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Hesperidin inhibits cell proliferation and induces mitochondrial-mediated apoptosis in human lung cancer cells through down regulation of β-catenin/c-myc

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ABSTRACT

The aim of this study is to evaluate the mechanism of actions involved in the anticancer effect of hesperidin against cell proliferation and inducing apoptosis in human lung cancer A549 cells. MTT assay were performed, by treating the cells with different concentrations of hesperidin ranges from 0, 6.25, 12.5, 25, 50 and 100 μM for 0, 6, 12, 24, and 48 h. Hesperidin treatment significantly inhibited the cell viability based on concentration and time dependent manner, which shows optimal LC 50 effective, dose as 12.5 and 25 μM for 24 h and this LC50 doses and optimized time point used for further studies. Hesperidin treated cells significantly inhibited the cell proliferation through down regulation of c-myc, β-catenin, PCNA protein expressions. An additional studies also shows that hesperidin significantly increased the p53 and p21 tumor suppressor proteins which subsequently inhibited the cyclin dependent kinase 4 (cdk4) and cyclin D protein expressions. Further studies supported that restoring of tumor suppressor proteins by hesperidin treatment directly affects the Bcl-2/Bax ratio, which limits the Bcl-2 levels and increased the pro-apoptotic Bax protein levels. Mitochondrial transmembrane potential studies, increased levels of cytochrome c, APAF-1, caspase-3 and DNA fragmentation was observed in hesperidin treated cells which clearly indicates apoptosis induction. Taken together, these findings suggest the anticancer effect of hesperidin in human lung cancer A549 cells through up regulation of p53, which triggers the proliferation arrest and mitochondrial dependent apoptosis activation.

1. Introduction

Lung cancer disease is the leading causes of malignant neoplastic associated with high risk of death rates in both the sexes accounting over one quarter of cancer deaths (Siegel et al., 2013). Non-small cell lung cancer (NSCLC) comprises 85%–90% of lung cancer diagnoses and despite advances in multimodality therapies, 5-year survival rates remain dismal with a median patient survival with metastatic diseases of one year (Duchemann et al., 2015). p53 is a master gene regulator controlling diverse cellular pathways, by either activating or repressing downstream genes. Among such genes c-myc, and β-catenin which is negatively regulated by p53 (Levy et al., 1993; Sadot et al., 2001). One of the important function of c-myc is to promote cell-cycle progression (Dang, 1999) by repressing genes as the cyclin-dependent kinase inhibitors p21/WAF1 (p21) and p27Kip1 (p27), which are involved in cell-cycle arrest (Gartel et al., 2001; Muller et al., 1997). Cell division relies on the activation of cyclins, which binds to cyclin-dependent kinases to induce cell-cycle progression towards mitosis. Following anti-mitogenic signals, p21 and p27 bind to cyclin-dependent kinase complexes to inhibit their catalytic activity and induce cell-cycle arrest (Sherr and Roberts, 1999).

Apoptosis is executed by caspases, a family of intracellular aspartate specific cysteine proteases which potentiates the apoptotic signal and proteolytically process numerous cellular target molecules with diverse functions (Cohen, 1997). Bcl-2 protein family members, including Bcl-2 and Bax function either to inhibit or promote apoptotic cell death (Scorrano et al., 2003). Once apoptosis was initiated, caspase-3 attenuates anti-apoptotic effect (Yang et al., 1997). The subtle balance of

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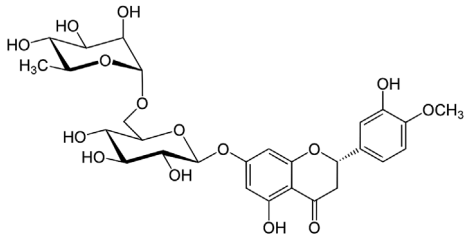

Fig. 1. Structure of hesperidin.

the Bcl-2/Bax complex might modulate the anti- or pro-apoptotic effect. AIF, apoptosis-inducing factor, is a mitochondrial flavoprotein that is released in response to death stimuli. It is reported that activation of poly (ADP-Ribose) polymerase-1 (PARP-1) is required for translocation of AIF from the mitochondria to the nucleus and AIF is necessary for PARP-1-dependent cell death (Yu et al., 2002).

