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Full Length Research Paper

Potential anticancer activity of the medicinal herb, *Rhazya stricta*, against human breast cancer

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Breast cancer is a global health problem being the most common and the leading cause of cancer death among women, which mandates the development of novel strategies for its treatment and prevention. *Rhazya stricta* Decne (Harmal) is an important medicinal plant used in traditional oriental medicine. It has anti-oxidant, anti-carcinogenic, and free radical scavenging properties. Our purpose was to confirm the potential anticancer activity of Harmal against human breast cancer cells *in vitro* and to elucidate the mechanism of its activity. We found that ethanol extract of Harmal potently inhibited cellular growth and colony formation of human breast cancer cell lines, MCF-7 and MDA-MB-231, in a dose- and time-dependent manner. Furthermore, it induced sequences of events marked by apoptosis, accompanied by a loss of cell viability, chromatin condensation, DNA fragmentation and proteolytic cleavage of poly (ADP-ribose) polymerase. Harmal-dependent apoptotic mechanisms involved an increase in the Bax/Bcl-2 ratio and down-regulation of all c-myc, human telomerase reverse transcriptase, and cyclin D1 proteins. From these results, we conclude that Harmal exerts antiproliferative action on breast cancer cells through apoptosis induction, and that it may be a potentially effective chemopreventive or therapeutic agent against breast cancer.

Key words: Chemoprevention, medicinal plants, breast cancers, apoptosis, cell cycle.

INTRODUCTION

Among women worldwide, breast cancer is the most common cause of cancer death. The latest statistics indicated that about 1.3 million women are diagnosed with breast cancer annually worldwide and about 465,000 die from the disease (Elangovan et al., 2008). Despite the fact that many tumors initially respond to chemotherapy, breast cancer cells can subsequently survive and gain resistance to the treatment (Campbell et al., 2001). Thus, there is a great need for new alternative agents for the prevention and treatment of breast cancers, especially for refractory phenotypes.

The elucidation and the molecular mechanisms underlying neoplastic transformation and progression have resulted in the understanding that breast cancer can be regarded as a genetic disease, which evolved from the accumulation of a series of acquired genetic lesions (Stoff-Khalili et al., 2006). These genetic lesions lead to inactivation of tumor suppressor genes and/or activation of oncogenes (Stoff-Khalili et al., 2006; Tan et al., 2008; Schlotter et al., 2008). An example of oncogene activation in breast cancer is that of c-Myc (Liao and Dickson, 2000), which is seen amplified and over-expressed in 15 to 25% of breast tumors and, in some series, has been associated with a worse prognosis or more aggressive clinical features (Osborne et al., 2004). Furthermore, c-Myc has been found to play a role in hormone responsiveness and chemotherapy resistance (Osborne et al., 2004). Another oncogene found to be highly active in breast cancer is telomerase, an enzyme

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Abbreviations: PARP, Poly (ADP-ribose) polymerase; **hTERT,** human telomerase reverse transcriptase; **RT-PCR,** reverse transcription-polymerase chain reaction; **HPRT1,** hypoxanthine-guanine phosphoribosyl transferase; **IC_{50},** 50% inhibitory concentration.

responsible for maintenance of the length of telomeres (Baykal et al., 2004; Herbert et al., 2001).

Increased expression of telomerase has been demonstrated to be strongly associated with neoplastic growth and its expression in cancer cells is a necessary step for tumor progression (Newbold, 2002; Kim et al., 1994). Moreover, the catalytic subunit of human telomerase reverse transcriptase (hTERT) has been found to be highly expressed in over 90% of breast cancer tumors, but the expression is low in normal breast tissue (Baykal et al., 2004; Herbert et al., 2001). Therefore, the development of agents having activity against telomerase has been found to be a productive approach to develop novel breast cancer therapies (Carey et al., 1999; Mokbel, 2000; Ahmed and Tollefsbol, 2003).

Similar to c-Myc and hTERT, cyclin D1 overexpression is found in more than 50% of human breast cancers and causes mammary cancer in transgenic mice (Arnold and Papanikolaou, 2005). Targeted deletion of the gene encoding cyclin D1 demonstrated an essential role in normal mammary gland development and defined a functional role for cyclin D1 overexpression in human breast cancer (Sutherland and Musgrove, 2002). Therefore, the development of agents having activity against c-Myc, telomerase and cyclin D1 may be a productive approach to develop novel breast cancer therapies and should be vigorously pursued.

Apoptosis or programmed cell death is an essential physiological process that plays a critical role in controlling the number of cells in development throughout an organism's life by removal of cells at the appropriate time. It is a strictly regulated pathway responsible for the ordered removal of superfluous, aged, and damaged cells (Reed et al., 2000). Morphological hallmarks of this process includes loss of cell volume, hyperactivity of the plasma membrane, and condensation of peripheral heterochromatin, followed by cleavage of the nucleus and cytoplasm into multiple membrane-enclosed bodies containing chromatin fragments (Sarastea and Pulkkic, 2000). Recently, the relationship between apoptosis and cancer has been emphasized, with increasing evidence suggesting that the related processes of neoplastic transformation, progression, and metastasis, involve the alteration of normal apoptotic pathways (Wong, 2011). Indeed, many studies strongly suggested that the loss of apoptotic control in favor of cell proliferation is responsible for breast cancer initiation and progression (Wu, 1996). In turn, one essential strategy for cancer therapy is to target the lesions that suppress apoptosis in the tumor cells. Consistent with this notion, it has been found that many cancer chemotherapy drugs exert anticancer effects on malignant cells (and breast cancer) by inducing apoptosis (Candi et al., 1995; Chien and Pihie, 2003).

Medicinal plants are excellent sources of new drug candidates and are gaining increasing momentum for cancer therapy (Da Rocha et al., 2001; Gupta et al., 2010). Therefore, the target of much current research has been the discovery of natural compounds that can be used in the prevention and/or treatment of cancer. Harmal (*Rhazya stricta* Decne), a member of the Apocynaceae family, is an important medicinal species used in indigenous medicinal herbal drugs to cure various diseases in South Asia and the Middle East (Gilani et al., 2007). The chemical constituents of the herb with known pharmacological activities have not yet been compiled, but some were reviewed (Gilani et al., 2007; Ali et al., 2000). Extract of Harmal leaves is prescribed in folkloric medicine for the treatment of various disorders such as diabetes, sore throat, helminthesis, inflammatory conditions and rheumatism (Ali et al., 1995, 1998). We found that the aqueous extract of Harmal leaves had genotoxic effect detected by cytogenetical and molecular assays against wide range of cell types including *Saccharomyces cervisae* (Baeshen et al., 2005), *Aspergillus terreus* (Baeshen et al., 2008), *Allium cepa* root tip meristem (Baeshen et al., 2008) and the primary culture of human lymphocytes (Baeshen et al., 2009). The last two studies (Baeshen et al., 2008, 2009) demonstrated pyknosis in *Allium* and necrosis in human lymphocytes which is considered indicative of anticancer activities. Additionally, the extract significantly decreased concentrations of triglycerides, low density lipoprotein, cholesterol, uric acid and creatinine, without affecting liver or kidney functions in rats (Baeshen et al., 2010). Finally, Iqbal et al. (2006) proved that Harmal may be a good source of antioxidants. This fact deserves attention since considerable laboratory evidences from chemical, cell culture, and animal studies indicate that antioxidants may slow or possibly prevent the development of cancer (Kaefer and Milner, 2008; Liu, 2004). Consistent with this, Mukhopadhyay et al. (1981) demonstrated the anticancer effects of the indole alkaloids in Harmal. Based on the preceding information and lack of any reported scientific data elucidating the role of Harmal in prevention of human breast cancers, we examined the impact of Harmal extracts on growth of breast cancer.

