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RESEARCH ARTICLE

# The pleiotropic effects of fisetin and hesperetin on human acute promyelocytic leukemia cells are mediated through apoptosis, cell cycle arrest, and alterations in signaling networks

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**Abstract** Fisetin and hesperetin, flavonoids from various plants, have several pharmaceutical activities including antioxidative, anti-inflammatory, and anticancer effects. However, studies elucidating the role and the mechanism(s) of action of fisetin and hesperetin in acute promyelocytic leukemia are absent. In this study, we investigated the mechanism of the antiproliferative and apoptotic actions exerted by fisetin and hesperetin on human HL60 acute promyelocytic leukemia cells. The viability of HL60 cells was evaluated using the MTT assay, apoptosis by annexin V/propidium iodide (PI) staining and cell cycle distribution using flow cytometry, and changes in caspase-3 enzyme activity and mitochondrial transmembrane potential. Moreover, we performed whole-genome microarray gene expression analysis to reveal genes affected by fisetin and hesperetin that can be important for developing of future targeted therapy. Based on data obtained from microarray analysis, we also described biological networks modulated after fisetin and hesperetin treatment by KEGG and IPA analysis. Fisetin and hesperetin treatment showed a concentration- and time-dependent inhibition of proliferation and induced G2/M arrest for both agents and G0/G1 arrest for hesperetin at only the highest concentrations. There was a disruption of mitochondrial membrane potential together with increased caspase-3 activity. Furthermore, fisetin- and hesperetin-triggered apoptosis was confirmed by annexin V/PI analysis. The microarray gene profiling analysis revealed some important biological pathways including mitogen-activated protein kinases (MAPK) and inhibitor of DNA binding (ID) signaling pathways altered by fisetin and hesperetin treatment as well as gave a list of genes modulated ≥2-fold involved in cell proliferation, cell division, and apoptosis. Altogether, data suggested that fisetin and hesperetin have anticancer properties and deserve further investigation.

**Keywords** Fisetin · Hesperetin · Apoptosis · Acute promyelocytic leukemia · Gene profiling

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## Introduction

Acute promyelocytic leukemia (APL), a distinct variant of acute myeloid leukemia (AML), is different from other types of AML with its molecular biology and clinical outcomes. APL is characterized by abnormal accumulation of immature granulocytes (promyelocytes) in the bone marrow and blood stream [1]. In the majority of APL cases, translocation, t(15;17), between the retinoic acid receptor-alpha (RARα) gene on chromosome 17 and the promyelocytic leukemia (PML) gene on chromosome 15 is very common, which is responsible for the pathogenesis of APL [2]. Even though several treatment strategies are available for APL such as chemotherapy including all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) and stem cell transplantation, they remain incurable due to their heterogenic nature, differences in their biological characteristics, drug resistance, and high recurrence rate and harmful side effects [3]. Thus, efforts to develop new treatments for APL are necessary.

Recently, dietary phytochemicals targeting cellular pathways in tumor cells have comprised great interest to develop novel apoptosis-based therapies [4]. Of these phytochemicals, flavonoids widely distributed in plants have received

increasing attention for their potential chemopreventive role, because they have been known to impair cancer cell growth by modulating various signaling pathways involved in different stages of cancer development as well as inducing apoptosis [5].

Fisetin (3,3′,4′,7-tetrahydroxyflavone), a bioactive flavanol molecule, is found in various fruits and vegetables, such as strawberry, apple, persimmon, grape, onion, and cucumber [6]. Fisetin has a broad range of biological activities including antioxidant, anti-inflammatory, and anticancer effects, and its chemotherapeutic potential has been studied on various in vitro and in vivo cancer models [7]. Despite that fisetin has been shown to induce apoptosis in a variety of cell lines, fisetin-mediated antiproliferative and apoptotic effects were specific for cancer cells based on differential modulation of cellular signaling pathways. The mechanisms behind fisetin's actions include induction of cell cycle arrest by altering cell cycle regulators [8], inhibition of signaling pathways such as NF-κB and PI3K/Akt pathways [9, 10], prevention of cell invasion and metastasis [11], and modulation of apoptotic and anti-apoptotic molecules [12]. These effects of fisetin have been evaluated on various cancer types such as colon, prostate, pancreas, and lung cancers; however, there is no study investigating the underlying mechanism(s) of fisetin in APL.

Citrus species have attracted significant attention for their flavonoid content which is thought to be promising in cancer therapy. Hesperidin and hesperetin are the most important examples of these flavonones found in citrus fruits such as lemon and orange with a wide range of pharmacological properties including antioxidant, anti-inflammatory, and anticancer effects [13]. Hesperidin could be considered as a pro-drug, since it is metabolized to hesperetin before absorption [14]. Promising results derived from a noticeable body of evidence displayed that hesperetin could mediate its anticancer effects in several cancer types by affecting several cellular targets. Some of these mechanisms include modulation of apoptotic proteins [15], increased activity of several caspases like caspase-3 and caspase-9 [16], and arrest of cell cycle progression [17]. On the other hand, the mechanism(s) responsible for the biological effects of hesperetin in APL cells remain unidentified yet.

The present study was undertaken to determine the molecular mechanism of fisetin- and hesperetin-induced cell death in HL60 APL cells by investigating their effects on cell proliferation, apoptosis, caspase-3 enzyme activity, loss of mitochondrial membrane potential, and cell cycle distribution. Moreover, whole-genome microarray analysis was performed to establish affected genes and genetic networks in response to fisetin and hesperetin. This is the first report that demonstrates the therapeutic potential of these flavanoids in APL cells via inducing apoptosis and cell cycle arrest and modulating multiple cellular targets.

## Materials and methods

### Chemicals

Fisetin (purity ≥98 %) and hesperetin (purity ≥95 %) were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Ten-millimolar stock solutions of these agents were prepared in DMSO and stored at −20 °C. The final concentration of DMSO did not exceed more than 0.1 % in culture. Penicillin-streptomycin, RPMI 1640, and fetal bovine serum were obtained from Invitrogen (Paisley, UK). MTT reagent was purchased from Sigma (St. Louis, MO).

### Cell culture

Human HL60 APL cells were kindly provided by Dr. Ali Ugur Ural from the Hematology Department of Gulhane Medical School, cultured in RPMI 1640 growth medium supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin at 37 °C in 5 % $CO_2$.

