Pathol. Oncol. Res. (2013) 19:763–770
DOI 10.1007/s12253-013-9641-1

RESEARCH

Naringenin (Citrus Flavonone) Induces Growth Inhibition, Cell Cycle Arrest and Apoptosis in Human Hepatocellular Carcinoma Cells

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Received: 12 February 2013 / Accepted: 11 April 2013 / Published online: 10 May 2013
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Abstract Search for new substances with antiproliferative activity and apoptosis inducing potential towards HepG2 cells is important since HCC is notoriously resistant to conventional chemotherapy. Dietary phytochemicals with significant anti-proliferative and apoptosis inducing potential are considered as agents promising for cancer therapy. Naringenin, a common dietary flavonoid abundantly present in fruits and vegetables, is believed to possess strong cytotoxic activity in numerous types of cancer cells. However, the detailed molecular mechanisms of its antiproliferative effects and apoptosis induction are still unclear. In this study, we investigated antiproliferative and apoptosis-inducing effect of naringenin in human hepatocellular carcinoma HepG2 cells. Naringenin was shown to inhibit the proliferation of HepG2 cells resulted partly from an accumulation of cells in the G0/G1 and G2/M phase of the cell cycle. Naringenin induced a rapid accumulation of p53, which might account for the naringenin-induced G0/G1 and G2/M phase arrests in Hep G2 cells. In addition, naringenin have been shown to induce apoptosis as evidenced by nuclei damage and increased proportion of apoptotic cells detected by flow cytometry analysis. Naringenin triggered the mitochondrial-mediated apoptosis pathway as shown by an increased ratio of Bax/Bcl-2, subsequent release of cytochrome C, and sequential activation of caspase-3. Our results showed that naringenin had inhibitory effect on the growth of HepG2 cell line through inhibition of cell proliferation and apoptosis induction. The elucidation of the drug targets of naringenin on inhibition of tumor cells growth should enable further development of naringenin for liver cancer therapy.

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Keywords Naringenin · Hepatocellular carcinoma cells · Cell proliferation · Apoptosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer related mortality. Its incidence has more than doubled during the last two decades in the western world, where it is the fastest growing cause of cancer related death [1, 2]. Evidence suggest that the incidence of HCC is rising in several countries [3–5]. Therefore, treatment of HCC increasingly requires a multidisciplinary approach and multiple treatment options.

In recent years, dietary phytochemicals receive the attention of various scientists to treat various cancers [6]. In particular, inhibition of cell proliferation and apoptosis induction by dietary phytochemicals represents significant potential for cancer preventive activity. Several studies have documented that naturally occurring dietary phytochemicals inhibit the growth of various cancer cells through the inhibition of cell proliferation and the activation of apoptosis induction [6–8]. In particular, Flavonoids (polyphenolic compounds) occurring naturally in the plant kingdom, display a wide range of pharmacological properties, including antioxidant and anti-carcinogenic activities [9–11].

Naringenin (Fig. 1) is a flavanoid predominantly found in citrus fruits has been found to exhibit hepato protective, antioxidant, anti-mutagenic and anti-carcinogenic effects [12–15]. Several earlier studies have indicated that naringenin has anticancer growth activities; however the mechanism underlying these activities was unclear and remained to be elucidated. In this study, we evaluated the molecular mechanism of the effect of naringenin on human hepatoma cell line, HepG2. Our results showed that the naringenin had inhibitory effect on the growth of HepG2 cell line possibly through inhibition of cell proliferation and apoptosis induction.

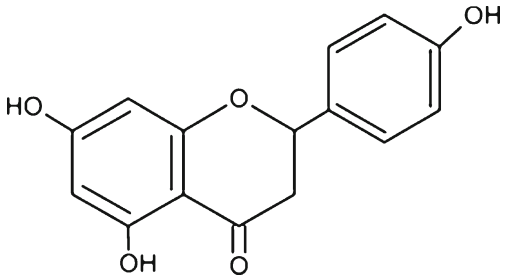

Fig. 1 Chemical Structure of Naringenin (4′,5,7-Trihydroxyflavanone)

Materials and Methods

Cell culture and Maintenance

Human hepatocellular carcinoma Hep G2 cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune India. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) as antibiotics (Himedia, Mumbai, India) in a humidified atmosphere of 5 % CO_2 and 95 % air in a CO_2 incubator.

