Fisetin inhibits various attributes of angiogenesis in vitro and in vivo—implications for angioprevention

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Studies have shown that fisetin, a small phytochemical molecule, has antitumor activity; however, its antiangiogenic activity has not yet been examined. Accordingly, herein, we investigated the antiangiogenic efficacy and associated mechanisms of fisetin in human umbilical vein endothelial cells (HUVECs). Fisetin (10–50 μM) strongly inhibited the regular serum plus growth supplement- and vascular endothelial growth factor (VEGF)-induced growth (up to 92%, P < 0.001) and survival (up to 16%, P < 0.001) of HUVEC in a dose- and time-dependent manner. Fisetin also caused cell cycle arrest at G1 (strong) and G2/M (moderate) phases together with a decrease in cyclin D1 and an increase in p53 levels. Fisetin-caused cell death was accompanied by decreased expression of survivin and an increase in cleaved levels of caspases-3 and -7 and poly-(ADP-ribose) polymerase along with an increased ratio of Bax to Bcl-2. Furthermore, fisetin inhibited capillary-like tube formation on Matrigel (up to 85%, P < 0.001) as well as migration (up to 66%, P < 0.001), which were associated with decreased expression of endothelial nitric oxide synthase (eNOS) and VEGF in HUVEC. It also decreased the expression of eNOS, VEGF, inducible nitric oxide synthase, matrix metalloproteinase-2 and -9 in A549 and DU145 human cancer cells. In vivo matrigel plug assay in mice showed significant decrease in size (up to 43%, P < 0.001), vascularization and hemoglobin content (up to 94%, P < 0.001) in the plugs from fisetin-treated, compared with control mice. Overall, these results suggest that fisetin inhibits various attributes of angiogenesis, which might contribute to its reported antitumor effects, and therefore, fisetin warrants further investigation for its angiopreventive potential toward cancer control.

Introduction

Tumor angiogenesis is the proliferation of existing blood vessels penetrating into the tumors to supply nutrients and oxygen and to remove metabolic wastes from them and is essentially required for the growth and metastasis of solid tumors (1,2). It is a complex process involving a network of many players, including tumor cells, endothelial cells, immune system cells and their secreted factors, which may act as promoters or inhibitors of angiogenesis; however, endothelial cells are the central players in this process. Several factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, interleukin-6/8, transforming growth factor alpha and prostaglandin E2 have been identified as proangiogenic factors secreted by tumor cells to regulate tumor growth and metastasis.

Herein, we evaluated for the first time the antiangiogenic efficacy and associated mechanisms of fisetin, a flavonol, on human endothelial cells, tumor cells and in vivo mouse model. Our results suggest that fisetin is a novel antiangiogenic chemopreventive agent that inhibits growth, survival and cell cycle progression and reduces apoptosis involving caspase and modulation of BAX to Bcl-2 ratio in human umbilical vein endothelial cell (HUVEC). Fisetin also suppressed the matrigel capillary tube formation and migration of HUVEC and angiogenic attributes in matrigel plug assay in mice.

Materials and methods

Cell lines, animals and reagents

HUVECs were procured from Clonetics (Walkersville, MD) and A549 human lung cancer and DU145 human prostate cancer cell lines were from ATCC (Manassas, VA). HUVECs were cultured in EGM-2 medium supplemented with 5% fetal bovine serum (FBS) and growth supplements (EGM-2 MV bullet kit) (Lonza, Walkersville, MD) under standard culture conditions (37°C, 95% humidified air and 5% CO2). Human embryonic kidney, HEK 293 cell line was procured from National Center for Cell Sciences, Pune, India and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and antibiotic/antimycotic as recommended. A549 and DU145 cells were cultured in RPMI-1640 complete media supplemented with 10% FBS (Lonza) under...
similar conditions. Swiss albino mice were obtained from Animal House Facility, Jawaharlal Nehru University, New Delhi, India and maintained at Central Animal House Facility at JNU as per the Institutional Animal Ethics Committee Guide lines. Complete cocktail of protease inhibitors was from Roche Molecular Biochemicals (Indianapolis, IN). Cleaved caspases and cleaved poly-(ADP-ribose) polymerase, a primary antibodies and peroxidase-conjugated secondary antibody were from Cell Signaling Technology (Beverly, MA). Anti-VEGF antibody was from Abcam (San Francisco, CA) and anti-endothelial nitric oxide synthase (eNOS) antibody was from Cell Signaling Technology. Propidium iodide was from Molecular Probes (Eugene, OR). Bradford assay kit was from Bio-Rad Laboratories (Hercules, CA) and Enhanced chemiluminescence detection system was from Amersham. Fisetin was obtained from Sigma–Aldrich (St Louis, MO) and dissolved in dimethyl sulfoxide (DMSO) as stock solution. Matrigel was from Fisher Scientific (Pittsburgh, PA). Deoxyribonucleic acid, reverse transcriptase enzyme and Taq polymerase reagents were from Bangalore Genie (India). Specific primers for semi-quantitative polymerase chain reaction (PCR) were from Sigma–Aldrich.