Flavonoids have been shown to inhibit the cancer cells proliferation in *invitro* (Nichenametla et al., 2006; Singh and Agarwal, 2006). The observed anti-proliferative property of flavonoids suggests that these compounds may inhibit the cell cycle or induce apoptosis (Casagrande and Darbon, 2001). However, molecular mechanisms underlying the potential anticancer activity of flavonoids are not completely understood. Hesperidin (Fig. 1) a flavanone glycoside present in citrus fruits which possesses various pharmacological effects, including anti-diabetic, hepatoprotective, anti-oxidant, anti-inflammatory and anti-tumor properties (Mahmoud, 2014; Mahmoud et al., 2012; Siddiqi et al., 2015). The antioxidative mechanism of hesperidin includes enhancement of endogenous antioxidant levels, scavenging of free radicals, repression of reactive oxygen species (Chen et al., 2010; Sahu et al., 2013). Although, previous work from my lab showed the ability of hesperidin to inhibit the benzo(a)pyrene induced lung carcinogenesis (Kamaraj et al., 2009; Kamaraj et al., 2010), the mechanism of action by which it exerts its anticancer activity in lung cancer is not known. In the present study, we elucidated the molecular mechanism action of hesperidin on human lung cancer A549 cells.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified eagle's medium (DMEM), 0.25% trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA), 3-[4,5-dimethythiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT), propidium iodide, ethidium bromide, acridine orange, Rhodamine 123, agarose, β-actin antibody and hesperidin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Fetal Bovine serum (FBS) and antibiotic/antimycotic solution were procurred from Gibco, USA. Primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and HRP conjugated secondary antibody was purchased from Genei Bengaluru, India.

2.2. Cell culture

The A549 cell line was procured from the National Centre for Cell Science (Pune, India). Cells were grown in T75 culture flasks containing DMEM supplemented with 10% FBS at 37 °C in humidified air with 5% CO_2. After reaching confluence, cells were detached using 0.25% of Trypsin-EDTA solution.

2.3. Viability assay

MTT assay were performed by the method of Safadi et al. (2003). The cells were seeded in 24 well plates at a density of 5×10^4 cells per well. After 12hrs of incubation, the cells were treated with different concentrations of hesperidin for different time points. At the end of treatment, media from control and hesperidin treated cells were discarded and 500 µl of MTT containing DMEM (0.5 mg/ml) was added to each well. MTT containing medium was then discarded and the cells were washed with 1x phosphate buffered saline (PBS; 1 ml). Crystals were then dissolved by adding 500 µl of solubilization solution and this was mixed effectively by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured using a micro plate reader at 620 nm. Based on MTT assay, we selected the doses of 12.5 µM and 25 µM hesperidin treatment for 24 h as effective LC50 doses, which is used for further studies.

2.4. Acridine orange/ethidium bromide dual staining

Ethidium bromide/acridine orange staining was carried out by the method of Gohel et al. (1999). A549 cells were plated at a density of 5×10^4 in 6 well plates containing sterile coverslips. They were allowed to grow at 37 °C in a humidified CO_2 incubator until they were reached 70–80% confluence. Then cells were treated with hesperidin (12.5 and 25 µM) for 24 h. The culture medium was aspirated from each well and the cells were gently rinsed twice with PBS at room temperature. Then the cover slips were taken out and put it on glass slides to be stained with 100 µl of dye mixture (1:1 of ethidium bromide and acridine orange) and were viewed immediately by fluorescence microscopy. Viable cells had green fluorescent nuclei with organized structure, early apoptotic cells had yellow chromatin in nuclei that were highly condensed or fragmented; apoptotic cells also exhibited membrane blebbing. Late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented; necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, unbiased manner. For each sample, at least 500 cells/well and 4 wells/conditions were counted, and the percentage of apoptotic cells was determined [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) x100].

2.5. Propidium iodide staining for nuclear morphology assessment

Propidium iodide staining was carried out by the method of Mohan et al., 2007). A549 cells were plated at a density of 5×10^4 in 6well plates containing sterile coverslips. They were allowed to grow at 37 °C in a humidified CO_2 incubator until they were reached 70–80% confluence. Then cells were treated with hesperidin (12.5 and 25 µM) for 24 h. The culture medium was aspirated from each well and the cells were gently rinsed twice with PBS at room temperature, before fixing in methanol: acetic acid (3:1 v/v) for 10 min, and stained with 50 µg/ml propidium iodide for 20 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined by fluorescence microscopy and at least 1×10^3 cells were counted for assessing apoptotic cell death.

2.6. Determination of mitochondrial membrane potential

Changes in mitochondrial transmembrane potential were measured by uptake of the mitochondrial specific lipophilic cation dye Rhodamine 123, by the method of Mohan et al. (2007). Approximately 1×10^6 cells were plated in 100 mm Petri dishes with DMEM containing 10% FBS, then they were incubated for 24 h in 5% CO_2 at 37 °C. Control cells received 0.1% DMSO containing DMEM and hesperidin treated cells received 12.5 and 25 µM of hesperidin containing DMEM. After 24 h, cells were trypsinized and used for mitochondrial membrane potential analysis using flow cytometry. Briefly, 1×10^6 cells were taken from the control and hesperidin treated plates, and was centrifuged at 1000 g for 5 min. Supernatant was removed, and cells were washed twice with PBS. The pellet was resuspended in approximately 900 µl of ice cold PBS and cells were mixed with aspiration 20 times

using a pipette. To this 100 µl of Rhodamine 123 (100 µg/ml) was added and all were incubated at room temperature for 30 min in the dark. Cells were pelleted again and the supernatant was discarded (to remove excess rhodamine 123) and cells were resuspended in PBS. The samples of 10^4 events were then immediately subjected to flow cytometric analysis at an excitation wavelength of 488 nm and emission wavelength of 545 nm. Mean fluorescence intensities were recorded and compared.