MATERIALS AND METHODS

Herbal material and extract preparation

The herb used in this study is *R. stricta*; it was collected from a nearby area of Jeddah, Kingdom of Saudi Arabia (KSA). The species was identified and authenticated to the genus and specie level by Professor Nabih A. Baeshen (Department Biology, Faculty of Science, King Abdulaziz University, Jeddah, KSA). The herb was collected either randomly or by following leads supplied by folk medicine-healers in geographical areas where the plants are found. Collection sites were chosen to reflect different ecological zones and diversity of species. The freshly picked parts of the herb were air-dried at room temperature for 2 weeks, with no direct sunlight. Once dried, the herb was ground and stored at -20°C. Ground herb was soaked in water (aqueous extract) or 70% ethanol (ethanol extract) for 24 h, then, the mixtures were filtered and passed sequentially through a 0.22 µm filter sterilization and kept in aliquots at 4°C. Before use, the stock (100 g/1000 ml) was further diluted in

tissue culture media to give the final indicated concentrations.

Cell culture

The human mammary carcinoma cells, MCF-7 and MDA-MB-231, were obtained from King Fahd Center for Medical Research, King Abdulaziz University, Kingdom of Saudi Arabia. The 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% CO_2 and 95% air at 37°C. The cells were subcultured at 3 to 4 days interval. They were seeded at a density of 5×10^4 cells/ml and passaged by washing the mono-layers 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. Cells growing at the exponential phase were used to perform all the experiments.

Cell viability and colony formation assays

The dose-dependent effects of the Harmal on the viability of the MCF-7/MDA-MB-231 cells were determined by trypan blue dye exclusion assay. Early log phase cells were trypsinized and re-grown 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 later, the medium was removed and replaced with fresh medium containing indicated concentrations of the aqueous/ethanol herbal extract for the indicated time intervals. At end of these treatment intervals, 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 harmal on growth inhibition was assessed as percent cell viability, where distilled vehicle-treated (control) cells were taken as 100% viable. For these studies, all experiments were repeated three or more times.

For clonogenic survival determination, log growth phase cells were trypsinized and plated onto 6-well plates at initial cell concentrations of 1×10^3 cells/ml for both MCF-7 and MDA-MB-231 cells. Twenty-four hours later, the medium was removed and fresh medium was added with the indicated concentrations of the ethanol Harmal extract for 10 days to allow cells to form colonies. The resulting colonies were stained with 0.4% trypan blue solution for 30 min and counted. Colonies with >50 cells were counted under a dissection microscope. Colony formation was calculated as a percentage of untreated control cultures. Each condition was repeated in duplicates.

DNA fragmentation assay

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. Therefore, DNA gel electrophoresis was used to determine the presence of internucleosomal DNA cleavage. Briefly, the MCF-7/MDA-MB-231 cells (10^6 cells/100 mm dish) was treated with the indicated concentrations of the ethanol Harmal extract for 24 h; then, cells were collected, washed in PBS and purified using a DNA purification kit, DNeasy Blood and Tissue Kit, from QIAGEN according to the manufacturer's recommendations. The DNA was resolved by electrophoresis on 1% agarose gel. After electrophoresis at 80 ~ 100 V, the gel was stained with ethidium bromide, and DNA was visualized by a UV trans-illuminator (BIO-RAD).

Apoptotic assay

The nuclear morphological changes associated with apoptosis was analyzed using DAPI staining. Briefly, cells (2×10^4) were plated on coverslips, allowed to attach overnight, and exposed to indicated concentrations of the ethanol Harmal extract 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. The cells were washed two more times with PBS and analyzed; the cells with condensed and fragmented DNA (apoptotic cells) were scored under a fluorescence microscope (Carl Zeiss, Germany) at ×40 objective lens magnification.

Reverse transcription-PCR

The MCF-7 and MDA-MB-231 cells were plated as earlier explained. Twenty-four hours later, the medium was removed and replaced with fresh medium containing the indicated concentrations of the ethanol Harmal extract for 24 h. 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. Total RNA was extracted, reverse transcribed and amplified by PCR using QIAamp® RNA Blood Mini Kits (QIAGEN) following the manufacturer's instructions. Expressions of Bcl-2, Bax, c-*myc*, cyclin D1 and hTERT were examined by PCR method using gene-specific primers. The sequences of the forward and reverse primers for Bax were 5'-ACCAAGAAGCTGAGCGAGTGTC-3' and 5'-AGAAAGATGGTCACGGTCTGCC-3', respectively. The sequences of the forward and reverse primers for hTERT were 5'-CGGAAGAGTGTCTGGIGCAA-3' and 5'-GGATGAAGCGGAGT-CTGGA-3', respectively. The sequences of other primers were previously described (El-Kady et al., 2011). The PCR was run as the follows, 25 to 30 cycles at 95°C for 5 min, 95°C for 20 s, 59°C for 40 s, 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were analyzed on a 1.0% 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 as previously desribed (El-Kady et al., 2011). The areas are reported in arbitrary units. The relative expression level was calculated by comparing the expression level of the sample with that of the internal standard (HPRT1). At least a 2-fold decrease in the relative expression level was considered to be reduced, which was verified by repeat RT-PCR assays.

Western blot analysis

The MCF-7 and MDA-MB-231 cells were plated as afore explained. Twenty-four hours later, the medium was removed and replaced with fresh medium containing indicated concentrations of the ethanol harmal extract for 24 h. 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 cell pellets were washed three times with PBS and lysed in cold lysis buffer containing 0.05 mmol/l Tris-HCl, 0.15 mmol/l NaCl, 1 mol/l EGTA, 1 mol/l EDTA, 20 mmol/l NaF, 100 mmol/l Na_3VO_4, 0.5% NP40, 1% Triton X-100, and 1 mol/l phenylmethylsulfonyl fluoride (pH 7.4) with freshly added protease inhibitor cocktail (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), as per the manufacturers protocol. The Western blot analysis was performed essentially as previously described (El-Kady et al., 2011). Briefly, equal amounts of protein (20 µg per lane) from the treated cells were loaded and electrophoresed on a 10% SDS–polyacrylamide gel and then transferred onto PVDF membranes. After transfer, the membranes were blocked and incubated with the primary antibodies (Spring Bioscience) against tested proteins, followed by incubation with secondary horseradish peroxidase–conjugated antibodies (Spring Bioscience). The membranes were developed by the enhanced chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ). Next, the membranes were imaged and autoradiograped, using X-ray film (Eastman Kodak). Equal loading of proteins was confirmed by stripping the blots and re-probing with *β*-actin (Spring Bioscience).

Statistical analyses

All values were expressed as the mean ± S.D. Statistical analyses were evaluated by Student's *t*-test. Probability values $P < 0.05$ were considered statistically significant.