Cell growth and death assays
HUVECs were seeded (5000 cells/cm²) in culture dishes in EGM-2 medium with or without 5% FBS and growth supplements. Next day, cells were treated with different concentrations of fisetin (0, 10, 25 and 50 μM) for 24 h and 48 h in presence or absence of VEGF. At the end, total cells were collected by a brief trypsinization and counted with hemocytometer after trypan blue staining. Protein concentration in lysates was determined using Bradford assay kit was from G-Biosciences (Maryland Heights, MO). Enhanced chemiluminescence detection system was from Amersham. Fisetin was obtained from Sigma–Aldrich (St Louis, MO) and dissolved in dimethyl sulfoxide (DMSO) as stock solution. Matrigel was from Fisher Scientific (Pittsburgh, PA). Deoxyribonucleic acid, reverse transcriptase enzyme and Taq polymerase reagents were from Bangalore Genie (India). Specific primers for semi-quantitative polymerase chain reaction (PCR) were from Sigma–Aldrich.

FACs analysis for cell cycle distribution
HUVECs were seeded and grown in EGM-2 medium containing FBS and growth supplements. Next day, cells were treated with different concentrations of fisetin. At the end of each treatment time, total cells were collected and processed for cell cycle analysis. Briefly, cells were suspended in saponin/propidium iodide solution [0.3% saponin (wt/vol), 25 mg/ml propidium iodide (wt/vol), 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 10 mg/ml RNase (wt/vol) in phosphate-buffered saline] and incubated over night at 4°C in dark. Cell cycle distribution was then analyzed by flow cytometry. Finally, percentage of cells in different phases of cell cycle was determined by ModFit LT cell cycle analysis software.

Reverse transcriptase–PCR
A549 and DU145 cells were seeded in culture plates under regular growth conditions, and at 70% confluency, they were treated either with DMSO vehicle control or with 50 μM fisetin in 10% serum-supplemented RPMI-1640 medium. Total RNA was isolated with TRIZOL, and first strand complementary DNA synthesis was carried out using 5 μg of total RNA template by reverse transcriptase enzyme (MBI Fermentas, India). This was followed by 20-25 cycles of regular PCR in a thermocycler using specific forward and reverse primers (Sigma–Aldrich). PCR products were analyzed on 1% agarose gel electrophoresis and stained with ethidium bromide. Total RNA was isolated with TRIZOL, and first strand complementary DNA synthesis was carried out using 5 μg of total RNA template by reverse transcriptase enzyme (MBI Fermentas, India). This was followed by 20-25 cycles of regular PCR in a thermocycler using specific forward and reverse primers (Sigma–Aldrich). PCR products were analyzed on 1% agarose gel electrophoresis and stained with ethidium bromide. Primer sequences are listed in Table 1. Fisetin inhibits HUVEC growth and proliferation

In vivo angiogenesis assay
Male Swiss albino mice (~8 weeks old) were approved by Institutional Animal Ethics Committee of Jawaharlal Nehru University and housed in the animal house under standard conditions. Mice were divided into three groups (each having five mice) and subcutaneously injected with 500 μl of Matrigel alone or Matrigel with VEGF (50 μg/ml) and/or fisetin (25 mg/kg body weight of mouse). Fourteen days later, mice were killed and the Matrigel plugs were removed. plugs were immediately photographed and weighed. To quantify the vascularization of the plug, the amount of hemoglobin (Hb) was measured using the HEMOCOR-D kit (Crest Biosystems, Tulip Group, India) following the manufacturer’s protocol step by step. Absorbance of samples was measured at 540 nm. Body weight and diet and water consumption were monitored every 3 days.

 statistical analysis
The data were analyzed using Jandel Scientific SigmaStat 2.03 software. Student’s t-test was employed to assess the statistical significance of difference between control and different treatment groups. A statistically significant difference was considered to be present at P ≤ 0.05.