2.7. DNA agarose gel electrophoresis

DNA extractions were performed using the following protocol. Briefly, 1×10^6 cells were plated in 100 mm Petri dishes with DMEM containing 10% FBS. Cells were incubated for 24 h in 5% CO_2 and 95% air at 37 °C. Control cells received 0.1% DMSO containing DMEM, and hesperidin treated cells received 12.5 and 25 µM of hesperidin containing DMEM. After 24 h, the cells were trypsinized and combined with the cells in the medium by centrifugation at 1500 rpm for 5 min, and then they were washed twice with PBS. The resulting pellet was resuspended in 0.25 ml of lysis buffer, transferred to a microfuge tube, and incubated for 1 h at 37 °C. To this 4 µl of proteinase K was added and tubes were then incubated at 50 °C for 3 h. To each tube, 0.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed and centrifuged at 13 000 rpm for 30 min at 4 °C to separate the DNA containing upper aqueous phase. To the resultant aqueous phase, two volumes of ice cold absolute ethanol and 1/10 the volume of 3 M sodium acetate was added and kept at −20 °C overnight to precipitate DNA. The DNA was pelleted by centrifuging at 13000 rpm for 10 min at 4 °C and the supernatant was aspirated and the pellet washed in 1 ml of 70% ethanol. After repeating the above centrifugation step and removing last traces of the supernatant fraction, the pellet was allowed to dry at room temperature for approximately 30 min before being resuspended in 50 µl of Tris-EDTA buffer. DNA was quantified by ultraviolet, visible spectroscopy and 10 µg of DNA was electrophoresed in 1.5% agarose gel containing ethidium bromide in a mini gel tank containing Tris-borate-EDTA buffer for 2 h at 90 V. The gel was then examined under ultraviolet light and photographed.

2.8. Western blot analysis

Using the following method assessed western blot analysis of protein expressions. Approximately 50 µg of protein was mixed with an equal volume of 2x sample buffer, boiled for 5 min at 100 °C, cooled, loaded in each lane of 8–15% polyacrylamide gel, and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at room temperature. Resolved proteins were electrophoretically transferred to PVDF membranes which were then blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature, and then probed with the following primary antibodies: APAF-1 (dilution 1:1000), Bcl-2 (rabbit polyclonal antibody at a dilution of 1:1000), Bax (rabbit polyclonal antibody at a dilution of 1:1000), p53 (rabbit polyclonal antibody at a dilution of 1:1000), cytochrome c (mouse monoclonal antibody at a dilution of 1:1000) active caspase-3 (goat polyclonal antibody at a dilution of 1:500), β-catenin (mouse monoclonal antibody at a dilution of 1:2000), cyclin D1 (mouse monoclonal antibody at a dilution of 1:1000), cdk4 (mouse monoclonal antibody at a dilution of 1:1000), p21 (mouse monoclonal antibody at a dilution of 1:1000), c-myc (rabbit polyclonal antibody at a dilution of 1:2000), PCNA (mouse monoclonal antibody at a dilution of 1:1000), β-actin (mouse monoclonal antibody at a dilution of 1:5000)incubated for overnight at 4 °C. Blots were then extensively washed with Tris-buffered saline with 0.1% Tween 20 (TBS-T) and were incubated with the respective (anti-goat, anti-rabbit and anti-mouse) horseradish peroxidase labelled secondary antibodies (Genei, Bangalore, India) at a dilution of 1:3000 for 1 h at room temperature. After thorough washes in TBS-T, bands were visualized by treating the membranes with 3,3′-diaminobenzidine tetrahydrochloride (Sisco Research Laboratories). Membranes were photographed and quantified with ImageJ image analysis software (National Institutes of Health, Bethesda, MD, USA).

2.9. Statistical analysis

All grouped data were significantly evaluated using SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference test. Significance values mentioned as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ were considered statistically significant when compared to untreated cells. All these results were expressed as mean ± standard deviation ($n = 3$).

3. Results

3.1. Inhibitory effects of hesperidin on the growth of A549 lung cancer cells

To evaluate the inhibitory effects of hesperidin on the growth of A549 cells were assessed. The cells were treated with different concentrations of hesperidin 0, 6.25, 12.5, 25, 50 and 100 µM of hesperidin for 0, 6, 12, 24, and 48 h and the cell viability were determined by MTT assay. Hesperidin significantly inhibited the growth of A549 in time and dose dependent manner. IC50 dose of 12.5 and 25 µM of hesperidin for 24hrs showed significance difference and optimal doses when compared to other doses/untreated cells (Fig. 2).

