Cellular & Molecular Biology
Cell. Mol. Biol. 2015; 61 (6): 92-99
Published online October 30, 2015 (http://www.cellmolbiol.com)
Received on October 2, 2015, Accepted on October 8, 2015.
doi : 10.14715/cmb/2015.61.6.12

Hesperetin exerts apoptotic effect on A431 skin carcinoma cells by regulating mitogen activated protein kinases and cyclins

T. P. Smina, A. Mohan, K. A. Ayyappa, S. Sethuraman and U. M. Krishnan✍

Centre for Nanotechnology & Advanced Biomaterials, School of Chemical & Biotechnology, SASTRA University, Thanjavur 613 401, India

Corresponding author: Dr. Uma Maheswari Krishnan, Centre for Nanotechnology & Advanced Biomaterials, School of Chemical & Biotechnology, SASTRA University, Thanjavur 613 401, India. E-mail: umakrishnan@sastra.edu

Abstract

Dietary agents and phytochemicals have been utilised for the management of cancer for many years. Hesperetin, a dietary flavonoid found abundantly in citrus fruits, was evaluated for its cytotoxic and pro-apoptotic activities in A431 human skin carcinoma cells. Effect of hesperetin in regulating MAPK (Mitogen-Activated Protein Kinase) signalling pathway and levels of various cyclins and other downstream apoptotic proteins were investigated. Its critical role in regulating other apoptotic proteins especially p21, Bcl-2 and Bax were also assessed. Hesperetin stimulated alterations in MAPK (Mitogen-Activated Protein Kinase) signalling pathway by modulating the expression levels of ERK (Extracellular signal Regulated Kinase), JNK (c-Jun NH_2-terminal Kinase) and p38; thereby induced apoptosis in A431 cells. Hesperetin regulated the levels of cyclin A_2, B_1, D_1, D_3 and E_1. It also modulated the levels of various proteins involved in apoptotic pathway especially p21, Bcl-2 and Bax. The study revealed the efficiency of hesperetin against human skin carcinoma cells and proposed its mechanism of action; there by opens up new avenues for the use of this dietary flavonoid against skin malignancies.

Key words: JNK, ERK, p38, p21, Bcl-2, Bax.

Introduction

Natural products, especially nutraceuticals, have a superior role in preventive and therapeutic treatment modes for cancer because of their reduced toxicity and capacity to alter multiple molecular targets and pathways (1). The occurrence of as many as 35% of all cancers has been attributed to diet and can be prevented by dietary modifications (2,3,4). The intake of large quantities of fruits and vegetables containing a variety of phytochemicals is considered to be beneficial for reducing the risk of cancer development (5).

Flavonoids are natural antioxidants that found abundantly in food and exert a wide range of therapeutic effects (6,7). The fact that flavonoids express cytotoxicity towards numerous human cancer cells with very little or no effect on normal cells (8) has garnered much interest towards the development of potential flavonoid-based chemotherapeutics for cancer. Hesperetin ((2S)-5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one) (Figure 1A) is a flavonoid found abundantly in citrus fruits. It has been reported to possess many medicinal properties including blood lipid lowering activity (9), anti-carcinogenic activities (10,11), antioxidant and free radical scavenging properties (12,13). But information regarding its molecular targets and the exact mechanism responsible for its pharmacological activities is still lacking. It was found that hesperetin induced cell cycle arrest at the G1-phase in human breast cancer (MCF-7) cells (11) and prompted Notch1 expression in carcinoid cells, resulting in suppression of tumour cell proliferation (10). Hesperetin induced apoptotic effect on human cervical cancer cells through cell cycle arrest, death receptors, and mitochondrial pathways (14). Hesperetin was also found to inhibit chemically induced mammary (15) and colon (16) carcinogenesis in laboratory animals. Although the effect of hesperetin on some of the signalling pathways has been studied, the key molecular mechanism accountable for its anti-cancer property is not yet established and this would require further investigation. Incidence of skin carcinoma has increased in the recent past (17). The pursuit for new anticancer agents against skin carcinoma is crucial owing to the limitations of the existing therapeutic strategies. The current study intends to investigate the anti-carcinogenic effect of hesperetin against human skin carcinoma cells and to evaluate the mechanism behind its anti-proliferative and apoptotic actions. To the best of our knowledge this is the first report investigating the pro-apoptotic activity and the corresponding mechanism of hesperetin in A431 human skin carcinoma cells.

Materials and methods

Reagents

Hesperetin,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2',7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell-culture medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco Life Technologies Inc. (Grand Island, NY, USA). Live-dead cell viability kit was purchased from Molecular Probes (Eugene, Oregon, USA). QIA Shredder Spin Column, Quantitect Reverse transcription kit, nuclease-free water and RNAse-free water were procured from Qiagen, USA. Antibodies for cyclins (A_2, B_1, D_1, D_3 and E_1), Bcl-2, Bax and p21 were obtained from Cell Signalling Technology Inc. (Danvers, MA,

USA). All other chemicals used were of reagent grade.

Maintenance of A431skin carcinoma cell line

A431 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL Penicillin and 100 µg/mL Streptomycin in 5% CO_2 atmosphere at 37°C.

Cytotoxicity screening

Cytotoxicity of hesperetin was assessed in terms of viability quantified by MTT assay (18). For the assay, cells in the logarithmic phase ($2x10^4$) were incubated in a 96-well plate in the presence of various concentrations of hesperetin (10-500µM) in a final volume of 200 µL. Twenty micro litres of MTT labelling agent (5 mg/mL in phosphate buffered saline) was added after 24h to each well. After an additional 4 h of incubation, the blue MTT formazan precipitate was dissolved by adding 100 µL of 10% SDS in 0.01M HCl and the absorbance at 550 nm was measured using a multimode plate reader (Infinite M200, Tecan, Austria).

