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Identification of Campesterol from *Chrysanthemum coronarium* L. and its Antiangiogenic Activities

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Campesterol, a plant sterol in nature, is known to have cholesterol lowering and anticarcinogenic effects. Since angiogenesis is essential for cancer, it was surmised that an antiangiogenic effect may be involved in the anticancer action of this compound. This study investigated the effect of campesterol on basic fibroblast growth factor (bFGF)-induced angiogenesis *in vitro* in human umbilical vein endothelial cells (HUVECs) and an *in vivo* chorioallantoic membrane (CAM) model. Campesterol isolated from an ethylacetate fraction of *Chrysanthemum coronarium* L. showed a weak cytotoxicity in non-proliferating HUVECs. Within the non-cytotoxic concentration range, campesterol significantly inhibited the bFGF-induced proliferation and tube formation of HUVECs in a concentration-dependent manner, while it did not affect the motility of HUVECs. Furthermore, campesterol effectively disrupted the bFGF-induced neovascularization in chick chorioallantoic membrane (CAM) *in vivo*. Taken together, these results support a potential antiangiogenic action of campesterol via an inhibition of endothelial cell proliferation and capillary differentiation. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: campesterol; *Chrysanthemum coronarium* L.; bFGF; HUVECs; CAM; angiogenesis.

INTRODUCTION

Campesterol is one of the most common plant sterols in nature along with β-sitosterol and stigmasterol (Rao and Janezic, 1992; Ikeda *et al*., 2006). Abundant in seeds, nuts, cereals, beans, legumes and vegetable oils (Ostlund *et al*., 2002; Phillips *et al*., 2005), campesterol is similar in structure to cholesterol and similarly metabolized by intestinal bacteria (Ikeda *et al*., 2006). Due to structural similarity to cholesterol, plant sterols including campesterol have cholesterol-lowering effects (Ling and Jones, 1995; Jones *et al*., 1997; Plat and Mensink, 2001; Tikkanen, 2005). Plant sterols also have many other biological functions, such as anticarcinogenic (Li *et al*., 2001), antiinflammatory, antibacterial and antifungal activities (Padmaja *et al*., 1993). There is accumulating evidence that campesterol exhibits chemopreventive effects against many cancers, including prostate (McCann *et al*., 2005), lung (Schabath *et al*., 2005) and breast (Awad *et al*., 2000) cancers.

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An anticarcinogenic activity of campesterol has been reported, although the mechanism by which campesterol affects vascular endothelial cells has not been elucidated. Thus, the effect of campesterol isolated from *Chrysanthemum coronarium* L. on basic fibroblast growth factor (bFGF) stimulated angiogenesis in HUVECs and chick chorioallantoic membrane (CAM) was studied, since bFGF is a typical angiogenesis promoting factor (Huh *et al*., 2005; Lee *et al*., 2006) employed in angiogenesis assays.

MATERIALS AND METHODS

Materials. *Chrysanthemum coronarium* L. was bought from Garak Vegetable Market, Seoul. Recombinant human basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN). M199, fetal bovine serum (FBS) and antibiotic-antimycotic were bought from Gibco (Grand Island, NY). 2,3-Bis [2-methoxy-4-nitro-5-sulfo]-2H-tetrazolium-5-carboxanilide (XTT), heparin and gelatin were purchased from Sigma Chemical (St Louis, MO). The thermanox coverslips were from Nunc (Napervile, IL) and the growth factor reduced-Matrigel was from Becton Dickinson (San Jose, CA). 48-well microchemotaxis chambers and polyester membrane (12 μm pores) were bought from Neuro Probe, Inc. (Cabin John, MD).

Isolation of campesterol. The fresh aerial parts of *Chrysanthemum coronarium* L. (80 kg) were extracted with