Treatment

Naringenin was purchased from Sigma Chemical Co., St. Louis, USA. A 100 mM solution of naringenin was prepared in dimethyl sulfoxide (DMSO), stored as small aliquots at −20 °C and then diluted as needed in cell culture medium. Dose–response studies were carried out to determine the suitable dose for the inhibition of cell growth and induction of apoptosis.

Cell Viability Assay

Cells were plated in 96-well plates at a density of 8×10^3 cells per well and incubated for 24 h with medium. The cells were rinsed with PBS and grown in a medium containing various concentrations of naringenin (50, 100, 150, 200, 250, 300 μM). The solvent DMSO treated cells were served as control. After 24 hrs of treatment, the medium was removed and replaced by another medium containing 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (1 mg/mL), and the cells were incubated for 2 h at 37 °C. To assess the proportion of viable cells, formazan was solubilized with 150 μL DMSO. Plates were then vortexed at room temperature for 30 mins, and the level of formazan was measured using a spectrophotometer at 575 nm.

Observation of Morphological Changes of Cells

Hep G2 cells grown on 6 cm dishes were treated with naringenin at different dosages of 100, 150 and 200 μM for 24 h. The morphological changes were observed under an inverted microscope.

Nuclear Staining with DAPI

After treating Hep G2 cells with naringenin (100, 150 and 200 μM) for 24 h, the cells were harvested, washed in ice-cold phosphate-buffered saline (PBS) and fixed with 3.7 % paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with a 4,6-diamidino-2-phenylindile (DAPI, Sigma) solution for 10 min at room temperature. The nuclear morphology of the cells was examined by fluorescent microscopy.

Annexin V Binding and Propidium Iodide Influx

The apoptotic cells were studied using flow cytometric analysis in accordance with the method of Koopman et al. [16]. The apoptotic and necrotic cells from the same samples were quantified using quantitative FACS analysis. This method utilizes the binding of FITC-labeled annexin V to phosphatidylserine (PS) in the cell membrane that surfaces only during the early phase of apoptosis, indicating the loss of cell membrane phospholipid asymmetry. However, these apoptotic cells with intact cell membranes do not stain with the propidium iodide. By utilizing the morphological changes that occur in both apoptotic and necrotic cells, the samples were stained simultaneously with annexin–FITC and propidium iodide. The samples were then subjected to flow cytometric analyses to detect the percentage of apoptotic (FITC-stained cells) and necrotic cells (PI-stained cells) in a given population. A minimum of 50,000 cells were maintained for all the samples. The samples were analyzed as per the protocol provided by the manufacturer (BD FACS, San Jose, CA).

Cell Cycle Analysis

HepG2 cells were treated for 24 h with increasing concentrations of naringenin (100, 150 and 200 μM). At the end of treatment, cells were trypsinized, and the resulting cell suspensions were centrifuged at 1000 rpm for 5 min. The cells were fixed overnight in 70 % ethanol at 4 °C and centrifuged at 1000 rpm for 5 min, and the pellets were washed twice with ice-cold PBS. Cell pellets were then resuspended in 0.5 ml of PBS containing 50 μg/ml propidium iodide (Sigma-Aldrich) and 100 μg/ml RNase A (Sigma-Aldrich), incubated at 37 °C for 30 min, and then analyzed by flow cytometer (Becton Dickson, Franklin Lakes, NJ). Cellular DNA content was analyzed by flow cytometr). At least 10 000 cells were used for each analysis, and the results were displayed as histograms. The percentage of cell distribution in G0/G1, G2/M and S phase were

