginger induced the proteolytic cleavage of PARP into its respective active form. All these changes are specific to impact of ginger extract and not fortuitous, due to differences in the amounts of proteins loaded on the gels, since reprobing stripped blots for β -actin (Bottom Gel) demonstrated the equivalent protein loading in all experiments. Taken together, these

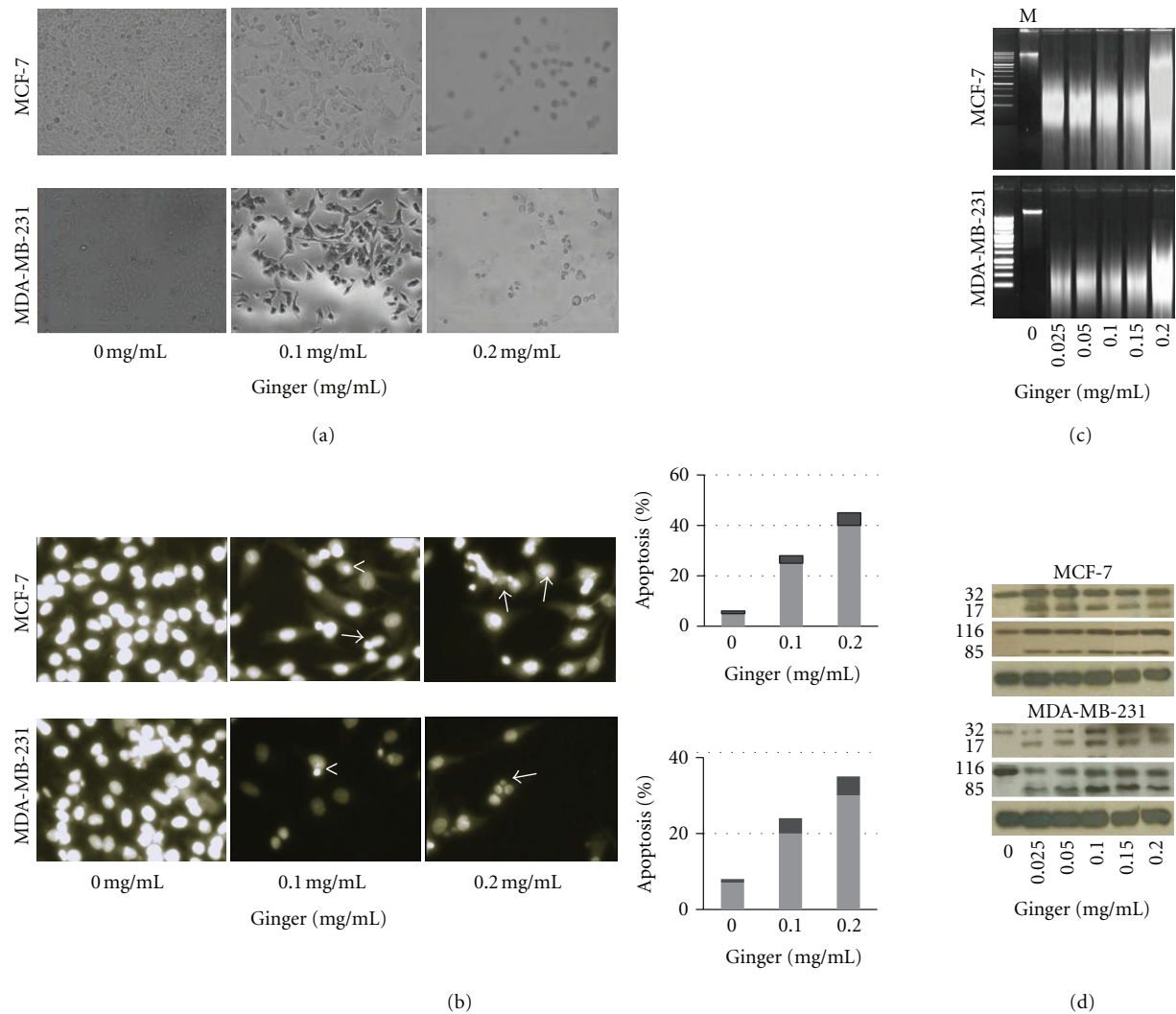


FIGURE 3: Ginger extract induces apoptotic cell death in MCF-7 and MDA-MB-231 cells. (a) Microphotographs showing the inhibitory effect of ginger extract on cellular growth of MCF-7 and MDA-MB-231 cells. Cells were plated onto 6-well plates and treated with indicated concentrations of ginger for 24 h. The photographs were taken directly from culture plates using a phase microscope. Note cell shrinkage, irregularity, and detachment in ginger-treated cultures. Representative pictures are shown from three independent experiments. (b) DAPI staining for MCF-7 and MDA-MB-231 cultures treated with ginger extract for 24 h. Note apoptotic cells with condensed chromatin (<) and fragmented nuclei (→) were clearly visible in ginger-treated, but not in control-treated, cultures. Histogram panels show quantitation of apoptotic cells with condensed chromatin in ginger-extract treated-MCF-7 and MDA-MB-231 cultures. Columns, mean of six to nine determinations. (c) The ginger extract treatment induced DNA fragmentation in MCF-7 and MDA-MB-231 cells. Cells were incubated with indicated concentrations of ginger extract for 24 h. Genomic DNA was isolated and electrophoresed in 1% agarose gel. M: 100 bp DNA ladder marker. (d) The ginger extract treatment increased the cleavage of caspase-3 (upper panel) and PARP (middle panels) in MCF-7 and MDA-MB-231 cells. Cells were treated with indicated concentrations of ginger extract for 24 h; thereafter, cell lysates were prepared and analyzed by Western blot as detailed in Section 2, for detecting the cleavage of caspase-3 and PARP using. Equal loading of samples was confirmed by β -actin (lower panels); representative blots are shown from three independent experiments with almost identical observations.

four independent methods of measuring apoptosis provided similar results, suggesting that the cytotoxic effect observed in response to the ginger extract treatment induces apoptosis in MCF-7 and MDA-MB-231 cells.

3.4. Ginger Treatment Modulates Expression of Apoptosis-Related Genes. Apoptotic cascades are tightly regulated by a variety of factors; among these factors, Bcl-2 protein