Live - Dead Assay

To study the influence of hesperetin on cell viability, live-dead assay was performed which determines intracellular esterase activity and plasma membrane integrity. Briefly, $1x10^5$ cells were seeded on glass slides in a 6-well plate and then incubated with different concentrations of hesperetin (10, 100 and 500µM) for 24 h. The medium containing hesperetin was then removed and cells were washed with PBS. Cells were stained with the live-dead cell viability kit (Molecular Probes, Oregon, USA) comprising 5µM ethidium bromide homodimer and 5µM calcein-AM followed by incubation at 37°C for 30 min. Cells were imaged using a laser scanning confocal microscope (FV1000, Olympus, Japan).

Clonogenic assay

For colony formation assay (19), 5×10^2 cells per well were seeded in 70mm petri plates and treated with different concentrations of hesperetin (10 and 100µM). Triplicate cultures of treated A431 cells were maintained at 37°C for 14 days in an atmosphere of 5% CO_2, with fresh medium being added after 4 days. Cells were then stained with 0.1% (w/v) crystal violet. Colonies, defined as groups of cells containing a minimum of 50 cells, were counted under an inverted phase contrast microscope (Axio Vert.A_1, Carl-Zeiss, Germany). The relative cell proliferation was expressed in percentage as:

$$\% \text{ Growth} = \frac{\text{number of colonies from treated cells}}{\text{number of colonies from controls}} \text{ X } 100$$

Estimation of apoptosis using DNA ladder assay

The ability of hesperetin to induce apoptosis in A431 cells was determined using DNA ladder assay. A431 cells (2×10^6) were incubated for 24 h with various concentrations of hesperetin (10, 100and 500µM) in a 5% CO_2 incubator at 37^{0}C. The cells were then washed with PBS and the pellet was re-suspended in 100µL lysis buffer (50mM Tris–HCl, pH 8, 10mM EDTA, 0.5% N-lauryl sarcosine, 0.5mg/mL proteinase K) followed by incubation at 50°C for 1 hour. 10µL of RNase (1mg/mL in Tris - NaCl buffer) was added to the lysate and further incubated at 50°C for 1 h. The DNA samples were separated in a 1.5% agarose gel containing 0.5µg/mL ethidium bromide in Tris–Borate–EDTA buffer. The bands were visualized using a Bioimaging System (ChemiDoc XRS, Bio-Rad Laboratories, India) and analyzed using Quantity One Software.

Measurement of intracellular ROS

The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was used to monitor the generation of ROS induced by hesperetin in A431 cells (20). To quantify intracellular ROS, A431 cells (2×10^6) were pre-incubated with 20 µM $DCFH_2$-DA for 20 min at 37°C prior to the addition of hesperetin. The cells were then incubated with various concentrations of the hesperetin (10, 100 and 500µM) for 30, 60 and 90min. The increase in fluorescence resulting from the oxidation of $DCFH_2$ to DCF was measured at excitation and emission wavelengths of 485 and 535 nm, respectively.

Gene expression studies of MAP kinases using PCR assay

The mRNA expression levels of MAP kinases (ERK, JNK and p38) were analysed using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). A431 cells (2×10^6) were incubated with various concentrations of hesperetin (100 and 500µM) for 24 h in a 5% CO_2 incubator at 37^{0}C. Total cellular RNA was isolated from treated cells using Trizol reagent (Gibco BRL, USA) following the procedure described by the manufacturer (Qiagen, 74106, USA) (21). Briefly, Trizol (1 mL) was added to the sample and left undisturbed at room temperature for half an hour. The solution was then used to extract RNA with chloroform (0.2 mL) and centrifuged at 12000 rpm at 4^{0}C for 15 min. The RNA extracted was stabilized in 70% ethanol prepared using nuclease-free water. QIA Shredder Spin Column (Qiagen, USA) was used to centrifuge RNA that was then dissolved in RNAse-free water. cDNA was synthesized from 2 µg of total isolated RNA by using QuantiTect Reverse Transcription Kit (Qiagen, USA). Real-time PCR was performed using Sybr® Green Jumpstart™ *Taq* Readymix (USA) using an Eppendorf AG22331 system, Germany. The primers used for the study were designed using Primer 3 software and

Table 1. Forward and reverse primers used for real-time RT-PCR.

Genes	Forward primers	Reverse primers
ERK1	5'-ACAGTCTCTGCCCTCCAAGA-3'	5'-CTCATCCGTCGGGTCATAGT-3'
ERK2	5'-CCACCCATATCTGGAGCAGT-3'	5'-CAGTCCTCTGAGCCCTTGTC-3'
JNK1	5'-GTGATCAATGGCTCTCAGCA-3'	5'-TGACTAACCGACTCCCCATC-3'
JNK2	5'-TTCATCCATGTCCACTGAGC-3'	5'-CCATCAACTCCCAAGCATTT-3'
p38	5'-CAGTGGGATGCATAATGGCC-3'	5'-GCATCTTCTCCAGCAAGTCG-3'
GAPDH	5'-AGGTCGGAGTCAACGGATTTG-3'	5'-GTGATGGCATGGACTGTGGT-3'

ERK -Extracellular signal Regulated Kinase, JNK - c-Jun NH_2-terminal Kinase, GAPDH- Glyceraldehyde-3-phosphate dehydrogenase.

are listed in Table 1. The expression levels of JNK, ERK and p38 genes in the treated cells were compared with those from the untreated control cells.

Analysis of cyclins and other apoptotic proteins expression using Western blot analysis

The effect of hesperetin on the expression levels of cyclins (A_2, B_1, D_1, D_3 and E_1) and other proteins involved in apoptotic pathway (Bcl-2, Bax and p21) was determined using Western blot analysis. A431 cells (2×10^6) were incubated with different concentrations of hesperetin (10, 100 and 500µM) for 24 h in a 5% CO_2 incubator at 37^{0}C. To determine the levels of protein expression, whole-cell extracts from treated A431 cells were prepared in lysis buffer (20mM Tris (pH 7.4), 250mM NaCl, 2mM EDTA, pH 8, 0.1% Triton X-100, 0.01µg/mL aprotinin, 0.005µg/mL leupeptin, 0.4mM PMSF, and 4mM sodium ortho vanadate). The lysates were spun at 14,000 rpm for 10 min to remove insoluble material and resolved by 10% SDS-PAGE. 80µg of protein was loaded in each well. After electrophoresis, the proteins were electro-blotted on to nitrocellulose membranes, probed with the appropriate primary (Cyclin A_2, B_1, D_1, D_3& E_1, Bcl-2, Bax and p21) and secondary (anti-rabbit/mouse IgG, HRP-linked) antibodies, and detected by enhanced chemi-luminescence (Thermo Scientific Pierce ECL Western blotting substrate). Rabbit polyclonal antibody to β-actin was used as loading control.