### MTT assay

Antiproliferative effects of fisetin and hesperetin on HL60 cells were assessed by the MTT cell proliferation assay, which depends on reduction of MTT dye to its insoluble formazan by NAD(P)H-dependent cellular oxidoreductase enzymes, giving a purple color in living cells. Briefly, $1\times10^4$ cells grown in 96-well plates were exposed to increasing concentrations of fisetin and hesperetin (1–200 μM) for 48 and 72 h. These cells were then incubated with 20 μl MTT for 3 h, and the resulting formazan crystals were dissolved in 100 μl DMSO. The absorbance values were read at 570-nm wavelength with an ELISA reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Finally, the $IC_{50}$ value (drug concentration which inhibits cell growth by 50 %) of these agents was calculated from cell proliferation plots.

### Assessment of apoptosis in human HL60 cells by annexin V-FITC/PI double staining

To examine apoptotic effects of fisetin and hesperetin on APL cells, the movement of phosphatidylserine (PS) from the inner leaflet of the membrane to the outer leaflet can be easily detected by using a fluorescent conjugate of annexin V (Annexin V-FITC Apoptosis Detection Kit, BioVision Research Products, USA). A total of $5\times10^5$ cells/well seeded into six-well plates were treated with increasing concentrations of agents for 72 h. Then, the collected cells were washed twice with cold PBS, suspended with 200 μl of 1× binding buffer, and stained with 2 μl of FITC Annexin V and 2 μl of propidium iodide (PI) for 15 min at room temperature in the dark. Afterwards,

samples were analyzed by flow cytometry (BD Facscanto Flowcytometry, Belgium) within 1 h.

### Detection of the changes in mitochondrial membrane potential

The effects of fisetin and hesperetin on mitochondrial membrane potential (MMP) of HL60 cells were examined by JC-1 Mitochondrial Membrane Potential Detection Kit (Cayman Chemicals, USA), which uses a unique cationic dye, JC-1. JC-1 has the ability to enter into mitochondria and change its color from green to red reversibly. In healthy cells with high MMP, JC-1 spontaneously forms complexes in mitochondria and gives intense red fluorescence. On the other hand, in apoptotic cells with low MMP, JC-1 remains in the monomeric form and gives green fluorescence. Briefly, $5\times10^5$ cells/well seeded into six-well plates were treated with agents for 72 h and then collected by centrifugation at 180*g* for 10 min. The pellets were homogenized with 300 μl medium containing 30 μl of JC-1 dye and incubated at 37 °C in 5 % $CO_2$ for 30 min. Then, the cells were collected by centrifugation at 400*g* for 5 min and homogenized with 200 μl assay buffer. Afterwards, 100 μl from each sample was added to a 96-well plate as triplicates. In healthy cells, the aggregate red form has absorption/emission maxima of 560/595 nm, whereas in apoptotic cells, the monomeric green form has absorption/emission maxima of 485/535 nm, which were read by a fluorescence ELISA reader (Thermo Varioskan Spectrum, Finland). The ratio of fluorescent intensity of JC-1 monomers (green) to fluorescent intensity of JC-1 aggregates (red) was calculated for each concentration as well as for the untreated control sample.

### Measurement of caspase-3 activity

Changes in caspase-3 activity of the cells were examined by caspase-3 colorimetric assay kit (BioVision Research Products, USA), which is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) released from the labeled substrate DEVD-*p*NA after cleavage by caspases. Briefly, the cells ($5\times10^5$ cells/well seeded into six-well plates) induced to undergo apoptosis by fisetin and hesperetin were collected by centrifugation at 180*g* for 10 min, which were then lysed by 50 μl of chilled cell lysis buffer on ice for 10 min. The reaction cocktail including 50 μl of 2× reaction buffer (containing 10 mM DTT), 50 μl of sample, and 5 μl of DEVD-pNA substrate was prepared and then added to 96-well plates, which was incubated for 2 h at 37 °C. Finally, the plate was read under 405-nm wavelengths by an ELISA reader. Total protein concentrations determined by the Bradford method were used to normalize the absorbance values.

### Cell cycle analysis by flow cytometry

dsDNA content and distribution of cell cycle phases are determined by flow cytometry. Briefly, $5\times10^5$ cells/well were treated with increasing concentrations of fisetin and hesperetin for 72 h. Then, the cells collected by centrifugation at 260*g* for 10 min were homogenized with 1 ml cold PBS and fixed with 4 ml of cold ethanol overnight at −20 °C for the analysis. The cells were centrifuged at 260*g* for 10 min, and pellets were homogenized with 1 ml cold PBS and centrifugation was repeated. Afterwards, cell pellets were homogenized with 1 ml PBS containing 0.1 % Triton X-100, and then 100 μl RNase A (200 μg/ml) was added and incubated at 37 °C for 30 min. One hundred microliters of PI (1 mg/ml) was added to the cells, which were incubated at room temperature for 15 min, and then analyzed by flow cytometry.

### Microarray analysis

Total RNA was isolated from HL60 cells treated with fisetin (20 and 50 μM) and hesperetin (100 and 150 μM) for 72 h using Nucleospin Total RNA isolation kit (Machery-Nagel, USA) according to the manufacturer's instructions. Only the RNA samples with an A260/A280 ratio between 1.9 and 2.1 were considered for further use. Expression profiling is accomplished using the Illumina Human HT-12v4 beadchip microarrays (containing ~47,000 transcripts: ~30,000 genes) (Illumina, Inc., San Diego, CA). Initially, 500 ng of total RNA converted to cDNA, followed by in vitro transcription to generate biotin-labeled cRNA using the Illumina Total Prep RNA Amplification Kit (Ambion, USA) based on the manufacturer's instructions for hybridization analysis. Then, 1.5 μg of labeled cRNA was hybridized to each array according to the Illumina whole-genome gene expression direct hybridization assay protocol. Then, arrays were imaged using the Illumina BeadArray Reader to measure fluorescence intensity at each probe. The images were processed and converted into signal intensities using the Illumina GenomeStudio software (Illumina, Inc.). Gene ontology and pathway analyses were performed to consider the biological meaning of differential expression of genes between the treated and untreated samples by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA).

### Statistical analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) for MTT analyses and two-way ANOVA for annexin V, MMP, caspase-3 activity, and cell cycle analyses. $P<0.05$ was considered to be significant. All

experiments were repeated in triplicate. Hierarchical cluster analysis (Illumina Genome Studio) applied to data sets to evaluate the "proximity" between genes and hierarchical clusters was constructed with the statistically significant ($P<0.05$) genes. Genes were considered differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 1.5 or less than 0.66 and when the $P$ values were less than 0.05. For each comparison, we obtained the list of differentially expressed genes constrained by $P$ value <0.05 and at least 2.0 fold change.