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80% aqueous methanol (40 L × 3), and the liquid was concentrated *in vacuo*. The extracts were partitioned with H_2O (2 L), EtOAc (2 L × 3) and *n*-BuOH (2 L × 3). The concentrated EtOAc fraction (CCE, 168 g) was subjected to silica gel (SiO_2) column chromatography (CC) (1500 **g**, Φ 8 × 20 cm) and eluted with a gradient of *n*-hexane–EtOAc (7:1→6:1→5:1→4:1→3:1→2:1→1:1, v/v, 1 L of each), resulting in 12 fractions (CCE1–CCE12). Fraction CCE6 [12.4 g, Ve/Vt (elution volume/total volume) 0.40–0.52] was subjected to SiO_2 CC(100 g, Φ 3 × 6 cm) and eluted with *n*-hexane–EtOAc (3:1, v/v, 2.0 L), resulting in 11 fractions (CCE6-1–CCE3-11). Fraction CCE6-4 (6.2 g, Ve/Vt 0.33–0.40) was subjected to silica gel CC (100 g, Φ 3 × 7 cm) and eluted with *n*-hexane–EtOAc (4:1, v/v, 2 L) yielding compound **1** [1.2 g, Ve/Vt 0.23–0.31; TLC (Keiselgel 60 F_{254}) R_f 0.45, *n*-hexane–EtOAc = 4:1]. The chemical structure of compound 1 was identified by IR, ^{1}H-NMR, ^{13}C-NMR and MS.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord veins according to a published protocol (Jaffe *et al*., 1973) and cultured in M199 supplemented with 20% heat-inactivated FBS, 3 ng/mL bFGF, 5 units/mL heparin and 100 units/mL antibiotic-antimycotic in 0.1% gelatin coated flasks. HUVECs were used in passage three to six. All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability assay under non-proliferating condition. The effect of campesterol on the viable cell number was assessed by XTT assay (Jost *et al*., 1992). Briefly, HUVECs (1×10^4) were seeded onto 0.1% gelatin coated 96-well plates and incubated in a humidified incubator for 24 h. Cells were washed with PBS (pH 7.4) and various concentrations (10, 20, 30, 40 and 50 μg/mL) campesterol were added to the cells in the absence of serum. After incubation at 37 °C in a humidified incubator for 24 h, a XTT working solution was added to each well. The cells were incubated at 37 °C for 2 h and the optical density was measured using a microplate reader (Molecular Devices Co.) at 450 nm.

bFGF stimulated proliferation assay. HUVECs (5×10^3) were seeded onto 0.1% gelatin coated 96-well plates and incubated in a humidified incubator for 24 h. The cells were starved for 6 h in M199 containing 5% heat-inactivated FBS and then treated with various concentrations (10, 20, 30, 40 and 50 μg/mL) of campesterol in M199 containing 5% heat-inactivated FBS, 10 ng/mL bFGF and 5 units/mL heparin. After 48 h incubation, a XTT working solution was added to each well. The cells were incubated at 37 °C for 2 h and the optical density was measured using a microplate reader (Molecular Devices Co.) at 450 nm.

bFGF stimulated migration assay. Migration assay was performed using modified 48-well microchemotaxis chambers (Nuero Probe, Inc., Cabin John, MD). Briefly, polyester membrane (12 μm pores) (Nuero Probe, Inc., Cabin John, MD) was coated with 0.1% gelatin for 30 min and dried. The lower chamber was filled with 30 μL of M199 containing 0.1% bovine albumin serum (BAS) (control medium) in the presence of bFGF (10 ng/mL). The coated membrane and upper chamber were laid over the lower chamber. HUVECs (5×10^4 cells/well) treated with various concentrations (1, 5, 10 and 20 μg/mL) of campesterol were loaded onto the upper chamber wells. After incubation for 4 h at 37 °C in a 5% CO_2 incubator, the membranes were fixed with Diff-Quick fixative and stained with Diff-Quick Sol. I and II (DADE Behring Inc., Newark, DE). Non-migrated cells on the upper surface of the membrane filter were wiped off with a swab and the migrated cells to the lower surface were randomly photographed in four fields under an Axiovert S 100 light microscope (Carl Zeiss, Inc., USA) at ×100 magnification and counted.

bFGF stimulated tube formation assay. The *in vitro* differentiation assay of HUVECs on Matrigel into capillary-like tubes was performed as described by Grant *et al.* (1989). HUVECs (2×10^5) were seeded onto Matrigel-coated 24-well plates and campesterol (1, 5, 10 and 20 μg/mL) was added in M199 with 1% FBS, 10 ng/mL bFGF and 5 units/mL heparin. After 24 h, randomly chosen fields were photographed under an Axiovert S 100 light microscope (Carl Zeiss, Inc., USA) at ×40 magnification and the formed tubes area was quantified by a NIH Scion image program.

bFGF stimulated CAM assay. *In vivo* angiogenic activity was assayed using CAMs as described previously (Marks *et al*., 2002). Campesterol (10 and 20 μg/egg) with bFGF (100 ng) was loaded onto 1/4 piece of thermonox disks (Nunc, Naperville, IL). The dried thermonox disk was applied to the CAMs of a 10-day-old embryo. After 48 h incubation, a fat emulsion was injected under the CAMs for better visualization of the blood vessels and the number of newly formed blood vessels was counted. The experiment was repeated twice and 15 eggs were used for each group.