Statistical analysis

All values were expressed as mean ± standard deviation (S.D.). Statistical evaluation of the data was done by one way analysis of variance (ANOVA) followed by Bonferroni's test using InStat Graph pad software. $P < 0.05$ was considered as significant with respect to control group.

Results

Effect of hesperetin on cell viability and cytotoxicity

Cytotoxicity of hesperetin in A431 cells was evaluated at various concentrations starting from nano- molar to milli-molar range and also at different time intervals. Selection of effective doses and incubation period for further experiments was based on this preliminary screening. The period in which hesperetin showed maximum cytotoxic effect was optimised as 24h. In treated wells, the percentage of surviving cells was found to be significantly reduced from the control in a dose-dependent manner, indicating the cytotoxicity of hesperetin in A431 cells. Figure 1b shows the results of MTT assay carried out to determine the cytotoxicity of hesperetin on A431 skin carcinoma cells at 24h. IC_{50} of hesperetin was calculated as 100 µM for 24h treatment. At the highest concentration (500 µM) of hesperetin, significantly reduced survival rate (5.6%) was observed after 24 h. These results indicate that hesperetin exhibits potent anti-proliferative effects against skin carcinoma cells.

Figure 1c shows the results of live/ dead assay, specifying the cytotoxicity of hesperetin in A431 cancer cells. The green fluorescence indicates the live cells stained by calcein and the red fluorescence shows the

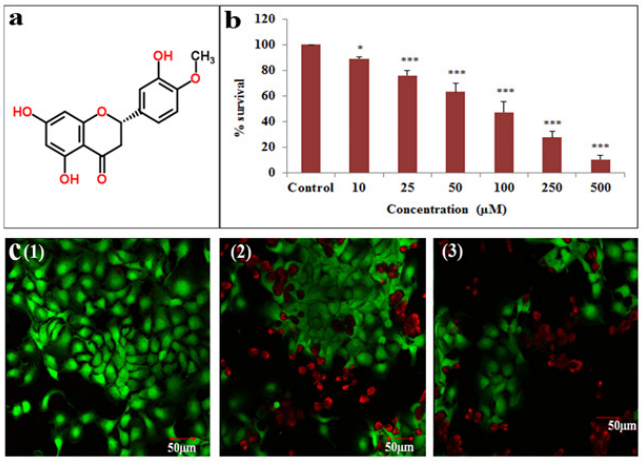

Figure 1. Cytotoxicity of hesperetin. a) Molecular structure of hesperetin. b) Cytotoxicity of hesperetin in A 431 cells at 24 h measured by MTT assay. c) Images showing the cytotoxicity of hesperetin analysed by live / dead assay (1) Control A 431 cells (2) A 431 cells treated with 10 µM hesperetin (3) A 431 cells treated with 100µM hesperetin. Values are mean ± S.D. ($n = 3$).

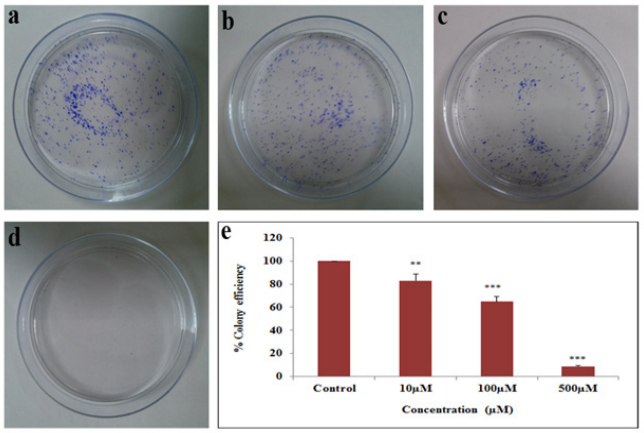

Figure 2. Clonogenic assay. a) Control A 431 cell colonies. b) Cells treated with 10 µM hesperetin. c) Cells treated with 100 µM hesperetin. d) Cells treated with 500 µM hesperetin. e) % Colony efficiency of various concentrations of hesperetin. Values are mean ± S.D. ($n = 3$). ***P < 0.001, **P < 0.01 as compared to control (Bonferroni test).

dead cells stained by ethidium bromide. A431 cancer cells when treated with 10 and 100µM of hesperetin for 24 hours induced cell death and detached dead cells started floating in between attached live cell colonies.

Figure 2 illustrates the results of clonogenic assay conducted by plating A431 cells after incubation with hesperetin. Figure 2a shows the control cells without hesperetin treatment. Figures 2b-d show the images of colonies formed from cells that survived the treatment with various concentrations of hesperetin. The percentage colony forming efficiency was reduced to 9.6% in the case of highest concentration of hesperetin (500µM) (Figure 2e).

