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Nutrition and Cancer

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/hnuc20>

Cat's Whiskers Tea (*Orthosiphon Stamineus*) Extract Inhibits Growth of Colon Tumor in Nude Mice and Angiogenesis in Endothelial Cells via Suppressing VEGFR Phosphorylation

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Version of record first published: 02 Dec 2011

To cite this article: Mohamed B. Khadeer Ahamed, Abdalrahim F. A. Aisha, Zeyad D. Nassar, Jamshed M. Siddiqui, Z. Ismail, S.M.S. Omari, C.R. Parish & A. M. S. Abdul Majid (2012): Cat's Whiskers Tea (*Orthosiphon Stamineus*) Extract Inhibits Growth of Colon Tumor in Nude Mice and Angiogenesis in Endothelial Cells via Suppressing VEGFR Phosphorylation, *Nutrition and Cancer*, 64:1, 89-99

To link to this article: <http://dx.doi.org/10.1080/01635581.2012.630160>

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Cat's Whiskers Tea (*Orthosiphon Stamineus*) Extract Inhibits Growth of Colon Tumor in Nude Mice and Angiogenesis in Endothelial Cells via Suppressing VEGFR Phosphorylation

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Cat's whiskers (*Orthosiphon stamineus*) is commonly used as Java tea to treat kidney stones including a variety of angiogenesis-dependent diseases such as tumorous edema, rheumatism, diabetic blindness, and obesity. In the present study, antitumor potential of standardized 50% ethanol extract of *O. stamineus* leaves (EOS) was evaluated against colorectal tumor in athymic mice and antiangiogenic efficacy of EOS was investigated in human umbilical vein endothelial cells (HUVEC). EOS at 100 mg/kg caused $47.62 \pm 6.4\%$ suppression in tumor growth, while at 200 mg/kg it caused $83.39 \pm 4.1\%$ tumor regression. Tumor histology revealed significant reduction in extent of vascularization. Enzyme-linked immunosorbent assay showed EOS (200 mg/kg) significantly reduced the vascular endothelial growth factor (VEGF) level in vitro (211 ± 0.26 pg/ml cell lysate) as well as in vivo (90.9 ± 2 pg/g tissue homogenate) when compared to the control (378 ± 5 and 135.5 ± 4 pg, respectively). However, EOS was found to be noncytotoxic to colon cancer and endothelial cells. In vitro, EOS significantly inhibited the migration and tube formation of human umbilical vein endothelial cells (HUVECs). EOS suppressed VEGF-induced phosphorylation of VEGF receptor-2 in HUVECs.

High performance liquid chromatography (HPLC) analysis of EOS showed high rosmarinic acid contents, whereas phytochemical analysis revealed high protein and phenolic contents. These results demonstrated that the antitumor activity of EOS may be due to its VEGF-targeted antiangiogenicity.

INTRODUCTION

Colorectal cancer is the second leading cause of death for cancer worldwide, and mortality resulting from this disease, much like many other solid tumors, is not from the primary tumor itself but from metastatic disease that occurs as a result of tumor vascularization, which is a major component of tumor metastasis (1). Highly vascular tumors are more susceptible to develop metastases at a higher rate than less angiogenic tumors (2). A number of angiogenic inhibitors have been developed and successfully been applied in syngenic and heterotransplant tumor models (3,4). Colorectal cancers are highly angiogenic and were the first tumor type to exhibit significant response to angiogenesis inhibitors (5). Many of these inhibitors are directed against the vascular endothelial growth factor (VEGF) or its receptors, which are considered to play a key role in angiogenesis (3).

Orthosiphon stamineus Benth. (Lamiaceae) is a medicinal herb widely distributed in Southeast Asia. Leaves of this plant are commonly used in Southeast Asia and Europe as

Submitted 13 March 2011; accepted in final form 10 August 2011.
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herbal tea, well known as “Java tea.” It is used as a traditional medicine for the treatment of a variety of angiogenesis-related diseases, including rheumatism, tumorous edema, obesity, diabetic blindness, and psoriasis (6,7). Studies have shown that *O. stamineus* leaves extracts possess strong antioxidant, antiinflammatory, and antibacterial properties with more than 20 phenolic compounds, 2 flavonol glycosides, 9 lipophilic flavones, 9 caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid (8,9), and nitric oxide inhibitory isopimarane-diterpenes (10). The reports on remedial uses of the herb to alleviate several angiogenesis-dependent diseases like rheumatism, cancer, and retinopathy (6), and the recent evidences that the plant has a significant antiangiogenic activity in rat aortic explants (11), have drawn attention toward hypothesizing that *O. stamineus* could be useful for treating angiogenesis dependent diseases if its antiangiogenicity is investigated in vitro and scientifically proven using an animal model of angiogenesis-related disease with a standard experimental protocol. Accordingly, the aim of this study is to evaluate the anticancer activity of ethanol extract of *O. stamineus* leaves (EOS) against colon tumor in athymic mice and to investigate and characterize the antiangiogenic efficacy of EOS in human umbilical vein endothelial cells (HUVECs). This work is an attempt to support its traditional use and further add to previous works on this plant in which suppression of prooxidants-induced VEGF expression mechanism is thought to play a role.