3.2. Hesperidin downregulated the proliferation associated proteins of β-catenin, c-Myc and PCNA in A549 lung cancer cells

To investigate further the cell growth inhibition by hesperidin (Fig. 3a and b) illustrates the protein expressions and densitometry analysis of β-catenin, c-Myc and PCNA in control and hesperidin treated cells for 24hrs. Hesperidin treatment significantly reduced the protein expressions of β-catenin, c-Myc and PCNA dose dependently when compared to untreated cells, which are clearly evident from immunoblotting and corresponding densitometry analysis (Fig. 3a and b).

3.3. Hesperidin upregulates the p53 expression and induces the cell cycle arrest in A549 cells

p53 plays an important role in both induction of cell cycle arrest (Ciciarello et al., 2001) and induce apoptosis in the cancer cells (Warin et al., 2014). Western blot analysis was performed to examine the expressions of p53, p21, cdk4, cyclin D, Bcl-2 and Bax during hesperidin treatment. The expression levels of p53 and p21 were significantly increased during hesperidin treatment. In contrast, the expressions of cyclin D and cdk 4 significantly decreased after treatment with 12.5 µM and 25 µM of hesperidin for 24hrs when compared to untreated cells (Fig. 4a and b). Hesperidin treatment significantly reduced the expression of Bcl-2, where as simultaneously increased the Bax expression

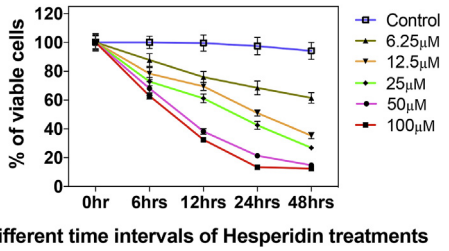

Fig. 2. Inhibitory effects of hesperidin on the growth of A549 cells. A549 cells were treated with various concentrations 0, 6.25, 12.5, 25, 50 and 100 µM of hesperidin for 0, 6, 12, 24, and 48 h. The viability was determined by the MTT assay. The data are presented as the mean ± S.D of the results for three independent experiments.

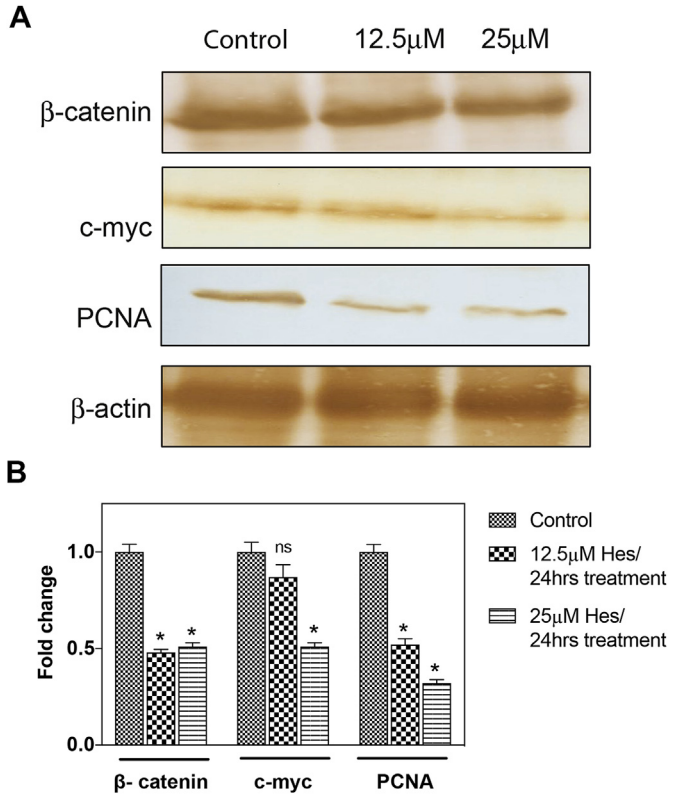

Fig. 3. Effects of hesperidin on proliferation of A549 cells. Hesperidin down regulated expression of proliferation associated proteins β-catenin, c-Myc and PCNA in A549 cells. Lanes l, 2 and 3 correspond to lysates of A549 control, 12.5 μM of hesperidin and 25 μM of hesperidin for 24 h treatment respectively. Significance values mentioned as *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant when compared to untreated cells. All these results were expressed as mean ± standard deviation (n = 3).

levels in a dose dependent manner when compared to untreated cells.

