Anti Angiogenic Effects of Isorhamnetin Isolated from *Persicaria thunbergii*

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ABSTRACT

***Persicaria thunbergii* has been utilized for the treatment of cancer as a folk medicine. We examined the effect of isorhamnetin, a flavonoid isolated from *Persicaria thunbergii*, on angiogenesis *in vitro* and *in vivo*. Basic fibroblast growth factor (bFGF) is a potent angiogenic factor found in various tumors. In this study, we found that the isorhamnetin decreased bFGF-induced human umbilical vein endothelial cells (HUVECs) proliferation and migration in a concentration-dependent manner (5, 10 and 20 μM) whereas, it did not inhibit bFGF-induced capillary-like formation of HUVECs. The chicken chorioallantoic membrane assay revealed that addition of isorhamnetin (10, 20 and 40 μM) displayed an antiangiogenic effect *in vivo*. These results suggest that the isorhamnetin inhibits the proliferation and migration of endothelial cells induced by bFGF, which may explain its anti-angiogenic properties.**

Key words : isorhamnetin, angiogenesis, *Persicaria thunbergii*

INTRODUCTION

Angiogenesis is a process by which new blood vessels are formed from pre-existing vessels. The induction process of angiogenesis is characterized by the degradation of the vascular basement membrane, endothelial cell migration, proliferation and tube formation. Angiogenesis is associated with diseases such as solid tumors, diabetes, rheumatoid arthritis, and atherosclerosis. In particular, the growth of solid tumors and metastasis are found to depend on tumor angiogenesis (Hanaha and Folkman, 1996).

The inhibition of angiogenesis *in vivo* can attenuate tumor growth and metastasis (Kim *et al.*, 1993). Thus, it is expected that angiogenesis inhibitors may be a useful drugs for the treatment of diseases related to

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angiogenesis, including tumors. Recently, phytochemicals derived from plants were reported to inhibit, reverse or retard tumorigenesis by inhibition of angiogenesis. (Surh., 2003; Lee *et al.*, 2004; Huh *et al.*, 2005). Isorhamnetin is a phytochemical isolated from the *Persicaria thunbergii* that has been traditionally utilized for cancer treatment (Kim *et al.*, 2005).Various pharmacological activities of isorhamnetin have been demonstrated including antioxidation by scavenging free radicals, prevention of atherosclerosis, and chronic inflammation (Zhang *et al.*, 2004). Isorhamnetin has various characteristics that make it a potential anticancer compound. These functions include cell cycle regulation and induction of tumor cell apoptosis (Yang *et al.*, 2004; Hibasami *et al.*, 2005). However, there is no data on the anti-angiogenic activity of isorhamnetin. Thus, in the present study, we evaluated the effects of isorhamnetin on anti-angiogenic activity in HUVECs.

MATERIALS AND METHODS

Plant materials

The aerial parts of *Persicaria thunbergii* were collected in October 2002 at Wanju, Chonbuk, Korea. A voucher specimen is deposited at the herbarium of college of pharmacy, Woosuk University, Korea (WSU-02-006).