Effect of hesperetin on induction of apoptosis

The induction of apoptosis by hesperetin in A431 cells was analysed by DNA ladder assay. DNA fragmentation is a characteristic feature of apoptosis and the fragmented DNA appear as a ladder when analysed with the aid of agarose gel electrophoresis. Figure 3a shows the laddered pattern of DNA induced by various concentrations of hesperetin in A431 cells. In control

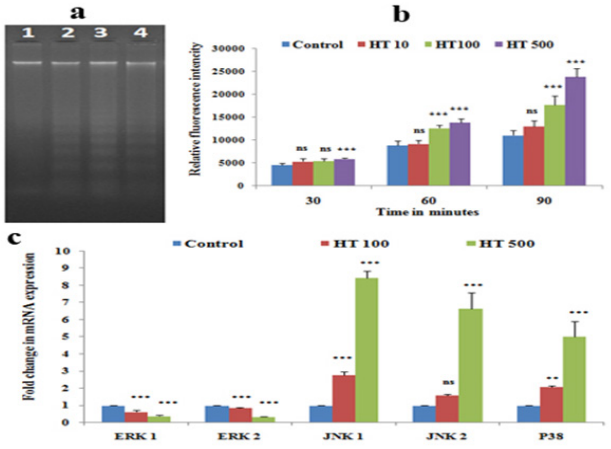

Figure 3. Induction of apoptosis by hesperetin and its mechanism. a) DNA laddering induced by hesperetin 1) Control A 431 cell 2) Cells treated with 10 µM hesperetin 3) Cells treated with 100 µM hesperetin 4) Cells treated with 500 µM hesperetin. b) Intracellular ROS generation by hesperetin. c) Gene expression levels of MAPK. Values are mean ± S.D. (n = 3). ***P < 0.001, **P < 0.01, ^{ns}P> 0.05 as compared to control (Bonferroni test).

cells there was no fragmentation of DNA signifying the absence of apoptosis. Treatment with hesperetin induced apoptosis in A431 cells, leading to the laddering of DNA. DNA fragmentation was visible even at low concentration of hesperetin (10µM).

Effect of hesperetin on intracellular ROS generation

Reactive Oxygen Species (ROS) generation is a commonly proposed toxicological mechanism of many anticancer compounds. Oxidation of $DCFH_2$ by intracellular ROS such as H_2O_2, $HO^{\bullet}$, $ROO^{\bullet}$, $NO^{\bullet}$ and $ONOO^{-}$ results in formation of fluorescent DCF (22). The relative fluorescence intensity exhibited by control cells indicates the level of intracellular ROS in A431 cells. Figure 3b shows the intracellular ROS levels of cells exposed to different concentrations of hesperetin (10, 100 and 500 µM). It is observed that exposure to hesperetin stimulates the production of intracellular ROS and thereby induces oxidative stress. There was not much variation in intracellular ROS levels with hesperetin at all the concentrations when compared to the control cells after a 30 min incubation period. But the levels of ROS progressively increased with time. Higher concentration of hesperetin (500µM) induces more ROS causing severe oxidative stress after 90 minutes.

Effect on MAP Kinase expression levels

Figure 3c displays the effect of hesperetin on mRNA expression of MAP kinases ERK, JNK and p38. It was found that mRNA expression levels of ERK1 and ERK2 were suppressed after 24h of treatment with hesperetin. In contrast, there was substantial increase in the expressions of JNK1, JNK2 and p38 after 24h treatment. The effect of hesperetin on the gene expression was maximum (p<0.001) at a concentration of 500µM. A more pronounced effect was observed on the mRNA levels of JNK1, JNK2 and p38.

Effect on Cyclins and apoptotic proteins levels

Figure 4 illustrates the effect of hesperetin at the transcriptional level of various cyclins and apoptotic genes investigated using Western blot analysis. There

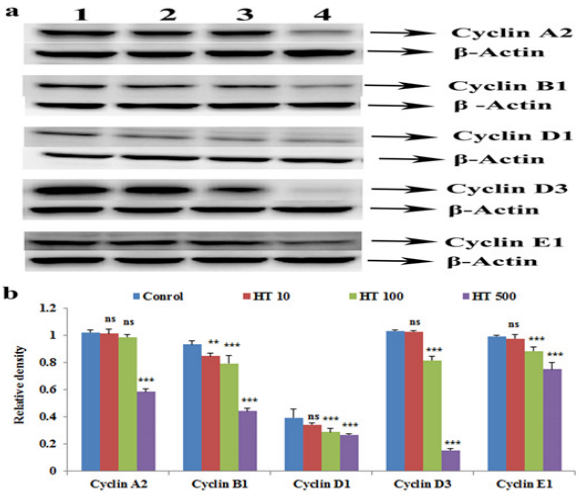

Figure 4. Effect of hesperetin on transcription levels of various cyclins. a) Representative images from three independent experiments are shown; 1) Control A 431 cell 2) Cells treated with 10 µM hesperetin 3) Cells treated with 100 µM hesperetin 4) Cells treated with 500µM hesperetin. b) Change in protein level expressed in terms of relative density over the control, normalized against β-actin (loading control), Values are mean ± S.D. (n = 3). ***P < 0.001, ***P < 0.01, ^{ns}P> 0.05 as compared to control (Bonferroni test).

was a significant decrease in the levels of cyclin B_1, D_1, D_3 and E_1 proteins by 1.76, 1.37, 1.27 and 1.12 fold respectively at 100µM concentration of hesperetin when compared to the untreated cells (p<0.001). The level of cyclin A_2 however did not show any significant (1.03 fold; p>0.05) difference in cells treated with 100µM hesperetin when compared to the control cells. A similar trend was observed in cells treated with 500µM hesperetin with 1.74, 2.11, 1.46, 6.75 and 1.32 fold (p<0.001) decrease in cyclins A_2, B_1, D_1, D_3 and E_1 respectively when compared to the control cells.

The expression level of Bcl-2 protein was however not significantly (p>0.05) altered in cells treated with 100µM and 500µM hesperetin when compared to control cells (Figure 5). In contrast, there was a significant increase in the expression of Bax protein in 100 and 500µM hesperetin-treated cells (Figure 5). The expression of p21 protein is also up-regulated in treated groups to 2.47 and 2.67–fold (p<0.001) respectively in 100 and 500µM hesperetin-treated cells (Figure 5), which ultimately causes apoptosis.

Discussion

Clinical trials have been enduring for a variety of dietary agents and phytochemicals as a remedy against various types of cancer (1, 23). Recent extensive studies on the flavonoids have established their remarkable spectrum of biological activities, particularly their anti-tumour and anti-carcinogenic effects by modulating different molecular targets and metabolic pathways (23, 24, 25, 26). However, the specific mechanisms accountable for the effects of flavonoids are still not completely investigated.