3.4. Hesperidin induced the apoptotic cell death in A549 cells

To determine the apoptosis induction by hesperidin were confirmed by different studies. The phase contrast microscope observation shows that the cells underwent reduced numbers of cells, marked morphological changes such as becoming round in shape and many dead cells were observed (Fig. 5a) during hesperidin treatment of 12.5 μM and 25 μM for 24hrs when compared with untreated control. Fluorescence microscopy morphological changes of ethidium bromide/acridine orange stained A549 cells (Fig. 5b) showed that the significant increase in the percentage of apoptotic cells after treatment with 12.5 μM and 25 μM of hesperidin for 24 h drastically to 21% and 32%, respectively. Propidium iodide stained A549 cells showed that the apoptotic cells had characteristic condensed nuclei (Fig. 5c). The percentage of apoptotic nuclei after treatment with 12.5 μM and 25 μM of hesperidin increased enormously to 37% and 59%, respectively, as revealed by nuclear condensation and fragmentation when compared to untreated cells. Hesperidin treated cells with 12.5 μM and 25 μM for 24hrs resulted in significant increased DNA fragmentation appearance than untreated cells (Fig. 5d).

3.5. Hesperidin induced the mitochondrial membrane depolarization and activation of apoptosis cascade in A549 cells

Western blot analysis was performed to examine APAF-1, cytochrome c and caspase 3 expressions during hesperidin treatment. The expression levels of APAF-1, cytochrome c and caspase 3 significantly

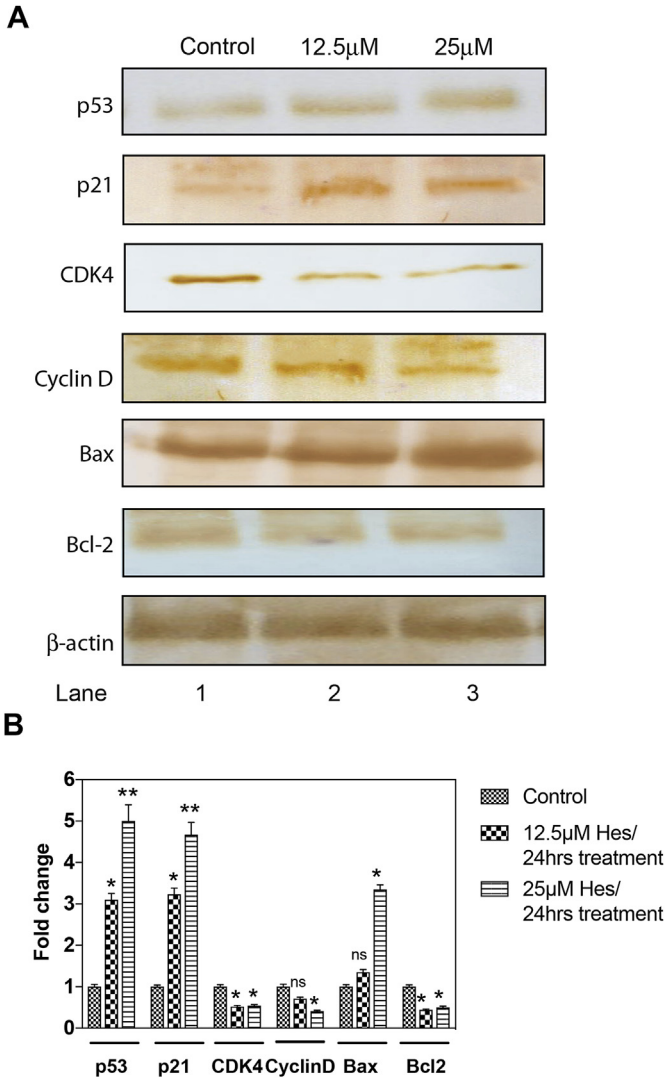

Fig. 4. p53 activation required for regulation of cell cycle and induce apoptosis. The immunoblotting analysis of p53, cyclin D, cdk4, p21, Bcl-2 and Bax in control and hesperidin treated A549 cells. Lanes l, 2 and 3 correspond to the lysates of A549 control, 12.5 μM of hesperidin and 25 μM of hesperidin for 24 h treatment respectively. Significance values mentioned as *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant when compared to untreated cells. All these results were expressed as mean ± standard deviation (n = 3).

increased in 12.5 μM and 25 μM of hesperidin treatments for 24hrs (Fig. 6a and b) when compared to untreated cells. Further, the effect of hesperidin on mitochondrial membrane potential was studied using flow cytometry analysis. Hesperidin treatment significantly depolarized the mitochondrial membrane that was evident from the significant decrease in mean fluorescence of rhodamine 123 (Fig. 6c) (peak shift move towards the left side) than untreated cells.