Extraction and isolation

The air-dried plant materials (1 kg) was extracted twice with MeOH under 50℃ The resultant MeOH extract (190 g) was suspended in water, and then fractionated successively with equal volumes of ethyl acetate and *n*-BuOH, leaving residual water soluble fraction. Each fraction was evaporated *in vacuo* to yield the residues of ethyl acetate soluble fraction (20 g) and *n*-BuOH soluble fraction (35 g). Ethyl acetate soluble fraction was chromatographed on the Sephadex LH-20 column (MeOH) to give three fractions (E1-E3). Silica gel column chromatography of the fraction E2 with EtOAc-MeOH (30:1) afforded compound 1 (60 mg, yield 0.06%). yellow powder (MeOH); mp. 303-304℃; UV λmax nm: (MeOH) 255, 268sh, 306sh, 327sh, 370; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 12.43 (1H, s, 5-OH), 7.73 (1H, d, J=1.9Hz, H-2'), 7.66 (1H, dd, J=8.0, 1.9Hz, H-6'), 6.91 (1H, d, J=8.0Hz, H-5'), 6.46 (1H, d, J=1.8Hz, H-8), 6.18 (1H, d, J=1.8Hz, H-6), 3.82 (3H, s, OCH_3); ^{13}C-NMR(100MHz, DMSO-d_6) δ: 146.8 (C-2), 136.0 (C-3), 176.0 (C-4), 160.8 (C-5), 98.4 (C-6), 164.1 (C-7), 93.8 (C-8), 156.4 (C-9), 103.2 (C-10), 122.1 (C-1'), 111.8 (C-2'), 148.9 (C-3'), 147.6 (C-4'), 115.7 (C-5'), 121.9 (C-6'), 56.0 (OCH_3).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord veins by collagenase treatment as described previously. The cells were cultured in M199 (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS), 3 ng/ml bFGF (R&D Systems, Minneapolis, MN), 5 units/ml heparin and 100 units/ml antibiotic-antimycotic in 0.1% gelatin coated flasks. Cells were grown at 37℃ in a humidified atmosphere containing 5% CO_2.

Proliferation assay

Proliferation assay was examined using a cell proliferation assay by XTT as descried previously (Jost *et al.*, 1992). HUVECs (5×10^3) cells seed onto 0.1% gelatin coated 96-well and incubated in a humidified incubator for 24 h. Cells were starved for 6 h in M199 containing 5% heat-inactivated FBS and then treated with various concentration of Isorhamnetin in M199 containing 5% heated-inactivated FBS, 5 ng/ml bFGF and 5 unit/ml heparin. After 48 h incubation, XTT working solution was added and then the optical density was measured using microplate reader (Molecular

Devices Co.) at 450 nm. Cell proliferation was calculated as a percentage compared with bFGF only treated-HUVECs.

Migration assay

Confluent HUVECs (3×10^5) monolayer was seeded onto 0.1% gelatin coated 6-well plates and "scratch" wounded using the tip of a universal 200 μl pipette tip. Then cells were treated with various concentration of isorhamnetin in M199 with 5% FBS, 5 ng/ml bFGF and 5 units/ml heparin. After 16 h incubation, rinsed with PBS and cells were stained Diff Quick solution and randomly chosen fields were photographed under a light microscope at $\times$100 magnification. The number of migration was counted.

Tube formation assay

Tube formation assay was performed on matrigel as described previously (*Grant et al.*, 1992). 24-well plates were coated with growth factor reduced matrigel by incubating at 37℃ for 1 h. HUVECs (3×10^4) were seeded onto matrigel coated 24-well plates and isorhamnetin were treated in M199 with 5% FBS, 5 ng/ml bFGF and 5 units/ml heparin. After 5 h incubation, cells were stained Diff Quick solution and randomly chosen fields were photographed under a light microscope at $\times$100 magnification. The number of tube formations was counted.

CAM assay

The *in vivo* angiogenic activity was assayed using CAM assay as described previously (*Gho and.,chae* 1997). Isorhamnetin (10, 20 and 40 μM / egg) and bFGF (100 ng) were loaded onto 1/4 piece of thermonox disk (Nunc, Naperville, IL). The dried thermonox disk was applied to the CAM of a 10-day-