MATERIALS AND METHODS

Chemicals, Cell Culture, and Reagents

Betulinic acid, oleanolic acid, and ursolic acid, bovine serum albumin (fraction V), glucose, quercetin, gallic acid, and Folin-Ciocalteu reagent were purchased from Sigma (Germany) and sinensetin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, and rosmarinic acid were purchased from Indofine Chemical Company (Hillsborough, USA). Other chemicals used were analytical grade.

Human VEGF assay kit was purchased from IBL (Japan). Phosphate buffered saline, trypsin, penicillin/streptomycin (PS), MTT reagent, suramin, and vincristine were purchased from Sigma (Germany). Matrigel was obtained from BD Bioscience (USA). HUVEC (ScienCell, USA) were maintained in endothelial cell medium (ECM) (ScienCell, USA) supplemented with endothelial cell growth supplements, 5% heat inactivated fetal bovine serum (HIFBS) and 1% PS. Human colon tumor (HCT 116) and normal colon fibroblast (CCD-18Co) cells were sourced from Rockville (USA), and maintained in RPMI 1640 and DMEM, respectively (Gibco, UK, supplemented with 10% HIFBS and 1% PS). NCI Nu/Nu mice (Jackson Laboratory, USA) were maintained in a sterile condition using individually ventilated cages.

Plant Material and Extraction

Leaves of *O. stamineus* were obtained from a contract farming facility in Penang, Malaysia. The specimen (Voucher No.: 11009) was deposited at the herbarium of School of Biology, Universiti Sains Malaysia. EOS was prepared by maceration process as previously described (12).

Phytochemical Analysis

The protein content was determined by Lowry et al. (13), and total polysaccharide content was measured by the phenol-sulphuric acid method (14) and glycosaponins content was analyzed by Siddiqui et al. (15). Total phenolics were determined using Folin-Ciocalteu reagent with gallic acid as a standard and the result is expressed as mg of gallic acid equivalent. Total flavonoids were determined using $AlCl_3$ colorimetric method with quercetin as standard and are expressed as μg of quercetin equivalent.

High Performance Liquid Chromatography (HPLC) Analysis

One hundred mg of EOS was dissolved in 25 mL mixture of methanol: water (1:1) and sonicated for 15 min. All samples were filtered through a $0.45 \mu m$ filter (Whatman). Similarly all reference compounds (5 mg) were dissolved in 5 mL of methanol and then filtered. Contents of sinensetin, eupatorin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, orthosiphonol A, rosmarinic acid, betulinic acid, oleanolic acid, and ursolic acid were determined by HPLC fingerprint analysis (12,16).

In Vivo Human Colorectal Tumor Xenograft in Athymic Mouse Model Antitumor Activity of EOS

HCT 116 cells (10^6 in $200 \mu l$ media) were injected subcutaneously into the right flank of each mouse (4–6 wk old, 20–22 g). Thirty mice randomly divided into 3 groups of 10 mice each. When the average tumor size reached approximately $100 mm^3$, in about 6 days post-cell inoculation, Group 1 received 0.1 ml distilled water (control), and Groups 2 and 3 received oral treatment with 100 and 200 mg/kg body weight of EOS, respectively. The regimens were administered by oral gavage once daily for a period of 4 wk. Body weights and size of tumors were recorded every 7 days and tumor volume was determined. Apart from the tumor volume, following parameters were also calculated: $\% \Delta T / \Delta C$, where, $\Delta T = T - \Delta_0$ and $\Delta C = C - \Delta_0$ (Δ_0 is the average tumor volume at the beginning of the treatment, T and C are the tumor volumes at a specified day for treated and control groups, respectively). The optimal $\% \Delta T / \Delta C$ value was used as a measure of test sample activity. In general, the $\Delta T / \Delta C$ value in percent is used as an indication of antitumor effectiveness, and a value of $\Delta T / \Delta C \leq 42\%$ is considered as significant antitumor activity by the Division of Cancer Treatment, NCI, NIH (17). After 28 days, the mice were sacrificed, and the tumors were removed and weighed. The present work was approved by the USM Animal Ethical Committee [Reference Number USM/PPSG/07(A)/044/(2009)(50)/159].

Analysis of VEGF in Colon Tumor Tissue Homogenates

Tumor tissues (100 mg) were homogenized in 1 mL lysis buffer (Ray Bio, USA). The homogenates were centrifuged for 10 min at 4°C (10,000 *g*), and the supernatants were assessed using VEGF-165 enzyme-linked immunosorbent assay (ELISA). The calibration curve of VEGF standard was used to calculate concentration of VEGF in the samples.

Cytotoxicity Assay

Cytotoxicity of EOS was evaluated using MTT assay (18) against HCT 116 and CCD-18Co cell lines. The assay plates were read using a microtiter plate reader (Hitachi U-2000, Japan) at 570 nm absorbance. One percent DMSO was used as a negative control.

In Vitro Antiangiogenic Activity of EOS:

HUVEC Proliferation Assay

HUVECs (2×10^4 cells/well) were allowed to attach in 96-well plates in 100 μ l growth media containing 1% VEGF. The cells were exposed to EOS (100 to 3.625 μ g/ml) for 48 h. Proliferation of HUVECs was assessed by MTT assay (18) using vincristine as the reference standard.