family plays central roles in apoptotic events. This family includes anti-apoptotic members (such as Bcl-2) and pro-apoptotic members (such as Bax) [30]. Therefore, Western blot analyses were conducted in order to determine whether ginger extract modulates the expression levels of Bcl-2 (apoptotic inhibitor) and Bax (apoptotic promoter) in MCF-7 and MDA-MB-231 cells. As shown in Figure 4(a), exposure of cells to increasing concentrations of ginger extract for 24 h resulted in a marked decrease in the protein expression of

anti-apoptotic Bcl-2, associated with a concomitant increase in the expression of pro-apoptotic Bax protein, in a dose-dependent manner. To substantiate these data, the mRNA expression levels of Bcl-2 and Bax were examined by RT-PCR. Figure 4(b) showed that ginger treatment decreased the level of Bcl-2 mRNA expression, but increased the level of Bax expression, which resulted in an increase in Bax/Bcl-2 ratio Figure 4(c) in both MCF-7 and MDA MB-231 cells. These findings indicate that the ginger extract mediating upregulation of Bax and downregulation of Bcl-2 might tip the balance toward apoptotic events.

3.5. Ginger Modulates Expression Levels Of The Cell Survival and Cell Cycle Regulators. It is already established that constitutive activation of NF- κ B supports progression of breast cancer to hormone-independent growth [31] and enhances survival of breast cancer cells during the development of tumor adaptive radio-resistance [32]. NF- κ B mediates these activities primarily through upregulation of its downstream targets that negatively regulate apoptosis in tumor cells [33, 34]. In order to define the significance of the expression patterns of NF- κ B-dependent genes as a response to ginger treatment, MCF-7 and MDA-MB-231 cells were treated with increasing concentrations of ginger for 24 h, then the possible alterations in the mRNA expression levels of various apoptosis-related genes were analyzed by RT-PCR using gene-specific primers. We determined expression levels of NF- κ B heterodimers (p50 and p65), the anti-apoptotic proteins, Bcl-xL and Mcl-1, the NF- κ B inhibitor, the I κ B- α and a member of the inhibitors of apoptosis protein family, Survivin. The results depicted in Figure 5(a) revealed that ginger treatment consistently downregulated, in a dose-dependent manner, expression of p50 and p65, Bcl-xL, Mcl-1, and Survivin. On the other hand, ginger treatment resulted in a concomitant increase in expression of the I κ B- α . These findings suggest that ginger treatment altered expression of cell survival genes at transcriptional level.