RESULTS

Harmal extract inhibits cellular growth

First, we determined the effect of Harmal extract on cell survival using a pair of well-characterized human breast cancer cell lines (MCF-7 and MDA-MB-231) as model. The MCF-7 cell line is an estrogen receptor positive, and estrogen responsive, and it was isolated from a pleural effusion of stage IV invasive ductal carcinoma. The MCF-7 cells are aneuploid with high chromosomal instability, partially defective for the G1 and mitotic spindle checkpoint and expresses normal p53 (Morse et al., 2005). The MDA-MB-231 cell line, which was originally derived from a stage IV invasive ductal carcinoma, is estrogen receptor negative, partially proficient for all cell cycle checkpoints, and expresses mutant p53 (Morse et al., 2005). The MCF-7 and MDA-MB-231 cells were incubated with increasing concentrations (0.05, 0.1, 0.2, 0.4 and 0.5 mg/ml) with either ethanol or aqueous extract of Harmal for 12, 24, 48 and 72 h before being harvested and cell viability was determined by trypan blue dye exclusion assay, as explained earlier. The results are summarized in Figure 1. Principally, the high concentration of the herbal complex induced lower cell viability; a relatively early inhibition was observed 12 h post-harmal treatment and ethanol extract was relatively more effective than aqueous one against proliferation of both cell types. This is because the IC_{50} (IC_{50} is the concentration of test compound that inhibits 50% of the cell growth) values of the ethanol extract for both cell types were seen as early as 24 h (nearly 18 h), and were lower than those of the aqueous extract. For example, the highest dose (0.5 mg/ml) of the ethanol extract decreased cell proliferation, in both cell types, to 35% back to its original value. On the other hand, the same dose of the aqueous extract decreased cell proliferation to 35% back to its original value after 24 (MCF-7) and 36 h (MDA MB-231). In addition, the 0.5 mg/ml of the ethanol extract decreased cell viability of MCF-7 and MDA-MB-231 cells down to 15 and 22%, respectively after 72 h. Meanwhile, the same dose of the aqueous extract reduced cell viabilities down to 35 (MCF-7) and 40% (MDA MB-231). Similarly, the IC_{50}% for 0.4 µg/ml of the ethanol extract appeared around 20 (MCF-7) and 24 h (MDA-MB-231). Meanwhile, the IC_{50} values for the medium dose (0.2 mg/ml) became visually noticeable barely before 48 h. Finally, the lowest doses (0.05 and 0.1 mg/ml) exhibited less cytotoxic effect.

When we monitored the effect of the aqueous extract of Harmal on growth of both cell types, we got parallel findings. Nonetheless, the effect of this extract was relatively less than that of the ethanol extract in this assay, since, whereas the IC_{50} value of the 0.5 mg/ml was around 24 h after treatment for both cell types, the IC_{50} value of 0.4 mg/ml inhibition became visually apparent only after 40 h. The lower doses (0.05, 0.1 and 0.2 mg/ml) were less effective to show IC_{50} over 72 h of treatment.

To further validate findings of the breast cell lines, we examined if the Harmal treatment was able to trigger similar growth-suppressive effects on human cervical carcinoma cell line, *HeLa*. For this purpose, we repeated the aforementioned experiments using *HeLa* cells; as demonstrated in Figure 1 (lower panels C), treatment of *HeLa* cells with either extract resulted in a significant reduction in cell viability in a concentration-dependent manner. This is because the IC_{50} values for 0.1 mg/ml of the ethanol, as well as aqueous, were 60 and 72 h, respectively. In addition, the 0.5 mg/ml of ethanol Harmal inhibited the proliferation of *HeLa* cells by 90% at 72 h. Overall, these observations indicated that ethanol and aqueous extracts of Harmal possess identical cytotoxic effects against both breast cell lines and *HeLa* carcinoma cells. Since the ethanol extract of Harmal was obviously more active than aqueous one in these assays, and we were interested in investigating the molecular mechanism underlying cytotoxic effects of Harmal, we decided to focus our study on the ethanol extract for further mechanistic studies.

Harmal extract suppresses colony formation in MCF-7 and MDA-MB-231 cells

Next, the anti-proliferative and cytotoxic effect of ethanol extract of Harmal 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

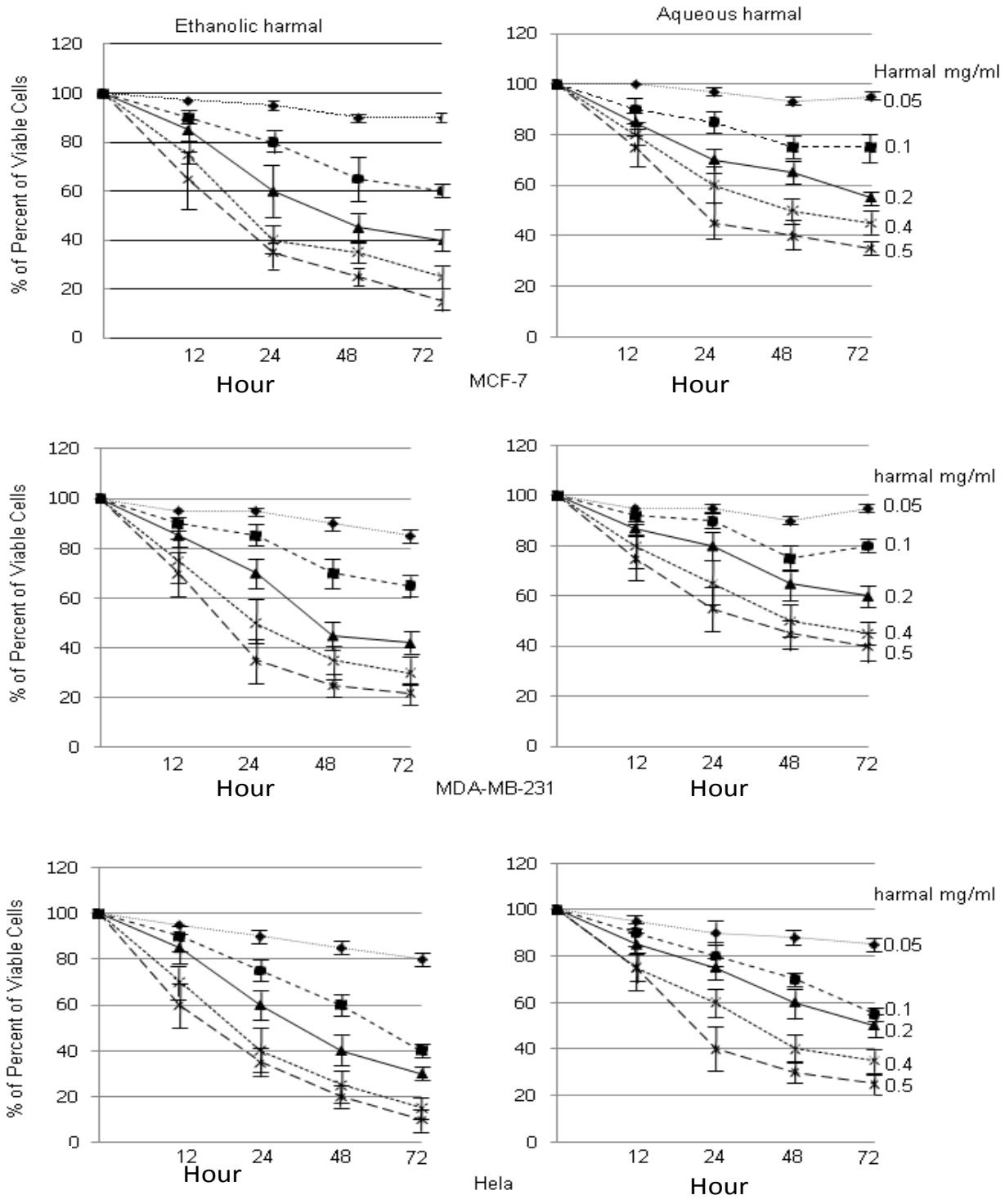

Figure 1. Dose- and time-dependent suppressive effect of harmal on cellular growth of MCF-7, MDA-MB-231 and *HeLa* cells. Each cell line ($10x10^4$ cells/ml) was incubated with indicated concentrations of ethanol/aqueous extract of harmal for indicated time intervals. The cell viability was measured by trypan bludescribed in Materials and methods. Results are presented as means ± SD of independent experiments performed in triplice dye exclusion assay, as ate.