As one of the most prevalent flavonoids extracted from orange, hesperetin was examined for its effect on skin carcinoma and an attempt to elucidate the molecular mechanism(s) by which it exhibits anticancer activity was made in the present study. Previous reports have

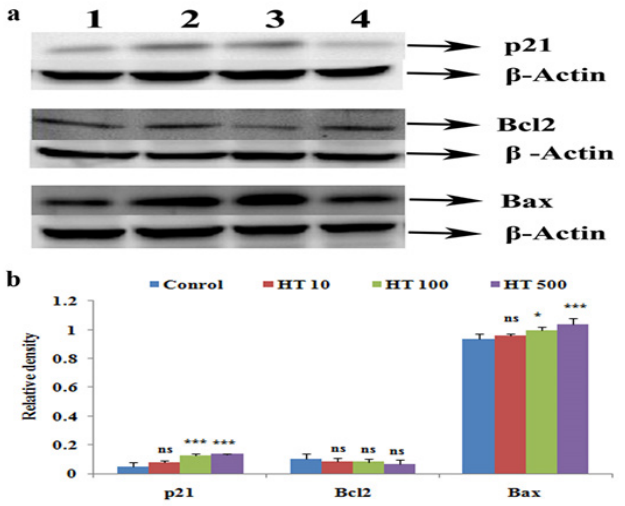

Figure 5. Effect of hesperetin on transcription levels of p21, Bcl-2 and Bax. a) Representative images from three independent experiments are shown; 1) Cells treated with 10 µM hesperetin 2) Cells treated with 100 µM hesperetin 3) Cells treated with 500 µM hesperetin 4) Control A 431 cell. b) Change in protein level expressed in terms of relative density over the control, normalized against β-actin (loading control), Values are mean ± S.D. (n = 3). ***P < 0.001, *P < 0.05, ^{ns}P> 0.05 as compared to control (Bonferroni test).

indicated that hesperetin can inhibit the proliferation of human breast cancer MCF-7 cells (11) and cervical cancer cells (14) *in vitro*. The results of our study have revealed the significant cytotoxicity of hesperetin towards A431 skin carcinoma cell line for the first time. A good agreement was observed between the results obtained from the MTT assay and live-dead assay suggesting that hesperetin causes significant reduction in the cell viability. In a previous study by Choi (11), treatment of hesperetin with MCF-7 cells for 24 h did not affect cell proliferation; but it significantly decreased cell proliferation after 48 h and 72 h. Difference in this effective time period might be due to the difference in the metabolic rates, doubling time and sensitivity of both the cell lines MCF-7 and A431. The effect of hesperetin to suppress the colony formation of A431 skin carcinoma cells also indicated its ability to inhibit cell proliferation and reduce the survival of skin cancer cells. Next, we examined the apoptotic potential of hesperetin by observing its ability to induce DNA damage in A431 cells. The observed laddered pattern of DNA confirmed the induction of apoptosis. The cytotoxicity and apoptosis inducing ability of hesperetin is in line with the earlier findings of similar effects in human gastrointestinal carcinoid (BON) (10) and cervical cancer SiHa cells (14).

As ROS - mediated mitochondrial dysfunction has been implicated in the induction of apoptosis (27); we examined the effect of hesperetin on intracellular ROS levels in A431 cells. Hesperetin-induced ROS generation in A431 cells which may have implications for the activation or suppression of other signalling and regulatory pathways. Earlier studies by Zhang et al. (28,29) also suggests that hesperetin could inhibit the proliferation and induce the apoptosis of hepatocellular carcinoma and gastric cancer cells via activating mitochondrial pathway by increasing reactive oxygen species.

To scrutinise the actual mechanistic pathways that are regulated by hesperetin, we examined its effect on MAPKs. MAPK signalling pathway serves a crucial role in the initiation and progression of malignancy and is associated with cell survival and apoptosis in stressful cellular conditions (30, 31). MAPK comprises of three members - ERK, JNK and p38 among which ERK is linked with cell survival and tumour progression whereas p38 and JNK are associated with either cell survival or apoptosis (30,31). ERK pathway is deregulated in approximately one-third of all human cancers and its inactivation may lead to cell cycle arrest in cancer cells (32). ERK plays a critical role in G1 progression by regulating the expression of cyclin D_1 (33). Furthermore, it is now recognised that p38 signalling also modulates the mammalian cell cycle by supressing cyclin D expression and thereby inducing the G1/S cell cycle arrest (34,35). JNK activation triggers G2/M arrest in cancer cells through subsequent up regulation of p21 and p27 proteins and simultaneous decrease in cyclin B, CDK2, CDK4 and E2F4 protein levels (36). Hesperetin down-regulated ERK 1/2 expression and up-regulated JNK1/2 and p38 expression levels promoting cell cycle arrest thereby supressing the cell proliferation. Previous studies conducted by Hwang and Yen (37) also reported the stimulation of signalling kinases such as JNK and p38 by hesperetin in hydrogen peroxide-treated PC12 cells. Our data also supported their proposition that flavonoids act more as signalling molecules than antioxidants in cancer cells.

To confirm that hesperetin regulated MAPKs in cell cycle progression, we further assessed the levels of various cyclins in the treated cells. Cyclin proteins are reported to be considerably up regulated in cancer cells leading to aberrant cell division (38,39). Hesperetin treatment significantly reduced the levels of the cyclins A_2, B_1, D_1, D_3 and E_1. The co-ordinated regulatory action exerted by hesperetin on the mRNA expression of ERK, JNK and p38 genes might be the reason for the perceived degradation of cyclin proteins and subsequent cell cycle arrest.

As a proliferation inhibitor, enhancing p21 activity is considered to have an important role in preventing tumour development (40). The p21 protein obstructs the activity of cyclin-CDK2, CDK1, and CDK4/6 complexes, and thus acts as a controller of cell cycle progression at G1 and S phase. Hesperetin was found to enhance the expression of p21 protein, mediated through enhanced JNK activity, leading the drive towards apoptosis. In order to confirm the downstream regulation of apoptosis proteins by hesperetin, we assessed the expression levels of genes encoding the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax. Hesperetin down regulated the level of Bcl-2 and up regulated the level of Bax as forecasted by the control mediated through MAPK and cyclins. The influence of hesperetin on the expression levels of these two genes could serve as the driving force that initiated the apoptotic cascade. Similar observations were recorded earlier by Choi (11) in MCF-7 human breast cancer cells, where hesperetin was found to induce cell cycle arrest at the G1 phase and apoptosis through the regulation of cyclin D, CDK4, p21 and also through the modulation of Bcl-2 and Bax expression.