Several reports suggest that cyclin D1 is overexpressed in breast cancer cells and plays a major role in tumorigenesis [35–37]. Therefore, we analyzed whether ginger modulates the expression of cyclin D1 protein in MCF-7 and MDA-MB-231 cells. Western blot analysis showed that ginger treatment resulted in a moderate to strong decrease in the expression of cyclin D1 (Figure 5(b)) in a dose-dependent manner; specifically, 0.1 and 0.2 mg/mL of ginger treatment showed 50% decrease in the protein level of cyclin D1. Reduction of cyclin D1 level was also accompanied by reduced expression of its regulatory partner, cyclin-dependent kinase-4 (CDK-4). Consistent with changes in the expression of cyclin D1 and CDK-4 proteins, ginger treatment, dose dependently, increased expression of p21, which is a universal inhibitor of CDKs, an important regulator of breast cancer cell proliferation [38] and responsible for the inhibition of MCF-7 cell proliferation by antioestrogens [39]. Next, we assessed the effect of ginger on the expression levels, the c-Myc and human telomerase reverse transcriptase (hTERT) proteins, which are among the most certain and commonly amplified oncogenes in breast cancer [40–42]. The data in

the Figure 5(b) illustrate that ginger treatment decreased, in a dose-dependent manner, the expression levels of the c-Myc and hTERT. Reprobing of the membranes with anti- β -actin antibody confirmed equal protein loading in each case. Collectively, these results suggest that ginger treatment modulated expression levels of the cell survival and cell cycle regulators.

4. Discussion

Ginger (*Zingiber officinale*) has been shown to be an important medicinal plant used in folkloric medicine to cure various diseases in South Asia and the Middle East [17]. Consistent with this notion, the results of the present study indicated that the ethanol extract of ginger potentially inhibited, in a dose- and time-dependent manner, the proliferation of MCF-7 (ER-positive breast cancer cells) and MDA-MB-231 (ER-negative breast cancer cells). The aqueous extract of ginger exhibited, albeit to a less extent, same potentiality. Therefore, our results demonstrate that ginger not only has a powerful inhibitory effect on the proliferation of ER-positive (MCF-7) breast cancer cells but also significantly inhibits the growth of ER-negative ones (MDA-MB-231). This conclusion holds promise for further *in vitro* and *in vivo* molecular target-oriented studies to examine the chemoprotective efficacy of ethanol extract of ginger, particularly for ER-negative breast cancers, which have a poorer prognosis and shorter survival [39]. On the other hand, ginger did not significantly affect viability of normal MCF-10A breast cells, which deserves attention, since selectivity toward cancer cells is a highly advantageous feature of potential cancer chemopreventive and therapeutic agents. Therefore, our ginger extract may selectively target breast cancer cells but save normal cells. The findings herein are consistent too with a recent report cited a crude methanol extract of ginger (*Zingiber officinale*) suppressing the proliferation of MCF-7 and MDA-MB-231 cells [43]. Consistent with its anti-proliferative and cytotoxic effect, ethanol extract of ginger efficiently ablated potentialities of the MCF-7 and MDA-MB-231 cells to form colonies as confirmed by the anchorage-dependent colony formation assay. Since this assay provides an indirect assessment of the propensity of tumor cells to undergo neoplastic transformation, thus, it adds further evidence demonstrating the antineoplastic potentiality of ginger.