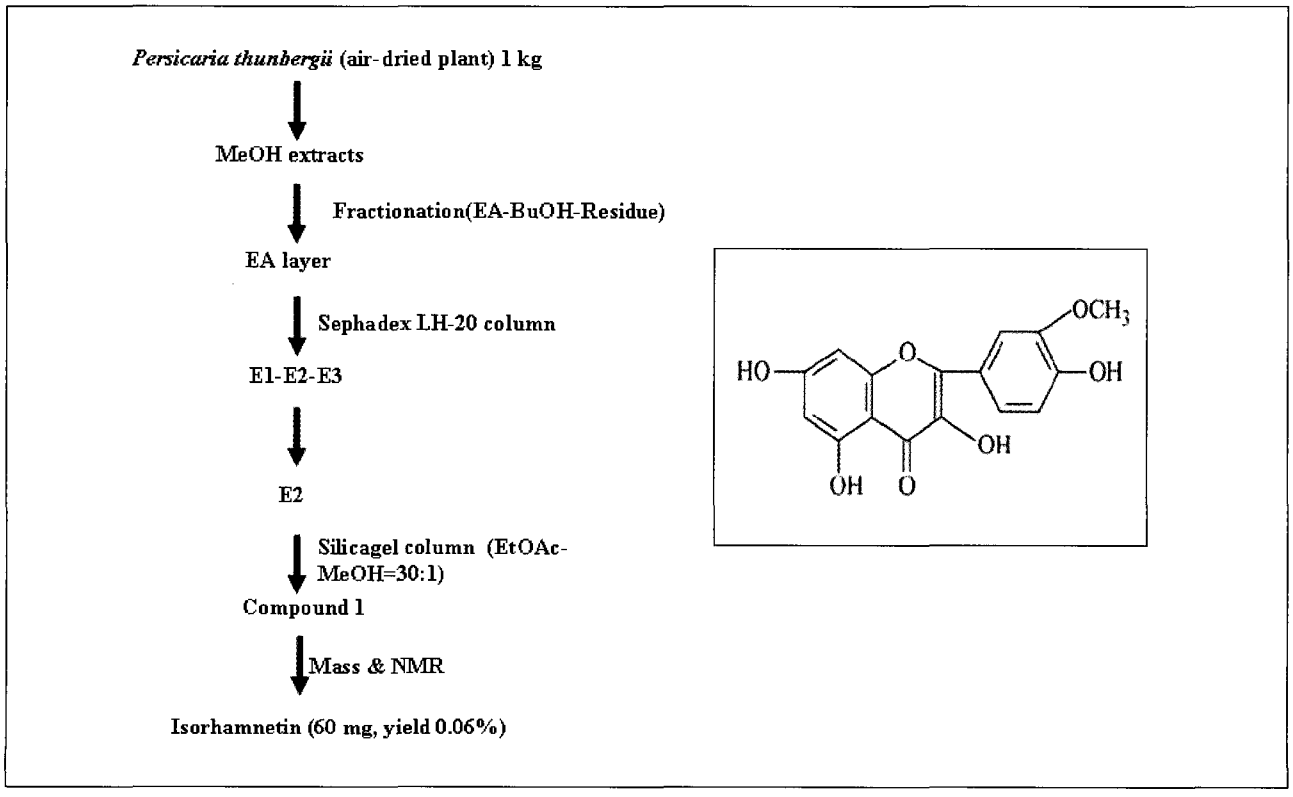


Fig. 1. Isolation and structure of isorhamnetin.

old embryo. After 72 h incubation, a fat emulsion was injected under the CAM for better visualization of the blood vessels. The number of newly formed blood vessels was counted. The experiment was repeated twice with 15 eggs for each group.

RESULTS AND DISCUSSION

Angiogenesis plays an important role in tumor growth, intrasavation, metastatic spread (Folkman and Klagsburn, 1987; Amore and Thompson, 1987). Inhibition of angiogenesis provides a good chance of preventing cancer from becoming malignant (Ingber *et al.*, 1990; Reilly *et al.*, 1994). Angiogenesis is composed of several process dissociations of pericytes from preexisting vessel, digestion of extracellular matrix with proteases growth, migration of endothelial cells, tube formation, then finally remodeling occurs. Among these processes, growth, migration and tube formation of endothelial cells are essential for angiogenesis. In the present study, we focused on the antiangiogenic and antitumor activities of isorhamnetin that was known to have anti-inflammatory (Zhang *et al.*, 2004), based on the reports that inflammantion is closely associated with angiogenesis (Huang *et al.*, 2001; Szekanecz and Koch., 2005).

