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Antiangiogenic Activity of Xanthomicrol and Calycopterin, Two Polymethoxylated Hydroxyflavones in Both *In Vitro* and *Ex Vivo* Models

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Our previous studies had shown xanthomicrol and calycopterin, two plant-derived flavonoids, to have selective antiproliferative activity against some malignant cell lines. The present study is focused on the investigation of antiangiogenic potential of these two flavonoids, using *in vitro* and *ex vivo* models. Xanthomicrol and calycopterin were found to have potent inhibitory effects on microvessel outgrowth in the rat aortic ring assay. Xanthomicrol was able to completely block microvessel sprouting at 10 μg/mL, and calycopterin suppressed microvessel outgrowth by 89% at 5 μg/mL. Suramin and thalidomide, used at 20 μg/mL as positive controls, inhibited microvessel formation by 23% and 64%, respectively. The flavones also inhibited endothelial cell tube formation and human umbilical vein endothelial cell proliferation at 0.5, 5, and 10 μg/mL. In order to delineate the underlying mechanisms of antiangiogenic activity of these flavones, we investigated the influences of xanthomicrol and calycopterin on expression of vascular endothelial growth factor (VEGF) and basic-fibroblast growth factor (b-FGF) in endothelial cells. These flavones were able to inhibit VEGF expression at 0.5, 5, and 10 μg/mL, but they had little or no effect on b-FGF expression. These findings suggest that xanthomicrol and calycopterin possess potent antiangiogenic activities, which may be due to their inhibitory influences on VEGF expression. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: xanthomicrol; calycopterin; antiangiogenic activity; rat aortic ring assay; endothelial tube formation assay; VEGF.

INTRODUCTION

Many flavonoids have been demonstrated to have *in vitro* cytotoxic activity against various cell lines and have therefore been proposed as anticancer drugs or precursors of such agents. However, a large number of the flavonoids studied have nonselective cytotoxic activity against many cell lines, malignant or otherwise (Chang *et al*., 2008). Studies involving structure–activity relationship of flavonoids have provided evidence that methoxylation of these compounds can change the pattern of their *in vitro* antiproliferative activity against various cell lines and, in some cases, such substitutions have rendered the molecules highly selective against specific cell lineages (Walle, 2007). Xanthomicrol and calycopterin (Fig. 1) are two such flavonoids isolated from *Dracocephalum kotschyii* Boiss (Jahaniani *et al*., 2005; Faham *et al*., 2008). *Dracocephalum kotschyii* Boiss is a native Iranian medicinal plant with analgesic, antispasmodic, and anticancer properties and has been used in traditional medicine for many years (Jahaniani *et al*., 2005). Research in our laboratory (Moghaddam *et al*., 2012) showed that many of the flavonoids obtained from this plant had some cytotoxic activity; however, those with one hydroxyl group and no methoxy groups, for example, luteoline and apigenine, were nonselectively cytotoxic against all cell lines tested. In contrast, xanthomicrol and calycopterin, which had no hydroxyl groups and were trimethoxylated and tetramethoxylated, respectively, showed selective cytotoxic activity against some cell lines with little effect on human fetal foreskin fibroblast cells used as normal, nonmalignant control. In the same study, we reported that these compounds showed little preferential cytotoxicity against WEHI-164 (murine fibrosarcoma) cells *in vitro*. However, both compounds inhibited tumor growth in mice transplanted with WEHI-164 cells (data not shown).

Sometimes, lack of correlation of *in vitro* and *in vivo* effects may induce research into alternative pharmacological effects. Genistein is an example in hand. It is consumed in large quantities in soya-containing products in the Far East, and it is thought to be the reason for lower incidences of some cancers in Japanese and Chinese populations compared with the rest of the world (Banerjee *et al*., 2008). Although research had shown it to have *in vitro* cytotoxic effects, pharmacokinetic studies had shown it to have lower serum levels than would be necessary for significant cytotoxic activity (Barnes, 1995). Further research showed that it inhibited proliferation of endothelial cells at pharmacokinetically observed serum concentrations (Barnes, 1995), suggesting an antiangiogenic mechanism of action for this isoflavone.

Because of structural similarities to genistein, other flavonoids could also act by inhibiting endothelial cell proliferation. Therefore, xanthomicrol and calycopterin may have an antiproliferative effect on endothelial cells and affect angiogenesis, *in vivo*. Angiogenesis, the generation of new blood vessels by sprouting of pre-existing

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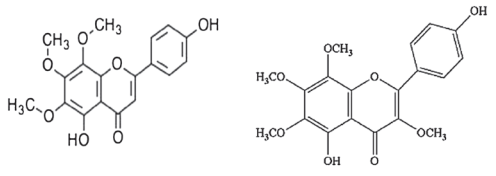

Figure 1. Chemical structures of xanthomicrol and calycopterin.

vessels, is an essential stage in both physiological processes including reproductive function, embryonic development, wound healing, and pathological states such as diabetic retinopathy, psoriasis, chronic inflammations, tumor growth, and metastasis (Carmeliet, 2003). On the basis of the fact that a tumor cannot grow over a few cubic millimeters without the generation of new blood vessels (Folkman, 1975), angiogenesis must play a critical role in the development of solid tumors and metastases. Therefore, inhibition of pathological angiogenesis may represent an effective strategy to suppress tumor progression. Angiogenesis, a complex and multistage process, is mediated by angiogenic factors, chemokines, angiogenic enzymes, adhesion molecules, and endothelial cell receptors. Each of these components can be targeted to develop agents with antiangiogenic activity (Liekens *et al*., 2001). Vascular endothelial growth factor (VEGF) appears to possess a central role in generation of new blood vessels. VEGF can interact with specific receptors (VEGFR) on endothelial cells to initiate the intracellular processes necessary for proliferation, adhesion, and migration of these cells, thus promoting angiogenesis. Basic-fibroblast growth factor (b-FGF) induces angiogenesis indirectly by increasing the release of VEGF from many cell types (Liekens *et al*., 2001; Cross and Claesson-welsh, 2001).