Most developed chemotherapeutic agents (e.g., Prodigiosin, Cimicifuga racemosa, Antrodia camphorata, etc.), which originate from natural products exert their anticarcinogenic activities by induction of apoptosis in breast cancer cells [44–46]. Likewise, the anti-proliferative and cytotoxic effects of ginger were linked to its ability to induce apoptosis in MCF-7 and MDA-MB-231 cells for many reasons. First, the mode of cell death was identified as apoptosis by a phase microscopic inspection, which revealed abundance of morphological alterations including a loss of cell viability, cell shrinkage, irregularity in cellular shape, and cellular detachment in ginger-treated MCF-7 and MDA-MB-231 cultures. These morphological alterations are hallmarks of

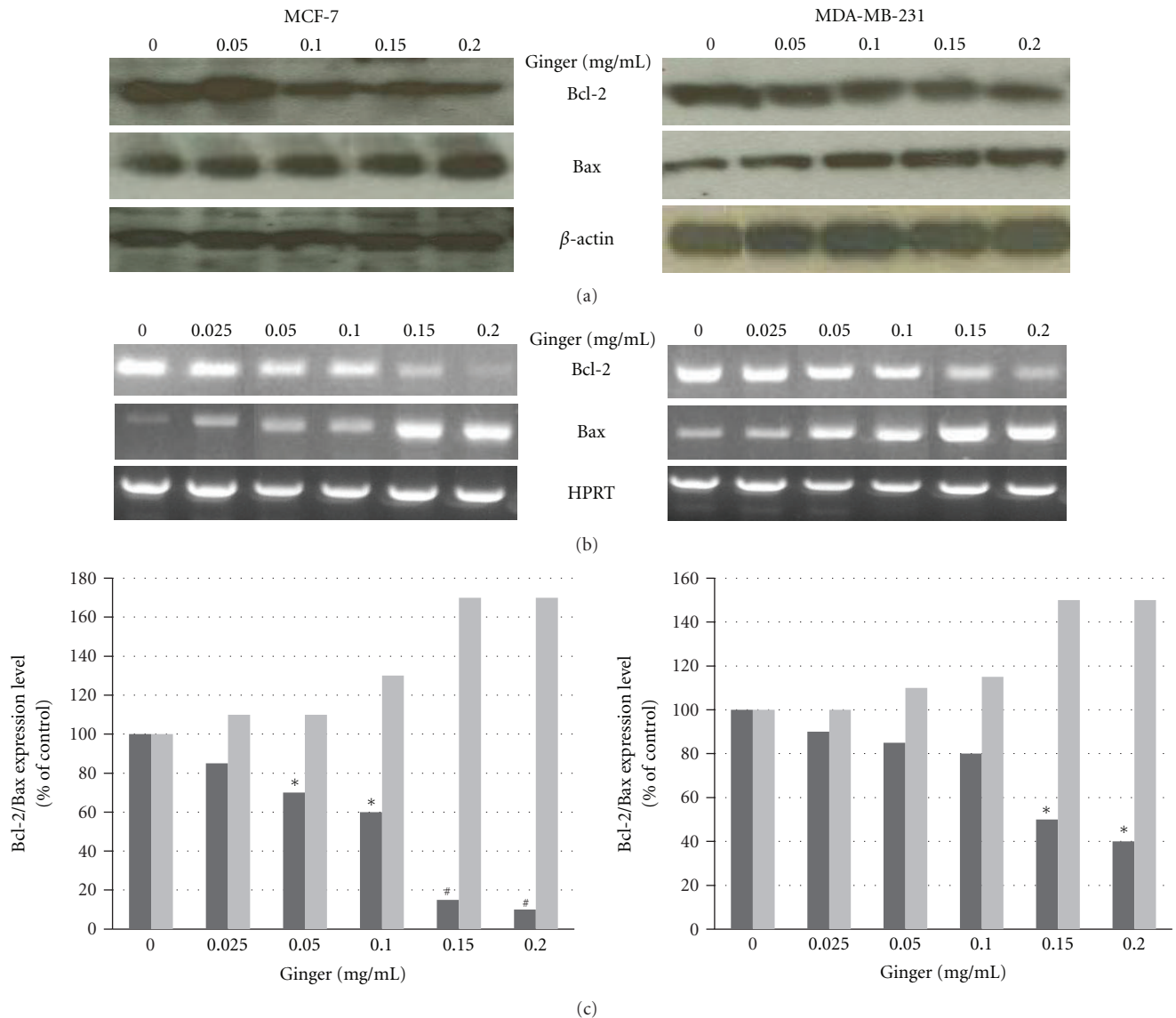


FIGURE 4: Ginger extract modulates expression of the apoptosis-related proteins. The MCF-7 and MDA-MB-231 cells were incubated with the indicated concentrations of the ethanol extract of ginger for 24 h. (a) Cell lysates were prepared and examined for the expression of Bcl-2 and Bax proteins by western blot as described in the Materials and Methods. β -actin was used as the internal control. Representative blots from three independent experiments are shown. (b) The total RNAs were isolated, reverse transcribed, and subjected to PCR with the Bcl-2- and Bax-specific primers. The PCR products were then subjected to electrophoresis in 1% agarose gel and visualized by EtBr staining. HPRT was used as the internal control. A typical result from three independent experiments is shown. (c) The histogram depicts the Bcl-2 (dark bars) and Bax (light bars) mRNA ratio in MCF-7 and MDA-MB-231 cells measured by using densitometric analysis, $P < 0.05$ (*); $P < 0.001$ (#).

cells undergoing apoptotic cell death [47]. Second, DAPI staining revealed the occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies in ginger-treated MCF-7 and MDA-MB-231 cultures. Third, fragmentation of DNA was isolated from ginger-treated cells. DNA fragmentation has been observed in cells undergoing apoptosis and is widely used as biochemical markers of apoptosis [27]. Finally, treatment of cells with ginger resulted in proteolytic cleavage both of procaspase-3 into its active form (a 17-kDa fragment) and a DNA repair enzyme, PARP,

into an 85-kDa fragment. These cleavages occur at the onset of apoptosis and considered to be biochemical markers of apoptosis [47]. Collectively, these findings demonstrated that the cytotoxic effect observed in response to the ginger treatment is associated with the induction of apoptosis in the breast cancer cell lines.

It is documented that the Bcl-2 family proteins play an important regulatory role in apoptosis and are considered as a target for anticancer therapy [48]. Of the Bcl-2 family members, the Bcl-2 and Bax protein ratio has been