Isorhamnetin inhibits of bFGF-induced proliferation

To elucidate anti-angiogenic activity of isorhamnetin, we investigated bFGF-induced proliferation and tube formation of HUVECs *in vitro*. bFGF treated HUVECs was exposed to various concentrations of isorhamnetin for 48 h. Isorhamnetin significantly inhibited bFGF-induced proliferation of HUVECs by 45%, 40% and 30% of untreated control at 10 μM, 20 μM and 40 μM, respectively (Fig. 2). To determine the growth inhibitory effect of isorhamnetin was not due to its mere cytotoxicity, the viability of

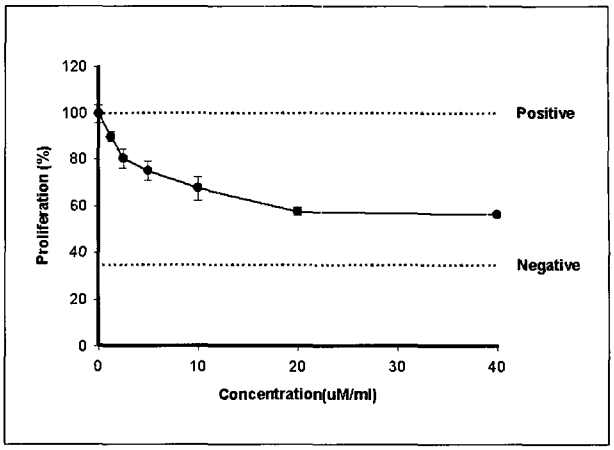


Fig. 2. Inhibitory effect of isorhamnetin on the proliferation of HUVECs.

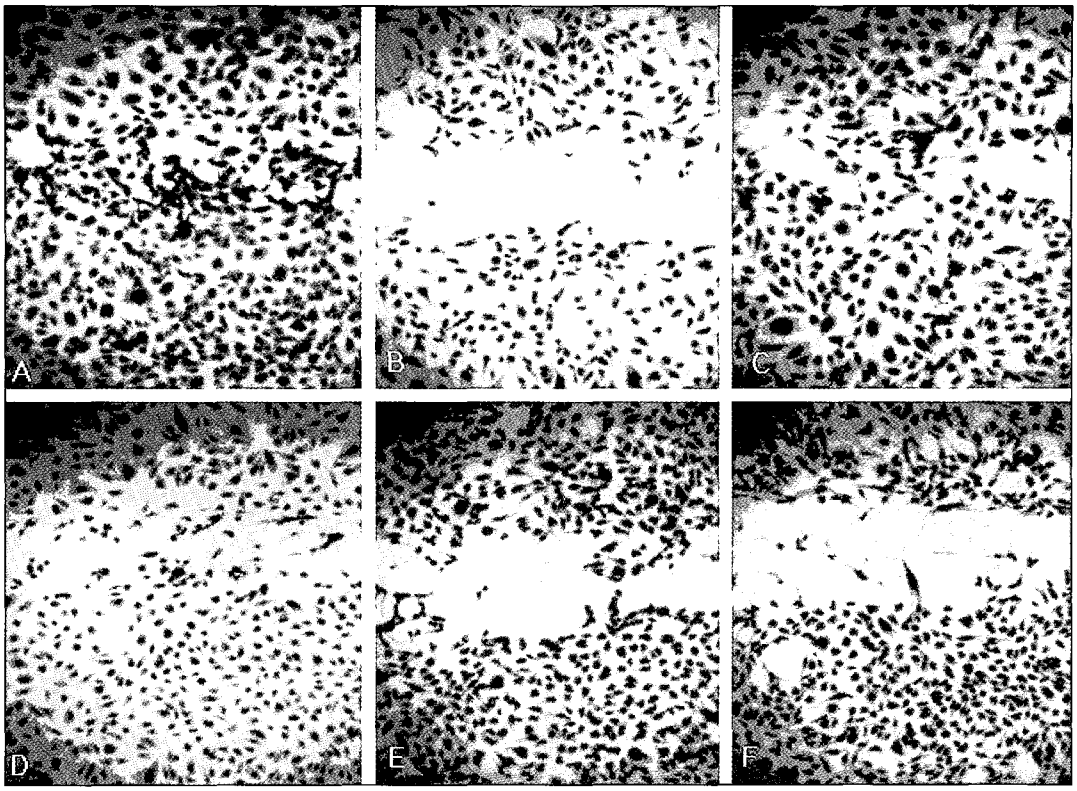
endothelial cells was determined by XTT assay. It is noteworthy that isorhamnetin may exhibit the antiangiogenic activity by specific inhibition of endothelial cell growth without cytotoxicity. In view of these findings, it was of interest to examine the effects of isorhamnetin on angiogenesis in endothelial cells.