## Results

### The growth inhibitory effects of fisetin and hesperetin on HL60 cells

To characterize the effects of fisetin and hesperetin on HL60 cells' growth, the viability of the cells treated with agents (1–200 μM) for 48 and 72 h was measured using an MTT assay. Results showed that fisetin was effective in inhibiting the growth in a dose- and time-dependent manner, whereas hesperetin showed significant effect on the proliferation of the cells in a dose-dependent way especially after 72 h of incubation. For 48 h of hesperetin treatment, growth inhibition was not significant up to 100 μM. $IC_{50}$ (inhibition of cell viability by 50 % at a particular concentration) values at 48 and 72 h were calculated from cell proliferation plots and were found to be 82 and 45 μM for fisetin (Fig. 1a) and 190 and 142 μM for hesperetin, respectively (Fig. 1b).

### Fisetin and hesperetin trigger apoptosis in HL60 cells

HL60 cells were incubated with increasing concentrations of fisetin and hesperetin for 72 h, and fisetin- and hesperetin-induced changes in apoptosis (late apoptotic plus early apoptotic population) were detected by flow cytometry using annexin V-FITC and propidium iodide (PI) staining. Figure 2 shows the fold changes in the percentage of apoptotic HL60 cells as compared to untreated controls. Fisetin treatment resulted in 1.6-, 6.2-, and 11.6-fold increases in apoptosis in response to 20, 50, and 100 μM fisetin, respectively (Fig. 2a). On the other hand, there were 1.2-, 2.1-, and 3.3-fold increases in response to 50, 100, and 200 μM hesperetin, respectively (Fig. 2c). Figure 2b, d indicates flow data representing the percentage of the cells in each flow quadrant. Fisetin was found to be the most effective flavonoid for the induction of apoptosis in HL60 cells based on the detection of PS on the outer membrane.

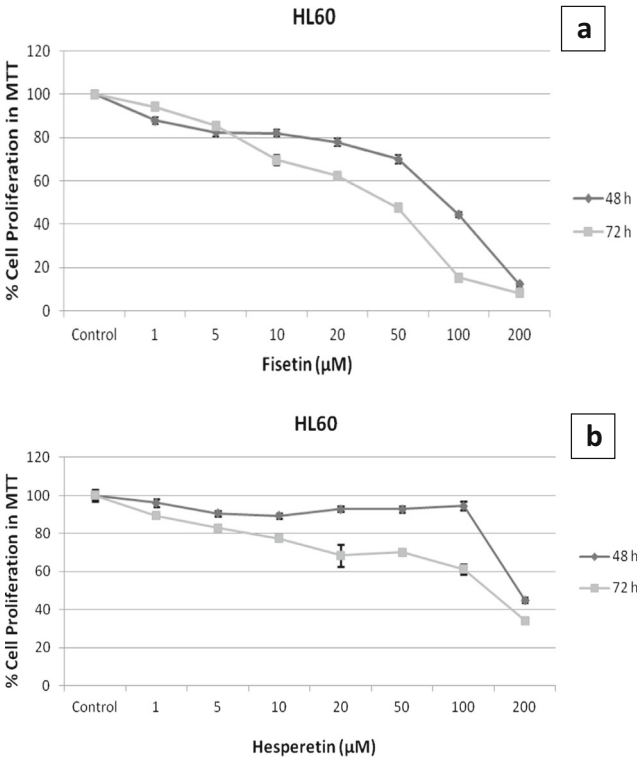


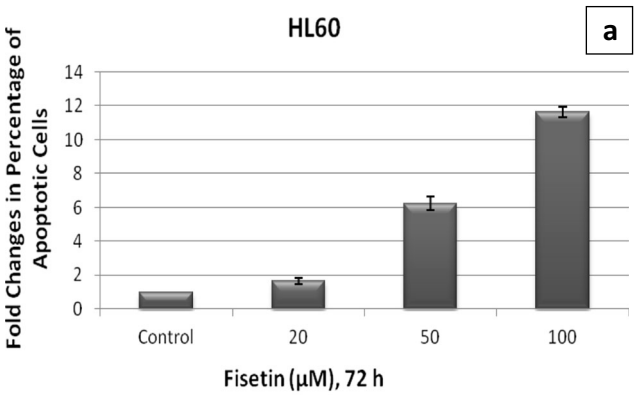

**Fig. 1** Antiproliferative effects of fisetin (**a**) and hesperetin (**b**) on HL60 cells by the MTT assay. The $IC_{50}$ values were calculated from cell proliferation plots. The results, the means of three independent experiments, are presented as mean±SEM; when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using one-way analysis of variance and $P<0.05$ was considered to be significant

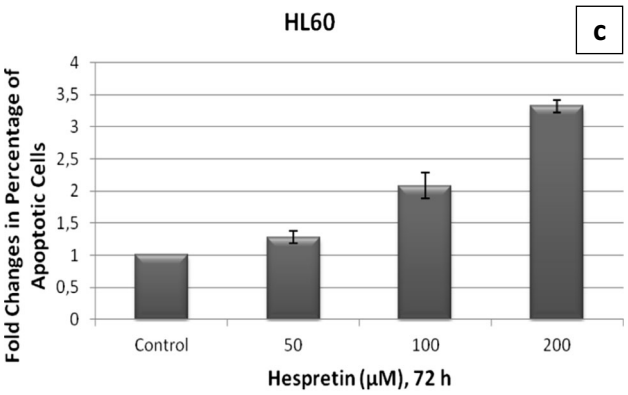
### Fisetin and hesperetin induce loss of mitochondrial membrane potential in HL60 cells in a dose-dependent manner

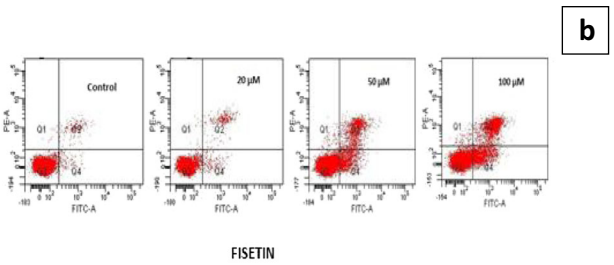
Loss of mitochondrial membrane potential is an important sign of apoptosis since it has been linked to initiation and activation of apoptotic cascades [18]. Therefore, it was determined whether these flavonoids cause the loss of MMP in APL cells exposed to increasing concentrations of them by using a JC-1 dye-based method. Treatment of HL60 cells with 20, 50, and 100 μM fisetin caused 1.01-, 1.15-, and 1.37-fold increases in loss of MMP, respectively (Fig. 3a). Figure 3b shows that hesperetin treatment resulted in 1.9-, 2.4-, and 5.4-fold increases in response to 50, 100, and 200 μM, respectively.