Migration Assay

The assay was performed according to Liang et al. (19) with minor modifications. Briefly, a wound was created using 200- μ l micropipette tip on a confluent monolayer of HUVECs in a 6-well plate. Two different concentrations of EOS were tested. After 12 h, the wounds were photographed and distances between one side of the scratch and the other were measured using inverted microscope supplied with Leica Quin computerized imaging system. Ten fields for each concentration were captured and minimum 10 readings of distance for each field were measured.

Tube Formation Assay

The ability of HUVECs to form tube-like structures was investigated on a matrigel matrix. In brief, the matrigel (150 μ l/well) was added to 48-well plate and allowed to polymerize for 45 min at 37°C and 5% CO₂. HUVECs were harvested and seeded onto the matrigel-coated plates. The cells were treated with various concentrations of EOS and incubated at 37°C for 24 h (20). Suramin was used as a positive control at 10 μ g/ml in the growth medium. The cells were imaged under an inverted fluorescence microscope at low magnification and the network length was quantified.

Western Blot Analysis

HUVECs were cultured in serum-free ECM for 24 h and then incubated with various concentrations of EOS (12.5, 25, and 50 μ g/ml) for 30 min before VEGF (100 ng/ml) stimulation for 5 min. Total cells extracts were prepared in ice-cold cell extraction buffer (Biosource, Camarillo, CA). Proteins were separated by electrophoresis on SDS gels, transferred onto polyvinylidene difluoride membranes, and incubated with the primary antibody,

antiphospho-VEGFR2. Binding of the primary antibody was detected with a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate (Pierce).

Statistical Analysis

The results were expressed as the mean \pm standard error (SEM), and the statistical significance was evaluated by using the Student's *t*-test. *P* values <0.05 implied significance.

RESULTS

HPLC Analysis of EOS

HPLC chromatograms of EOS (absorbance at 330 nm) along with the mixed standards are shown in Fig. 1A and 1B. Results from this study are consistent with previous reports, suggesting that rosmarinic acid is one of the major chemical constituents of *O. stamineus* extracts along with other flavonoids (12). The analytical plots of standards, betulinic acid, oleanolic acid, and ursolic acid along with the sample are shown in Fig. 1C and 1D. Chemical structures of 8 biomarkers used in the study are given in Fig. 1E. Good linearity, retention time, and method validation of 5-point calibration curves were obtained for all replicates (Table 1). The quantitative result of bioactive markers (% dry weight) is illustrated in Fig. 1F.

Phytochemical Analysis

The result of quantitative analysis of total proteins, polysaccharides, flavonoids, phenolics, and glycosaponins present in EOS is depicted in Fig. 1G.

Effect of EOS on Colorectal Tumor Growth

To evaluate the antitumor effect of EOS, HCT 116 cells were injected into nude mice. EOS exhibited dose-dependent suppression in HCT 116 tumor growth relative to vehicle-treated control (Fig. 2A–2C). Morphology of excised tumors (Fig. 2D–2F) showed significant reduction in density of blood vessels in the tumors from treated animals compared to the tumors from control group. Fig. 2G shows the tumor growth profile in the respective test groups. A significant $\Delta T/\Delta C$ (13.6%, *P* < 0.01) antitumor activity of EOS (100 mg/kg) on 28th post-cell inoculation day (Table 2). At a dose of 200 mg EOS/kg on 28th post-cell inoculation day, the result was far more obvious showing potent activity of EOS (1.45%, *P* < 0.001). Fig. 2H shows the average tumor weight from different test groups.

Histopathological Examination

In histological study, the antiangiogenic effect of EOS was even more pronounced. Fig. 3A and 3B show the typical vehicle-treated tumor in which there is no or minimal necrosis and the tumor cells showing compact sheets of polygonal cells with abundant supply of blood vessels (Fig. 3B). In the animals treated with EOS, many tumors showed histological evidence of EOS's antitumor effect. The tumors from these animals showed significant loss of compactness of the cells in the tumor with moderate to severe necrosis, with areas of decreased cell density, many

TABLE 1

The linearity, correlation coefficient (R^2), limit of detection (LOD), and limit of quantification (LOQ) of the compounds studied

Standards and [retention time (min)]	Linear range ($\mu\text{g/mL}$)	Regression equation	r^2	LOD (ng/mL)	LOQ (ng/mL)
Rosmarinic acid (5.8)	0.01–1000	$y = 36.88x - 18.54$	0.9998	2.0	20
Orthosiphon A (7.0)	0.01–500	$y = 23.076x - 12.36$	0.9999	2.0	20
TMF (11.2)	0.01–500	$y = 49.878x - 11.69$	0.9999	2.0	20
Sinensetin (14.0)	0.01–500	$y = 52.728x - 16.82$	0.9999	2.0	20
Eupatorin (17.4)	0.01–500	$y = 48.338x + 4.83$	0.9996	2.0	20
Betulinic acid (11.7)	0.01–500	$y = 39.039x + 13.88$	0.9993	10	100
Oleanolic acid (13.6)	0.01–500	$y = 54.44x - 24.93$	0.9991	10	100
Ursolic acid (13.9)	0.01–500	$y = 49.15x + 7.07$	0.9992	10	100

Note. y indicates peak area; x, concentration (mg/mL).

small islands of tumor cells, and calcification (an indicator of past necrosis) (Fig. 3C and 3D). The most remarkable change observed in tumor histology was the treatment with 200 mg/kg of EOS induced reduction in tumor vascular density. In these animals, histology of the tumor was highly disorganized with severe necrosis (Fig. 3E and 3F). In many of these, much of the tumor mass that remained, and was measured, was actually necrotic debris and calcification, and in several cases only a few islands of tumor cells remained.