In this study, effects of xanthomicrol and calycopterin on proliferation of human umbilical vein endothelial cells (HUVECs) were first studied. In order to demonstrate whether xanthomicrol and calycopterin had any antiangiogenic effect, we used the rat aortic ring assay. The inhibitory effects of these flavones on the formation of tubular network of endothelial cells were also studied. To determine if the effects of xanthomicrol and calycopterin on the production of VEGF and b-FGF mirrored the effects they had on cellular models of angiogenesis, we measured the expression of VEGF and b-FGF in endothelial cells.

MATERIALS AND METHODS

Chemicals and reagents. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), suramin, thalidomide, bovine serum albumin (BSA), Tween 20 were purchased from Sigma-Aldrich (St. Louis, MD, USA). HUVECs were supplied by Pasteur Institute (Tehran, Iran). Fetal bovine serum (FBS) was purchased from Gibco (Life Technologies, Dan Allen Way, Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). Rabbit polyclonal anti-VEGF, rabbit polyclonal anti-b-FGF, rabbit polyclonal anti-β-actin, and goat anti-rabbit IgG-horseradish peroxidase were obtained from Santa Cruz Co. (Santa Cruz, Biotechnology, Dry Creek Rd, Paso Robles, CA, USA). All other reagents were of ANALAR grade. All solvents used were of HPLC grade.

Extraction and purification of xanthomicrol and calycopterin from *Dracocephalum kotschyii* Boiss. Xanthomicrol and calycopterin were extracted from *Dracocephalum kotschyii* Boiss as reported previously (Moghaddam *et al*., 2012). Briefly, 50 g of powdered leaves of *Dracocephalum kotschyii* Boiss were extracted with 400 mL of ethyl acetate overnight by the Soxhlet method. The resulting extract was concentrated at 40 °C under vacuum. The dried extract was dissolved in chloroform, and then aliquots of the filtered chloroform fraction were further extracted with equal volumes of ammonia solution (pH 11). The resulting aqueous phase was acidified using concentrated HCl. The acidified solution was extracted with ethyl acetate. Finally, the resulting organic phase was concentrated at 40 °C under vacuum, and the residue was kept at −20 °C until needed.

The resulting dried substance after liquid–liquid extraction was subjected to semi-preparative reversed-phase HPLC on a Macherey–Nagel 10 μm Nucleosil, column (2.5 × 5 cm), using an isocratic solvent system consisting of 56% HCl 0.01 M, 25% acetonitrile, and 19% methanol. The flow rate was set at 7 mL/min. The injection volume was 1.7 mL, and the detector was set to 226 nm. Data acquisition was performed using an Adventec PCI 1719 data acquisition card and an in-house developed software. The identities of xanthomicrol and calycopterin were confirmed by Nuclear Magnetic Resonance (NMR) Spectroscopy, Mass Spectrometry (MS) and Infrared Spectroscopy (IR) as reported previously (Moghaddam *et al*., 2012).

Endothelial cell proliferation assay. The MTT assay was used to determine the proliferation of endothelial cells treated with xanthomicrol or calycopterin. HUVECs were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 5% FBS. Cells were seeded onto 96-well culture plate at a density of 10,000 cell per well and allowed to attach overnight at 37 °C under 5% CO_2. The culture medium was aspirated, and fresh medium containing vehicle (0.1% DMSO), xanthomicrol (0.5, 5, and 10 μg/mL), or calycopterin (0.5, 5, and 10 μg/mL) was added to each well. The plate was then incubated for 72 h at 37 °C under 5% CO_2. Thereafter, 20 μL of MTT (Sigma) solution in deionized water (5 mg/mL) was added to each well and incubated at 37 °C under 5% CO_2 for 4 h. The resulting MTT formazan was dissolved by the addition of 100 μL of DMSO per well. The absorbance was measured at 570 nm using a Dynex MRX microplate reader (Sullyfield Circle, Chantilly, VA, USA). Independent MTT tests were repeated three times.

Rat aortic ring assay. Briefly, 48-well culture plates were coated with 100 μL of Geltrex (Gibco), which were allowed to gel for 30 min at 37 °C under 5% CO_2. Thoracic aortas were carefully excised from juvenile male Wistar rats. After removal of fibroadipose tissue, the aortas were dissected into 1-mm-long cross sections. Each aortic ring was set on one Geltrex-coated well and was then covered with an additional 100 μL of Geltrex. After the second layer of Geltrex had been placed, the rings were covered with EGM-II Bulletkit medium

(Clonetics, Lonza Pharma, Maryland, USA) and incubated overnight at 37 °C under 5% CO_2. Afterwards, the culture medium was replaced with EBM-II (Clonetics, Lonza Pharma, Maryland, USA) supplemented with 2% FBS, 0.25 μg/mL amphotericin B, and 10 μg/mL gentamicin (no Bulletkit was added). Each aortic section was exposed to vehicle (0.5% DMSO), xanthomicrol (0.25–10 μg/mL), or calycopterin (0.25–5 μg/mL). Control rings were treated with 20 μg/mL suramin or thalidomide for 4 days and photographed under an inverted microscope on the fifth day using an X4 objective. Pixel-integrated density of the area of microvessel sprouting was quantified by image analysis using Image J software (developed at National Institutes of Health, USA). Independent tests were repeated three times, using aortas from three different rats.