Isorhamnetin inhibits of bFGF-induced migration

Endothelial cell migration is critical events for angiogenesis. To examine whether isorhamnetin has *in vitro* antiangiogenic activity, bFGF-induced migration assay was carried out. In the presence of isorhamnetin at concentrations above 2.5 μM, the migratory process was significantly inhibited in a concentration dependent manner. Treatment with 2.5, 5, 10 and 20 μM isorhamnetin resulted in 40%, 50%, 60% and 80%, respectively, inhibition of the endothelial cell migration compared with the control group (Fig. 3).

Isorhamnetin inhibits of bFGF-induced tube formation

Differentiation assay, tube formation assay, was performed in HUVECs. The presence of isorhamnetin at concentration up to 40 μM did not show any inhibitory effect on bFGF-induced capillary-like tube



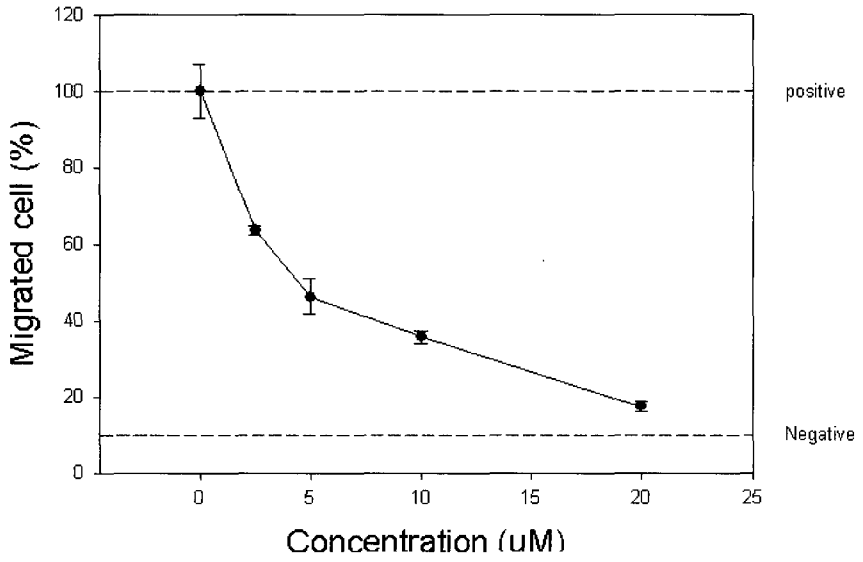
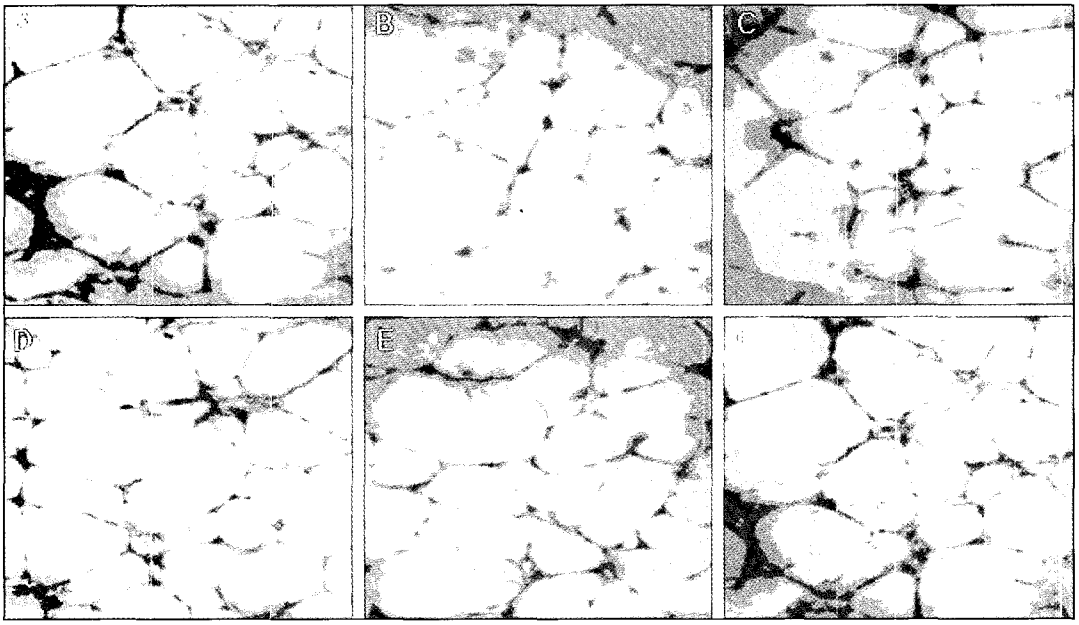