Inhibition of HUVEC Proliferation and Tube Formation

In order to confirm the possible mechanism by which EOS inhibits the growth of tumors, an investigation on a series of in vitro angiogenesis models was conducted. We first determined whether EOS inhibited endothelial cell proliferation. Results showed that EOS caused inhibition of HUVEC proliferation at higher concentrations with IC_{50} 48.23 $\mu\text{g/ml}$ (Fig. 4A), whereas the standard reference vincristine exhibited potent cytotoxicity with IC_{50} 0.13 $\mu\text{g/ml}$. To characterize the antiangiogenic activity of EOS, endothelial cell tube formation assay, a well-established in vitro angiogenesis assay, was conducted. HUVECs cultured on matrigel formed tubelike networks (Fig. 4B) within 8 h, which might, in part, reflect the process of angiogenesis. At a concentration of 25 $\mu\text{g/ml}$, 98.26% inhibition was observed (Fig. 4C), in which EOS absolutely abrogated endothelial tube

formation, reducing the tubelike structure both in width and in length. Noteworthy, 56.22% inhibition in tube formation was observed at 12.5 $\mu\text{g/ml}$ EOS concentration (Fig. 4D), which is much lower than the IC_{50} value of EOS on HUVEC proliferation. It can be seen clearly that the HUVECs rounded up and rendered network structures incomplete and broken in the presence of EOS. The activity of EOS was more pronounced than the standard drug, suramin, which showed 49.76% inhibition at a concentration of 10 $\mu\text{g/ml}$ (Fig. 4E).

Inhibitory Effect of EOS on HUVECs Migration

HUVEC migration assay represents an important step in the formation of new blood vessels and is a straightforward and economical method to study the cell migration phenomenon (19). A scratch wound was created on the monolayer of cells (Fig. 4F), and the effect of EOS on closure of the wound was studied. EOS (25 $\mu\text{g/ml}$) inhibited HUVECs migration ($P < 0.001$) by 97.33% after 12 h. Even at a lower concentration of EOS (12.5 $\mu\text{g/ml}$), a significant ($P < 0.05$) inhibitory effect was observed, with 76.8% inhibition after 12 h.

Effect of EOS on VEGF-Induced Tyrosine Phosphorylation of VEGFR-2

Serum-starved HUVECs were treated with various concentrations of EOS for 30 min, followed by VEGF stimulation. Phosphorylation state of VEGFR-2 was assessed by Western blot with anti-phospho-VEGFR2 antibody. Fig. 5A depicts the inhibitory effect of EOS on VEGF-induced tyrosine phosphorylation of VEGFR2 in a dose-dependent manner.

In Vitro Effect of EOS on VEGF level in HCT 116 Cell Lysate

The level of VEGF in treated and untreated HCT 116 cells was determined by human VEGF 165 ELISA kit. Result showed that the treatment caused a significant decrease in VEGF content in cell lysates when compared to the control (378 ± 5 pg/ml). The extract showed dose-dependent reduction of VEGF levels (Fig. 5B). At 25 $\mu\text{g/ml}$, the VEGF level was 310 ± 0.82 pg,

TABLE 2

% $\Delta\text{T}/\Delta\text{C}$ of HCT 116 tumor growth in immunocompromised mice

Dose of EOS (mg/kg)	% $\Delta\text{T}/\Delta\text{C}$ value with respect to the post-cell inoculation period		
	1st wk	2nd wk	3rd wk
100	19.9*	16.46*	13.6**
200	<6.41**	<2.83**	<1.45**

$P < 0.05$. ** $P < 0.01$.