Results
Fisetin inhibits HUVEC growth and proliferation

To examine whether fisetin has any antiangiogenic activity, first, we assessed its antiproliferative and death-inducing effects on HUVEC cultured in serum-supplemented EGM-2 media containing angiogenic growth factors, including VEGF, basic fibroblast growth factor, insulin-like growth factor-1, platelet-derived growth factor, etc. Fisetin treatment strongly inhibited HUVEC growth in a time- and dose-dependent manner. Fisetin treatment at 10, 25 and 50 μM concentrations resulted in 34–82%, 70–98% and 87–99% (P < 0.001) decrease in total HUVEC number as compared with control after 24, 48 and 72 h, respectively (Figure 1A). HUVEC survival under the similar conditions was also moderately reduced in a dose- and time-dependent manner. Fisetin treatment at 10–50 μM concentrations showed decrease in cell survival after 24–72 h of treatment (Figure 1B). Cell death was accounted for 16% at 50 μM concentration of fisetin after 72 h of treatment as compared with 6% in control. Since VEGF activity is central to the angiogenesis process (4), next we examined whether fisetin also inhibits VEGF-stimulated HUVEC growth and survival. After 24 h of fisetin treatment (10, 25 and 50 μM), VEGF-induced HUVEC cell proliferation was inhibited by 52, 81 and 92%, respectively (P < 0.001) (Figure 1C). Moreover, HUVEC cell survival under similar conditions also decreased significantly with increasing concentration. At 10–50 μM fisetin concentrations, the number of live cells was decreased by 50–95% (P < 0.001) as compared with VEGF treatment alone (data not shown). Furthermore, we studied the effect of fisetin on growth and survival of non-neoplastic HEK 293 cell line. Fisetin decreased total cell number by 25 and 38% at 25 and 50 μM concentrations after 48 h of treatment as compared from control; however, cell death compared with control (4.6%) was only 3.5 and 4.7%, respectively (Figure 1D). This finding suggests that fisetin is largely non-toxic and relatively less growth inhibitory to non-neoplastic human embryonic cells even after 48 h of treatment.
Fisetin inhibits cell cycle progression in HUVEC

Inhibition of cell cycle progression in highly proliferating cells like cancer cells or endothelial cells during tumor angiogenesis is an important strategy to slow down tumor growth and progression (30–34). Since we observed a strong growth inhibitory effect of fisetin on HUVEC, we next analyzed its possible inhibitory effect on cell cycle progression. Twenty four hours of 10 μM fisetin treatment caused 77% HUVEC in G1 phase (P ≤ 0.001) as compared with 54% cells in control, whereas 25 μM concentration of fisetin did not cause G1 arrest but showed 24% cells in G2/M phase (P = 0.005) as compared with 19% cells in control (Figure 2A). Similarly, 48 h of 10 μM fisetin treatment showed 85% cells in G1 phase (P ≤ 0.001) as compared with 65% cells in control, whereas 25 μM fisetin did not cause G1 arrest but induced a mild G2/M arrest (36% cells with fisetin, P < 0.001 versus control) (Figure 2A). A fifty micromolar concentration of fisetin did not show considerable effect on cell cycle phase distribution. An increase in G1 cell population at both time points was at the expense of S phase and G2/M phase cells, whereas G2/M arrest at 24 h was mainly at the expense of S phase population but at 48 h, it was at the expense of both G1 and S phase populations (P < 0.05–0.001). Thus, fisetin induced both a strong G1 arrest (at lower concentration) and a mild G2/M arrest.