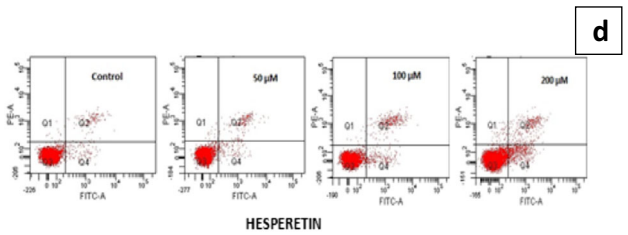
### Fisetin and hesperetin increase caspase-3 activity in HL60 cells

To determine whether apoptosis induced by fisetin and hesperetin was associated with activation of caspase-3, a hallmark of apoptosis, the cells were treated with increasing concentrations of these agents for 72 h and the changes in




| | Q1 | Q2 | Q3 | Q4 |
|---|---|---|---|---|
| Control | 0.2 | 1.9 | 96.7 | 1.2 |
| 20 | 0.2 | 3.1 | 94.8 | 2 |
| 50 | 0.7 | 10.4 | 80 | 8.9 |
| 100 | 1 | 20.1 | 63 | 15.9 |

| | Q1 | Q2 | Q3 | Q4 |
|---|---|---|---|---|
| Control | 0,1 | 1,3 | 97,4 | 1,2 |
| 50 | 0,2 | 1,8 | 96,6 | 1,4 |
| 100 | 0,1 | 3,2 | 94,7 | 2 |
| 200 | 0,5 | 3 | 91,1 | 5,3 |

**Fig. 2** Apoptotic effects of fisetin (**a**, **b**) and hesperetin (**c**, **d**) on HL60 cells by FACS analysis via annexin V-FITC/PI staining. *Cells in the lower right quadrant* indicate annexin-positive/PI-negative (*Q4*), early apoptotic cells. *Cells in the upper right quadrant* indicate annexin-positive/PI-positive, late apoptotic (*Q2*) (**b**, **d**). The results, the means of three independent experiments, are presented as mean±SEM; when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance and $P<0.05$ was considered to be significant

caspase-3 enzyme activity were detected through the cleavage of the labeled substrate DEVD-*p*NA. As indicated in Fig. 4a, there were 1.05-, 2.0-, and 2.2-fold increases in caspase-3 activity of fisetin-treated HL60 cells in response to 20, 50, and 100 μM, respectively, as compared to untreated controls. On the other hand, treatment of HL60 cells with 50, 100, and 200 μM hesperetin caused 1.08-, 1.1-, and 2.8-fold increases in caspase-3 enzyme activity, respectively (Fig. 4b).

## Fisetin arrested cell cycle at the G2/M phase while hesperetin at both G0/G1 and G2/M in HL60 cells

In order to determine the possible mechanism of antiproliferative activity of the agents, cell cycle profiles of APL cells in response to 72 h of exposure to fisetin and hesperetin were examined by flow cytometry in the presence of DNase-free RNase and PI dye. Treatment of HL60 cells with fisetin resulted in dose-dependent increases in the percentage of cells in the G2/M phase, which was accompanied by a reduction in the percentage of cells in the G0/G1 phase. The G2/M phase cell cycle distribution was 0.89, 8, and 29.5 % at 20-, 50-, and 100-μM concentrations of fisetin, respectively (control value 0.57 %) (Fig. 5a). On the other hand, hesperetin treatment was found to result in a dose-dependent increase of cell population in the G2/M phase. The G2/M phase cell cycle distribution was 1.64, 3.65, and 13 % at 50-, 100-, and 200-μM concentrations of hesperetin, respectively (control value 0.46 %). Moreover, the percentage of the cells in the G0/G1 phase was found to increase especially at 100 (34.8 %) and 200 (44.3 %) μM hesperetin as compared to control (31.13 %) while there was a dose-dependent decrease in the S phase population (Fig. 5b).

## Microarray analysis detects differentially expressed genes affected by fisetin and hesperetin in HL60 cells

We examined the changes in expression levels of the genes following treatment of HL60 cells with 20 and 50 μM fisetin and 100 and 150 μM hesperetin. Isolated total RNA was amplified and converted to biotin-labeled cRNA, which was hybridized to a microarray system containing approximately 30,000 genes. Hierarchical clustering of gene expression in untreated (control), fisetin-treated, and hesperetin-treated HL60 cells is shown in Fig. 6.

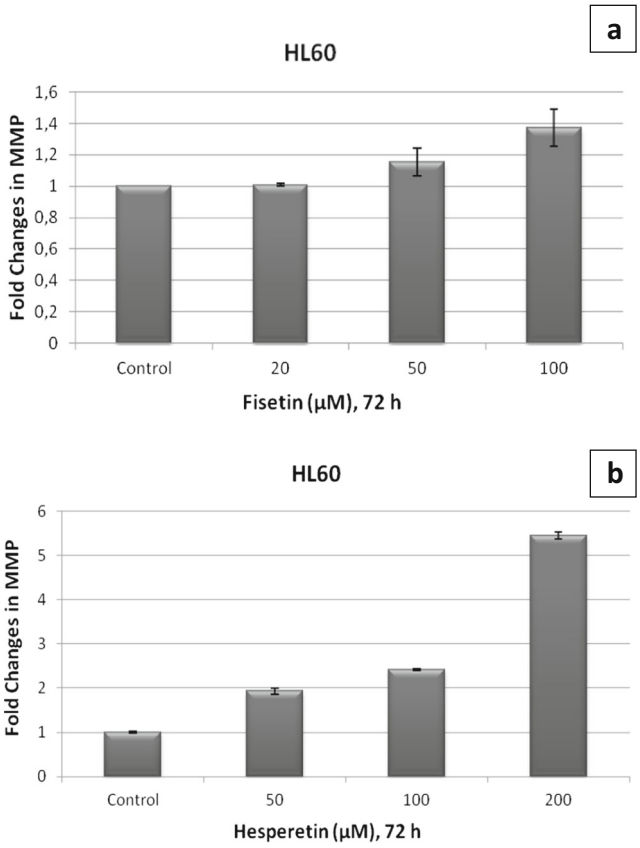



**Fig. 3** Changes in MMP of HL60 cells treated with increasing concentrations of fisetin (**a**) and hesperetin (**b**). The results, the means of three independent experiments, are presented as mean±SEM; when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance and $P<0.05$ was considered to be significant