Endothelial cell tube formation assay. Culture plates with 48 wells were coated with 50 μL of Geltrex and allowed to gel at 37 °C under 5% CO_2 for 30 min. HUVECs pre-treated with xanthomicrol or calycopterin in EGM-II medium containing the vehicle (0.1% DMSO), xanthomicrol (0.5, 5, and 10 μg/mL), or calycopterin (0.5, 5, and 10 μg/mL) were seeded on Geltrex-coated wells at 40,000 cell per well and incubated at 37 °C under 5% CO_2 for 20 h. Suramin as positive control was used at the concentration of 40 μg/mL. Afterwards, the cells were stained with calcein acetoxymethyl ester (Gibco) and incubated at 37 °C under 5% CO_2 for 30 min. To analyze the resulting capillary-like structures, the area of tubular structures in each well was photographed under a fluorescence microscope using an X20 objective. The corresponding area was measured as the length of endothelial cell tube at five random positions using Image J software (Yamamizu *et al*., 2013). Independent experiments were repeated three times.

Preparation of cell lysates. The HUVECs were grown to 80% confluency in 75-cm^2 tissue culture flasks in DMEM medium supplemented with 5% FBS at 37 °C under 5% CO_2. Cells were exposed to DMEM with vehicle (1% DMSO), xanthomicrol (0.5, 5, and 10 μg/mL), or calycopterin (0.5, 5, and 10 μg/mL) and incubated at 37 °C under 5% CO_2 for 48 h. Thereafter, HUVECs were serum starved and treated with xanthomicrol (0.5, 5, and 10 μg/mL) or calycopterin (0.5, 5, and 10 μg/mL) and incubated at 37 °C and 5% CO_2 for 24 h. The cells were trypsinized and washed in phosphate-buffered saline (pH 7.4). Cells were lysed by homogenizing in lysis buffer containing proteases inhibitors and then centrifuged to pellet cell debris. The resulting supernatants were frozen at −80 °C. To determine protein concentrations of cell lysates, bicinchoninic acid assay kit (Pierce) was applied using BSA as standard.

Western blotting. The resulting cell lysates were used for VEGF, b-FGF, and β-actin analysis. Briefly, proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 15% w/v acrylamide gel. Protein bands were transferred to a polyvinylidene difluoride membrane. The blot was blocked for 1 h in tris-buffered saline containing 0.1% v/v Tween 20 (TBS-T) and 5% non-fat dry milk. Membranes were incubated with primary antibodies: rabbit polyclonal anti-VEGF, rabbit polyclonal anti-b-FGF, and rabbit polyclonal anti-β-actin in TBS-T containing 5% non-fat dry milk, overnight at 4 °C. After washing several times in TBS-T, the blot was incubated with goat anti-rabbit IgG-horseradish peroxidase in TBS-T for 1 h at room temperature. After extensive washing, the bands were detected using enhanced chemiluminescence plus kit (Pierce Protein Biology Products, Thermo Scientific, Rockford, IL, USA). The resulting chemiluminescence was visualized by developing the X-ray-film. Densitometric analysis of protein bands was performed using Quantity One Analysis software (Bio Rad, Alfred Nobel Drive, Hercules, CA, USA). Independent tests were repeated for each protein three times.

RESULTS

Inhibition of HUVECs proliferation by xanthomicrol and calycopterin

The MTT as an *in vitro* assay was applied to assess the effects of xanthomicrol and calycopterin on proliferation of endothelial cells. Xanthomicrol and calycopterin inhibited endothelial cells growth in a concentration-dependent manner after 72 h of treatment as illustrated in Fig. 2. At 10 μg/mL, xanthomicrol and calycopterin attenuated endothelial cells proliferation by 51% and 75%, respectively.

Xanthomicrol and calycopterin blocked microvessel sprouting in *ex vivo* rat aortic ring assay

In order to evaluate the antiangiogenic properties of xanthomicrol and calycopterin, the influences of these flavones on microvessel outgrowth from rat aorta were examined using the aortic ring assay. Xanthomicrol and calycopterin showed potent inhibitory effects on microvessel sprouting in a concentration-dependent manner as presented in Fig. 3. The antiangiogenic effects of these flavones were stronger than suramin and thalidomide used as positive controls. Xanthomicrol completely inhibited microvessel outgrowth at 10 μg/mL, whereas calycopterin suppressed microvessel outgrowth by 89% at 5 μg/mL. Suramin and thalidomide at 20 μg/mL inhibited sprouting of new vessels by 23% and 64%, respectively.

Xanthomicrol and calycopterin inhibited the endothelial cell tube formation

To further characterize whether xanthomicrol and calycopterin could block endothelial cell migration and tubulogenesis, we examined their effects on capillary-like structures formed by endothelial cells seeded on Geltrex. Tubulogenesis was inhibited by these flavones in a concentration-dependent fashion as shown in Fig. 4. At 10 μg/mL, xanthomicrol and calycopterin inhibited three-dimensional tubular structures by 65% and 70%, respectively, whereas suramin at 40 μg/mL, used as a positive control, inhibited tube formation by 80%.