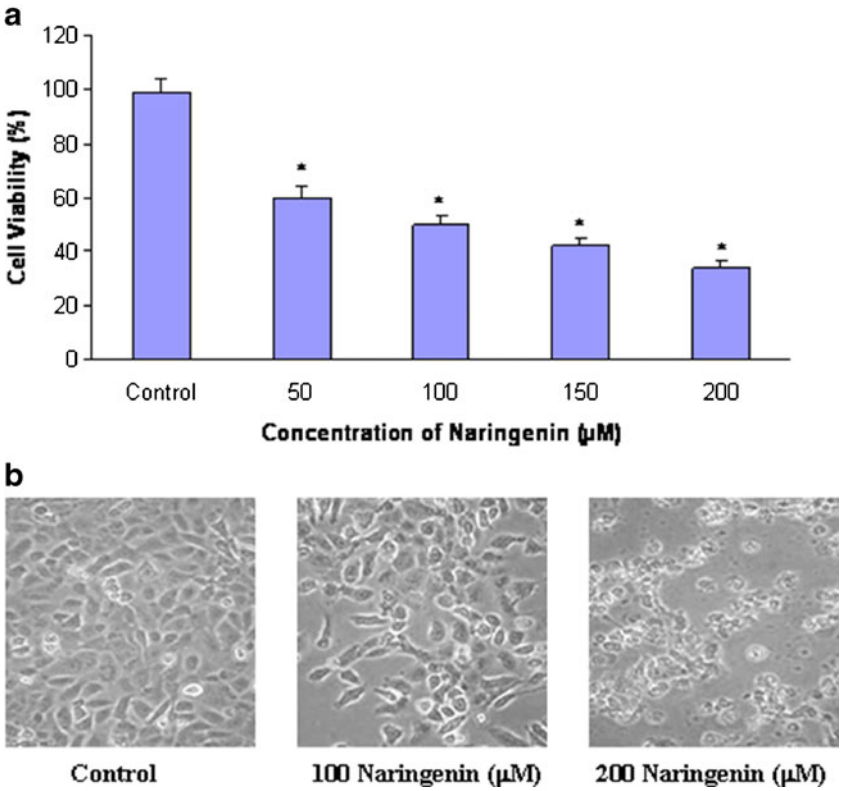

Fig. 2 **a** Effect of naringenin on viability of Hep G2 cells. Data were expressed as mean ± SD of 3 replicates (*$p<0.001$ vs control cells). **b** Naringenin caused morphological changes to Hep G2 cells in a dose-dependent manner after treatment for 24 h

measured and the results were analyzed by the Modfit LT version 5.2 software (Verity Software House, Topsham, ME) for cell cycle profile.

Western Blot Analysis

Cells were treated with DMSO (control) and with different concentrations of naringenin (100, 150 and 200 µM). After 24 h of stimulation, floating cells and adherent cells were collected and washed three times with ice-cold PBS and harvested in lysis buffer. The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was collected. The protein concentration of lysates was determined by Bradford method [17]. SDS-PAGE was performed using equivalent protein extracts (55 µg) from each sample according to Laemmli [18]. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Sartorius, Germany). The membranes were incubated in 1X PBS containing 5 % non-fat dry milk for 2 h to block non-specific binding sites. The blots were incubated with 1:400 dilutions of rabbit polyclonal antibody for p53, Bcl-2, Bax, mouse monoclonal antibody for cytochrome C and Goat polyclonal antibody for active caspase-3 overnight at 4 °C. The blots were washed three times with high salt buffer (2.18 g NaH_2PO_4, 7 g Na_2HPO_4, 23.37 g NaCl and 200 µl Triton X-100 in 400 ml distilled water) followed by low salt buffer (2.18 g NaH_2PO_4, 7.03 g Na_2HPO_4, 1.2 g NaCl and 200 µl Triton X-100 in 400 ml distilled water). The blots were then incubated with 1:1000 dilutions of horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 45 min at room temperature. After extensive washes with high and low salt buffers, the immunoreactive proteins were visualized using enhanced chemiluminescence (ECL)

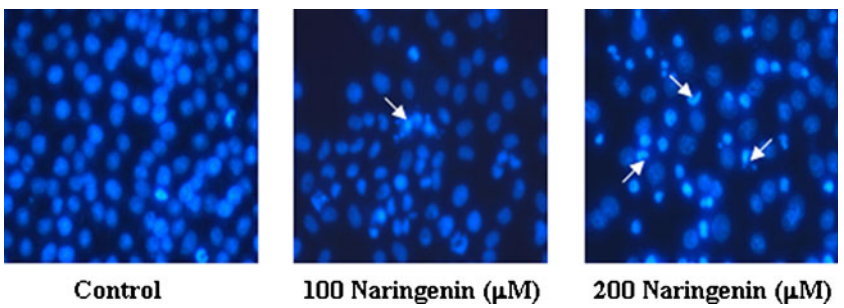

Fig. 3 Fluorescent microscopic analysis of cells stained with DAPI. 24 h after naringenin treatment the cells were fixed, stained with DAPI and analysed for morphological characteristics associated with apoptosis. Apoptosis bodies are indicated by white arrows

detection reagents (Sigma). Densitometry was performed on IISP flat bed scanner and quantitated with Total Lab 1.11 software.