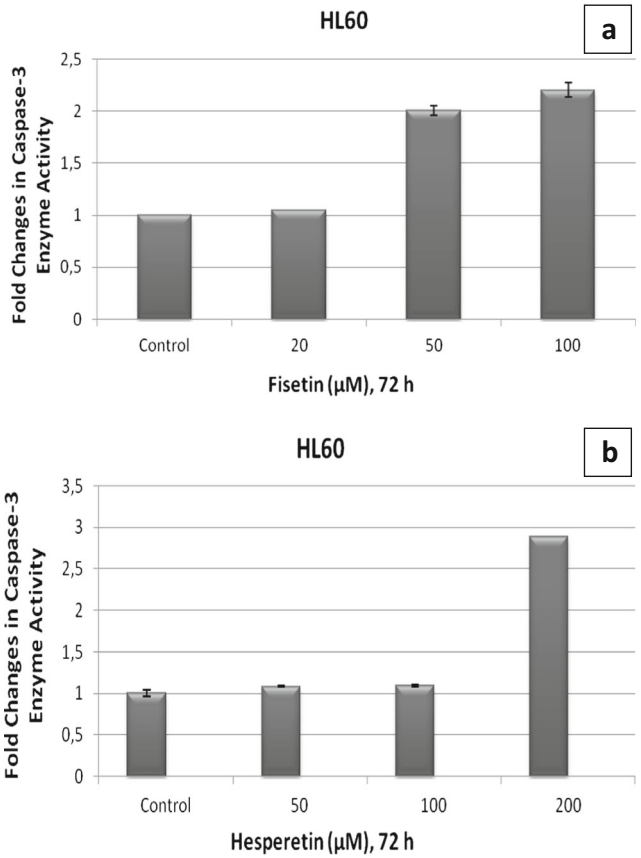



**Fig. 4** Changes in caspase-3 enzyme activity in response to fisetin and hesperetin in HL60 cells. The results, the means of three independent experiments, are presented as mean±SEM; when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance and $P<0.05$ was considered to be significant

A total of 54 and 1608 genes were significantly regulated ($P<0.05$) in 20- and 50-μM-fisetin-treated HL60 cells, respectively. The number of genes upregulated was 30 whereas that of genes downregulated was 24 in 20-μM-fisetin-treated HL60 cells. In 50-μM-fisetin-treated HL60 cells, 527 genes were upregulated and 1081 genes were downregulated. Fold change analysis displayed that TXNIP (thioredoxin-interacting protein), TFPI (tissue factor pathway inhibitor), miRNA1974, ID1 and ID3 (inhibitor of DNA binding 1 and 3), HSPA1B (heat shock protein 1B), and IDH1 (isocitrate dehydrogenase 1, NADP+) were altered genes in both 20- and 50-μM-fisetin-treated HL60 cells (Table 1). It was clear that MAP3K1 (mitogen-activated protein kinase kinase kinase 1), caspase-4, LASS6 (longevity assurance gene 6), and CBLB (E3 ubiquitin-protein ligase CBL-B) were the examples of upregulated genes, while LONP1 (Lon protease 1), STAT5A and STAT3 (signal transducer and activator of transcription 5A and 3), and JAK1 (Janus kinase 1) were some of the downregulated genes in 50-μM-fisetin-treated HL60 cells (Table 1).

On the other hand, a total of 130 and 691 genes were significantly altered ($P<0.05$) in 100- and 150-μM-hesperetin-treated HL60 cells, respectively. Treatment with 100 μM hesperetin resulted in the upregulation of 36 genes and downregulation of 94 genes. The number of genes upregulated was 288, whereas that of genes downregulated was 403 in 150-μM-hesperetin-treated HL60 cells. SASH1 (SAM and SH3 domain-containing protein 1), MT1F (metallothionein 1F), and SPRR2D (small proline-rich protein 2D) were representative common upregulated genes, while TUBB1 (tubulin beta-1 chain), ID3, ID1, NMU (neuromedin U), FGFR3 (fibroblast growth factor receptor 3), and S100P (calcium-binding protein P) were common downregulated genes in both 100- and 150-μM-hesperetin-treated HL60 cells (Table 2) based on fold change analysis. Furthermore, 150 μM hesperetin induced more genes that were either upregulated or downregulated as compared to 100 μM hesperetin (Table 2). TXNIP, MT1A (metallothionein 1A), MAP3K1, and SPRR2F (small proline-rich protein 2F) were some of the upregulated genes, while RPS25 (ribosomal protein S25), C-MYC, and tubulin family members (TUBA1C and TUBB2C) were examples of downregulated genes (Table 2).

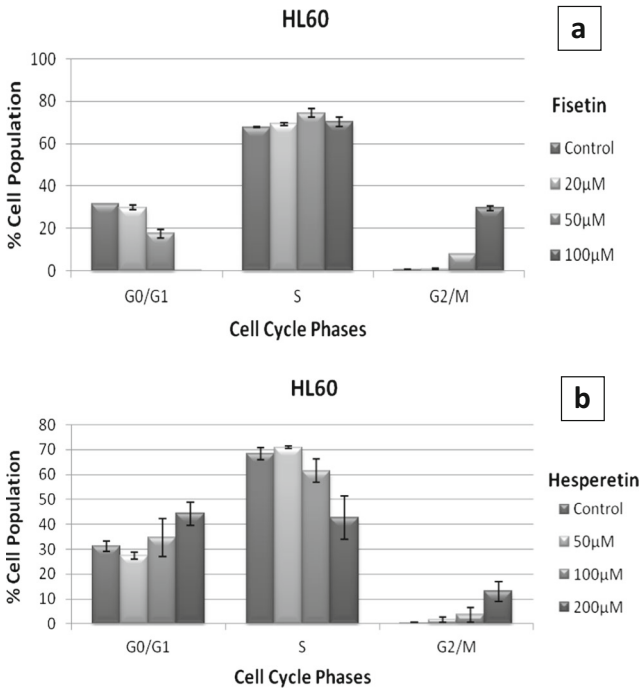



**Fig. 5** Effects of fisetin (**a**) and hesperetin (**b**) on cell cycle progression of HL60 cells after treatment. The results, the means of three independent experiments, are presented as mean±SEM; when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance and $P<0.05$ was considered to be significant

### Genetic networks affected by fisetin and hesperetin in HL60 cells

To examine the affected genetic networks after fisetin and hesperetin treatment, we carried out pathway analysis using the IPA tool. These networks describe functional relationships between gene products based on known interactions in the literature. The results showed that the MAPK signaling pathway, ID signaling pathway, cell cycle pathway, JAK/STAT signaling pathway, cell cycle checkpoint pathways, and PI3K/AKT signaling pathways were examples of the most altered networks in fisetin-treated HL60 cells (Table 3).

Based on the data obtained from the IPA analysis, the ID signaling pathway, eukaryotic ribosome and translation-related networks, gluconeogenesis-related and mitosis-related networks, and TGF-β and MAPK pathways were the affected signaling networks in hesperetin-treated HL60 cells (Table 4).