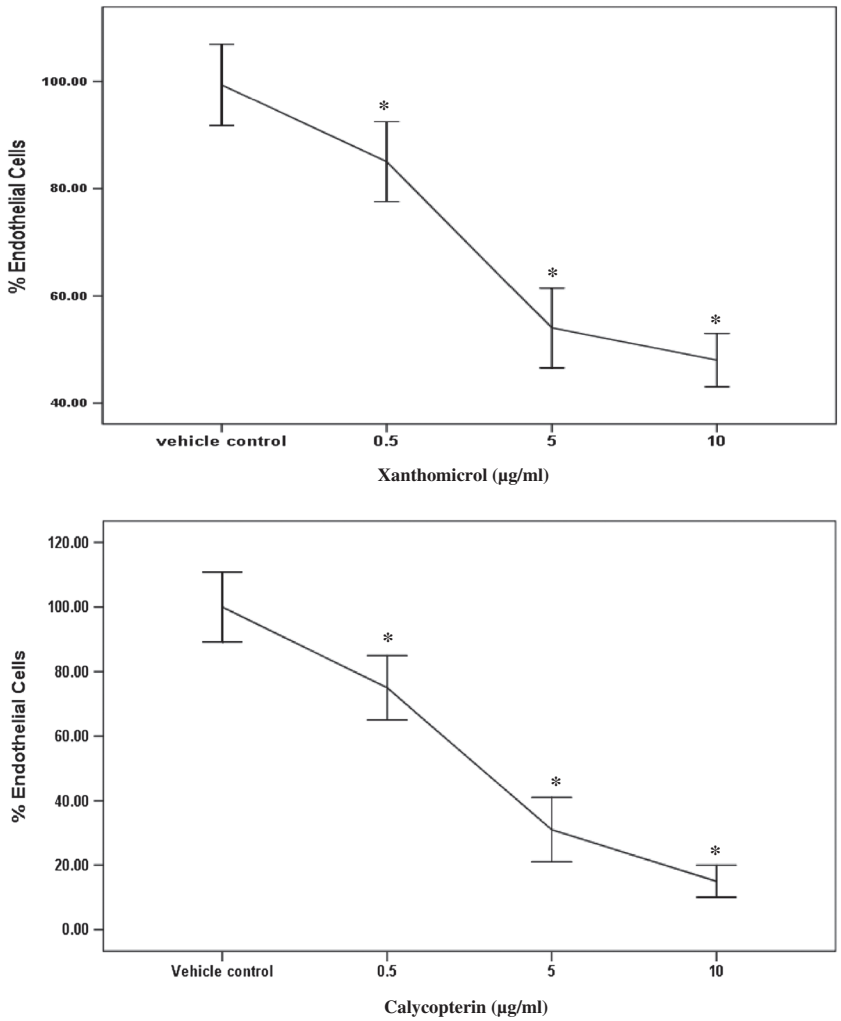

Figure 2. Concentration-dependent inhibition of HUVECs proliferation by xanthomicrol and calycopterin. The percentage of endothelial cell proliferation for each xanthomicrol and calycopterin concentration was calculated relative to the vehicle control (0.1% DMSO). Each point represents mean ± SE, $n = 3$ (*, $p < 0.05$, compared with vehicle control).

Xanthomicrol and calycopterin reduced VEGF expression in endothelial cells

To obtain an insight into the mechanism of antiangiogenic activities of xanthomicrol and calycopterin, the effects of these flavones on VEGF expression in HUVEC cells were evaluated. After 72 h incubation with xanthomicrol (0.5, 5, and 10 µg/mL) or calycopterin (0.5, 5, and 10 µg/mL), the intracellular VEGF expression was decreased in a concentration-dependent manner (Fig. 5). Compared with vehicle control, xanthomicrol and calycopterin, at 10 µg/mL, reduced VEGF expression by 38% and 44%, respectively. These flavones had little or no effect on b-FGF expression in endothelial cells at the same concentrations used for VEGF (Fig. 6).

DISCUSSION

Many flavonoids have been tested for their cytotoxic effects, and some have been found effective enough to be of interest for developing new anticancer agents (Chang *et al*., 2008). Although many flavonoids such as 3-hydroxyflavone, apigenine, and luteoline are cytotoxic across the board (Cao *et al*., 2002), that is, having nonselective effects, it is increasingly clear that some compounds in this vast group affect various cell lines differently, suggesting selective mechanisms of action. Also, there are discrepancies between the cytotoxic activities seen *in vitro* and the effectiveness of some of these compounds as potential antineoplastic drugs *in vivo* (Cao *et al*., 2002). Xanthomicrol and calycopterin are two examples of such flavonoids. They are both polymethoxylated and appear to have selective activity against some cell lines but have little effect on many others. More interestingly, these compounds showed *in vivo* activity against tumors induced in mice using cell lines, against which they had little *in vitro* activity. One possible mechanism that could explain this was a putative antiangiogenic activity exhibited by these compounds.

Tumor angiogenesis is essential for the progression of a neoplasm from a dormant localized tumor to a developing tumor with the potential to metastasize (Folkman, 2006). Therefore, agents with antiangiogenic activity could be effective in controlling primary growth and secondary metastatic tumors. Many bioactive plant-derived compounds have been tested for their antiangiogenic properties (Cao *et al*., 2002; Singh and Agarwal, 2003; Sagar *et al*., 2006; Mojzis *et al*., 2007; Mojzis *et al*., 2008). Consistent with previous preclinical studies, plants that are rich in polyphenols tend to have antiangiogenic properties.