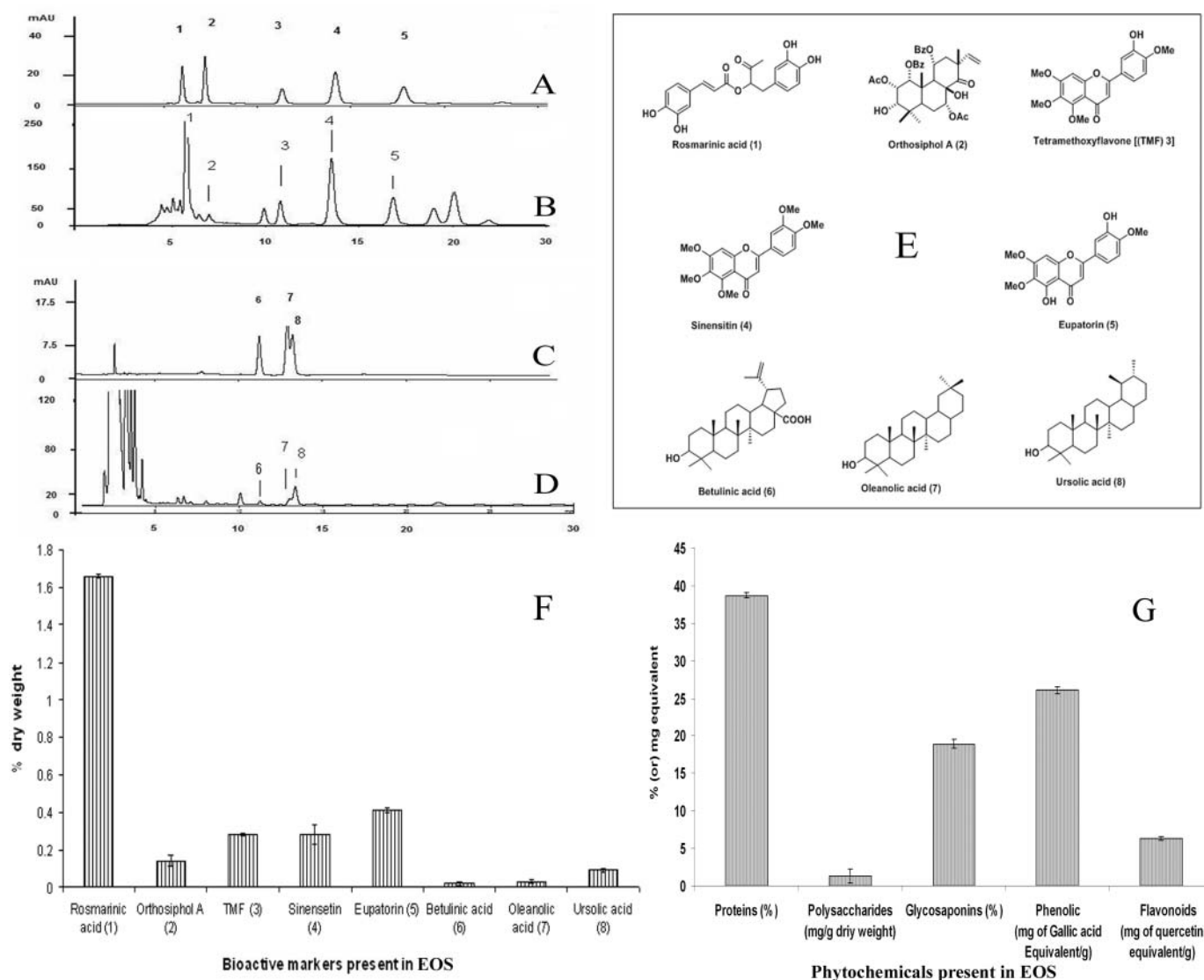


FIG. 1. A, C: High performance liquid chromatography (HPLC) chromatograms of standard markers, rosmarinic acid (1); orthosiphon A (2); 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (3); sinensetin (4); eupatorin (5); betulinic acid (6); oleanolic acid (7); and ursolic acid (8). B, D: HPLC chromatograms of ethanol extract of *O. stamineus* leaves (EOS) showing different proportions of respective markers. E: Chemical structures of marker compounds in EOS. F: Illustration depicts the contents of bioactive markers (% dry weight) present in EOS. G: Graphical representation of the levels of total proteins, polysaccharides, glycosaponins, phenolic and flavonoid content in EOS. All values are expressed as mean \pm SEM ($n = 6$).

whereas at 50 $\mu\text{g/ml}$ concentration, VEGF level was decreased drastically to $211 \pm 0.26 \text{ pg/ml}$.

In Vivo Effect of EOS on VEGF Level in Colon Tumor Tissue Homogenates

Having examined the effect of EOS on tumor vasculature, the level of VEGF in tumor tissues harvested from the treated and control animals was determined. Results showed that the treatment caused a significant decrease in VEGF content in homogenates when compared to the control ($135.5 \pm 4 \text{ pg/100 g}$ of tissue homogenate). EOS showed dose-dependent reduction of VEGF levels (Fig. 5C). At 100 mg/kg dose, the VEGF

level was $112.9 \pm 2 \text{ pg/100 g}$ of tissue homogenate, whereas at 200 mg/kg VEGF level was $90.9 \pm 3 \text{ pg/100 g}$ of tissue homogenate.

Effect of EOS on Proliferation of Non-Endothelial Cells

In order to evaluate the potential cytotoxic activity of EOS, MTT cell proliferation assay was conducted. HCT 116 and CCD-18Co were exposed to different concentrations of EOS. The results showed no significant cytotoxicity as compared to DMSO alone. The IC_{50} value of EOS against both cell lines was found to be $> 100 \mu\text{g/ml}$ (data not shown).