## Discussion

APL accounts for 10 % of AML. It evolves very rapidly and causes sudden hemorrhages; thus, it is extremely malignant [1]. Currently, the treatment for APL includes chemotherapy, ATRA, ATO, monoclonal antibodies, and hematopoietic stem cell transplantation [2]. Harmful side effects, high recurrence rate and development of resistance to chemotherapeutics and other agents, and heterogeneity in APL cases make APL treatment difficult; thus, they remain incurable in most cases [3]. Thus, new compounds treating APL are urgently needed to be developed.

Flavonoids are known to block the initiation, promotion, and progression of cancer by modulating various signaling pathways that have significant roles in cell proliferation, differentiation, apoptosis, angiogenesis, and metastasis [5]. Thus, flavonoids (both natural and synthetic analogs) are being investigated for their potentials in cancer therapy. Fisetin and hesperetin, plant-derived flavonoids, have been shown to produce a wide range of pharmacological effects including antioxidant, anti-inflammatory, and anticancer effects and found to induce apoptosis in various tumor cells by causing alterations in various cellular processes [7, 13]. The potential anticarcinogenic effects of fisetin and hesperetin, naturally occurring flavonoids, have been considered in various cancer types especially solid tumors including lung, colon, and breast cancers through several different mechanisms [11, 12, 16, 17]. However, to our knowledge, there is no detailed study investigating their mechanisms of action in APL.

In this study, we measured the time- and dose-dependent effects of fisetin and hesperetin on APL cell viability using the MTT assay. As indicated in the "Results" section, the growth of APL cells was inhibited by fisetin in a dose- and time-dependent way while hesperetin was most effective in a longer incubation time. The evaluation of changes in MMP and caspase-3 enzyme activity showed that fisetin and hesperetin treatment results in increases in the loss of MMP and caspase-3 enzyme activity with different extents. Induction of apoptosis was also confirmed by localization of phosphatidylserine to the outer membrane after treatment by flow cytometry. We detected increased percentage of cells undergoing apoptosis in a dose-dependent manner for both flavonoids. Therefore, we display that fisetin and hesperetin not only inhibit the proliferation but also induce the apoptosis of APL cells in vitro via mitochondrial membrane depolarization and increased caspase-3 enzyme activity, which suggested that they may have potential to treat APL.

The results of our study are in accordance with the data in the literature derived from various cancer types treated with fisetin. For instance, in LNCaP prostate cancer cells, fisetin-mediated apoptosis was related to activation of caspase cascade [19]. In a recent study, Kang et al. [20] displayed that fisetin had a dose-dependent cytotoxic effect on NCI-H460 human non-small cell lung cancer cells, and apoptosis induction in these cells was related to mitochondrial membrane depolarization and caspase-3 activation. Fisetin was also responsible for growth inhibition of U266 multiple myeloma cells via caspase-3 activation [21]. Similarly, hesperetin was displayed to trigger cytotoxicity in MCF-7 breast cancer cells

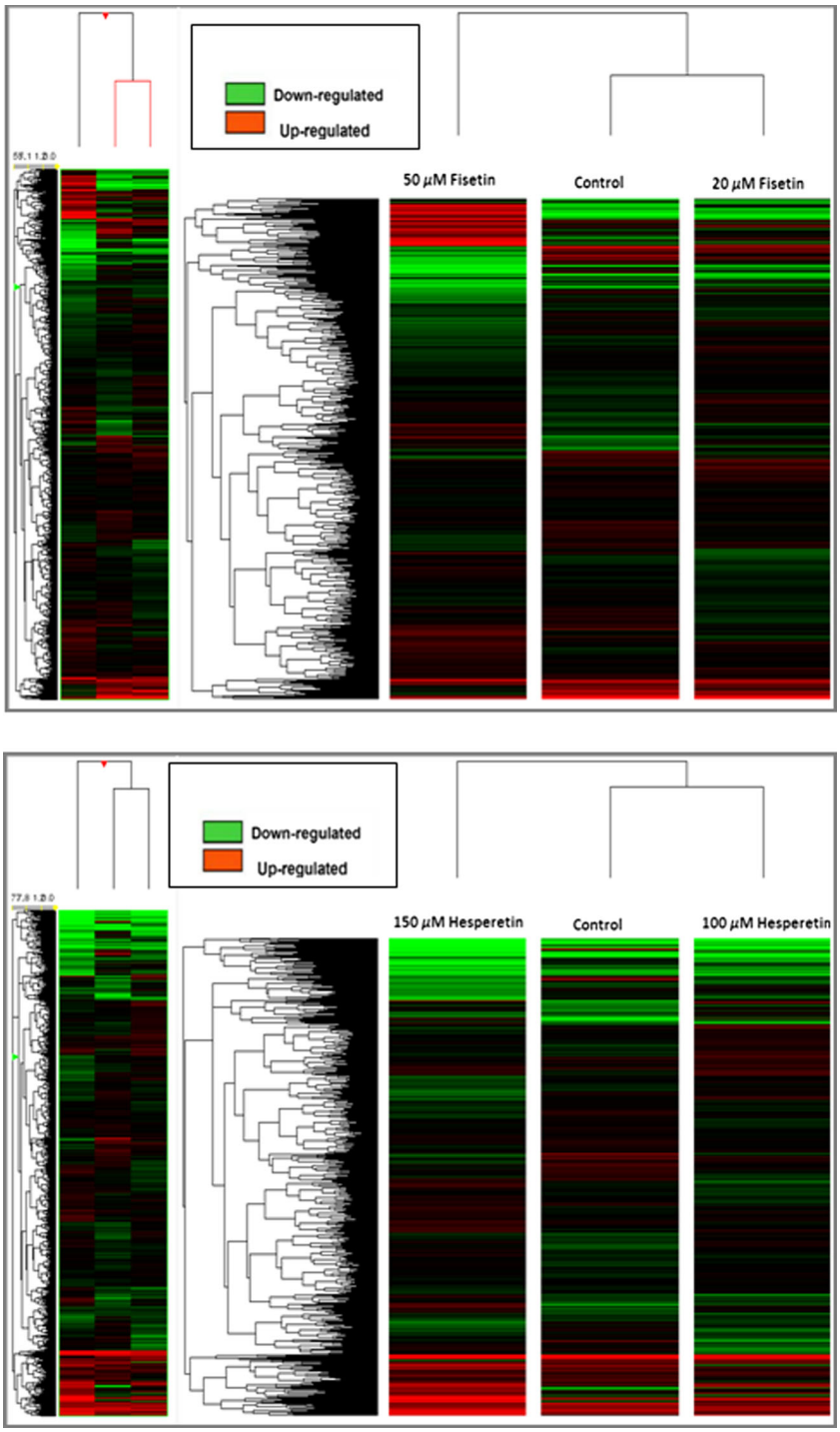



**Fig. 6** Hierarchical clustering based on gene expression data from HL60 cells exposed to fisetin (*upper panel*) and hesperetin (*lower panel*)

via phosphatidylserine externalization and MMP loss [22]. In another study, SiHa cervical cancer cells treated with hesperetin underwent apoptosis evidenced by disruption of MMP and caspase-3 activation [23].