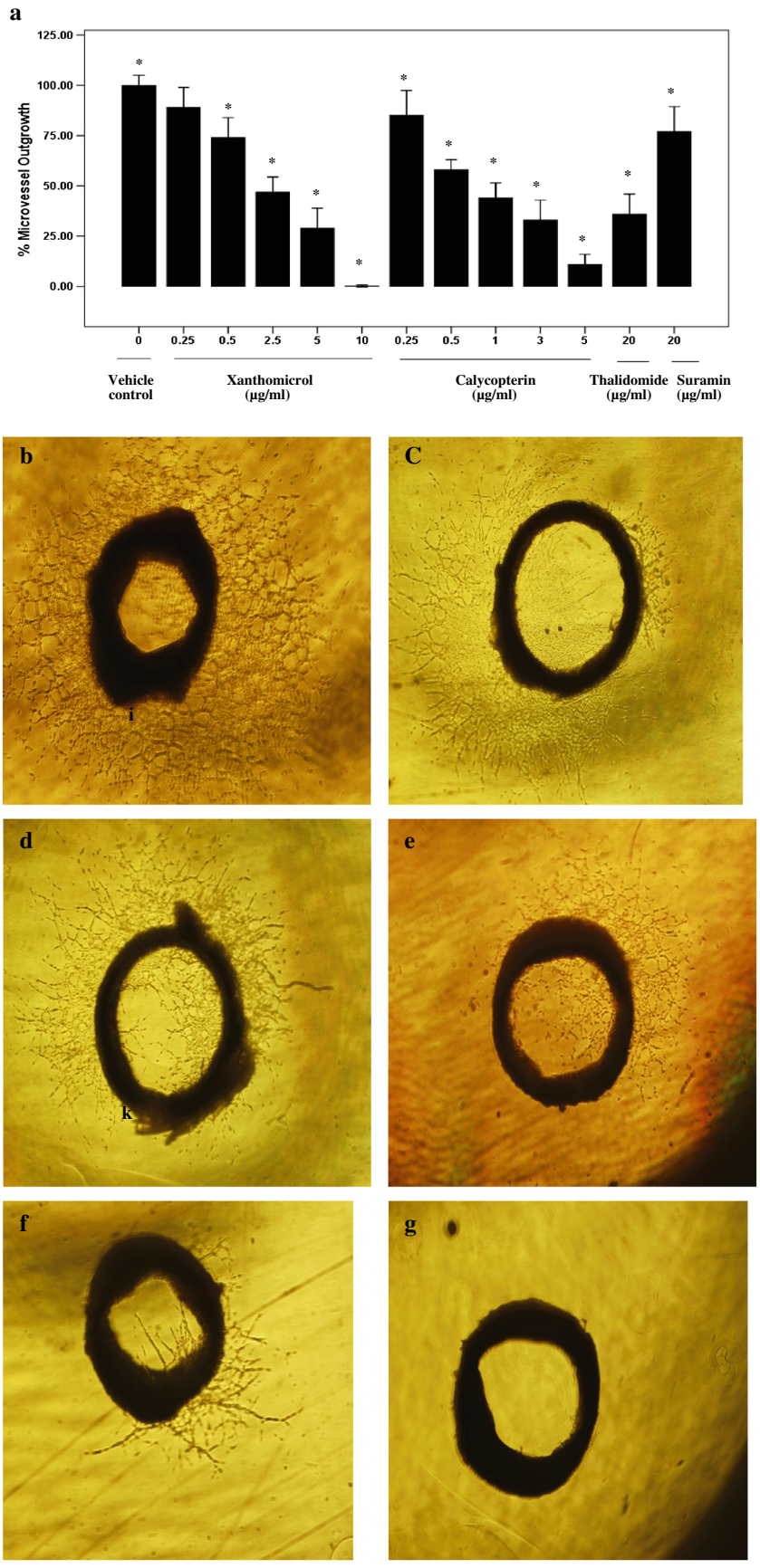

Figure 3. Concentration-dependent antiangiogenic activity of xanthomicrol and calycopterin in the rat aortic ring model. (a) Bar graphs of the percentage of microvessel outgrowth relative to the vehicle control (0.5% DMSO) after treatment with xanthomicrol (0.25–10 µg/mL), calycopterin (0.25–5 µg/mL), suramin (20 µg/mL), or thalidomide (20 µg/mL). Bars represent mean ± SE, $n = 3$ (*, $p < 0.05$, compared with vehicle control). Representative images of rat aortic rings treated with the vehicle control (b); xanthomicrol at (c) 0.25, (d) 0.5, (e) 2.5, (f) 5, and (g) 10 µg/mL; calycopterin at (h) 0.25, (i) 0.5, (j) 1, (k) 3, (l) 5 µg/mL; 20 µg/mL thalidomide (m); or 20 µg/mL suramin (n). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