Modulation of cell cycle-related proteins like cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors is observed in proliferating cells. For example, cyclin D1 is overexpressed in rapidly growing cells and pushes them from G1 to S phase of cell cycle (34). Since we observed a strong G1 arrest with fisetin treatment in HUVEC, next we assessed its effect on the expression levels of the molecules, which regulate G1–S phase transition. Fisetin decreased the levels of cyclin D1 protein after 24 h of treatment without any considerable effect on CDK2 and CDK4 (Figure 2B). Lower concentrations (10 and 25 μM) of fisetin increased Cip1/p21 expression level without any considerable effect on Kip1/p27 level. Interestingly, higher concentration (50 μM) of fisetin drastically decreased the levels of both Cip1/p21 and Kip1/p27 (Figure 2C). These results suggest that inhibition of cell cycle progression largely at G1–S transition could be a potential antiproliferative mechanism of fisetin.

Tumor suppressor proteins like p53 can repress cell cycle progression, promote apoptosis and inhibit tumor angiogenesis (27,35). p53 suppresses angiogenic process by inducing antiangiogenic factor thrombospondin-1, down-regulating VEGF and inducible nitric oxide synthase (iNOS) expression as well as hypoxia-induced molecular events and by induction of apoptosis in endothelial cells (36,37). Fisetin treatment of HUVEC for 24 h strongly induced p53 expression in a dose-dependent manner (Figure 2C), suggesting its potential role in inhibition of cell cycle progression at lower concentration and induction of apoptosis at higher concentration.

Fisetin induces apoptosis and down-regulates the expression of survivin

Mitochondria-regulated apoptosis is mediated by caspase activation and subsequent PARP cleavage, which is known as the hallmark of early apoptosis induction leading to apoptotic cell death (38–40).
Since fisetin induced cell death as well as p53 protein expression in HUVEC, we assessed its effect on molecules involved in the regulation of apoptosis. Fisetin treatment (10–50 μM) for 24 h induced the cleavage of caspase-3 and caspase-7 as well as PARP (Figure 3A). These molecular effects were quite prominent at higher concentrations of fisetin and also in accordance with their cell death effects. In mammalian system, an inhibitor of apoptosis protein family member, survivin is a unique protein, which is known to regulate cell death (41). Survivin directly regulates caspases-3 and -7 by inhibiting their activation; however, down-regulation of survivin can also cause caspase-independent apoptosis via mitotic catastrophe (42). We observed that fisetin treatment dose-dependently decreases survivin expression with almost no detectable level at higher concentration (50 μM) (Figure 3B). Hence, fisetin-mediated inhibition of HUVEC survival could be associated with down-regulation of survivin expression.

Fig. 2. Fisetin causes G1 and mild G2/M arrest in HUVECs and modulates levels of cell cycle-related proteins. (A) In total, 1 × 10⁵ cells were cultured in complete medium and treated next day with DMSO vehicle control or 10, 25 and 50 μM concentrations of fisetin. After 24 and 48 h of these treatments, cells were collected, processed and analyzed for cell cycle distribution flow cytometric analysis as detailed in Materials and methods. The figure shows percentage of cell cycle distribution data for each treatment group. (B) and (C) HUVECs were treated with indicated doses of fisetin for 24 h and total cell lysates were prepared as described in Materials and methods. Cell lysates were analyzed by immunoblotting using specific antibodies for (B) Cdk4, Cdk2, cyclin D1 and β-actin and (C) Cip1/p21, Kip1/p27 and p53 and β-actin protein levels as described in Materials and methods. Numerical bottom panel of each band indicates the fold change in the band intensity compared with that of control. Data represented in (A) are mean ± SE of three independent samples. The experiments were repeated with similar results.

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Fig. 3. Fisetin induces apoptotic cell death in HUVEC. Cells were treated with the indicated doses of fisetin for 24 h and whole-cell lysates were prepared as described in Materials and methods. Lysates were analyzed by immunoblotting using specific primary and secondary antibodies for (A) cleaved caspase-3, cleaved caspase-7 and cleaved PARP; (B) Bax and survivin and (C) Bcl-2 and pBcl-2 protein levels. (D) Bax to Bcl-2 ratio was determined as described in Materials and methods. β-actin was probed after stripping the membrane as protein loading control. Numerical bottom panel of each band indicates the fold change in the band intensity compared with that of control. The experiments were repeated with similar results.