It is very well known that the cell cycle plays a critical role in cell proliferation, growth, and division [24], and cell cycle arrest could be a reason for growth inhibitory effects of fisetin and hesperetin. Therefore, we evaluated cell cycle distribution of APL cells after fisetin and hesperetin treatment as compared to untreated control cells. Our study shows that fisetin and hesperetin treatment leads to G2/M arrest. The cell cycle progression was also arrested at the G0/G1 phase at 100 and 200 μM hesperetin. Previous studies demonstrated that fisetin and hesperetin inhibited the proliferation of cancer cells by inducing G2/M or G0/G1 arrest. For instance, fisetin induced G2/M arrest in human epidermoid carcinoma A431 cells [25],

**Table 1** The list of genes altered ≥2-fold after fisetin treatment of HL60 cells together with their key roles (italic type represents upregulated genes)

| Altered genes in fisetin-treated HL60 cells | Fold change 20 μM | 50 μM | Function |
|---|---|---|---|
| *TXNIP* | 11.76 | 12.61 | Apoptosis, tumor suppressor |
| *miRNA1974* | 2.496 | 4.23 | Inhibits cell growth |
| *TFPI* | 2.055 | 3.680 | Inhibitor of invasion and metastasis |
| ID3 | −7.798 | −8.667 | Dominant negative regulator of basic helix-loop-helix transcription regulators |
| ID1 | −3.685 | −7.005 | Dominant negative regulator of basic helix-loop-helix transcription regulators |
| HSPA1B | −3.313 | −3.305 | Heath shock response |
| IDH1 | −2.685 | −5.940 | Cytosolic enzyme |
| Altered genes in 50-μM-fisetin-treated HL60 cells | Fold change | | Function |
| *MAP3K1* | 3.310 | | Apoptosis, caspase-3 substrate |
| *CBLB* | 3.121 | | E3 ubiquitin ligase, autophagy |
| *LASS6* | 2.728 | | Ceramide synthase, apoptosis |
| *Caspase-4* | 2.422 | | Apoptosis |
| LONP1 | −6.017 | | Tumor bioenergetic |
| STAT5A | −3.604 | | Cell proliferation |
| PHB2 | −3.582 | | Cell survival through the Ras pathway |
| XRCC6 | −3.325 | | DNA repair |
| PIM2 | −2.960 | | Kinase in cell survival |
| PRKCB | −2.920 | | Protumorigenic |
| MKNK2 | −2.883 | | MAP kinase pathway |
| MAP2K5 | −2.477 | | MAP kinase pathway |
| PIK3CB | −2.445 | | PI3 kinase pathway, cell proliferation |
| CHECK2 | −2.382 | | Cell cycle |
| STAT3 | −2.390 | | Cell proliferation |
| GAB2 | −2.168 | | PI3 kinase pathway, cell proliferation |
| JAK1 | −2.045 | | Cell proliferation |

HT-29 colon cancer cells [8], and PC3 prostate cancer cells [26]. Human cervical SiHa cells treated with hesperetin displayed G2/M arrest in a dose-dependent manner as well [23]. Fisetin and hesperetin blocked cell cycle progression at G0/G1 in T24 and E7 bladder and MCF-7 breast cancer cells by decreasing cyclin-dependent kinases and cyclins involved in the regulation of G0/G1 passage [27, 17]. Therefore, our data together with literature knowledge explain that these two flavonoids may arrest cell cycle in a cell-type-specific manner and, thus, act as a non-specific cell cycle blocker.

We also performed genome-wide gene profiling analysis to establish genes affected in response to fisetin and hesperetin. Tables 1 and 2 summarize differentially expressed genes constrained by $P$ value <0.05 and at least 2.0 fold change. As clearly seen in Table 1, TXNIP, miRNA1974, and TFPI genes were common upregulated genes, which are known to be involved in the inhibition of cell growth, invasion, and metastasis. For instance, TXNIP or thioredoxin-interacting protein plays a crucial role in apoptosis. Its expression was found to be downregulated in many tumor cells [28], and overexpression of TXNIP inhibits cancer cell growth [29]. Another upregulated gene, TFPI, an inhibitor of plasmin-activating matrix metalloproteinases, blocks invasion and metastasis [30]. Downregulation of some common genes such as ID1, ID3, and IDH1 in fisetin-treated HL60 cells could be responsible for its apoptotic effects as well. In a recent study, suppression of ID1 and ID3, transcription regulators involved in proliferation and apoptosis, was related to reduced tumor growth [31, 32]. Downregulation of IDH1 after fisetin treatment might cause cell death due to the accumulation of reactive oxygen species (ROS) that damage proteins, lipids, and DNA in the cells, since it is involved in the production of NADPH required to scavenge ROS [33]. It is also understood from Table 1 that additional genes encoding proteins with significant functions in important cancer-related signaling pathways such as the MAPK pathway (MKNK2, MAP2K5), PI3K pathway (PIK3CB, GAB2), and JAK/STAT pathway (STAT5A, STAT3, and JAK1) were downregulated in response to higher fisetin concentration, thus leading to suppression of leukemic cell growth and induction of apoptosis. LONP1 gene expression was significantly downregulated at higher fisetin concentration, and it could be also an important

**Table 2** The list of genes altered ≥2-fold after hesperetin treatment of HL60 cells including their functions in the cell (italic type represents upregulated genes)