Fig. 3. Inhibitory effect of isorhamnetin on the migration of HUVECs.
A; Positive control, B; Negative control, C; isorhamnetin 2.5 μM, D; isorhamnetin 5 μM, E; isorhamnetin 10 μM. F; isorhamnetin 20 μM.

formation on HUVECs (Fig. 4).

Isorhamnetin inhibits of bFGF-induced angiogenesis in vivo

The ability of Isorhamnetin *in vivo* bFGF-induced angiogenesis was examined using chick choriollantoic membrane assay. Isorhamnetin inhibited the bFGF-induced angiogenesis without any visible effect on the pre-existing blood vessels. The presence of 10, 20 and 40 μM/egg caused 30 %, 50 % and 60% reduction in the



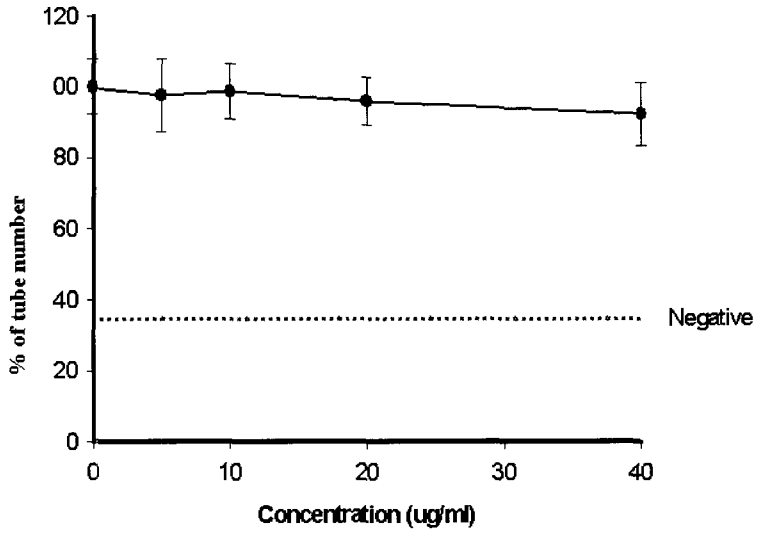


Fig. 4. Effect of isorhamnetin on bFGF-induced capillary tube formation of HUVECs.
A; Positive control, B; Negative control, C; isorhamnetin 5 μM, D; isorhamnetin 10 μM, E; isorhamnetin 20 μM F; isorhamnetin 40 μM.

infiltration of blood vessels, respectively (Fig. 5).

We suggest the inhibitory effects of isorhamnetin on angiogenesis in vitro and the necessity of further studies on the angiogenesis *in vivo*.