Fisetin enhances BAX to Bcl-2 ratio of expression level in HUVEC

BAX interacts with Bcl-2 protein through heterodimerization and negates its anti-apoptotic action and induces apoptosis (43). BAX expression is up-regulated by p53 that can mediate p53-dependent apoptosis (44,45). In the present study, fisetin (10–50 μM) increased the BAX expression level in a dose-dependent manner (Figure 3B). Fisetin (25 μM) also strongly decreased both phospho-Bcl-2 and total...
Bcl-2 protein levels (Figure 3C). At 25 μM fisetin concentration, the bax to bcl-2 ratio was increased by ~7-fold as compared with control (Figure 3D). These results suggest that fisetin perturbs BAX to Bcl-2 ratio in favor of apoptosis induction.

_Fisetin inhibits expression of VEGF and eNOS in HUVEC_

VEGF and eNOS are critical angiogenic regulators, which stimulate endothelial cells for new blood vessel formation (4,7,46). Herein, VEGF protein expression was down-regulated in a dose-dependent manner, whereas eNOS protein expression was strongly suppressed at all the fisetin concentrations (Figure 4A). Therefore, fisetin could inhibit angiogenesis by down-regulating the expression of VEGF and eNOS in endothelial cells.

_Fisetin inhibits in vitro angiogenesis by HUVEC_

Capillary-like tube formation by HUVEC on matrigel is an established procedure for assessing the effect of potential antiangiogenic or proangiogenic agents (30). Figure 4B depicts capillary-like tube formation by HUVEC following 16 h treatment with DMSO (control) or different concentrations of fisetin, and its quantitative representation is shown in Figure 4C. The inhibitory effect of fisetin on capillary-like tube formation by HUVEC under similar condition

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**Fig. 4.** Effect of fisetin on VEGF protein expression and HUVEC tube-like structure formation. (A) HUVECs were treated with the indicated concentrations of fisetin for 48 h and whole-cell lysates were prepared and analyzed by western immunoblotting for eNOS and VEGF as described in Materials and methods. β-actin was probed after stripping the membrane as protein loading control. Numerical bottom panel of band indicates the fold change in the band intensity compared with that of control. (B) Representative images depicting formation of capillary-like tube structures on matrigel by HUVEC following a 16 h treatment with DMSO (control) or the indicated concentrations of fisetin. (C) Quantitative data for the effect of fisetin on the formation of capillary-like tube structures by HUVEC after 6 and 16 h of treatment determined as detailed in Materials and methods. (D) Fisetin treatment was given after 6 h of HUVEC seeding for 16 h as discussed in Materials and methods. The quantitative data shown are mean ± SE of three samples. Results were reproducible in at least three experiments. *P < 0.05, **P < 0.001 versus DMSO control.
was observed as early as 6 h of fisetin treatment (pictures not shown) as quantitatively shown in Figure 4C. Fisetin (10–50 μM) treatment for 6 h started with cell seeding showed 31–61% decrease in tube formation (P < 0.005–0.001) as compared with control. When this treatment continued for a total of 16 h, the decrease in tube formation accounted for 47–85% (P < 0.001) (Figure 4C). In another protocol, HUVECs were seeded for 6 h, which formed rudimentary tubular network and then treated with fisetin (10–50 μM) for 16 h. In this protocol, 10–50 μM concentrations of fisetin resulted in 50–81% (P < 0.001) inhibition of capillary-like tube formation in a dose-dependent manner (Figure 4D). These observations suggest that fisetin inhibits capillary-like tubular organization as well as disrupts preformed capillary tubes by HUVEC on matrigel, which could have greater implications in inhibiting tumor angiogenesis.

Fisetin suppresses HUVEC cell migration in wound closure assay

Endothelial cell migration is critical for angiogenesis process. In tumor angiogenesis, endothelial cell migration is necessary for the penetration of new blood capillaries deep into the tumor mass and for invasion of new spaces/tissues/organs to positively affect tumor growth and metastasis (7). Scratch wound closure assay has been accepted as a reliable method to show cell migration (47). In the present study, fisetin effectively inhibited HUVEC cell migration showing slower closure of the wound as compared with control (Figure 5A). Compared with control, 25 and 50 μM concentrations of fisetin inhibited HUVEC migration by 37 and 66% (P < 0.001) after 24 h of treatment, respectively (Figure 5B). These results suggest that fisetin can inhibit cell migration needed for vessel sprouting and growth.