provides an indirect assessment of the propensity of tumor cells to undergo neoplastic transformation. To measure clonogenicity, MCF-7 and MDA-MB-231 cells were plated onto multiple well tissue culture dishes and incubated with 0.0, 0.05, 0.1, .2, 0.4 and 0.5 mg/ml of ethanol extract of Harmal. Control and treated cultures

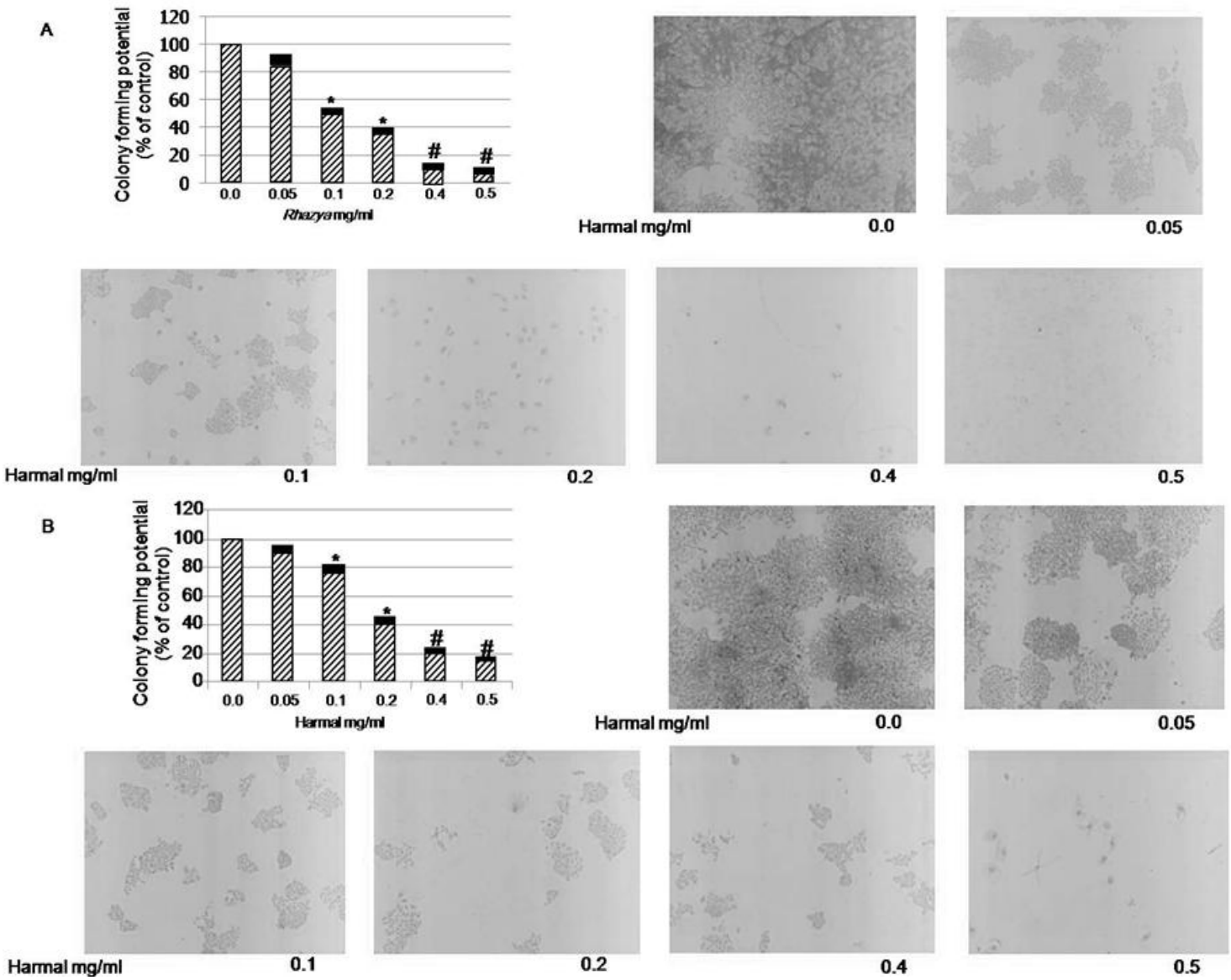

Figure 2. Effects of Harmal on clonogenicity of MCF-7 and MDA-MB-231 cells. Cells were seeded onto a 6-well plate at 300/well and treated with indicated concentrations of the ethanol extract of harmal for 10 days. Colonies were counted under a dissection microscope. A survival of 100% corresponds to the number of colonies obtained with cells that were not treated with harmal. Dose-dependent colony formation inhibition was found. The experiment was repeated three times, and the colony forming potential of the cells at each dose of harmal is expressed in terms of percent of control and is reported as the mean ± SD. $P<0.05$ (*); $P<0.001$ (#).

were maintained in culture for an additional 10 days to allow formation of colonies. Then, size and number of colonies were visually inspected by fixing and staining in 0.5% trypan blue. As shown in Figure 2, during the 10-day culture period, Harmal treatment did not only reduce the number of growing colonies, but it also 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 Harmal at the concentrations of 0.5 mg/ml.

Harmal treatment induces apoptosis in MCF-7 and MDA-MB-231 cells

Having established the growth-suppressive effect of Harmal, we raised the question of whether the Harmal-mediated suppression of MCF-7 and MDA-MB-231 cell viability was due to apoptosis induction. This is because the anti-proliferative effect of many naturally occurring cancer chemopreventive agents is tightly linked to their ability to cause apoptosis (Khan et al., 2007). To address this question, we investigated the morphology of the cells under microscope, which is a well-accepted technique for