Statistical Analysis

Data were analysed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a statistically software package (SPSS for Windows, V. 13.0, Chicago, USA). Results were presented as mean ± S.D. p-values<0.05 were considered as statistically significant.

Results

Naringenin Reduces Hep G2 Cell Viability

To investigate the effect of naringenin on human hepatocellular carcinoma Hep G2 cells, the viability of cells was studied using the MTT assay. As shown in Fig. 2a, naringenin treatment significantly inhibited the proliferation of cells in dose-dependent manner (50–300 μM) after 24 h of incubation. Since the greatest inhibition was seen after 24 h treatment with 100–200 μM of naringenin (The IC50 was 100 μM). Based on this study, we selected the dose at

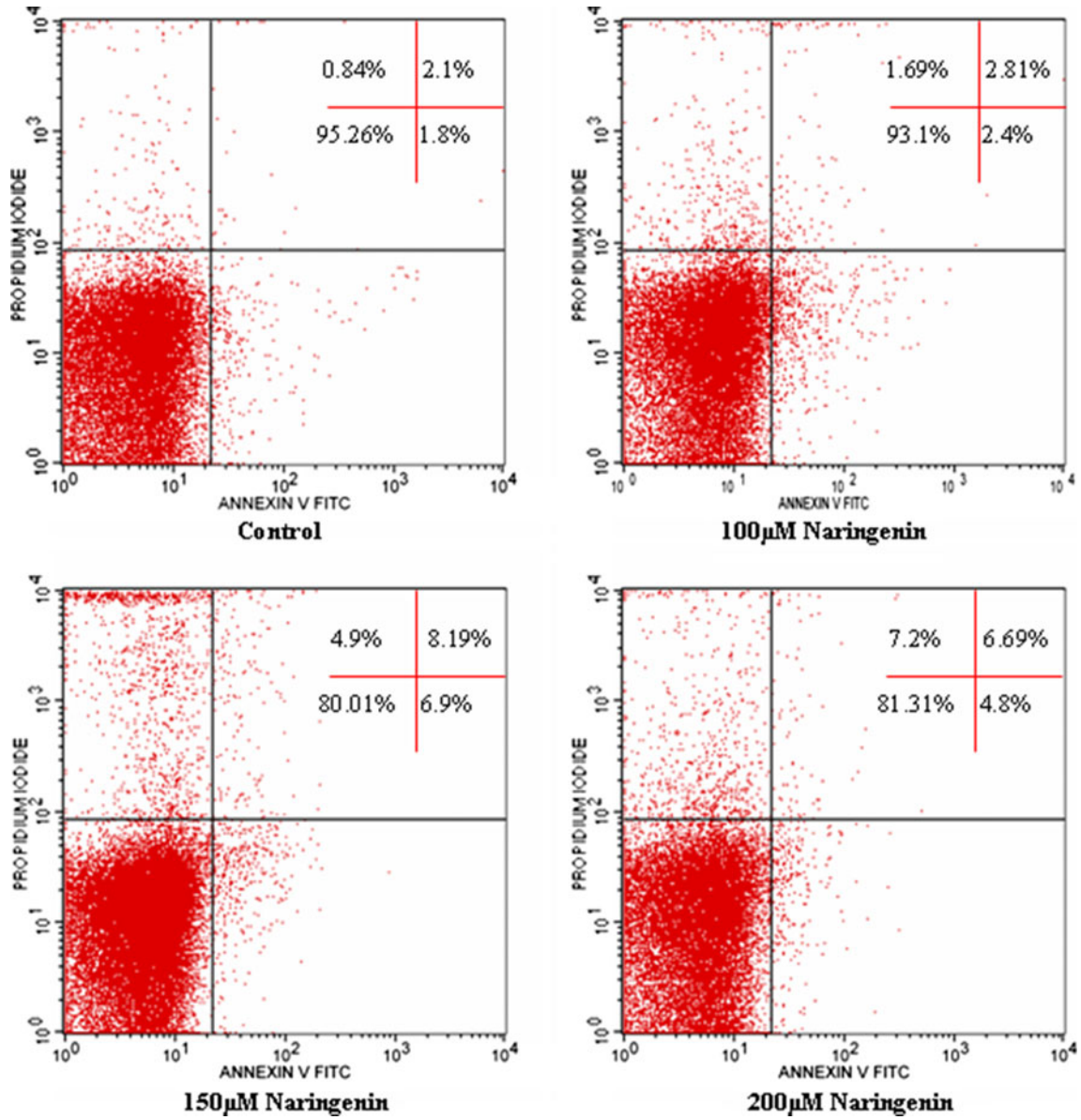

Fig. 4 Hep G2 cells were treated with control and naringenin (100,150,200) for 24 h. The cells were then permeablized, stained for Annexin V, and stored on ice until analyzed by FACS. Increased Annexin V staining was seen in Hep G2 cells in the presence of naringenin. In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, in the upper right are in late apoptosis, and cells in the upper left quadrant are dead. Percentage of total signal within the quadrant is indicated

100, 150 and 200 μM naringenin for 24 h incubation for further experiments.

Morphological changes induces by naringenin were observed naringenin- concentration dependently at 100 and 200 μM under inverted microscope. After exposure to naringenin for 24 h, Hep G2 cells shrank and retracted from their neighbors, accompanied with floating apoptotic cells in the culture medium (Fig. 2b).

Nuclear Staining with DAPI

Figure 3 has depicted the details of DAPI staining of Hep G2 cells. It should be noted that the number of cells showing signs of apoptosis (cells that have brightly fluoresced and fragmented nucleus) are more in extract treated groups than the control group. It is clearly evident that different doses of naringenin have induced apoptosis and DNA condensation in cells with varying intensity depending up on the dosage of exposure

Annexin V assay

Annexin V is a Ca2+−dependent phospholipid binding protein possessing high affinity for PS, a membrane bound component localized to the inner surface of the cell membrane. An indicator of early-stage apoptosis is the detection