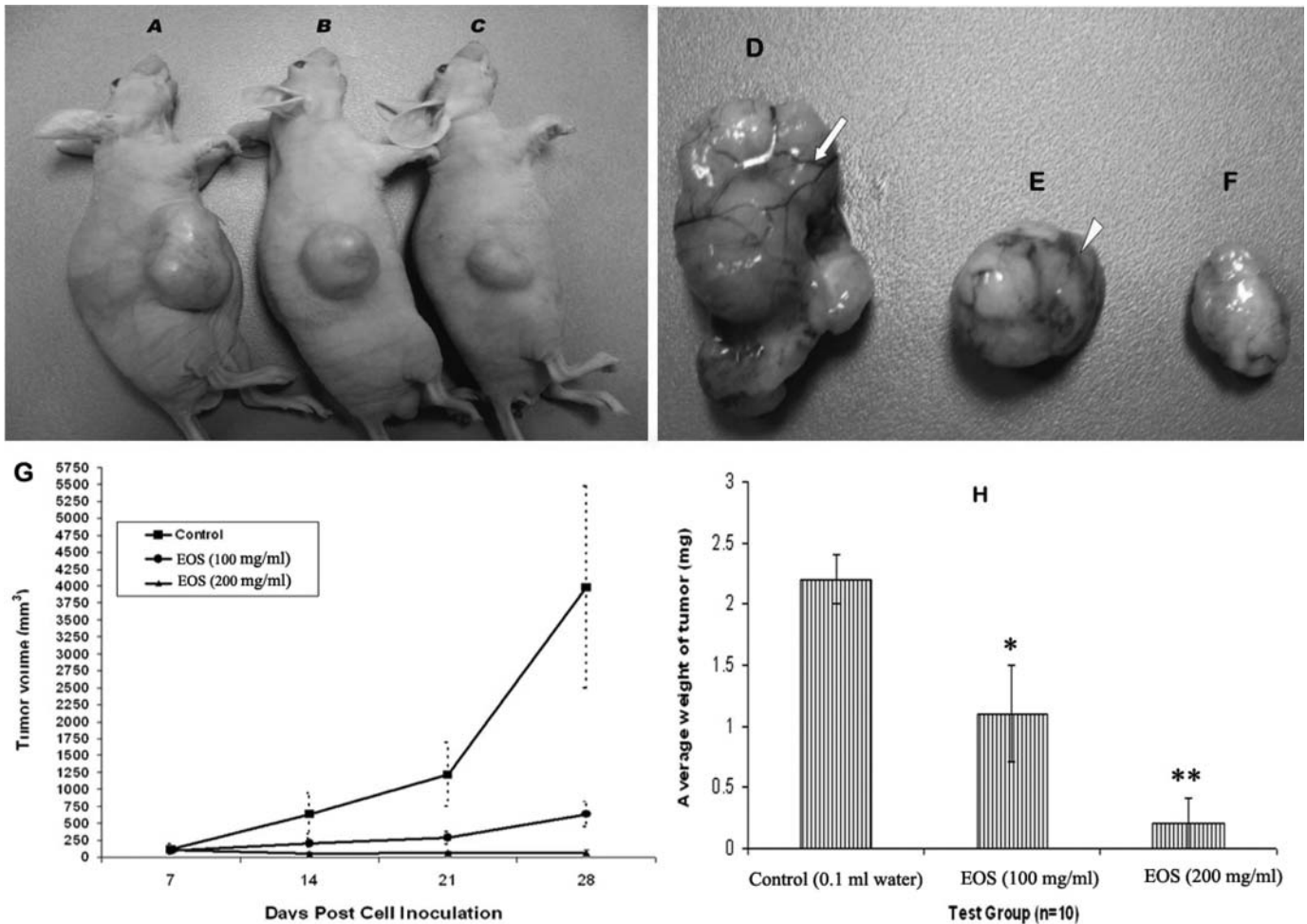


FIG. 2. In vivo antitumor activity in which immunocompromised mice bearing HCT 116 tumors at 28th post-inoculation day. A: Animal without treatment; animal treated with 100 mg/kg (B) and 200 mg/kg (C) of ethanol extract of *O. stamineus* leaves (EOS). D: Tumor harvested from control animal (arrow indicates prompt and well-developed blood vessels in control). E: Tumor harvested from animal treated with 100 mg/kg EOS (arrowhead points toward the reduction in tumor vasculature caused by EOS). F: Tumor harvested from animal treated with 200 mg/kg EOS. Here the density of vascularization is nil or negligible. G: Illustrates the comparative effect of 2 different doses of EOS with control group on HCT 116 tumorigenesis in nude mice. H: Effect of EOS on the average tumor weight. All values are expressed as mean \pm S.E.M ($n = 10$). * $P < 0.05$, ** $P < 0.01$.

DISCUSSION

Dietary antioxidants with antiangiogenic activity have been extensively studied as the core constituents responsible for cancer prevention. Among those that have been identified include vitamin C, vitamin D, vitamin E, vitamin A, betulinic acid, and 3-hydroxyflavone, 3',4'-dihydroxyflavone, 2',3'-dihydroxyflavone (21). In the present work, the antiangiogenesis efficacy of EOS was shown to be mediated via blocking the VEGF signaling pathway. The oxidants participate actively in the VEGF signaling pathway, which is not just limited to induction of VEGF signal. When VEGF binds to its specific receptors, especially VEGFR-2, oxidants are required for the signaling cascade that leads to the angiogenic response of VEGF (22,23). Reactive oxygen species are responsible to drive the mitogenic cascade initiated by the tyrosine kinase receptor of VEGF (24). EOS effectively inhibited the in vitro and in

vivo release of the key angiogenic factor VEGF. On the one hand, EOS strongly inhibited the level of VEGF mitogen in tumor cells; on the other hand, it suppressed the phosphorylation of VEGFR-2 in HUVECs. These effects of EOS could be ascribed mainly to its antioxidant-rich polyphenolic contents, the caffeic acid derivatives, polymethoxylated flavonoids and terpenes, particularly rosmarinic acid, eupatorin, sinensetin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, and betulinic acid (25). Flavonoids and their metabolic precursors, phenolics, possess very promising antiangiogenic properties (26). However, other non-flavonoid polyphenols, such as rosmarinic acid and betulinic acid also significantly contribute to the antiangiogenic effect of polyphenols-rich plants (27,28). The blockade of the angiogenic process through the inhibition of VEGF signaling has been shown to result in significant tumor growth delay in a wide range of preclinical models and the clinical benefit of this