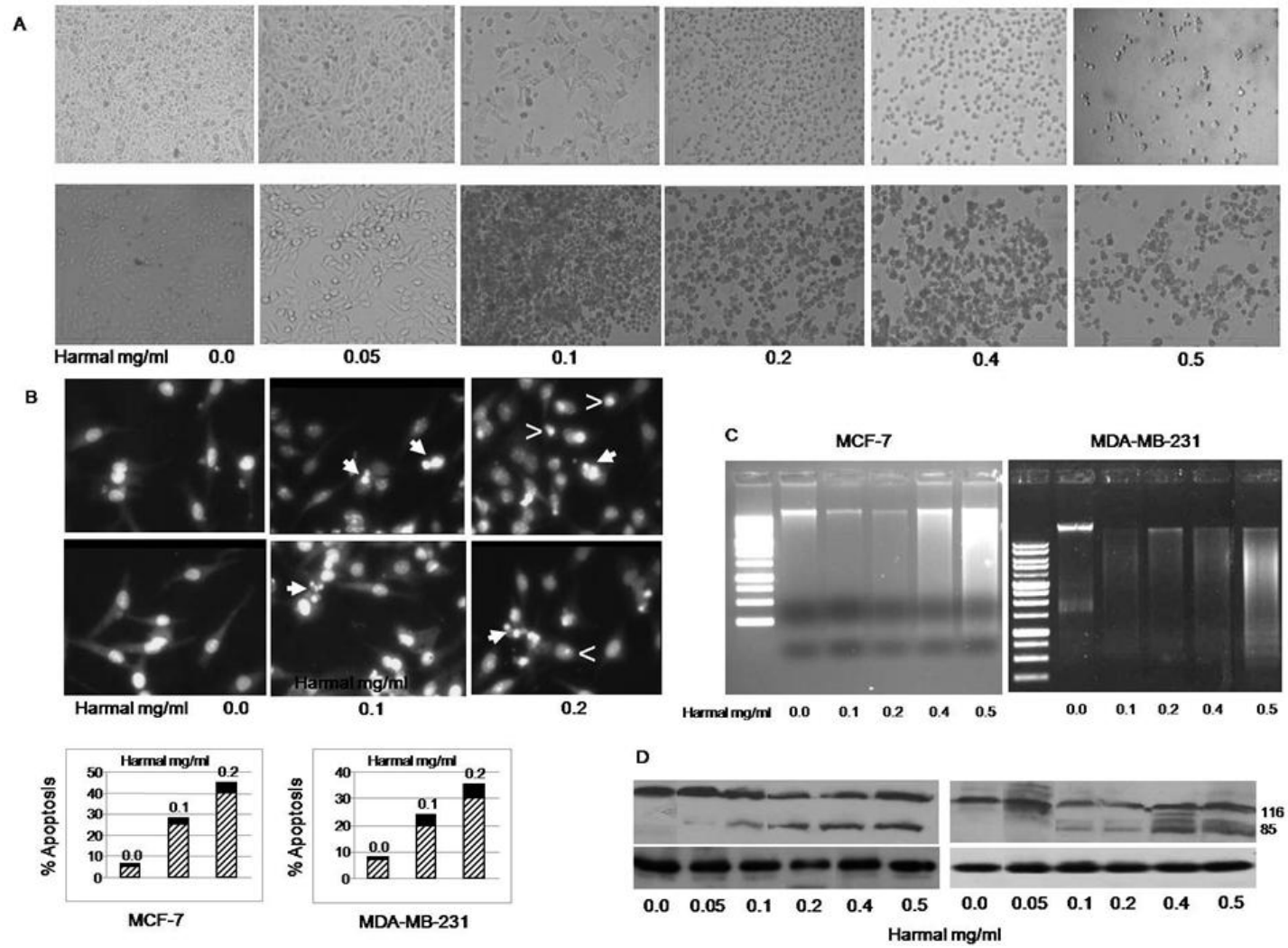

Figure 3. Harmal induces apoptotic cell death in MCF-7 and MDA-MB-231 cells. (A**)** Microphotographs showing the inhibitory effect of ethanol extract of Harmal on cellular growth of MCF-7 (upper panels) and MDA-MB-231 (lower panels) cells. Cells were plated onto 6-well plates and treated with indicated concentrations of Harmal for 24 h. The photographs were taken directly from culture plates using a phase microscope. Cell shrinkage, irregularity in cellular shape, and cellular detachment are seen in the Harmal-treated cultures. Representative pictures are shown from three independent experiments. (B) DAPI staining for MCF-7 and MDA-MB-231 cultures treated ethanol extract of Harmal for 24 h. Apoptotic cells with condensed chromatin (**>**) and fragmented nuclei (◄) were clearly visible in Harmal-treated cultures but much less frequent in controls. The lower panels (Histograms) display quantitation of apoptotic cells with condensed chromatin (DAPI assay) in MCF-7 and MDA-MB-231 cultures following 24 h treatment with ethanol extract of harmal. Bars, mean of six to nine determinations. (C) The Harmal treatment induced DNA fragmentation in MCF-7 (Left) and MDA-MB-231 (Right) cells. Cells were incubated with indicated concentrations of Harmal for 24 h. Genomic DNA was isolated and electrophoresed in 1% agarose gel. M: 100 bp DNA ladder marker. (D) The Harmal treatment increased the cleavage of PARP (upper panels) in MCF-7 (left) and MDA-MB-231 (right) cells. Cells were treated with indicated concentrations of Harmal for 24 h; thereafter, cells were harvested and samples were prepared to analyze the cleavage of PARP using western blotting, as detailed in the Materials and Methods. Equal loading of samples was confirmed by *β*-actin (lower panels); representative blots are shown from three independent experiments with almost identical observations.

detection of apoptosis. The data in Figure 3a explains that the treatment of MCF-7 and MDA-MB-231 cells with Harmal (≥0.1 mg/ml) for 24 h resulted in obvious cell shrinkage and cellular detachment which were determined to be apoptotic events (Cruchten and Broeck, 2002).

Next, we further ascertained proapoptotic effect of Harmal by DAPI assay, which is widely used as yet another sensitive technique for detection of apoptosis. The Harmal treatment caused a statistically significant increase in apoptotic cells, which were rarely seen in control-treated MCF-7 and MDA-MB-231 cultures (Figure 3b). DAPI assay revealed occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies in Harmal-treated MCF-7 and MDA-MB-231 cultures, which were rare in control cultures. We scored

apoptotic cells with condensed and fragmented DNA from control and Harmal-treated cultures of MCF-7 and MDA-MB-231 cells. The percentage of apoptotic cells was increased by ~25- (MCF-7) and 20-fold (MDA-MB-231) upon a 0.1 mg/ml ethanol extract treatment and ~40- (MCF-7) and 30-fold (MDA-MB-231) upon a 0.2 mg/ml ethanol extract, compared with control-treated cultures. These results indicate that Harmal treatment caused apoptotic cell death in both MCF-7 and MDA-MB-231 cell lines.

Finally, 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 (Nagata, 2000). Therefore, DNA gel electrophoresis was used to determine the presence of internucleosomal DNA cleavage. The data depicted in Figure 3c illustrates that DNA fragmentations were clearly detectable in MCF-7 and MDA-MB-231 cells treated with 0.1, 0.2, 0.4 and 0.5 mg/ml of Harmal for 24 h.

Currently, there are two known pathways that activate the apoptotic cascade, the intrinsic and extrinsic pathways. Following the initial activation of caspase-9 and -8 in the intrinsic and extrinsic pathways (Reed, 2000), respectively each caspase cleaves procaspase-3. Once activated, caspase-3 proteolytically cleaves the 116-kDa poly (ADP-ribose) polymerase (PARP) protein into an 85-kDa fragment, which is considered to be a biochemical characteristic of apoptosis. Western blot analysis results showed that Harmal induced the proteolytic cleavage of PARP into its respective active form, as depicted in Figure 3d. Taken together, these four independent methods of measuring apoptosis provided similar results, suggesting that the cytotoxic effect observed in response to the Harmal treatment induces apoptosis in MCF-7 and MDA-MB-231 cells.

Harmal treatment modulates expression of Bcl-2 and Bax genes

The mitochondrial apoptotic pathway has been described as an important signaling of apoptotic cell death for mammalian cells (Wong, 2011). A key event in mitochondrial-dependent apoptotic pathway is the release of cytochrom *c* from the inter-membrane spaces of the mitochondria into the cytosol (Wong, 2011). The release of cyt *c* from mitochondria is tightly regulated by a variety of factors. Among these factors, Bcl-2 family proteins, including anti-apoptotic members (such as Bcl-2) prevent the release of cyto *c* from mitochondria and pro-apoptotic members (such as Bax) increase the release of cyto *c* from mitochondria (Wong, 2011). Therefore, we raised the question of whether the Harmal-mediated apoptosis of MCF-7 and MDA-MB-231 cells was due to modulation of the expression levels of Bcl-2 and Bax proteins. We addressed this question by determining the effect of Harmal on expression of the Bcl-2 family proteins. To this end, MCF-7 and MDA-MB-231 cells were treated with increasing concentrations (0.0, 0.05, 0.1, 0.2, 0.4 and 0.5 mg/ml) of Harmal for 24 h. Then, RT-PCR analysis was used to determine the ratio of Bcl-2 and Bax mRNA expression. As shown in Figure 4a, the Harmal treatment decreased the level of Bcl-2 mRNA expression in MCF-7 cells, but increased the level of Bax expression. On the other hand, in MDA-MB-231 cells, the Harmal treatment decreased the level of Bcl-2 mRNA expression, but had little effect on the level of Bax expression. These findings suggest that Harmal induced apoptosis in MCF-7 and MDA MB-231 cells through modulating expression of the Bcl-2 and Bax (only in MCF-7) proteins at transcription level.