4. Discussion

In this study, we have assessed that hesperidin inhibited the cell growth, altered proliferation associated proteins, and induced apoptosis in A549 cells. Constitutive activity of β-catenin can exert both proliferative and anti-apoptotic effects (Sadot et al., 2001). Hence, the down-regulation of β-catenin by activated p53 is likely to contribute to the anti-proliferative effects of p53 and possibly also facilitate p53-mediated apoptosis. Down-regulation of c-Myc and β-catenin by antioxidants has been proposed as a promising pharmacological treatment

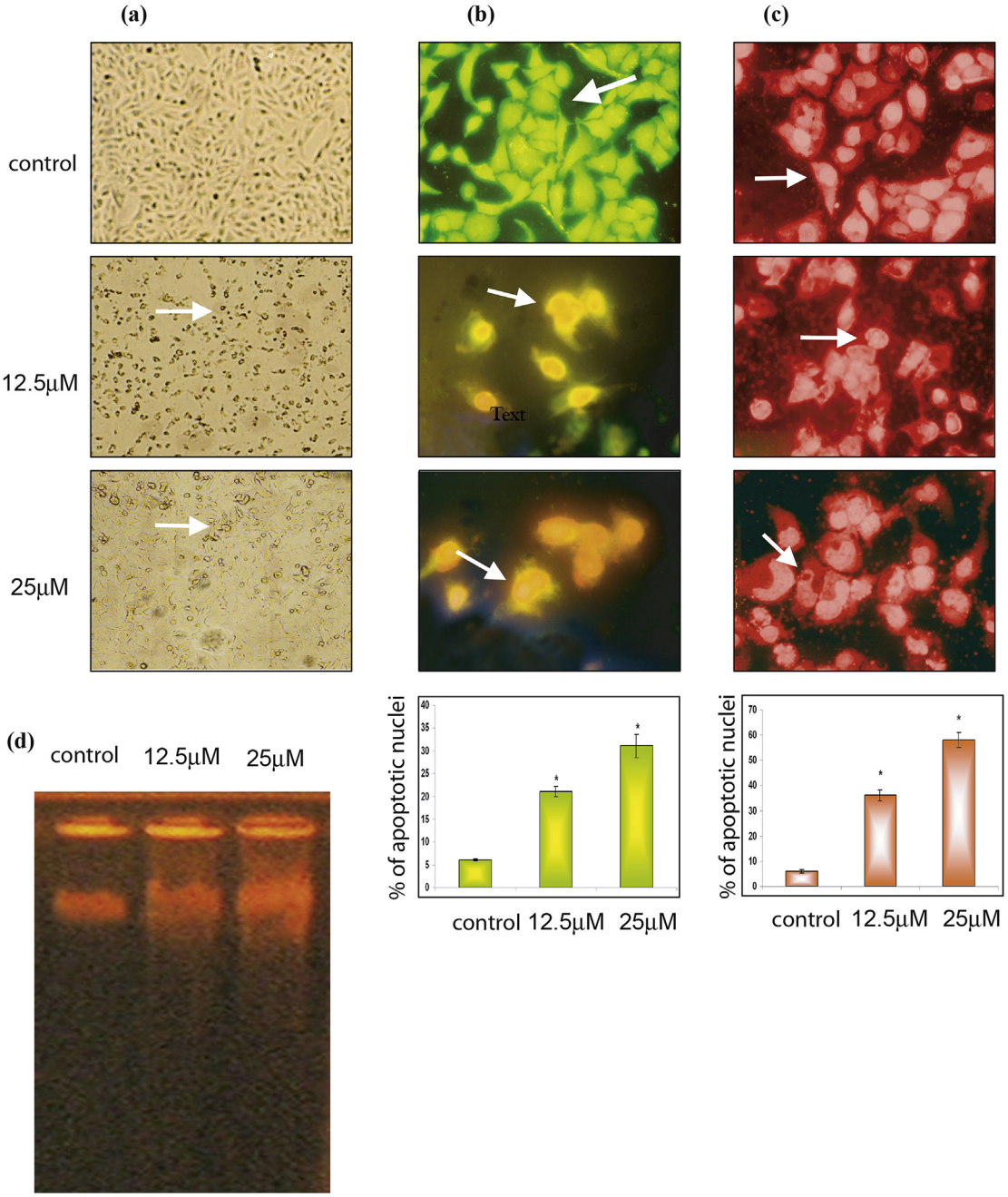

Fig. 5. Hesperidin induced apoptosis in A549 cells. (a) Morphological changes of A549 cells by light microscopy (× 20): A549 controls, treatment with 12.5 μM and 25 μM of hesperidin for 24 h treatment. Hesperidin increased percentage of apoptosis of A549 cells as viewed by (b) ethidium bromide/acridine orange staining, (× 40), ((1) A549 control cells (showing viable green fluorescent nuclei), (2) treatment with 12.5 μM of hesperidin (early apoptotic cells are yellow fluorescent nuclei), (3) treatment with 25 μM of hesperidin (late apoptotic cells with orange fluorescent nuclei), and (4) representative bar chart showing percentage of apoptotic cells. Results are expressed as mean ± standard deviation ($n = 3$). *$P < 0.05$ compared with A549 control, 12.5 μM and 25 μM of hesperidin). (c) By propidium iodide staining, (× 40). ((1) A549 control (normal nuclear pattern), (2) treatment with 12.5 μM hesperidin (nuclear condensation and fragmentation), (3) treatment with 25 μM hesperidin (nuclear condensation and fragmentation) and (4) representative bar chart showing percentage of apoptotic nuclei. Results are expressed as mean ± standard deviation ($n = 3$). *$P < 0.05$ compared with A549 control, 12.5 μM of hesperidin and 25 μM of hesperidin), (d) by agarose gel electrophoresis pattern of nuclear DNA. (Lanes 1, 2, 3 and M representing A549 control 12.5 μM hesperidin, 25 μM hesperidin and marker, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

for cancer (Ramakrishnan et al., 2009). In our experiments, hesperidin significantly induced the p53 expressions consequently suppression of c-myc and β -catenin expressions. PCNA is a useful marker of proliferative activity and an important marker for evaluating the proliferation of several cancers, including lung cancer (Zhou and Ho, 2014). Hesperidin treatment significantly decreased the PCNA expressions thereby reduced proliferation.

The p53 protein is a regulator of cell cycle progression and mediator of apoptosis in many cell lines, and the cell cycle progression was accelerated by cdk and decelerated by p53 and cdk inhibitors including p21 (Bunz et al., 1998). Cdk4 and its cyclin D/cdk4 complex play a role of in regulating either G1 cell cycle progression or cell growth (Datar et al., 2000). The p53 gene is often mutated in many tumor cells and the mutations contribute to clonal cellular expansion and genomic