Fisetin inhibits angiogenic attributes in various cancer cell lines

Solid tumors secrete various proangiogenic factors such as VEGF, eNOS, iNOS, HIF1-alpha, etc. to activate the nearest endothelial cells in the host tissue for neoangiogenesis needed for the growth of tumors (7,48). In the present study, we investigated whether fisetin could also inhibit angiogenic attributes in cancer cells by employing human lung carcinoma A549 and prostate carcinoma DU145 cell lines. Fisetin (50 μM) treatment for 24 h strongly suppressed VEGF protein expression in both A549 and DU145 cells (Figure 5C). Also, similar treatment of fisetin strongly reduced the messenger RNA transcript levels of VEGF-A, eNOS, iNOS and matrix metalloproteinase (MMP)-2 and MMP-9 in both these advanced cancer cell lines (Figure 5D). These results suggest that fisetin strongly suppresses the expression of factors that initiate and promote tumor angiogenesis in cancer cells and therefore, it could inhibit tumor-induced angiogenesis.

Fisetin inhibits in vivo angiogenesis

Since fisetin strongly inhibited various attributes of angiogenesis in HUVEC and cancer cells, next we examined whether it could inhibit angiogenesis in vivo by employing matrigel plug implant in mice. Matrigel plug was subcutaneously implanted with or without VEGF/fisetin for 2 weeks in Swiss albino mice. Fisetin (25 mg/kg body weight of mice) strongly suppressed the VEGF-induced angiogenesis after 2 weeks of matrigel implantation as depicted in Figure 6A. Since VEGF attracts blood vessel growth into the matrigel, therefore, plug weight as well as Hb content increased in plugs having VEGF as compared with control groups. Plug weight per size and Hb content were taken as parameters to quantitate angiogenesis. Fisetin decreased plug weight by 43% (P < 0.05) and Hb content by 94% (P < 0.001) as compared with VEGF only control (Figure 6B and C). These results strongly support the antiangiogenic efficacy of fisetin in vivo condition.

Discussion

The central findings in the present study are that fisetin exhibits strong antiangiogenic potential by inhibiting various angiogenic attributes in HUVEC as well as in cancer cells. Fisetin inhibited proliferation and survival of HUVEC in regular as well as VEGF-induced conditions and also suppressed matrigel tube formation and cell migration by HUVEC. The potential mechanisms behind these inhibitory effects in HUVEC observed were (i) strong cell cycle arrest in G1 phase along with mild G2/M arrest; (ii) down-regulation of cyclin D1, survivin and Bcl-2 expression and induction of p53, p21 and Bax expression; (iii) induction of cleavage of caspases-3 and -7 and PARP and (iv) inhibition of VEGF expression. In cancer cells, fisetin down-regulated the expression of various angiogenic genes and also inhibited angiogenic attributes in matrigel implants in mice. It showed relatively less growth inhibitory effect on non-neoplastic HEK 293 cells without any considerable cytotoxicity. Fisetin, a natural flavonol, found commonly in strawberries and other fruits and vegetables has been shown to possess strong efficacy against many cancers (23–27), suggesting that fisetin is a promising and potential anticancer agent. Based on our central findings summarized here, we suggest that antiangiogenic effects of fisetin in part play a potential role in its overall anticancer activity.

Solid tumor growth and progression heavily rely on the angiogenesis processes mediated by the tumor cell and microenvironment-derived proangiogenic factors, which induce blood vessel formation in the tumor mass (1,2,4,48). Thus, targeting of these processes could be a potential strategy in suppressing tumor growth and metastasis (13–18). Consistent with these observations, in the present study, fisetin showed strong antiangiogenic activity by targeting many molecular events associated with the HUVEC cell proliferation, survival, tube formation, migration and in vivo angiogenesis. These findings are relevant as the HUVEC angiogenesis model used in present study is the well accepted and the most used cell line to study the direct proangiogenic or antiangiogenic activities of a given compound (8,30,47).