Harmal treatment modulates expression of c-Myc, hTERT and cyclin D1 genes

Among the most certain and commonly amplified oncogenes in breast cancer, are the c-Myc, cyclin D1 (Stoff-Khalili et al., 2006; Tan et al., 2008; Schlotter et al., 2008) and hTERT, (human telomerase reverse transcriptase gene) that encodes the catalytic subunit of telomerase (Ahmed and Tollefsbol, 2003). To investigate whether Harmal extract extracts down-regulates expression of these oncogenes at the transcriptional level, MCF-7 and MDA-MB-231 cells were treated with harmal extract for 24 h, and the level of hTERT gene expression was determined by RT–PCR analysis. As shown in Figure 4a, Harmal treatment, dose-dependently, decreased the mRNA expression levels of all c-Myc, cyclin D1 and hTERT. To substantiate these data, we repeated the previously stated experiment and assessed expression of these oncogenes using Western blot analysis. As shown in Figure 5, Harmal extract, dose-dependently, decreased expression of all proteins. Taken together, these finding demonstrate that Harmal treatment modulated expression levels of these oncogenes. In particular, down-regulation of hTERT by Harmal is a significant outcome for Harmal treatment, since hTERT is a promising target for cancer therapeutics and an important marker for the diagnosis of malignancy (Ahmed and Tollefsbol, 2003).

DISCUSSION

Throughout medical history, plant-derived products have been shown to be valuable sources for the discovery and development of unique anticancer drugs (Da Rocha et al., 2001; Gupta et al., 2010). Harmal (*R. stricta* Decne) is an important medicinal plant used in indigenous medicinal herbal drugs to cure various diseases in South Asia and the Middle East (Gilani et al., 2007). Previously, we demonstrated that the aqueous extract of Harmal

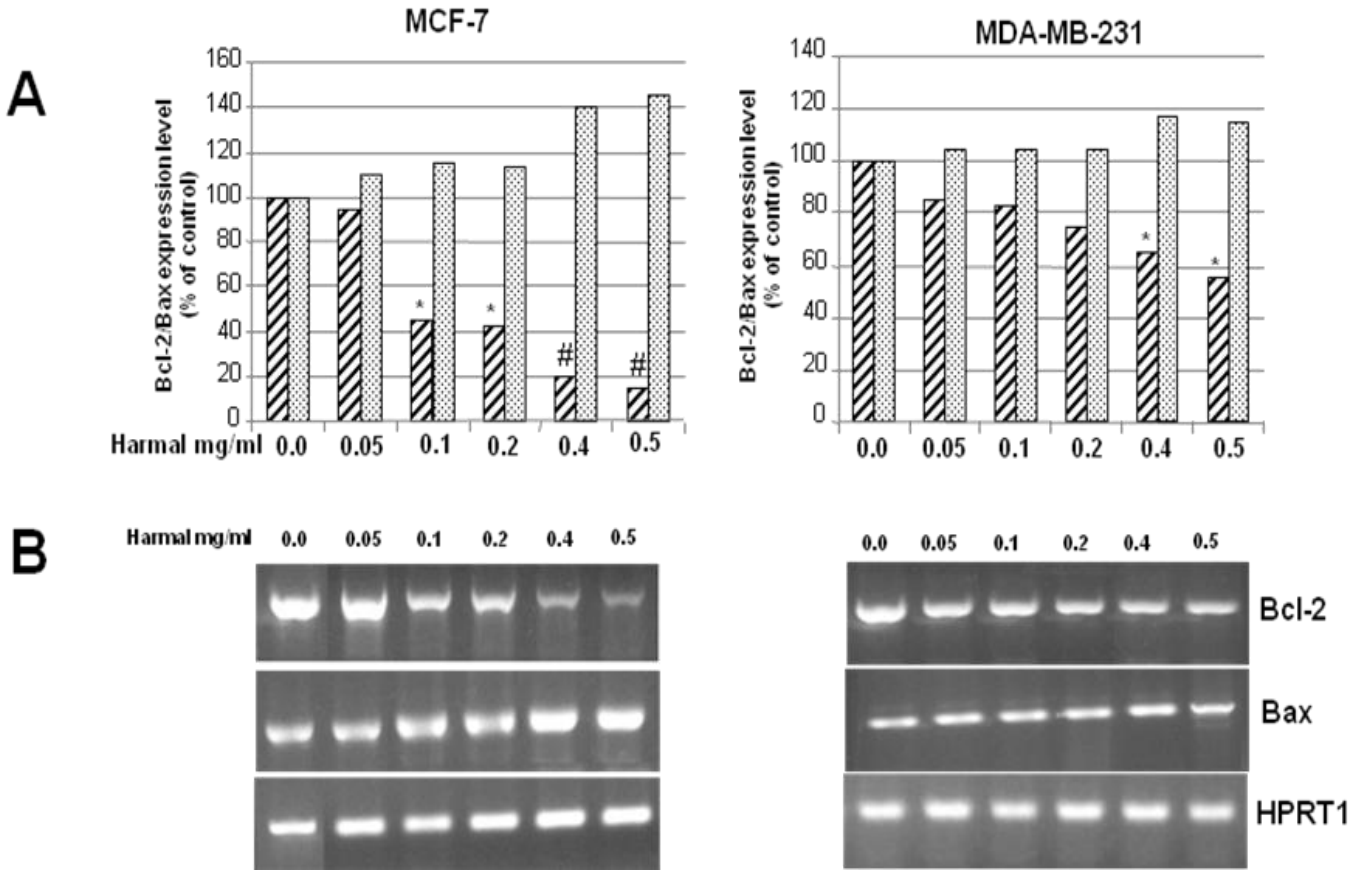

Figure 4. Effect of harmal on the mRNA expression of the apoptosis- and cell cycle-related genes. The MCF-7 and MDA-MB-231 cells were incubated with the indicated concentrations of the ethanol extract of harmal for 24 h. The total RNAs were isolated, reverse transcribed and subjected to PCR with the Bcl-2- and Bax-specific primers. The PCR products were then subjected to electrophoresis in 1% agarose gel and visualized by EtBr staining. (A) The histogram depicts the Bcl-2 (hatched bars) and Bax (dotted bars) mRNA ratio in MCF-7 and MDA-MB-231 cells measured by using densitometric analysis. (B) The gel graphs show the mRNA expression levels of the Bcl-2- and Bax genes. HPRT1 was used as the internal control. A typical result from three independent experiments is shown. $P<0.05$ (*); $P<0.001$ (#).