| Altered genes in hesperetin-treated HL60 cells | Fold change 100 μM | 150 μM | Function |
|---|---|---|---|
| *SPRR2D* | 4.574 | 5.985 | Differentiation |
| *MT1F* | 2.758 | 4.851 | Tumor suppressor |
| *SASH1* | 2.508 | 3.023 | Tumor suppressor |
| S100P | −8.285 | −10.153 | Cell proliferation |
| ID1 | −5.467 | −6.361 | Dominant negative regulator of basic helix-loop-helix transcription regulators |
| NMU | −5.062 | −6.065 | Invasion, metastasis |
| ID3 | −3.913 | −4.256 | Dominant negative regulator of basic helix-loop-helix transcription regulators |
| FGFR3 | −3.270 | −3.623 | Cell proliferation |
| TUBB1 | −3.010 | −3.327 | Cell division, tubulin isotype |
| Altered genes in 150-μM-hesperetin-treated HL60 cells | Fold change | | Function |
| *TXNIP* | 2.977 | | Apoptosis, tumor suppressor |
| *MT1G* | 2.939 | | Tumor suppressor |
| *TPM1* | 2.734 | | Tropomyosin 1, tumor suppressor |
| *SPRR2F* | 2.673 | | Differentiation |
| *RERG* | 2.629 | | Ras-related growth inhibitor |
| *ST7* | 2.609 | | Tumor suppressor, cell cycle arrest |
| *MAP3K1* | 2.606 | | Apoptosis, caspase-3 substrate |
| *RASA1* | 2.438 | | Inhibitor of mitogenic signal |
| *MT1A* | 2.434 | | Tumor suppressor |
| *SIN3A* | 2.400 | | Tumor suppressor |
| RPS25 | −8.177 | | Ribosome component |
| TUBA1C | −3.544 | | Cell division, tubulin isotype |
| EEF1A1 | −3.042 | | Protein translation |
| RPS6P1 | −3.029 | | Ribosome component |
| PRKCB1 | −2.432 | | Cell proliferation |
| C-MYC | −2.157 | | Proto-oncogene transcription factor |
| PIM1 | −2.330 | | Proto-oncogene kinase |
| TUBB2C | −2.226 | | Cell division |
| RPS9 | −2.208 | | Ribosome component |
| PCK2 | −2.206 | | Enzyme involved in gluconeogenesis |
| RXRA | −2.122 | | Differentiation |

**Table 3** Affected networks in fisetin-treated HL60 cells by IPA analysis (italic type represents networks modulated in both 20- and 50-μM fisetin treatment)

| Network/pathway | *P* value |
|---|---|
| MAPK signaling pathway (50 μM) | 0.04 |
| *ID signaling pathway* | 2.71E−05 (20 μM) |
| | 3.62E−04 (50 μM) |
| Nucleotide excision repair (50 μM) | 1.48E−06 |
| Cell cycle pathway (50 μM) | 2.47E−05 |
| JAK/STAT signaling pathway (50 μM) | 0.002 |
| *Protein folding* | 0.01 (20 μM) |
| | 2.01E−09 (50 μM) |
| Cell cycle checkpoint pathways (50 μM) | 2.57E−04 |
| PI3K/AKT pathway (50 μM) | 0.04 |

**Table 4** Affected networks in hesperetin-treated HL60 cells by IPA analysis (italic type represents networks modulated in both 100- and 150-μM hesperetin treatment)

| Network/pathway | *P* value |
|---|---|
| *ID signaling pathway* | 4.52E−04 (100 μM) |
| | 0.008 (150 μM) |
| *TGF-β pathway* | 7.45E−04 (100 μM) |
| | 3.32E−04 (150 μM) |
| Cytoplasmic ribosomal components (150 μM) | 1.27E−10 |
| Translation factors (150 μM) | 4.24E−06 |
| Gluconeogenesis (150 μM) | 4.29E−04 |
| Mitosis (150 μM) | 0.001 |
| MAPK signaling pathway (150 μM) | 0.01 |

target to induce leukemic cell death because this gene is found to be overexpressed in different tumors and cell lines [34].

The genome-wide changes in the expression levels of genes after hesperetin treatment in HL60 APL cells are summarized in Table 2. SPRR2D, MT1F, and SASH1 were upregulated genes that are known to play roles in cell proliferation, apoptosis, and differentiation. It is known that cancer cells have no or lower expression of SPRRs compared to normal cells [35]. Therefore, increased expression of SPRR2D is thought to induce cell growth suppression with an unknown mechanism. MT1F, a member of the metallothionein family, is expressed transiently in response to stimulation and plays an important role in cell growth by modulating gene transcription [36]. In a recent study, exogenous overexpression of the MT1F gene increased apoptosis in colon cancer cells [37], which supports our data. SASH1, a potential tumor suppressor, may function in various signaling pathways due to its SH3 and SAM domains; thus, its downregulation is related to tumor growth [38]. Therefore, its increased expression after hesperetin treatment might induce apoptosis and growth suppression. Similar to fisetin treatment as discussed above, ID1 and ID3 genes were downregulated in hesperetin treatment, thus inducing apoptosis. The other common downregulated gene was NMU, which was found to be increased in AML cells, and its knockdown caused growth arrest [39]. Therefore, it may be a reason for hesperetin-related antiproliferative effect. Another significantly downregulated gene was S100P, whose upregulation results in increased cell proliferation in several tumors such as endometrial cancer and oral squamous cell carcinoma [40, 41]. As well as these common altered genes, higher hesperetin treatment also induced either up- or downregulation of several genes as indicated in Table 2. It is interpreted from Table 2 that increased hesperetin concentration resulted in downregulation of additional tubulin-encoding genes, TUBB2C and TUBA1A, which could cause problems in cell division and then induce apoptosis [42]. Hesperetin impaired protein synthesis by targeting cytoplasmic ribosomal proteins such as RPS6P1, RPS9, and RPS25 and factors involved in translation initiation like EEF1A1. Another important downregulated gene was C-MYC which is described as a proto-oncogenic transcription factor and upregulated in various cancer types such as leukemia, lung, and breast cancers, thus inducing cell proliferation and inhibition of apoptosis via altering the expression of several target genes [43]. Therefore, its downregulation by hesperetin could be a strategy to inhibit HL60 cell growth. PIM1, a member of serine/threonine kinases involved in cell survival and proliferation, is overexpressed in a variety of cancer types such as leukemias, lymphomas, and pancreatic and prostate cancers [44]. It is also known that PIM1 functions together with C-MYC to induce malignancy. In our study, hesperetin downregulates both C-MYC and PIM1, therefore inducing significant growth inhibition and apoptosis.

We also characterize differentially modulated networks in response to fisetin and hesperetin using the IPA tool. Genes associated with biological functions are taken into consideration for analysis to identify networks, showing physical or functional relationships among differentially expressed genes. As clearly seen in Table 3, IPA analysis of fisetin-induced pathways includes ID signaling, JAK/STAT, and MAPK kinase signaling pathways. On the other hand, Table 4 displays networks/pathways modulated after hesperetin treatment, which include networks involved in protein synthesis (cytoplasmic ribosomal components, translation factors) and cell division. All these data are consistent with gene expression profiles.

## Conclusions

The present study has revealed that fisetin and hesperetin have significant antiproliferative effects on HL60 APL cells evidenced by induction of apoptosis and cell cycle arrest. Based on the data presented, fisetin and hesperetin trigger apoptosis and growth suppression via affecting various significant targets as in HL60 cells, and these new targets could open the usage of new strategies together with fisetin and hesperetin to overcome difficulties in APL treatment. These data also provide significant insights about the mechanisms of action of fisetin and hesperetin in HL60 cells for the first time. Further in vivo investigations are needed to confirm its antitumor activity and toxicity profiles.

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**Conflicts of interest** None

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