ACKNOWLEDGEMENTS

This study was supported by grants of Biogreen 21and Agricultural Research &Development Promotion Center project

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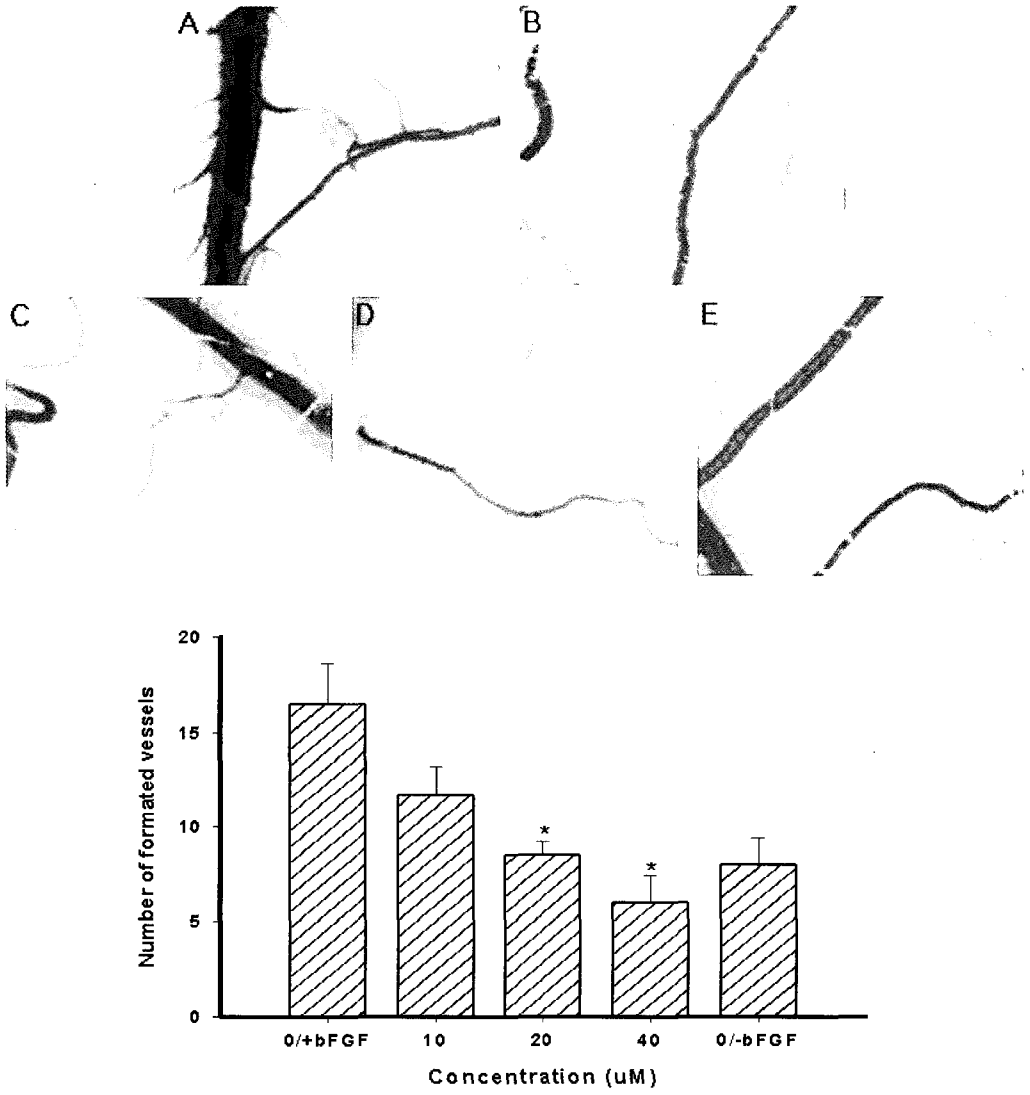

Fig. 5. Isorhamnetin inhibits bFGF-induced angiogenesis *in vivo*.*; Statistically significant value compared with untreated control (* $p<0.05$).
A; Positive control, B; Negative control, C; isorhamnetin 10 μM, D; isorhamnetin 20 μM, E; isorhamnetin 40 μM.

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(Received May. 14, 2005)
(Accepted Aug. 20, 2005)