leaves induced genetic lesions in wide range of cell types. For example, it induced genotoxicity in *S. cervisae* (Baeshen et al., 2005), *A. terreus* (Baeshen et al., 2008), *A. cepa* root tip meristems (Baeshen et al., 2008) and in the primary culture of human lymphocytes (Baeshen et al., 2009). The last two studies (Baeshen et al., 2008, 2009) demonstrated pyknosis in *Allium* and necrosis in human lymphocytes considered indicative of anticancer activities. All these prompted us to examine the anticancer activity of Harmal *in vitro*. In the present study, we investigated for the first time the potential anticancer activities of aqueous and ethanol extracts of Harmal on the growth of human breast cancer cell lines, MCF-7 and MDA-MB-231. Both extracts (in particular ethanol extract) potently inhibited, in a dose- and time-dependent manner, proliferation of MCF-7 and MDA-MB-231 cells. Therefore, our results demonstrate that Harmal not only has a powerful inhibitory effect on the proliferation of ER-positive but also significantly inhibits the growth of ER-negative breast cancer cells. These results highlight Harmal as a promising chemopreventive agent, particularly for ER-negative breast cancers, which have a poorer prognosis and shorter survival (Sheikh et al., 1995). Furthermore, both Harmal extracts suppressed growth of human cervical carcinoma cell line, *HeLa*, indicating that the cytotoxic effect of Harmal is not restricted to breast cancer cell lines, but extends to a wide range of cancerous lines. Coherent to its anti-proliferative and cytotoxic effect, ethanol extract of Harmal 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 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, therefore, this assay is further evidence demonstrating the anti-cancerous potentiality of harmal.

Most developed chemotherapeutic agents (*Prodigiosin*, *Cimicifuga racemosa*, *Antrodia camphorata*, etc), which

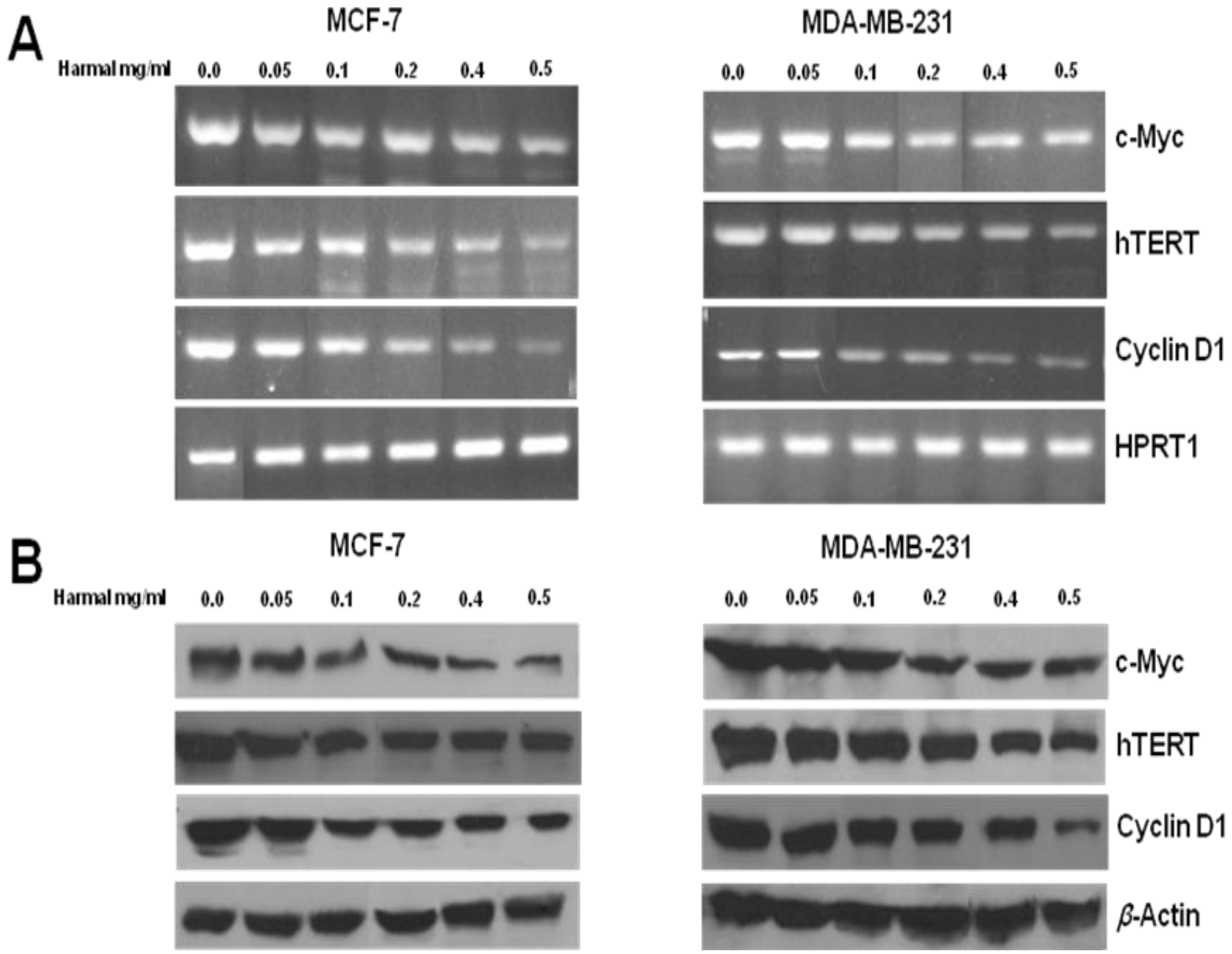

Figure 5. Harmal modulated the expression c-Myc, hTERT and cyclin D1 genes. (A) The MCF-7 and MDA-MB-231 cells were incubated with the indicated concentrations of the ethanol extract of Harmal for 24 h. The total RNAs were isolated, reverse transcribed and subjected to PCR with the c-Myc-, cyclin D1- and hTERT-specific primers. The PCR products 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. $P<0.05$ (*); $P<0.001$ (#). (B) Cell lysates were prepared from Harmal treated MCF-7 and MDA-MB-231 cells and 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.

originate from natural products exert their anti-carcinogenic activities by induction of apoptosis in breast cancer cells (Soto-Cerrato et al., 2004; Hostanska et al., 2004; Yang et al., 2006). Likewise, the anti-proliferative activity of Harmal was linked to its ability to induce apoptosis in MCF-7 and MDA-MB-231 cells for many reasons. First, microscopic inspection revealed abundance of morphological alterations including a loss of cell viability, cell shrinkage, irregularity in cellular shape, and cellular detachment in Harmal-treated MCF-7 and MDA-MB-231 cultures. These morphological alterations are hallmarks of cells undergoing apoptotic cell death (Cruchten and Broeck, 2002). Additionally, DAPI staining, another well-accepted technique for quantitation of apoptosis (Gorman et al., 1996), revealed occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies in Harmal-treated MCF-7 and MDA-MB-231 cultures. A further proof explaining apoptotic activity of Harmal is the fragmentation of DNA isolated from Harmal-treated cells. Fragmentation of cellular DNA at the inter-nucleosomal linker regions has been observed in cells undergoing apoptosis induced by a variety of agents and is widely used as biochemical markers of apoptosis (Nagata, 2000). Finally, treatment of cells with Harmal resulted in cleavage of a DNA repair enzyme, poly (ADPribose) polymerase (PARP), which has also been used as early markers of apoptosis (Cruchten and Broeck, 2002). Overall, these findings

demonstrate that the cytotoxic effect observed in response to the Harmal treatment is associated with the induction of apoptosis in breast cancer cell lines.