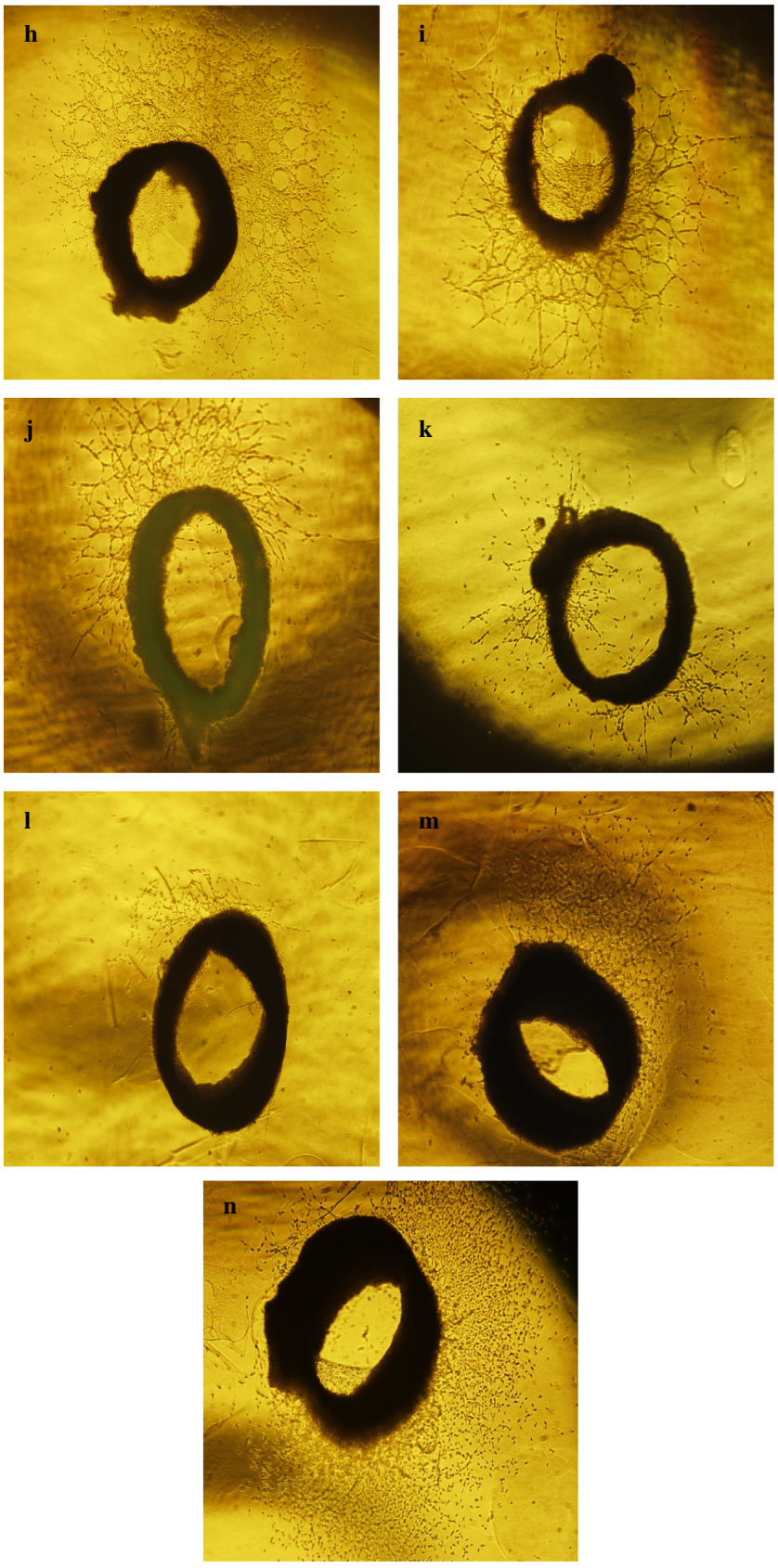

Figure 3. (Continued)

Xanthomicrol and calycopterin inhibited HUVEC proliferation in culture medium in a concentration-dependent manner. The antiproliferative activity of xanthomicrol and calycopterin appeared to reach a plateau around 10 μg/mL. The inhibition of endothelial cell proliferation at the highest concentration of xanthomicrol and calycopterin used was not complete, which could be an indication of cytostatic rather than cytotoxic activity of these flavones. This could be of great significance for an antiangiogenic agent because cytotoxicity against endothelial cells would mean damage to blood vessels in non-tumor tissue. Also, the inhibition of HUVEC cell proliferation by xanthomicrol