Statistical analysis. All data are presented as mean ± SD. The statistically significant differences between control and campesterol groups were calculated by the Student's *t*-test.

RESULTS

Identification of compound 1 isolated from *Chrysanthemum coronarium* L.

Compound **1** isolated from *Chrysanthemum coronarium* L. was a white amorphous powder. $[\alpha]^{23}_{D}$ −33° (c 1.0, $CHCl_3$); IR (CaF_2 window in $CHCl_3$) ν_{max} 3400, 1640, 1050, 845, 830, 802 cm^{-1}; mp 157–158 °C; ^{1}H-NMR (400 MHz, pyridine-d_5) δ5.34 (1H, brd, J = 5.2 Hz, H-6), 3.51 (1H, brdd, J = 7.3, 7.3 Hz, H-3), 2.28 (1H, d, J = 7.3 Hz, H-4), 1.01 (3H, s, H-19), 0.92 (1H, d, J = 6.6 Hz, H-21), 0.84 (3H, d, J = 7.2 Hz, H-27), 0.81 (3H, d, J = 7.2 Hz, H-26), 0.80 (3H, d, J = 7.0 Hz, H-28), 0.68 (3H, s, H-18); ^{13}C-NMR (100 MHz, pyridine-d_5) δ140.7 (C-5), 121.7 (C-6), 71.8 (C-3), 56.9 (C-14), 56.1 (C-17), 50.1 (C-9), 45.8 (C-24), 42.3 (C-4), 42.3 (C-13), 40.0 (C-12), 37.3 (C-1), 36.5 (C-10), 36.1 (C-25), 33.9 (C-22), 31.9 (C-7), 31.6 (C-20), 29.2 (C-8), 28.2 (C-2), 26.1 (C-16), 24.4 (C-28), 23.1 (C-15), 21.1 (C-23), 20.0 (C-18), 19.8 (C-11), 19.4 (C-21), 19.0 (C-27), 18.8 (C-26), 11.8 (C-19); EIMS *m/z* [rel int. (%)]: 400 $[M]^+$ (40), 385 (78), 382 (13), 367 (97), 315 (32), 289 (13), 213 (100). Based on the above data,

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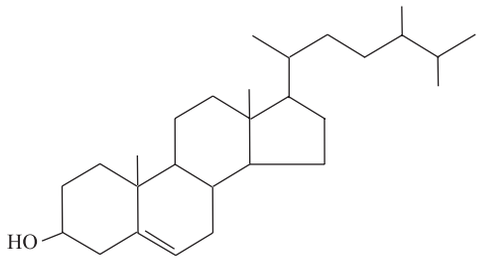


Figure 1. The chemical structure of campesterol.

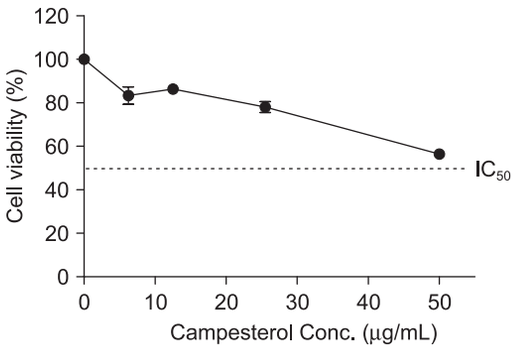


Figure 2. Effect of campesterol on cell viability of HUVECs. HUVECs were seeded onto 0.1% gelatin coated 96-well plate and treated with campesterol in serum-free medium with or without bFGF. The effect of campesterol on viable cell numbers was measured by XTT assay.

compound **1** was finally identified as 24(R)-methylcholesta-5-en-3β-ol (campesterol) by the comparison of several physical and spectral data with the literature (Kircher and Rosenstein, 1974) as shown in Fig. 1.

Effect of campesterol on cell viability of non-proliferating HUVECs

The cytotoxic effect of campesterol on non-proliferating HUVECs, XTT assay was first examined with the XTT assay. Under a serum-free condition, 50 μg/mL of campesterol decreased the cell viability up to about 56% of control (IC_{50} of over 50 μg/mL) as shown in Fig. 2. Thus, campesterol caused weak cytotoxicity at concentrations below 20 μg/mL. Thus, all subsequent experiments were performed at nontoxic concentrations of equal or below 20 μg/mL.