Next, we conducted our effort to elucidate the molecular mechanism underlying apoptotic activity of Harmal. Apoptosis is governed by a complex network of anti-apoptotic and pro-apoptotic effector molecules; the proteins of Bcl-2 family play an important role in apoptosis and are considered as a target for anticancer therapy (Wong, 2011). Bcl-2 protein functions as a suppressor of apoptosis while Bax is a pro-apoptotic protein of the Bcl-2 family (Wong, 2011; Reed, 2000). Increased expression of Bax can induce apoptosis, while Bcl-2 protects cells from apoptosis (Reed, 2000). Numerous studies have shown that the ratio of Bax: Bcl-2 proteins increases during apoptosis (Lai and Thomas, 1999; Tudor et al., 2000). In line with these observations, we found that Harmal treatment decreased Bcl-2 expression, but increased Bax expression in MCF-7 cells; this indicates that the increased ratio of Bax to Bcl-2 proteins might underlie the induction of apoptosis by Harmal in these cells. On the other hand, Harmal decreased expression of Bcl-2, but did not alter expression of Bax in MDA-MB-231 cells. This raises a possibility that Harmal may exploit different apoptotic pathways to impart its apoptotic effect in MDA-MB-231 cells. This is reminiscent to induction of apoptosis in MCF-7 and MDA-MB-231 cells by another oriental medicinal herb namely, *Siegesbeckia glabrescens* (SG), where SG induced apoptosis associated with a decrease in the level of Bcl-2 mRNA expression and an increase in the level of Bax mRNA expression in MCF-7 cells, but did not induce detectable change in the MDA-MB-231 cells (Jun et al., 2006). The authors suggested that SG might exert anti-proliferative action in human breast carcinoma cells via two different apoptotic pathways, namely, an intrinsic signal (Bax:Bcl-2 ratio-dependent) in MCF-7 cells and an extrinsic signal (Bax:Bcl-2 ratio-independent) in MDA-MB-231 cells. However, to draw a conclusion, whether, alike to SG, Harmal utilizes different apoptotic pathways in MCF-7 versus MDA-MB-231 cells needs further investigations.

One seminal finding in this study is that Harmal treatment decreased expression of c-Myc mRNA and protein. c-Myc is a master transcriptional factor; it participates in most aspects of cellular function, including replication, growth, metabolism, differentiation, apoptosis and carcinogenesis (Dang, 2006). The c-Myc oncoprotein is expressed in a high proportion of most human cancers including breast (Nesbit et al., 1999) and clinical studies have indicated that c-Myc is important in the development and progression of breast cancer (Liao and Dickson, 2000). In addition, a frequent genetic abnormality seen in breast cancer is the elevated expression of c-Myc (Liao and Dickson, 2000; Doisneau-Sixou et al., 2003). Moreover, previous studies demonstrated that the continued presence of c-Myc was required for cancer development and not just for initiation, and inactivation of c-Myc resulted in the sustained regression of tumors. For example, it has been demonstrated that over-expression of c-Myc by an inducible system in the mammary epithelium of transgenic mice resulted in the formation of invasive mammary adenocarcinomas, many of which regressed fully after c- Myc deinduction (D'Cruz et al., 2001). Therefore, specific down-regulation of c-Myc might be a potential therapeutic strategy against human cancers, including breast cancer. In fact, the antagonists of c-Myc, including full-length antisense mRNA (Steiner et al., 1998), oligonucleotides against c-*myc* mRNA (Watson et al., 1991) or a dominant-negative mutant (Sawyers et al., 1992) were previously reported to inhibit proliferation of cancer cell lines *in vitro*. However, it was only successful in some situations; these technologies have been difficult to apply universally (Braasch and Corey, 2002). In these regards, the data in this study showing Harmal down-regulated expression of c-Myc explain that Harmal can rectify deregulated expression of c-Myc and hold promise for exploiting Harmal as a breast cancer-preventive functional food.

A hallmark of cancer cells is limitless proliferative potential (Hanahan and Weinberg, 2011) which entails the maintenance of telomeres. Telomerase is the enzyme responsible for maintenance of the telomeres and increase of telomerase activity is known in a variety of human carcinogenesis (Shay and Bacchetti, 1997). It has been cited that in over 85% of human tumors, and more than 90% of breast carcinomas, telomerase is active whereas in normal tissues telomerase is active at low levels or is undetectable (Carey et al., 1998, 1999). The hTERT promoter is a target for c-Myc (Latil et al., 2000) and over-expression of c-Myc increases transcriptional activity of the hTERT core promoter (and hence telomerase activity) which is necessary for cancer cell viability. Since Harmal extract down-regulated expression of c-Myc mRNA and protein along with those of hTERT, then, at least in part, this down-regulation might confirm the anti-proliferative activity of Harmal, through a telomerase inhibitory pathway. Similar to Harmal, sulforaphane (in cruciferous vegetables) has been shown to inhibit cellular growth of MCF-7 and MDA-MB-231 cells through down-regulation of hTERT mRNA (Meeran et al., 2010). Furthermore, concomitant down-regulation of c-Myc and hTERT by chemopreventive agents has been found in other cancer cell line, where it has been confirmed that ginger extract suppressed growth of A549 lung carcinoma cells through inhibition of hTERT and c-Myc expression (Tuntiwechapikul et al., 2010). Other point deserves attention, it has been reported that down-regulation of Bcl-2 and telomerase is considered to play a role in drug induced apoptosis (Ji et al., 2002a, b, 2004). Since Harmal treatment resulted in concurrent inhibition of Bcl-2 and hTERT mRNA expression, therefore, our results directly corroborate conclusions of these previous studies and suggest that coincident down-regulation of

Bcl-2 and hTERT may play a causative role in Harmal-induced apoptosis.

The loss of normal cell cycle control has been implicated in tumor development (Hanahan and Weinberg, 2011). c-Myc oncoprotein is located upstream of the G0/1 to S transition and promotes cell cycling through targeting several cell cycle regulators (Dang, 2006). Cyclin D1 is another central player in cell cycle, overexpressed in up to 50% of human breast cancers, acts as a driving force in human breast cancer (Arnold and Papanikolaou, 2005) and plays an essential role in malignant transformation of breast cancers induced by other oncogenes (Yu et al., 2001). Therefore, cyclin D1 is a potential target for the rational design of new drugs to prevent/treat breast cancer and it has been cited that phytochemicals prevented breast cancer cell growth via down-regulation of cyclin D1 (Meeran and Katiyara, 2008). In this study, Harmal treatment down-regulated expression of both cyclin D1 mRNAs and protein, which coincided with the reduction of c-Myc's. However, whether down-regulation of mRNA expression by Harmal is due to transcriptional down-regulation, mRNA stabilization or both remains to be determined. Based on these findings and on their crucial roles in cell cycle progression, we propose that Harmal-mediated concomitant down-regulation of c-Myc and cyclin D1 expressions might induce cell cycle arrest, which underlies the antiproliferative activity of Harmal, in MCF-7 and MDA-MB-231 cells. Our ongoing research will support this conclusion by cell cycle (flow cytometry) analysis.

Conclusion

In conclusion, we proved that Harmal has an anti-proliferative effect in MCF-7 and MDA-MB-231 cells. The anticancer activity of Harmal could be attributed in part to its inhibition of proliferation and apoptosis induction of cancer cells through upregulation (Bax) and down-regulation of Bcl-2 proteins. Other Harmal-dependent growth-inhibitory mechanisms entailed, at least in part, the down-regulation of c-Myc, hTER and cyclin D1. Our ongoing study further validates the effective cancer-preventive potential of Harmal in other cancer cell lines as well as in a xenograft assays. If Harmal is indeed proven to exert an effective cancer preventive potential *in vivo*, it may be an ideal chemopreventive or therapeutic agent for breast cancer.

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