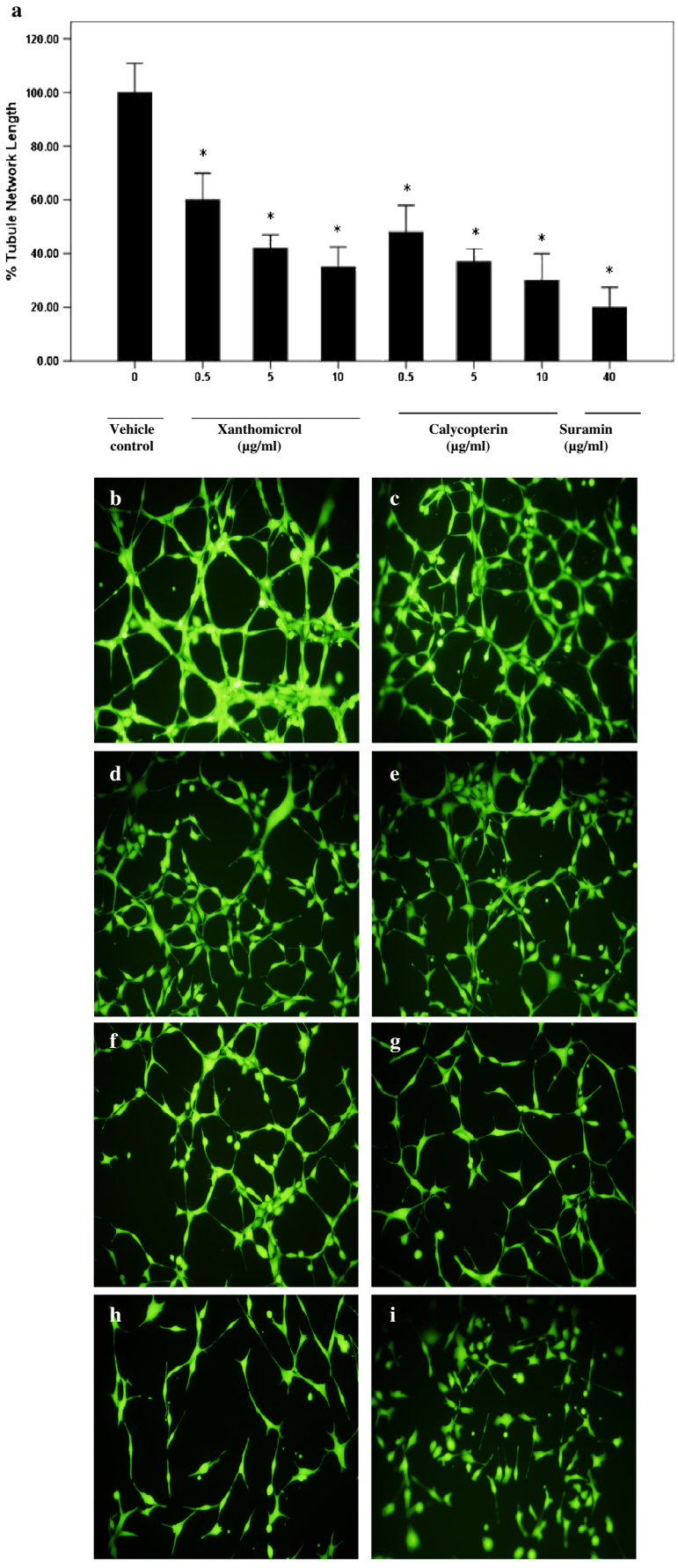

Figure 4. Concentration-dependent inhibition of capillary-like tube formation in HUVECs by xanthomicrol and calycopterin. (a) Bar graphs of the percentage of tubule network length relative to the vehicle control (0.1% DMSO) after treatment with xanthomicrol and calycopterin at 0.5, 5, and 10 μg/mL or suramin (40 μg/mL). Bars represent mean ± SE, $n = 3$ (*, $p < 0.05$, compared with vehicle control). Representative images of the effects of treatment with vehicle control (b); xanthomicrol at (c) 0.5, (d) 5, and (e) 10 μg/mL; calycopterin at (f) 0.5, (g) 5, and (h) 10 μg/mL; or 40 μg/mL suramin (i) on endothelial cell tubule formations. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

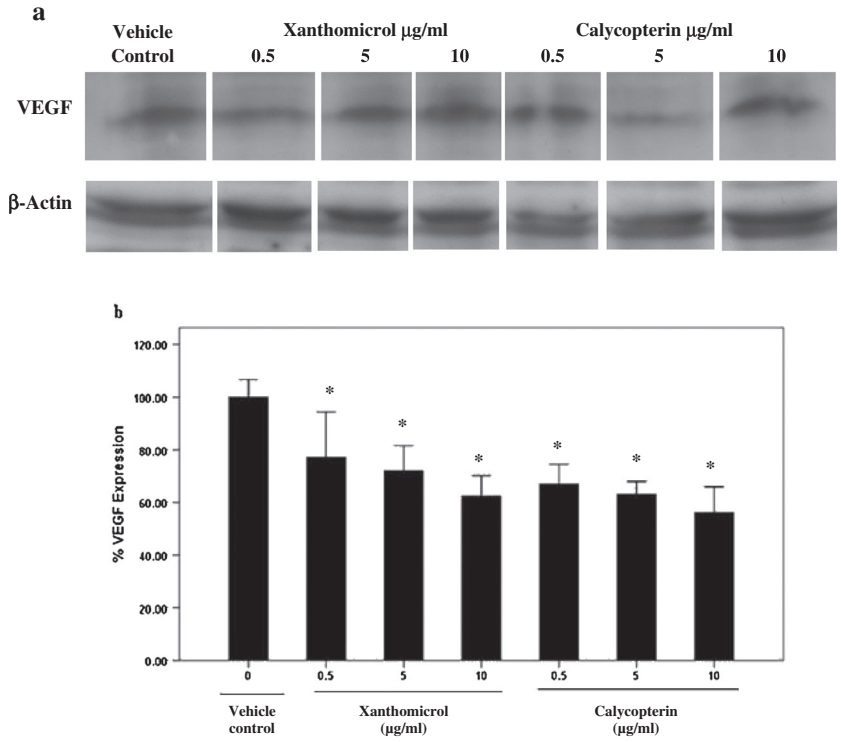

Figure 5. Concentration-dependent inhibitory effects of xanthomicrol and calycopterin on VEGF expression in HUVECs. (a) Western blot of HUVEC lysates treated with vehicle control (0.1% DMSO), xanthomicrol (0.5, 5, and 10 μg/mL), or calycopterin (0.5, 5, and 10 μg/mL), for 72 h and stained for VEGF using rabbit polyclonal anti-VEGF antibody followed by chemiluminescent visualization. (b) Bar graphs of the percentage of VEGF expression relative to the vehicle control (0.1% DMSO) versus treatment of endothelial cells with xanthomicrol or calycopterin at 0.5, 5, and 10 μg/mL. Bars represent mean ± SE, $n = 3$ (*, $p < 0.05$, compared with vehicle control).