Effect of campesterol on bFGF-stimulated proliferation of HUVECs

The effect of campesterol on endothelial cell proliferation was investigated using HUVECs stimulated by 10 ng/mL bFGF for 48 h. As shown in Fig. 3, campesterol inhibited the proliferation of HUVECs in a concentration-dependent manner at a non-cytotoxic range of 20 μg/mL and less.

Effect of campesterol on bFGF-stimulated migration of HUVECs

Chemotactic migration was performed using a Boyden chamber containing a polycarbonate membrane coated

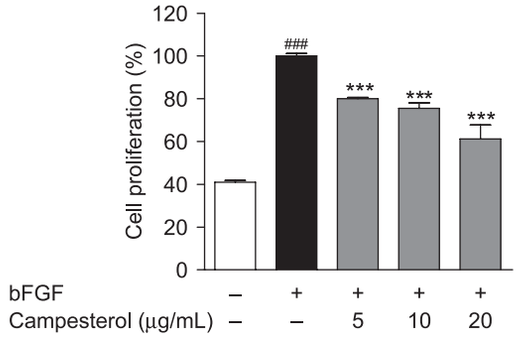
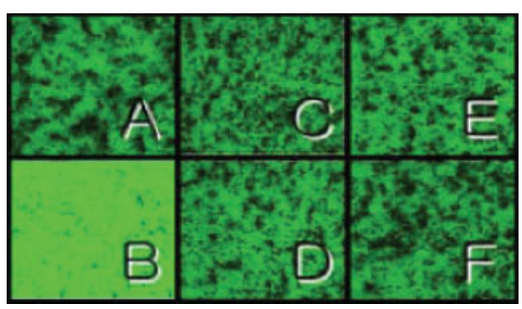


Figure 3. Effect of campesterol on bFGF-induced proliferation of HUVECs. HUVECs were plated onto 96-well plates and treated with campesterol with or without bFGF for 48 h. Cell proliferation was determined by XTT assay. All data were presented as mean ± SD of three experiments. The statistically significant differences between control and sample groups were calculated by the Student's *t*-test. ### $p < 0.001$ versus untreated control; *** $p < 0.001$ versus bFGF control.

with 0.1% gelatin. As shown in Fig. 4, few cells were found in the untreated control in the absence of bFGF in the lower chamber, whereas many migrated cells were detected in the positive control. However, campesterol did not affect the migration of bFGF-stimulated HUVECs.



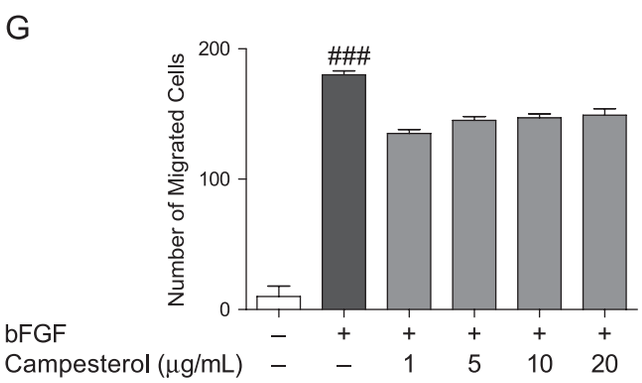


Figure 4. Effect of campesterol on bFGF-induced migration of HUVECs. The 12 μm pore trans-membrane was coated with 0.1% gelatin. HUVECs (5×10^4 cells/well) treated with various concentrations (1, 5, 10 and 20 μg/mL) of campesterol were loaded onto the upper chamber wells. After incubation for 4 h at 37 °C in 5% CO_2 incubator, the membranes were fixed with Diff-Quick fixative and stained with Diff-Quick Sol. I and II (DADE Behring Inc., Newark, DE). Non-migrated cells on the upper surface of the membrane filter were wiped off with a swab and the migrated cells to the lower surface were randomly photographed under an Axiovert S 100 light microscope. (A) positive control (0.1% BSA in M199 and bFGF), (B) negative control (only M199 with 0.1% BSA), (C) campesterol (1 μg/mL), (D) campesterol (5 μg/mL), (E) campesterol (10 μg/mL), (F) campesterol (20 μg/mL). (G) The migrated cells were quantified. The significant differences between control and campesterol groups were calculated by the Student's *t*-test. ### $p < 0.001$ versus untreated control.