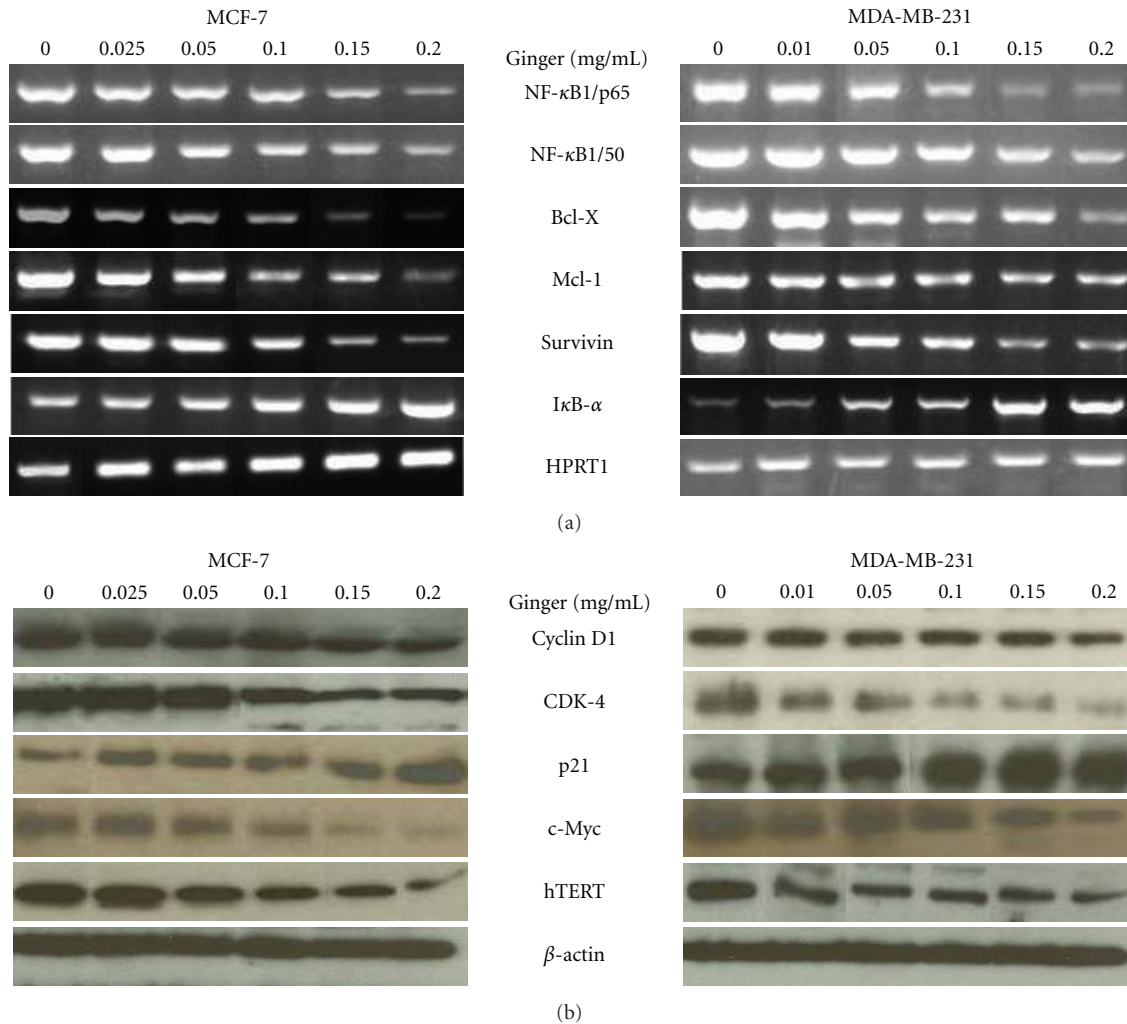


FIGURE 5: Ginger extract modulates expression levels of the cell survival and cell-cycle regulators. The MCF-7 and MDA-MB-231 cells were incubated with the indicated concentrations of the ethanol extract of ginger for 24 h, then cells were harvested and assayed. (a) The total RNAs were isolated, reverse transcribed, and subjected to PCR with the gene-specific primers. The PCR products of the depicted genes were then subjected to electrophoresis in 1% agarose gel and visualized by EtBr staining. HPRT1 was used as the internal control. A typical result from three independent experiments is shown. (b) Cell lysates were examined for the displayed proteins by western blot as described in the Materials and methods. β -actin was used as the internal control. Representative blots from three independent experiments are shown.

recognized as a key factor in regulation of the apoptotic process, where the ratio of Bax:Bcl-2 proteins increases during apoptosis [49]. The results herein displayed that ginger extract obviously decreased expression levels of Bcl-2 mRNA and protein and increased those for Bax. This modulation would result in increased ratio of Bax to Bcl-2 proteins, which might underscore the induction of apoptosis by ginger in MCF-7 and MDA-MB-231 cells. However, how ginger modulated expression of these proteins at levels of transcription and translation is not clear at the moment.

There is considerable evidence that NF- κ B is constitutively active in breast cancer and its activation is correlated to tumor progression [31]. Moreover, it is one of the major antiapoptotic signal transduction pathways that confer radioresistance to breast carcinoma cells [32]. The peculiarity of NF- κ B proteins is that they are ubiquitously