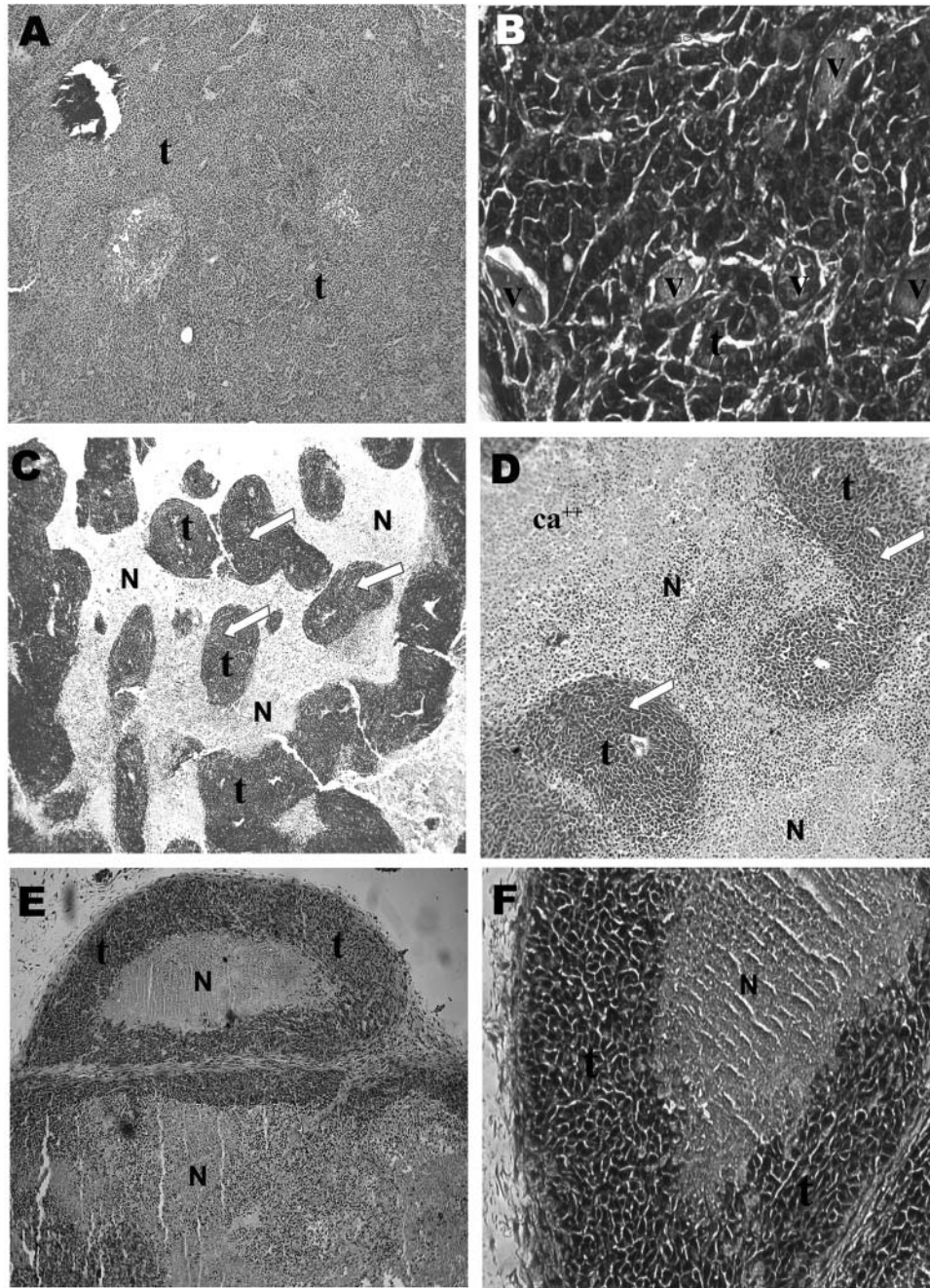


FIG. 3. Necrotic changes in tumor histology and evidence of antiangiogenesis by treatment of ethanol extract of *O. stamineus* leaves (EOS). At the termination of the study, hematoxylin and eosin (H&E)-stained tumor sections were examined histologically. A: An H&E-stained tumor section of a representative animal in the vehicle control group is shown, original magnification of $\times 10$. This tumor is composed of viable tumor cells (designated by "t") and virtually no necrosis. B: Magnified ($\times 100$) tumor section of vehicle control shows well organized tumor with the densely packed viable tumor cells (t) and large number of blood vessels (v). C, D: Significant changes in tumor histology were noted in immunocompromised animals treated with EOS (100 mg/kg), such as loss of compact arrangement of tumor cells, islands (indicated by arrows) of live tumor cells (t) surrounded by necrosis (indicated by "N"), calcification (Ca^{++}) evidence of previous necrosis, and decreased density of remaining tumor cells (magnification: Fig. 3C $10\times$ and