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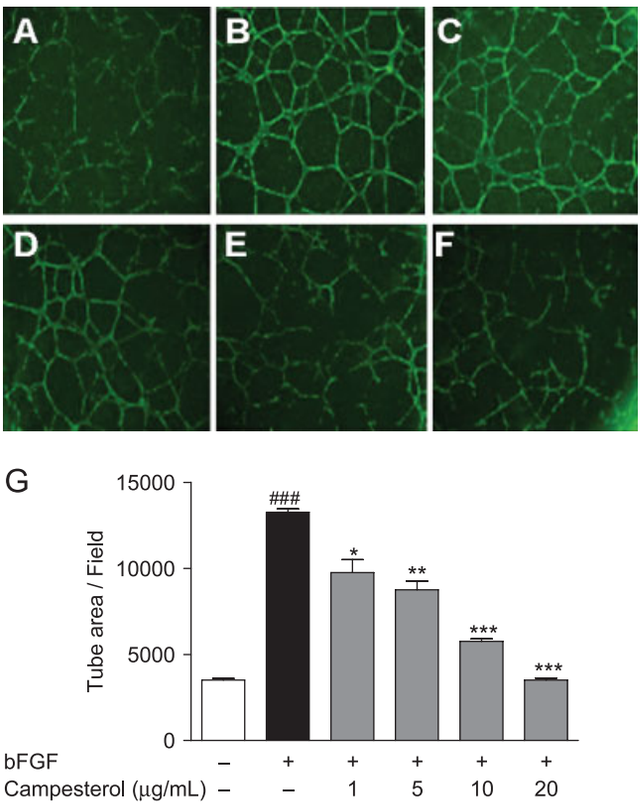

Figure 5. Effect of campesterol on bFGF-induced capillary-like tube formation of HUVECs. HUVECs were plated on Matrigel and treated with campesterol. After incubation for 24 h, the cells were observed under microscope (×100 magnification) and photographed. (A) negative control (only M199 without bFGF), (B) positive control (M199 with bFGF), (C) campesterol (1 μg/mL) with bFGF, (D) campesterol (5 μg/mL) with bFGF, (E) campesterol (10 μg/mL) with bFGF, (F) campesterol (20 μg/mL) with bFGF. (G) Formed tube areas analysed by NIH Scion image program were quantified. All data were presented as mean ± SD. The statistically significant differences between control and campesterol treated group were calculated by the Student's *t*-test. ### $p < 0.001$ versus untreated control; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus bFGF control.

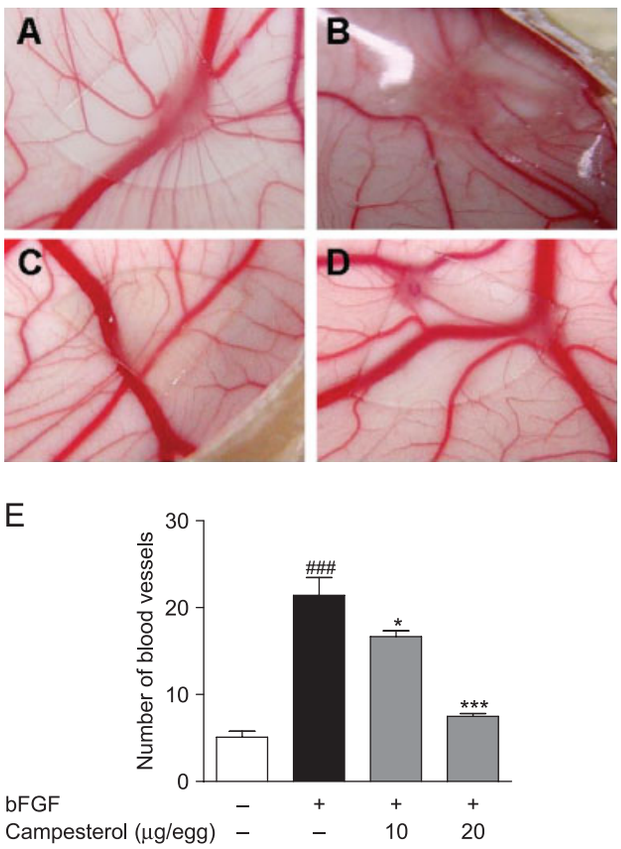

Figure 6. Effect of campesterol on bFGF-induced neovascularization in CAM assay. Campesterol was loaded on the CAMs of 10-day-old fertilized eggs. After 48 h incubation, a fat emulsion was injected into the CAMs for better visualization of the blood vessels. (A) positive control (100 ng bFGF alone in/embryo), (B) negative control (distilled water), (C) 10 μg campesterol + 100 ng bFGF/embryo) and (D) 20 μg campesterol + 100 ng bFGF/embryo). (E) Numbers of newly formed blood vessels per field were counted and quantified. All data were presented as mean ± SD, $n = 15$. The statistically significant differences between control and campesterol treated group were calculated by the Student's *t*-test. ### $p < 0.001$ versus untreated control; * $p < 0.05$ and *** $p < 0.001$ versus bFGF control.