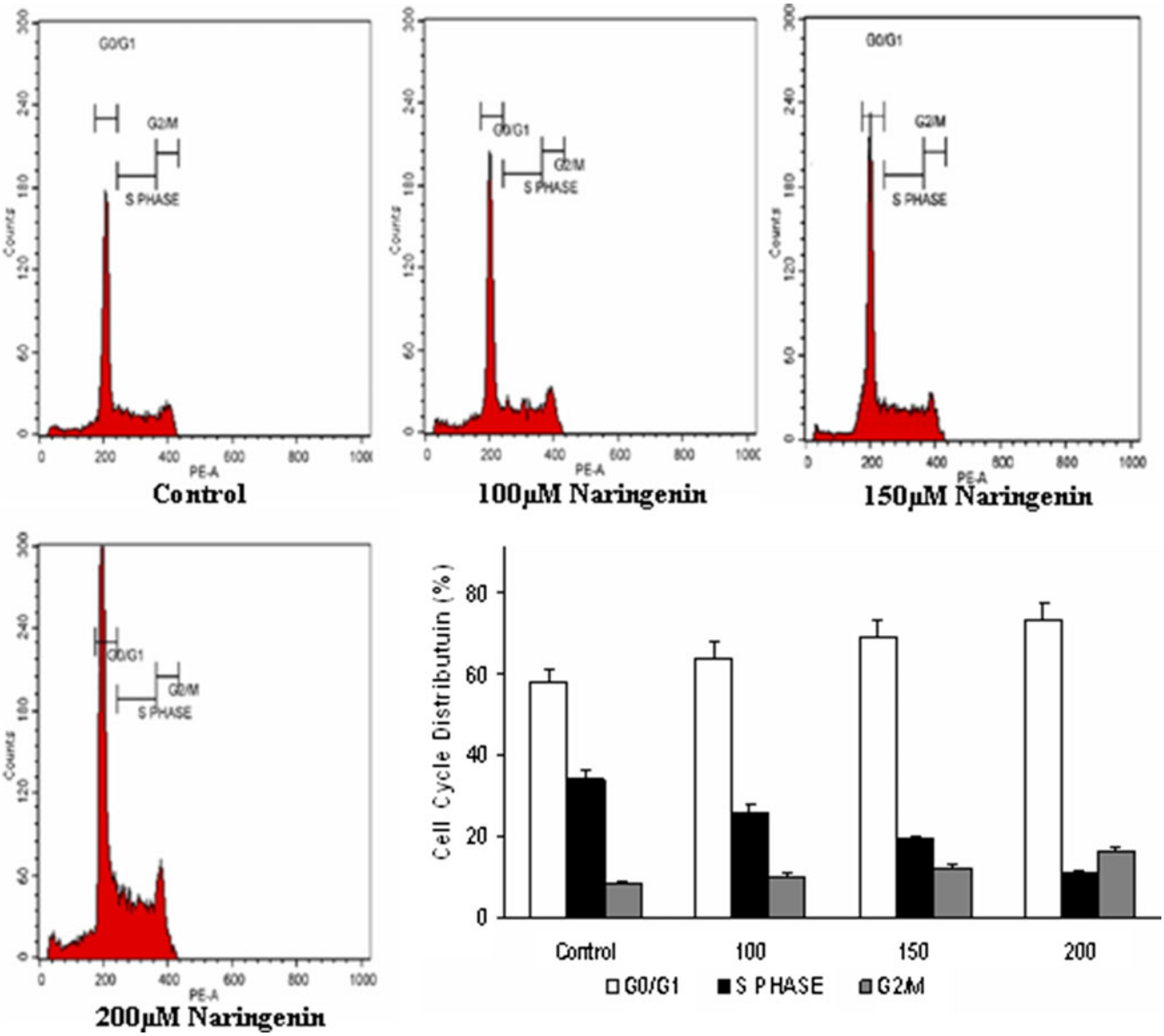

Fig. 5 Cells were treated with naringenin for up to 24 h and stained with propidium iodide and analyzed by FACS can as described. The proportions of cells in the G0/G1, S and G2/M-phase of the cell-cycle are calculated. The data are from one representative experiment (mean ± SD, n=3). Similar results were obtained in three other independent experiments

of exposed PS residues that have translocated to the cell surface. The Annexin V Assay permits simultaneous detection of early apoptotic events based on Annexin V binding to exposed PS and late apoptotic/dead events through uptake of propidium iodide. From (Fig. 4), it is clear that the vast majority of HepG2 cells in the untreated control were healthy and thus unstained for Annexin V and propidium iodide. By contrast, treatment with naringenin dose dependently resulted in marked apoptotic induction.

Cell Cycle

The effect of naringenin on the cell cycle was illustrated in (Fig. 5). The flow cytometric assay showed that naringenin induced Hep G2 cell cycle arrest at the Go/G1 and G2/M phase in a dose-dependent manner. A 24 h exposure to 100,150 and 200 μM Naringenin, the fraction of Hep G2 cells at the Go/G1 phase increased from 58.37 % to 73.24 %, while the fraction of cells in S phase decreased from 34.27 % to 11.93 %, and the fraction in G2/M phase increased from 8.36 % to 16.89 % (Fig. 5).

Effect of Naringenin on the Expressions of Protein Involved In Apoptosis

To obtain further support for the induction of apoptosis by naringenin in Hep G2 cells, the expression of apoptosis-related proteins, such as p53, Bcl-2, Bax, cytochrome c Caspase-3, were examined (Fig. 6). Cultured Hep G2 cells showed very low level of p53 expression, and naringenin treatment significantly increased the expression of p53 in dose dependent manner. We further demonstrated that naringenin treatment significantly decreased the expression of Bcl-2 and increased the expression of Bax, caspase-3 and cytochrome C compared to control (Fig. 6).