and constitutively expressed, whilst their activities (nuclear translocation/DNA-binding) are rapidly and transiently regulated [50]. Some purified components derived from ginger have been found to inhibit nuclear translocation [51] or DNA-binding activity [52] of NF- κ B. Nonetheless, a growing number of studies explain that expression levels of the NF- κ B subunits might be modulated by phytochemical agents. For example, couarmin [53], flavonoid quercetin [54], benzyl isothiocyanate [55] and isoflavone genistein [56] all have been confirmed to exert their anti-apoptotic activity through downregulation expression of NF- κ B heterodimers (p50 and p65). The results herein explain that ginger treatment may target NF- κ B, through downregulation constitutive mRNA expression of the NF- κ B heterodimers (p50 and p65). Therefore, these findings are in accord with these previous reports and explain that ginger treatment may mediate

activation of some mediators leading to downregulation of NF- κ B expression. To the best of our knowledge, this is the first study demonstrating that ginger targets NF- κ B at transcriptional level. However, whether decreased expression level of the mRNAs is due to transcriptional downregulation, mRNA stabilization, or both remains to be determined. Consistent with the data showing downregulation of NF- κ B expression, ginger treatment downregulated expression of genes known to be NF- κ B effectors and have prosurvival role, such as Bcl-X, Mcl-1, and Survivin [50]. On the other hand, it upregulated expression of the NF- κ B inhibitor, the I κ B- α . Therefore, it is tempting to speculate that ginger treatment could effectively target survival advantage rendered by NF- κ B, as well as NF- κ B-downstream effectors, which could sensitize MCF-7 and MDA-MB-231 cells to apoptosis induction.