Cancers are caused by deregulation of multiple pathways due to the cross-talk between different signal-

ling pathways. Most anti-cancer drugs fail because they are mono-targeted, and toxic to normal cells (1). Thus, agents that can suppress multiple pathways are likely to be more effective chemotherapeutic agents. The present study reveals that hesperitin regulated tumour cell growth through multiple cell signalling pathways such as protein kinases (MAPK), cell proliferation (cyclin A_2, B_1, D_1, D_3 and E_1), tumour suppression (p21), cell survival and apoptotic (Bcl-2, Bax) pathways. The functional interplay among the pathways in melanoma suggests that their simultaneous targeting by hesperetin could result in efficacious inhibition of cancer cell growth. Earlier investigations on the biological activity of hesperetin using *in vivo* models have also demonstrated the potential antineoplastic ability of this dietary flavonoid against 1,2- dimethyl hydrazine-induced rat colon carcinogenesis (41) and MCF-7-induced tumour in ovariectomized athymic mice (42). The effectiveness of hesperetin against skin carcinoma cells *in vitro*, hitherto unexplored and revealed for the first time in this study implies that hesperetin can serve as a therapeutic agent against this form of malignancy. However, further *in vivo* studies and clinical trials are required to establish the effect of hesperetin against skin carcinoma.

The present study revealed that hesperetin exhibited anticancer activity against A431 skin carcinoma cancer cell lines through the induction of apoptosis. By monitoring the molecular changes associated with hesperetin treatment, we have demonstrated the crucial role of oxidative stress-induced MAP kinase signalling and altered levels of cyclins as the predominant pathways responsible for the anticancer effects of hesperetin. The expression of ERK was down regulated while the expression levels of JNK and p38 were up regulated on treatment with hesperetin. The down regulation of the levels of many cyclins including cyclins A_2, B_1, D_1, D_3 and E_1also implies their role in induction of cell death. Hesperetin-induced apoptosis was also mediated via up-regulation of p21, Bax and down-regulation of Bcl-2. Thus our findings suggest that hesperetin could serve as a chemotherapeutic agent that can also be incorporated in everyday diet as a curative measure against skin malignancies.

Other articles in this theme issue include references (43-54).

Acknowledgements

Authors acknowledge FIST (SR/FST/LSI-327/2007& SR/FST/LSI-058/2010), Department of Science & Technology, India for the financial support and cell culture facility. Authors also thank SASTRA University for financial (T. R. Rajagopalan research fund) and infrastructural support.

References

1. Pratheeshkumar, P., Sreekala, C., Zhang, Z., Budhraja, A., Ding, S., Son, Y., Wang, X., Hitron, A., Hyun-Jung, K., Wang, L., Lee J. and Shi, X., Cancer Prevention with Promising Natural Products: Mechanisms of Action and Molecular Targets. *Anticancer Agents in Med. Chem.* 2012, **12(10)**: 1159-84. doi: 10.2174/187152012803833035.

2. Anand, P., Kunnumakara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., Sung B. and Aggarwal, B. B., Cancer is a Preventable Disease that Requires Major Lifestyle Changes. *Pharm. Res*. 2008, **25(9)**: 2097–2116. doi: 10.1007/s11095-008-9661-9.

3. Bialy, T.L., Rothe, M.J. and Grant-Kels, J.M., Dietary factors in the prevention and treatment of nonmelanoma skin cancer and melanoma. *Dermatol Surg*. 2002, **28(12)**: 1143-52. pii: dsu02114.

4. Millen, A. E., Tucker, M.A., Hartge, P., Halpern, A., Elder, D.E., Guerry, D., Holly, E.A., Sagebiel, R.W., and Potischman N., Diet and Melanoma in a Case-Control Study. *Cancer Epidemiol Biomarkers Prev.* 2004, **13(6)**: 1042-51. pii: 13/6/1042.

5. Block, G., Patterson B. and Subar, A., Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer.* 1992, **18(1)**: 1–29. doi: 10.1080/01635589209514201.

6. Miller, A.L., Antioxidant Flavonoids: Structure, Function and Clinical Usage. *Altern. Med. Rev.* 1996, **1(2)**: 103-111.

7. Selvaraj, S., Krishnaswamy, S., Devashya, V., Sethuraman S. and Krishnan, U.M., Flavonoid-Metal Ion Complexes: A Novel Class of Therapeutic Agents. *Med. Res. Rev.* 2014, **34(4)**: 677–702. doi: 10.1002/med.21301.

8. Hirano, T., Abe, K., Gotoh M. and Oka, K., Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. *Br. J. Cancer*. 1995, **72**: 1380–88. oid: NLM: PMC2034105.

9. Borradaile, N.M., Carroll K.K. and Kurowska, E.M., Regulation of HepG2 cell apolipoprotein B metabolism by the citrus flavanones hesperetin and naringenin. *Lipids*. 1999, **34**: 591-98. PMID:10405973.

10. Zarebczan, B., Pinchot, S.N., Kunnimalaiyaan M. and Chen, H., Hesperetin, a potential therapy for carcinoid cancer. *Am. J. Surg*. 2011, **201**: 329–33. doi: 10.1016/j.amjsurg.2010.08.018.

11. Choi, E.J., Hesperetin Induced G1-Phase Cell Cycle Arrest in Human Breast Cancer MCF-7 Cells: Involvement of CDK4 and p21. *Nutr. Cancer*. 2007, **59(1)**: 115–19. doi: 10.1080/01635580701419030.

12. Choi, E.J., Antioxidative effects of hesperetin against 7, 12-dimethylbenz(a)anthracene-induced oxidative stress in mice. *Life. Sci*. 2008, **82**: 1059-64. doi: 10.1016/j.lfs.2008.03.002.