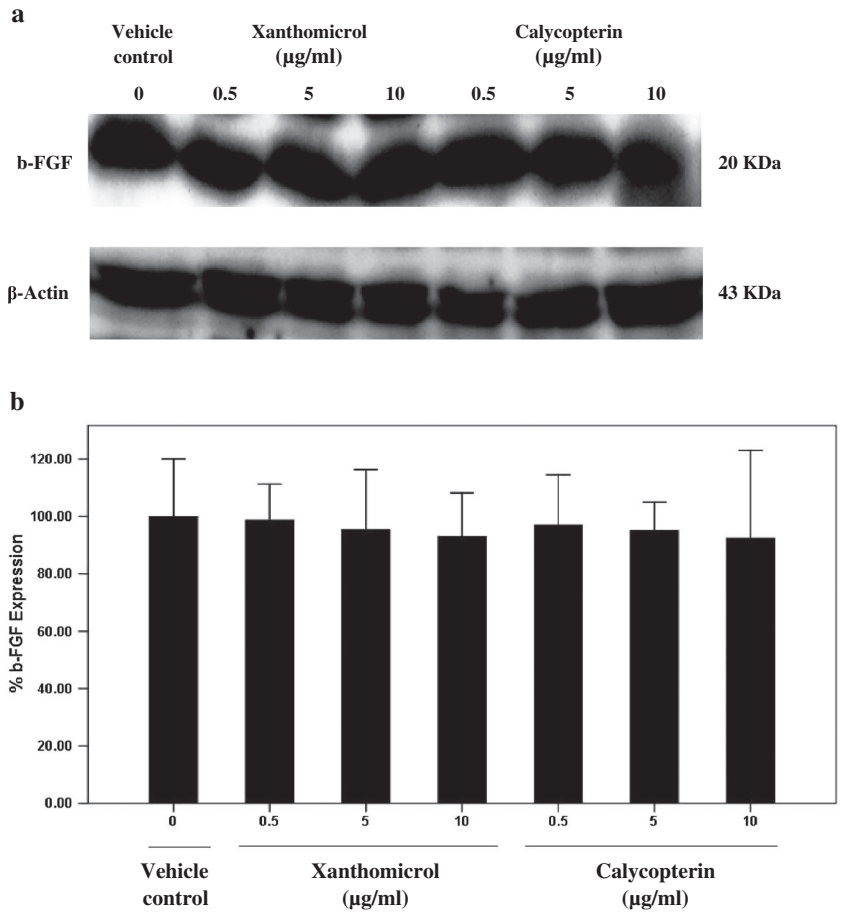

Figure 6. The effect of xanthomicrol and calycopterin on b-FGF expression in HUVECs. (a) Western blot of HUVEC lysates treated with vehicle control (0.1% DMSO), xanthomicrol (0.5, 5, and 10 μg/mL), or calycopterin (0.5, 5, and 10 μg/mL), for 72 h and stained for b-FGF using rabbit polyclonal anti-b-FGF antibody followed by chemiluminescent visualization. Bar graphs of the percentage of b-FGF expression relative to the vehicle control (0.1% DMSO) versus treatment of endothelial cells with xanthomicrol or calycopterin at 0.5, 5, and 10 μg/mL. Bars represent mean ± SE, $n = 3$.

and calycopterin occurred at much lower concentrations than needed to inhibit proliferation cancer cell lines as reported by Moghaddam *et al.* (2012). This is a similar situation to that seen with genistein (Cao *et al.*, 2002).

In order to see if this inhibitory effect on HUVEC proliferation could induce angiogenesis inhibition in a more pathologically or physiologically relevant situation, we studied the effects of these two flavonoids using the rat aortic ring assay. For various reasons, rat aortic ring assay is a good model of angiogenesis: the endothelial cells within the aortic explants are not in a proliferative condition compared with when cell culture models are used; thus, they are in a physiological state very similar to a blood vessel in the body. In addition, there are multiple cell types in aorta section. Therefore, the aortic ring assay is an *ex vivo* model that simulates the real-life environment in which angiogenesis occurs and involves all of the essential steps seen in angiogenesis, including matrix degradation, migration, proliferation, and reorganization (Staton *et al.*, 2004; Auerbach *et al.*, 2003; Staton *et al.*, 2009). Xanthomicrol and calycopterin inhibited microvessel outgrowth in the rat aortas in a concentration-dependent manner. Their effects on new vessel formation were seen in a concentration range over which they inhibited endothelial cell proliferation. These flavones were more potent than suramin and thalidomide in inhibiting microvessel sprouting in rat aortas, which have been approved for clinical use as inhibitors of angiogenesis for cancer treatment.

Many factors modulate new blood vessel sprouting from rat aortic rings including presence of immune cells, for example, phagocytes, smooth muscle cells, and also cells from connective tissue (Zorzi *et al.*, 2010). However, as endothelial cells have a central role in the angiogenic process, the effects of xanthomicrol and calycopterin on migration and proliferation of these cells were studied next. Endothelial cell tube formation assay was used to study the effects of xanthomicrol and calycopterin on cell migration. These flavones were able to inhibit cell migration in a concentration-dependent manner, although these effects appeared to reach a maximum at 10 μg/mL. The effects of both these flavonoids on tube formation occurred over a similar concentration range as their inhibitory effects on HUVEC proliferation. Overall, the data presented so far support the idea that these flavonoids can exert an antitumor effect over a concentration range that is lower than that needed for cytotoxic effects on most cancer cell lines tested.

At the molecular level, recent investigations have clarified how many compounds with antiangiogenic properties inhibit this physiological/pathological process. There are many angiogenic factors and enzymes involved in angiogenesis. VEGF is the most specific angiogenic factor with a potent effect on the proliferation, migration, survival, and differentiation of endothelial cells. b-FGF is one of the first angiogenic factors identified with an influence on proliferation, migration, and differentiation of endothelial cells (Liekens *et al.*, 2001). We found that xanthomicrol and calycopterin inhibited VEGF expression in endothelial cells over the same concentration range that had antiangiogenic effects in the rat aortic ring and endothelial cell tube formation assays. As VEGF is essential for endothelial cell proliferation and tube formation, this 'selective' effect of xanthomicrol and calycopterin on VEGF expression may suggest a mechanism for antiproliferative and capillary tube formation inhibitory activity of these flavones. The influences of these flavones on b-FGF expression were negligible over the same concentration range used for VEGF studies. This suggests that these flavones change VEGF expression and thus affect angiogenesis independently of their effects on b-FGF expression.

It appears that a few polyphenolic compounds selectively inhibit endothelial cell proliferation at low concentration (Cao *et al.*, 2002). This study also provides a mechanistic insight into how polymethoxylated flavones may inhibit angiogenesis.

Further studies are warranted to fully investigate the mechanisms of antiangiogenic effect of xanthomicrol and calycopterin. Such studies will indicate whether the use of xanthomicrol and calycopterin is an effective approach to control tumor progression and metastasis. Especially important are studies on the *in vivo* antiangiogenic activity of xanthomicrol and calycopterin on tumor models.

The authors declare that all the experiments were carried out in compliance with the regulations set out by Iran University of Medical Sciences' Ethical Committee on the use of cell lines and also the humane use of laboratory animals.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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