Effect of campesterol on bFGF-stimulated capillary-like tube formation of HUVECs

When HUVECs were plated on the Matrigel with bFGF (10 ng/mL), the cells formed a capillary-like network within 24 h. As shown in Fig. 5, campesterol dramatically decreased the total tube formation in each of four randomly chosen fields in a concentration-dependent manner.

Effect of campesterol on bFGF-induced neovascularization in CAM assay

In vivo chick CAM angiogenesis by bFGF (100 ng/embryo) was examined after 48 h treatment with campesterol. As shown in Fig. 6, bFGF induced a pronounced angiogenic response in the control, whereas the formation of new blood vessels under disks on chick embryo CAMs could not be observed in the negative control (distilled water instead of bFGF). In contrast, campesterol significantly inhibited the development of vascularization in the CAMs without any sign of thrombosis and hemorrhage, suggesting that campesterol significantly disrupted bFGF-induced angiogenesis compared with the positive control.

DISCUSSION

There is now considerable evidence from *in vitro* and *in vivo* animal studies to suggest an inhibitory effect of plant sterols on tumorigenesis (Ovesna *et al*., 2004). Dietary plant sterols were shown to inhibit both the growth of some kind of tumor cells and their metastasis *in vivo* and *in vitro* (Awad *et al*., 2000; Janezic and Rao, 1992; Raicht *et al*., 1980). One of the components of plant sterols with anticancer effects is campesterol (Rao and Janezic, 1992; Ikeda *et al*., 2006). However, the effects of campesterol on angiogenesis and mechanisms have not been investigated thoroughly.

Angiogenesis is the formation of new capillary blood vessels from pre-existing vessels. It is a tightly regulated process including normal reproductive function, embryonic development and wound healing (Folkman, 1971; Zetter, 1998). However, aberrant angiogenesis

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is a crucial factor in the pathogenesis of numerous diseases including rheumatoid arthritis, diabetic retinopathy and cancer (Carmeliet and Jain, 2000; Bodolay *et al*., 2002; Lee *et al*., 1998). Angiogenesis involves multi-step processes including endothelial cell proliferation, migration and differentiation, degradation of the extracellular matrix, tube formation and sprouting of new capillary branches (Folkman, 1995). Because each step of these angiogenesis processes usually serves as a potential target for the therapeutic intervention of the angiogenesis, antiangiogenic or proangiogenic effect of any compound is usually evaluated at each individual step of the angiogenic process.

In the present study, compound 1 was isolated from *Chrysanthemum coronarium* L. through activity based fractionation and identified as campesterol. Thereafter, the effects of campesterol on the endothelial cell proliferation, migration and tube-like formation in HUVECs stimulated by bFGF were investigated. Campesterol showed weak cytotoxicity against HUVECs. However, within non-cytotoxic concentrations (less than 20 μg/mL), campesterol inhibited bFGF-induced proliferation of HUVECs in a concentration-dependent manner. These data imply that campesterol can suppress the bFGF induced pathological angiogenesis in HUVECs. Campesterol also dramatically suppressed capillary-like tube formation, whereas the inhibitory effect of campesterol on the migration of HUVECs stimulated by bFGF was not significant. These data suggest campesterol may inhibit the differentiation of HUVECs rather than the motility of HUVECs. Its *in vivo* antiangiogenic activity was affirmed using the CAM assay, which is a widely adopted *in vivo* model for studying angiogenesis. Campesterol significantly disrupted the development of new embryonic blood vessels without affecting the pre-existing vasculature. However, no toxicity was recognized in any of the chick embryos at doses of 10–20 μg/embryo. These findings indicate that campesterol may be a potent angiogenesis inhibitor with no toxicity.

In summary, campesterol inhibited endothelial cell proliferation and differentiation *in vitro* as well as neovascularization in CAM *in vivo* with no toxicity. These results strongly suggest that campesterol could be an antiangiogenic candidate for the prevention and treatment of angiogenesis related diseases.

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