13. Kim, J.Y., Jung, K.J., Choi, J.S. and Chung, H.Y., Hesperetin: a potent antioxidant against peroxynitrite. *Free Radic. Res*. 2004, **38 (7)**: 761–69. PMID: 15453641.

14. Alshatwi, A.A., Ramesh, E., Periasamy V.S. and Subash-Babu, P., The apoptotic effect of hesperetin on human cervical cancer cells is mediated through cell cycle arrest, death receptor, and mitochondrial pathways. *Fundam. Clin. Pharmacol*. 2013, **27(6)**: 581-92. doi: 10.1111/j.1472-8206.2012.01061.x.

15. So, F.V., Guthrie, N., Chambers, A.F., Moussa M. and Carroll, K.K., Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr. Cancer*. 1996, **262**: 167-81. doi: 10.1080/01635589609514473.

16. Aranganathan S. and Nalini, N., Antiproliferative efficacy of hesperetin (citrus flavanoid) in 1,2-dimethylhydrazine-induced colon cancer. *Phytother. Res.* 2013, **27(7)**: 999-1005. doi: 10.1002/ptr.4826.

17. Panda, S., Non-melanoma skin cancer in India: Current scenario. *Indian J. Dermatol*. 2010, **55(4)**: 373–78. doi: 10.4103/0019-5154.74551.

18. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*. 1983, **65**: 55-63. doi: 0022-1759(83)90303-4.

19. Franken, N.A.P., Rodermond, H.M., Stap, J., Haveman J. and Bree, C.V., Clonogenic assay of cells *in vitro*. *Nat. Protoc.* 2006, **1(5)**: 2315-9. doi: 10.1038/nprot.2006.339.

20. Shao, Z.H., Xie, J.T., Hoek, T.L.V., Mehendale, S., Aung, H., Li, C.Q., Qin, Y., Shumacker, P.T., Becker L.B. and Yuan, C.S.,

Antioxidant effects of American ginseng berry extract in cardio myocytes exposed to acute oxidant stress. *Biochim. Biophys. Acta*. 2004, **1670**: 165–71. doi: 10.1016/j.bbagen.2003.12.001

21. Radhakrishnan, J., Kuppuswamy, A.A., Sethuraman S. and Subramanian, A., Topographic cue from electrospun scaffolds regulate myelin-related gene expressions in Schwann cells. *J. Biomed. Nanotechnol*. 2014, **10**: 1–10. doi: http://dx.doi.org/10.1166/jbn.2015.1921.

22. LeBel, C.P., Ischiropoulos H. and Bondy, S.C., Evaluation of the probe 29,79- dichloro fluorecein as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol*. 1992, **5**: 227–31. doi: http://escholorship.org/uc/item/19r571vt.

23. Sak, K., Cytotoxicity of dietary flavonoids on different human cancer types. *Pharmacogn. Rev*. 2014, **8(16)**: 122–46. doi: 10.4103/0973-7847.134247.

24. Chiu, F.L. and Lin, J.K., Downregulation of androgen receptor expression by luteolin causes inhibition of cell proliferation and induction of apoptosis in human prostate cancer cells and xenografts. *Prostate*. 2008, **68**: 61–71. doi: 10.1002/pros.20690.

25. Wang, L., Wang, J., Fang, L., Zheng, Z., Zhi, D., Wang, S., Li, S., Ho, C.T. and Zhao, H., Anticancer activities of citrus peel polymethoxy flavones related to angiogenesis and others. *Bio Med Research Int.* 2014, **2014**: 1-10. doi: 10.1155/2014/453972.

26. Patil, J.R., Chidambara-Murthy, K.N., Jayaprakasha, G.K., Chetti, M.B. and Patil, B.S., Bioactive compounds from Mexican lime (Citrus aurantifolia) juice induce apoptosis in human pancreatic cells. *J Agric Food Chem*. 2009, **57**: 10933–42. doi: 10.1021/jf901718u.

27. Liu, H., Peng, H., J,i Z., Zhao, S., Zhang, Y., Wu, J., Fan, J. and Liao, J., Reactive oxygen species-mediated mitochondrial dysfunction is involved in apoptosis in human nasopharyngeal carcinoma CNE cells induced by Selaginella doederleinii extract. *J. Ethnopharmacol.* 2011, **138(1)**: 184-91. doi: 10.1016/j.jep.2011.08.072.

28. Zhang, J., Wu, D., Vikash, Song, J., Wang, J., Yi, J. and Dong, W., Hesperetin induces the apoptosis of gastric cancer cells via activating mitochondrial pathway by increasing reactive oxygen species. *Dig. Dis. Sci*. 2015, doi: 10.1007/s10620-015-3696-7.

29. Zhang, J., Song, J., Wu, D., Wang, J. and Dong, W. Hesperetin induces the apoptosis of hepatocellular carcinoma cells via mitochondrial pathway mediated by the increased intracellular reactive oxygen species, ATP and calcium. *Med. Oncol*. 2015, **32(4):** 101. doi: 10.1007/s12032-015-0516-z.

30. Dhillon, A. S., Hagan, S., Rath O. and Kolch, W., MAP kinase signalling pathways in cancer. *Oncogene*. 2007, **26**: 3279–90. doi: 10.1038/sj.onc.1210421.

31. Orlowski, R.Z., Small G.W. and Shi, Y.Y., Evidence that inhibition of p44/42, mitogen-activated protein kinase signalling is a factor in proteasome inhibitor-mediated apoptosis. *J. Biol. Chem*. 2002, **277**: 27864–71. doi: 10.1074/jbc.M201519200.

32. Lavoie, J.N., L'Allemain, G., Brunet, A., Muller R. and Pouyssegur, J., Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J. Biol. Chem*. 1996, **271**: 20608–16. doi: 10.1074/jbc.271.34.20608.

33. Liu, J.J., Chao, J.R., Jiang, M.C., Ng, S.Y., Yen J. J. and Yang-Yen, H.F., Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3 T3 cells. *Mol. Cell. Biol*. 1995, **15**: 3654–63. doi: 10.1128/MCB.15.7.3654.