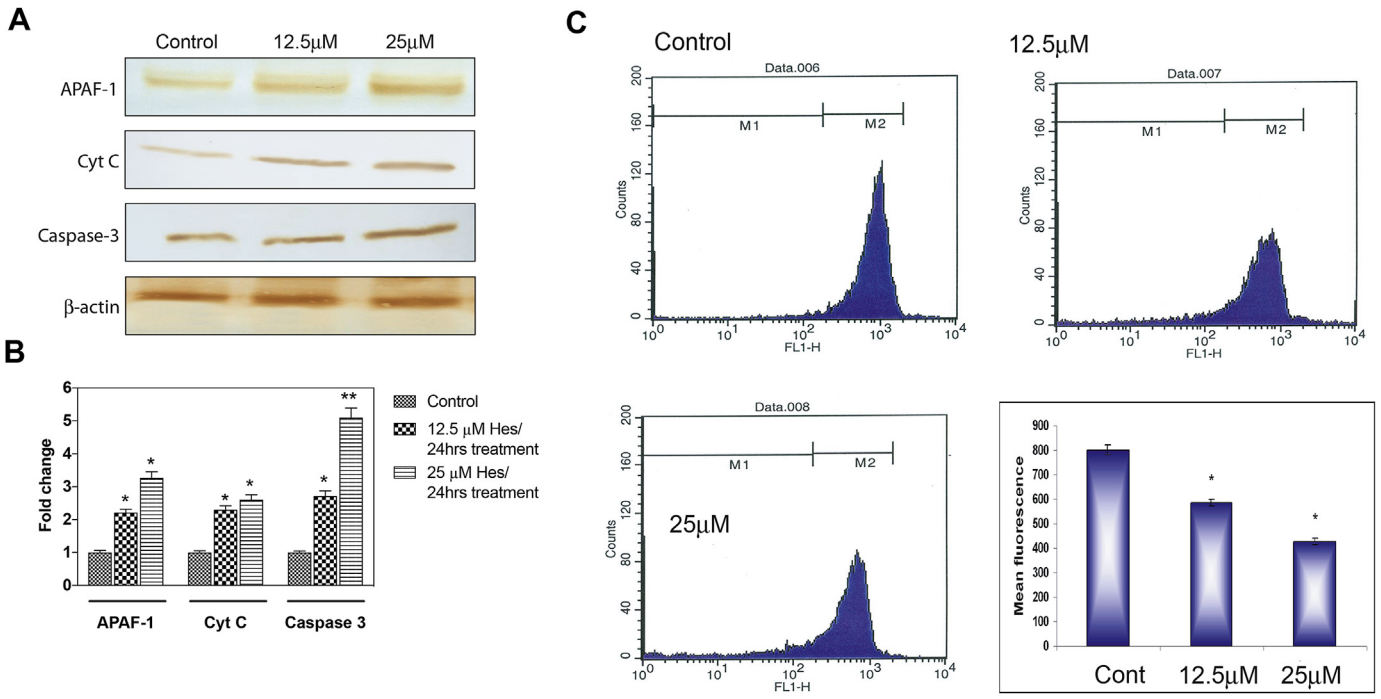

Fig. 6. Hesperidin treatment decreased mitochondrial transmembrane potential of A549 cells. (a & b) Immunoblotting analysis of APAF-1, cytochrome c and caspase-3 in control and hesperidin treated A549 cells. Lanes l, 2 and 3 correspond to the lysates of A549 control, 12.5 μM of hesperidin, and treatment with 25 μM of hesperidin for 24 h treatment, respectively. (b) Mitochondrial membrane potential as assessed by staining with rhodamine 123. Control A549 cells, treatment with 12.5 μM and 25 μM of hesperidin for 24 h treatment, respectively. Bar chart representing mean fluorescence of rhodamine 123 in A549 control, treatment with 12.5 μM and 25 μM of hesperidin. Significance values mentioned as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ were considered statistically significant when compared to untreated cells. All these results were expressed as mean ± standard deviation ($n = 3$).

instability because of diminished regulation of cell cycle checkpoints, DNA repair, and apoptosis (Ciciarello et al., 2001). It was reported that human lung cancer cells A549 did not show mutation in the p53 gene (Ahmed et al., 1996). In addition, hesperetin induced a decrease in cyclin D and cdk 4, together with an increase in p21Cip1and p27Kip1 associated with cdk 4. Cdk 4 appears to play a key role in the G1 cell cycle arrest observed in response to hesperetin (Choi, 2007). The present results indicated that hesperidin caused alteration in cell cycle progression through up-regulation of p53, p21 thereby down regulation of cyclin D and cdk 4, which clearly indicates that hesperidin, possess potent antiproliferative property against cancer cells.

Hesperidin treatment dose dependently increased the level of p53 in our experiment. p53 might have directly facilitated the cytochrome c release (Mihara et al., 2003), from mitochondria and initiated apoptosis. Bcl-2 and Bax protein are members of a large family of proteins called 'the Bcl-2 family' and both are important regulators of apoptosis. Thus, ratio between Bcl-2/Bax might be one of the critical factors of a cell's threshold for undergoing apoptosis (Cory and Adams, 2005). In the present study, there was increased expression of Bcl-2 and a subsequent decreased in expression of Bax was seen in A549 cells; hence, an increase in the Bcl-2/Bax ratio, which is an indication of diminished apoptosis. Hesperidin treatment decreased the Bcl-2/Bax ratio, which might be due to the ability of hesperidin able to up regulated p53 expression because p53 is a positive transcriptional activator for Bax and a negative transcriptional activator for Bcl-2(Xiao et al., 2004), thus the activation of the p53 pathway by hesperidin might lead to the down-regulation of Bcl-2 and up-regulation of Bax.