Discussion

In the present study, we demonstrated the antiproliferative and apoptotic events of naringenin in Hep G2 cell lines. Hep G2 cells were cultured in the presence of naringenin at various concentrations for 24 h and the percentage of cell viability was evaluated by MTT assay. MTT showed that

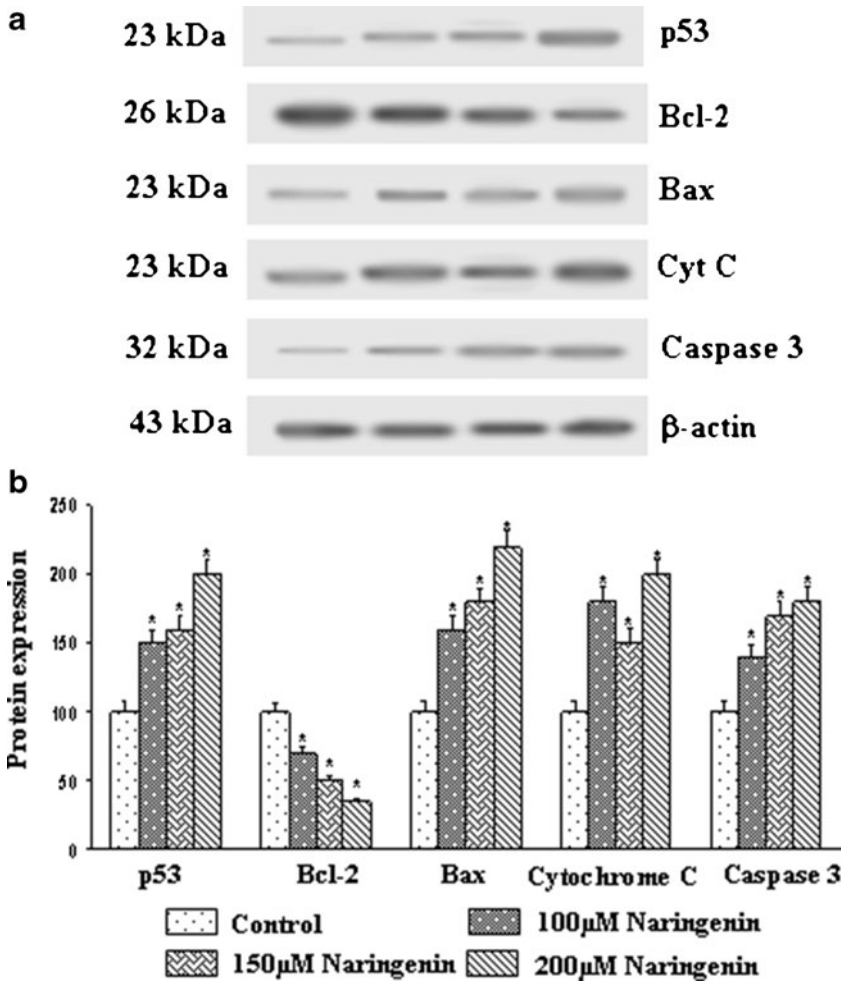

Fig. 6 Effect of naringenin on the protein expression of p53, Bax, Bcl-2, Cytochrome C, Caspase 3 in control and treatment groups. **a** Representative immunoblot and their corresponding densitometric analysis of key molecules has been represented here. β-actin served as internal control. Lane 1: control; Lane 2:100 μM naringenin; Lane 3: 150 μM naringenin; Lane 4: 200 μM naringenin. **b** Densitometric analysis. Values are expressed as mean ± S.D. * symbol represents statistical significantly different from Control ($p<0.001$) ANOVA followed by LSD

naringenin inhibited the growth of Hep G2 cells in concentration dependent manner. Several studies have also documented that naringenin was shown to inhibit the proliferation of various cancer cell lines [19, 20]. Dysregulation of the cell cycle mechanism has also been shown to play an important role in the growth of various types of cancer cells [21, 22]. In the present study, Naringenin was shown to inhibit the proliferation of HepG2 cells resulted partly from an accumulation of cells in the G0/G1 and G2/M phase of the cell cycle.