The antiproliferative effect of fisetin in regular as well as VEGF-induced conditions in HUVEC could be attributed to the G1 phase cell cycle arrest and mild G2/M arrest. Decrease in cyclin D1 expression and an increase in Cip1/p21 (at lower doses) and p53 expression could contribute to the cell cycle arrest caused by fisetin. The apoptotic effect of fisetin could be due to the down-regulation of survivin protein expression leading to the activation of caspase pathway as a potential mechanism to inhibit the survival of human endothelial cells (30). PARP is involved in DNA repair, stability and other cellular events and is cleaved (89 kDa) by the caspase family members mainly involved in the onset of apoptosis pathway (89). Apoptosis induction is a dynamic process relying on the balance between pro- and anti-apoptotic factors in which Bcl-2 family members like pro-apoptotic BAX and anti-apoptotic Bcl-2 play vital roles and whose ratio determines the fate of a cell. Also, phosphorylation of Bcl-2 at serine70 in post-translational mode activates Bcl-2 and leads to its degradation (45). Consistent with these reports, fisetin induced cleavage of caspasas and PARP, and increased Bax expression and down-regulated Bcl-2 expression showing many fold increase in BAX to Bcl-2 ratio. Fisetin also showed a correlation in p53 induction along with BAX, as former is known to increase the Bax expression. Overall, these molecular alternations could partly explain the growth inhibitory and cell death effects of fisetin on HUVEC. It could be of further importance that fisetin did not show any considerable cytotoxicity in HEK 293 cells in the present study, together with a similar observation on normal human prostate epithelial PrEC cells when compared with prostate cancer cells (23).

VEGF and eNOS are important regulators of angiogenesis that are highly expressed in growing tumors (4,5,7,46,48), and their down-regulation has been suggested as a potential strategy in cancer prevention and control (7,46). In the present study, VEGF expression was almost abrogated at 25–50 μM concentrations of fisetin, whereas eNOS expression was strongly down-regulated in a dose-dependent manner. Therefore, it can be anticipated that fisetin would inhibit angiogenesis. HUVEC tube-like structure formation and migration capabilities are among the prime parameters in tumor angiogenesis. As anticipated, fisetin effectively inhibited HUVEC tube-like structure formation as well as preformed tubular organization and
migration in a dose- and time-dependent manner. This implies that fisetin inhibits both de novo and rudimentary capillary formation.

Tumor-induced angiogenesis is a well-known target in cancer control and prevention, therefore, inhibition of proangiogenic signals in cancer cells is an important aspect of this strategy (reviewed in refs. 7,49). In this regard, fisetin strongly suppressed the transcript levels of various proangiogenic factors like VEGF, eNOS, iNOS, MMP-2 and MMP-9 in prostate carcinoma DU145 and lung carcinoma A549 cells. The down-regulation of VEGF, the most important angiogenic factor, was also confirmed at protein level. These findings suggest that fisetin can effectively modulate the angiogenic regulators toward inhibition of angiogenesis in cancer cells. Furthermore, we evaluated the in vivo antiangiogenic efficacy of fisetin using well-known matrigel plug angiogenesis model in Swiss albino mice. Using one time 25 mg/kg body weight of fisetin treatment for 2 weeks, we found that fisetin strongly inhibited VEGF-stimulated angiogenesis in matrigel implant. Plug weight and Hb content (taken as angiogenic parameters) were significantly decreased in fisetin-treated group. This implies that fisetin could inhibit in vivo angiogenesis.

Taken together, the present study shows that fisetin is a potent and promising antiangiogenic small molecule, which inhibits HUVEC cell growth, cell cycle progression and survival and strongly
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Conflict of Interest Statement: None declared.

References


Fig. 6. Effect of fisetin on VEGF-induced in vivo angiogenesis. Mice were randomly divided into three groups as control, VEGF and VEGF + fisetin groups (n = 5) and subcutaneously received either matrigel only, Matrigel + VEGF (50 ng/ml) or Matrigel + VEGF (50 ng/ml) + fisetin (25 mg/kg body weight), respectively. DMSO was added to each treatment group at 0.1% (vol/vol) final concentration. After 14 days of matrigel plug implantation, mice were killed and plugs were retrieved, weighed, photographed for blood vessel formation and Hb content was determined by Hemoglobin measuring kit as described in Materials and methods. (A) Photographs of representative matrigel plugs in each group, (B) Hb content (g/dl) and (C) Weight of matrigel plugs (mg/plug). Body weight and diet/water consumption did not change among groups (data not shown). *P < 0.05, **P < 0.001.

suppresses both in vitro and in vivo angiogenesis along with down-modulation of many proangiogenic regulators in cancer cells. Thus, additional studies of fisetin employing in vivo tumor models could further support its clinical usefulness in cancer prevention and control.


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