34. Rastogi, N. and Mishra, D.P., Therapeutic targeting of cancer cell cycle using proteasome inhibitors. *Cell Div*. 2012, **7(1)**: 26. doi: 10.1186/1747-1028-7-26.

35. Thoms, H.C., Dunlop, M.G. and Stark, L.A. p38-Mediated Inactivation of Cyclin D1/Cyclin-Dependent Kinase 4 Stimulates Nucleolar Translocation of RelA and Apoptosis in Colorectal Cancer Cells. *Cancer Res*, 2007, **67(4)**: 1660- 1669. doi: 10.1158/0008-5472.CAN-06-1038.

36. Yin, D., Zhou, H., Kumagai, T., Liu, G., Ong, J.M., Black K.L. and Koeffler, H.P., Proteasome inhibitor PS-341 causes cell growth arrest and apoptosis in human glioblastoma multiforme (GBM). *Oncogene*. 2005, **24**: 344–54. doi: 10.1038/sj.onc.1208225.

37. Hwang S.L. and Yen, G.C., Modulation of Akt, JNK, and p38 activation is involved in citrus flavonoid-mediated cytoprotection of PC12 cells challenged by hydrogen peroxide. *J. Agric. Food Chem*. 2009, **57**: 2576–82. doi: 10.1021/jf8033607.

38. Masamha C.P. and Benbrook, D.M., Cyclin D1 degradation is sufficient to induce G1 cell cycle arrest despite constitutive expression of cyclin E2 in ovarian cancer cells. *Cancer Res.* 2009, **69**: 6565–72. doi: 10.1158/0008-5472.CAN-09-0913.

39. Alao, J.P., The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol. Cancer.* 2007, **6**: 24. doi: 10.1186/1476-4598-6-24.

40. Gartel A.L. and Tyner, A.L., The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol. Can. Ther.* 2002, **1**: 639–49. doi: http://mct.aacrjournals.org/content/1/8/639.

41. Nalini, N., Aranganathan S. and Kabalimurthy, J., Chemopreventive efficacy of hesperetin (citrus flavonone) against 1,2- dimethylhydrazine-induced rat colon carcinogenesis. *Toxicol. Mec. Methods.* 2012, **22(5)**: 397–408. doi:10.3109/15376516.2012.673092.

42. Ye, L., Chan, F., Chen S. and Leung, L.K., The citrus flavononehesperetin inhibits growth of aromatase-expressing MCF-7 tumor in ovariectomized athymic mice. *J. Nutr. Biochem*. 2012, **23(10)**: 1230–37. doi: 10.1016/j.jnutbio.2011.07.003.

43. Li, Y., Ahmad, A., Sarkar and F. H., ASPP and iASPP: Implication in cancer development and progression. *Cell. Mol. Biol.* 2015, **61(6)**: 2-8.

44. Masood, N.,, Qureshi, M. Z. and Yasmin, A., Association of NOTCH with different microRNAs in head and neck cancer. *Cell. Mol. Biol.* 2015, **61(6)**: 9-16.

45. Amirkhah, R., Farazmand, A., Irfan-Maqsood, M., Wolkenhauerand, O. and Schmitz, U., The role of microRNAs in the resistance to colorectal cancer treatments. *Cell. Mol. Biol.* 2015, **61(6)**: 17-23.

46. Wang, Z., Chen, J. and Capobianco, A. J., The Notch signaling pathway in esophageal adenocarcinoma. *Cell. Mol. Biol.* 2015, **61(6)**: 24-32.

47. Limami, Y., Pinon, A., Riaz, A. and Simon, A., TRAIL and targeting cancer cells: between promises and obstacles. *Cell. Mol. Biol.* 2015, **61(6)**: 33-38.

48. Silva Galbiatti-Dias, A. L., Pavarino, É. C., Kawasaki-Oyama, R. S., Maniglia, J. V., Maniglia, E. J. V. and Goloni Bertollo, E. M., Cancer stem cells in head and neck cancer: A Mini Review. *Cell. Mol. Biol.* 2015, **61(6)**: 39-43.

49. Musella, A., Marchetti, C., Gasparri, M. L., Salerno, L., Casorelli, A., Domenici, L., Imperiale, L., Ruscito, I., Abdul Halim, T., Palaia, I., Di Donato, V., Pecorini, F., Monti, M., Muzii, L. and Panici, P. B., PARP inhibition: A promising therapeutic target in ovarian cancer. *Cell. Mol. Biol.* 2015, **61(6)**: 44-61.

50. Attar, R., Tabassum, S., Fayyaz, S., Ahmad, M. S., Nogueira, D. R., Yaylim, I., Timirci-Kahraman, O., Kucukhuseyin, O., Cacina, C., Farooqi, A. A. and Ismail, M., Natural products are the future of anticancer therapy: Preclinical and clinical advancements of *Viscum album* phytometabolites. *Cell. Mol. Biol.* 2015, **61(6)**: 62-68.

51. Hsu, Y-C., Hsieh, Y-H., Liao, C-C., Chong, L-W., Lee, C-Y., Yu, Y-L. and Chou, R-H., Targeting post-translational modifications of histones for cancer therapy. *Cell. Mol. Biol.* 2015, **61(6)**: 69-84.

52. Chong, L-W., Chou, R-H., Liao, C-C., Lee, T-F., Lin, Y., Yang, K-C. and Hsu, Y-C. Saturated fatty acid induces cancer stem cell-like properties in human hepatoma cells. *Cell. Mol. Biol.* 2015, **61(6)**: 85-91.

53. Ahmadi, M., Jafari, R., Marashi, S. A. and Farazmand, A., Indirect role of microRNAs and transcription factors in the regulation of important cancer genes: A network biology approach. *Cell. Mol. Biol.* 2015, **61(6)**: 100-107.
54. Zahoor, A., Mansoor, Q., Farooqi, A. A., Fayyaz, S., Naz, G. and Ismail, M., Genetic variants in the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and death receptor (DR4) genes contribute to susceptibility to colorectal cancer in pakistani population. *Cell. Mol. Biol.* 2015, **61(6)**: 108-112.