In the present study, ethidium bromide/acridine orange staining, propidium iodide staining and DNA fragmentation assessed induction of apoptosis by hesperidin in A549 cells. All the above clearly indicated that hesperidin treatment induced apoptosis. Substantial evidence implicates mitochondria in apoptotic cell death and there is a direct relationship between mitochondrial depolarization and cytochrome c release. Cytochrome c is undoubtedly one of the prominent actors in the apoptotic scene. Here, hesperidin treatment significantly decreased mitochondrial membrane potential of A549 cells, thus, leading to opening of mitochondrial permeability transition pores and consequently, release of cytochrome c from the intermembrane space into the cytosol. This initiates the apoptotic cascade, which is well correlated with earlier studies of hesperidin on other types of cancer cell(Sivagami et al., 2012; Tamilselvam et al., 2013). In response to apoptotic stimuli, APAF-1 binds to cytochrome c and procaspase-9 in the presence of adenosine triphosphate to form a multi protein complex called the apoptosome. This results in activation of procaspase-9 by autocatalytic cleavage, initiating a cascade of downstream effector caspases, especially caspase-3, which cleave several cell proteins, ultimately leading to apoptosis (Hickman and Helin, 2002). Here, hesperidin treatment resulted in increased APAF-1 and activated caspase-3 in the A549 lung cancer cells. Treatment with hesperidin inducing expression of APAF-1 might have interacted with released cytochrome c forming apoptosomes and therefore further activates the caspase-3 expressions which triggers apoptosis that strongly suggest hesperidin possess apoptosis inducing property.

Thus, our present results clearly demonstrate that hesperidin inhibited A549 lung cancer cell proliferation and arrested the cell cycle progression through down regulation of c-myc and β-catenin expressions. Simultaneously, hesperidin restores the tumor suppressor proteins p53 and p21, which induces the apoptosis by up regulation of Bax and down regulation of Bcl-2 expressions. These results in the release of cytochrome c, apaf-1 complex and caspases 3 activation ultimately lead to induced cell death by apoptosis. Thus, our overall present study reveals that regulation of apoptosis and cell cycle progression could be one of the plausible molecular mechanisms actions for the chemotherapeutic effect of hesperidin against human lung cancer A549 cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101065.

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