It is well established that a single, most defining feature of cancer is uncontrolled growth [8]. Cell-cycle progression is tightly controlled by a network of cyclins and cyclin-dependent kinases complexes, and deregulated expression of these complexes frequently impacts on breast cancer outcome, including response to therapy [57]. In particular, cyclin D1 has been found to be overexpressed in up to 50% of human breast cancers, acting as a driving force in human breast cancer and playing an essential role in malignant transformation of breast cancers induced by other oncogenes [58]. Therefore, cyclin D1 is a potential target for the rational design of new drugs to prevent/treat breast cancer. Indeed, it has been cited that phytochemicals prevented breast cancer cell growth via downregulation of cyclin D1 [59–61]. Consistent with these reports, ginger treatment decreased the protein expression of cyclin D1 and CDK-4, which raises a possibility that the anti-proliferative effects of ginger may be due to inhibition of cyclin D1-CDK-4 complex expression. On the other hand, ginger treatment upregulated expression of p21, which is a universal inhibitor of CDKs and involved in the regulation of cell cycle restriction points [57]. Furthermore, numerous studies demonstrated that the p21 is responsible for the inhibition of various cancerous cell lines [59], as well as breast cancer cells [62], by dietary agents. Taken as a whole, these results suggest that ginger treatment may suppress cell-cycle progression in MCF-7 and MDA-MB-231 cells, at least in part, through the downregulation of the cyclin D/CDK4 expression and the induction of the p21 protein.

The hTERT plays an essential role in the immortalization of most cancer cells [63] and in progression of breast cancer [64, 65]. In addition, it has been cited that the regulation of apoptosis is correlated with the telomerase activity and Bcl-2 expression, where downregulation of Bcl-2 and telomerase is considered to play a role in drug-induced apoptosis [66–68]. Myc is a master regulator in a number of cellular pathways [69] and is found at elevated levels in most cancers [70]. It plays essential role in initiation of carcinogenesis [71] and in development of breast cancer [72]. The hTERT promoter contains binding sites for c-Myc, and there evidence that c-Myc may increase hTERT expression [73–75]. Recently, it has been demonstrated that the ethyl acetate fraction of ginger extract inhibited growth of A549 lung carcinoma cells through downregulation of

hTERT and c-Myc expression [76]. The findings in this study directly recapitulate these earlier studies, since treatment of MCF-7 and MDA MB-231 cells with ginger extract resulted in a significant reduction in the protein level of Bcl-2, which coincided with a parallel reduction in the protein levels of c-Myc and hTERT. Therefore, it is tempting to speculate that, at least in part, the decreased expression of Bcl-2, c-Myc and hTERT by ginger could be candidate mechanism underlies induction of apoptosis in MCF-7 and MDA MB-231 cells, through a telomerase inhibitory pathway. Related to this, it has been suggested that telomerase inhibition is useful in chemoprevention after chemotherapy treatment [77]; since the ginger extract used in this study downregulated hTERT, ginger might prove to be beneficial as a complementary agent in cancer prevention.

In conclusion, our findings underscore that ethanol extract of ginger suppressed, in a dose- and time-dependent manner, the proliferation and colony formation in the breast cancer cell lines, MCF-7 and MDA-MB-231. The anti-proliferative potentiality of ginger could be attributed to its induction of apoptosis through increasing Bax/Bcl-2 ratio. Ginger-dependent growth-inhibitory mechanisms may involve, at least in part, the downregulation of key cellular molecules including NF- κ B, Bcl-X, Mcl-1, Survivin, cyclin D1, CDK-4, c-Myc, and hTERT and upregulation of the I- κ B- α and p21. As both c-Myc and hTERT inhibition are cancer-specific targets for cancer therapy, ginger extract might prove to be an ideal chemopreventive or therapeutic agent for breast cancer.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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