In addition, p53 activation has also been implicated in triggering cell cycle arrest, including both G_1 and G_2 phases of the cell cycle. The mechanism of cell cycle arrest within G_1 phase of the cell cycle is understood by p53 stimulating transcription of the gene for the cyclin-dependent kinase inhibitory protein p21. Elevated levels of p21 then inhibit the cyclin E/cyclin-dependent kinase 2 and cyclin A/cyclin-dependent kinase 2kinases, thus preventing these kinases from promoting cell cycle progression [23–25]. In the present study, naringenin induced a rapid accumulation of p53 which occurred within 24 h of naringenin treatment in a dose-dependent manner, which might account for the naringenin-induced G0/G1 and G2/M phase arrests in Hep G2 cells. Emerging evidences from literature have demonstrated that the antiproliferative effect of natural products is known to be associated with apoptosis induction [22, 24, 25]. Our results are in line with these findings.

To determine whether the antiproliferative effects of naringenin was due to apoptosis, HepG2 cells were treated for 24 h with various concentrations of naringenin. However, naringenin treatment has been shown to induce apoptosis as evidenced by nuclei damage of human hepatocellular carcinoma Hep G2 cells [26, 27]. Flow cytometry with Annexin V-FITC/PI staining showed that the drug treatment significantly increased the proportion of apoptotic cells, confirming that naringenin induced apoptosis in HepG2 cells. The accumulated data suggest that natural products induce apoptosis through the mitochondria-initiated death pathway that plays an important role in triggering apoptosis in response to various stimuli [26, 28, 29].

Naringenin triggered the mitochondrial-mediated apoptosis pathway as evidenced by an increased ratio of Bax/Bcl-2, subsequent release of cytochrome C, and sequential activation of caspase-3 [30, 31]. The increase in the percentage of apoptosis cells was accompanied by the modification of the expression of Bcl-2 family of proteins, Bax and Bcl-2. Bcl-2 is a known anti-apoptotic protein that is frequently examined for potential clinical use as a prognostic biomarker in cancer and its over-expression is associated with resistance to cytotoxic drugs such as cisplatin and 5-fluorouracil [5-Fu]. Also, studies have shown that aberrant expression of this protein facilitates tumor progression in the early stages of hepatocarcinogenesis [30–33]. Our results showed that naringenin resulted in a dose-dependent reduction in the expression of Bcl-2 proteins in Hep G2 cancer cells. This reduction on the expression of anti-apoptotic Bcl-2 is concomitant with increased expression of the pro-apoptotic Bax protein. Naringenin at 200 μM resulted in a seven fold increased in the expression of Bax by Hep G2 cancer cells. Bax is essential mitochondrial mediated apoptosis as its insertion in the mitochondrial membrane resulted in the release of cytochrome C into the cytosol leading to the activation of caspases thereby committing the cells to apoptosis [30, 32]. Our results showed that naringenin is known to induce apoptosis through modification of Bcl-2 family of proteins involved in apoptotic mitochondrial pathway. Our results showed that naringenin caused an increase in the activity of caspase-3 in Hep G2 hepatocellular carcinoma cells. Previous studies suggested a direct activation of capase-3 by naringenin in various cancer cell lines. Moreover, several studies have shown the capability of naringenin to stimulated apoptosis in different cancer cell lines [34–36]. These results suggested that naringenin induced apoptosis of Hep G2 cells by suppressing anti-apoptotic proteins. Taken together, dysregulation of the cell cycle mechanism and the induction of cancer cell apoptosis are recognized as an important target in cancer therapy.

In conclusion, we are the first to provide evidence that naringenin is highly effective in inhibiting cell proliferation and inducing apoptosis cell death in human hepatocellular carcinoma Hep G2 cells and naringenin may be a promising candidate for hepatocarcinogenesis treatment.

Acknowledgements We wish to thank Prof. C.M. Elinos-Báez, Universidad Nacional Autónoma de México, Mexico for her gift of antibodies against Bcl-2 and Bax; Prof. S. Soddu, Regina Elena Cancer Institute, Italy for her kind gift of anti p53; Prof. Haider Raza, UAE University, UAE for her kind gift of antibodies against caspase-3; Prof. Ronald Jemmerson, Department of Microbiology, Northwestern University for her kind gift of the antibody against cytochrome C.

Conflict of interest The authors declare that there are no conflicts of interest.

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