Fig. D $40\times$). E, F: A representative tumor from EOS (200 mg/kg)-treated group is shown, original magnification of $\times 10$ (Fig. 3E). Central necrosis is markedly observed in this group of tumors. In these animals, tumor architecture was highly disorganized and with moderate to severe necrosis (N). In approximately half of these animals, only a small rim of viable tumor cells (t) remained at the periphery and the majority of the mass was actually necrotic debris, as represented by the necrotic tumor from the treatment group shown here, original magnification of $\times 100$ (Fig. 3F).

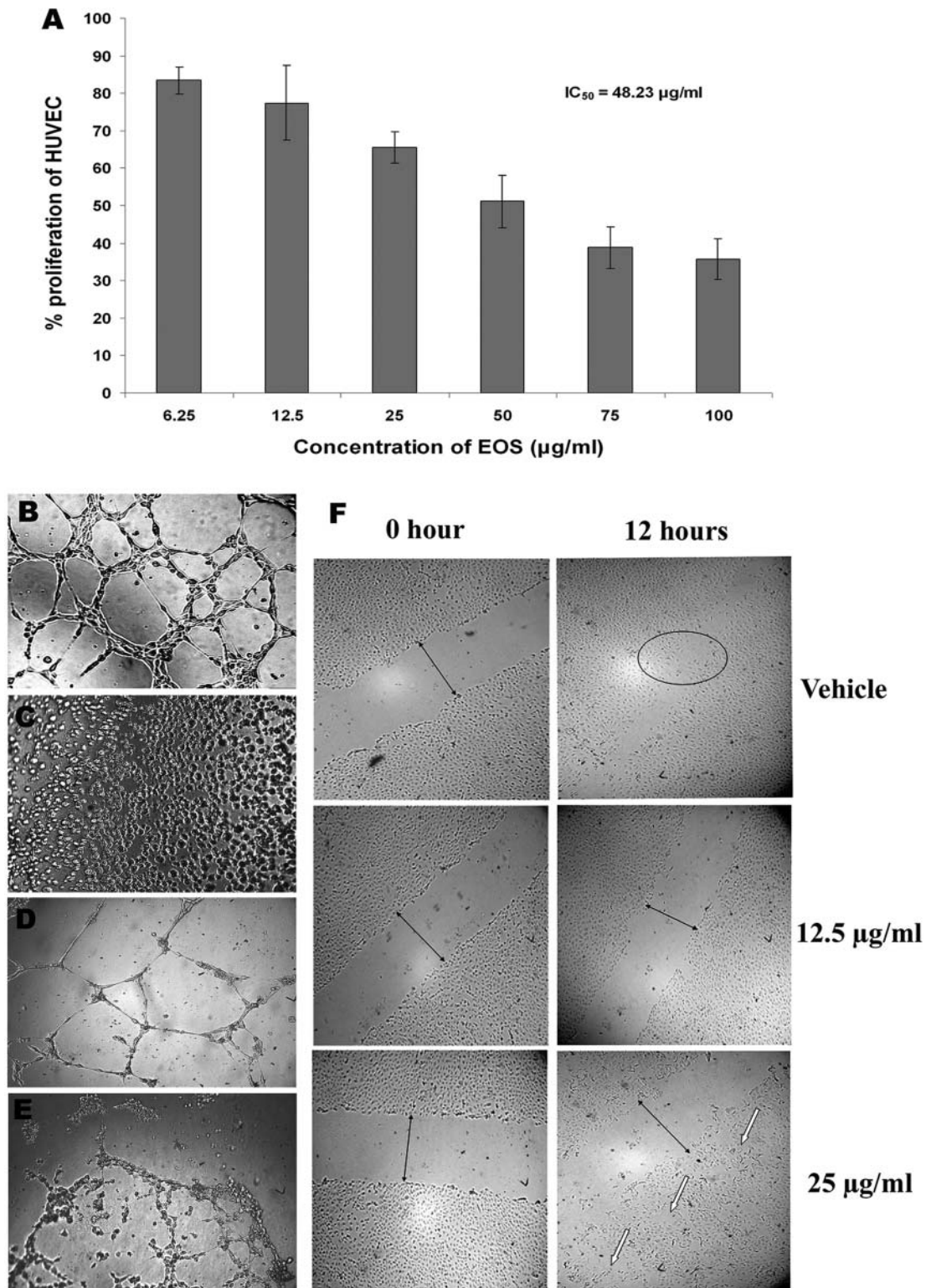


FIG. 4. Effect of ethanol extract of *O. stamineus* leaves (EOS) on proliferation, tube formation and migration of human umbilical vein endothelial cells (HUVECs). A: Effect of EOS on HUVEC proliferation. EOS inhibited the proliferation in dose-dependent manner with IC_{50} 48.23 $\mu\text{g/mL}$. ($n = 6$, values are in mean \pm SEM). In tube formation assay, HUVECs (2×10^4 cells/well) were plated on Matrigel precoated 96-well plates and treated with different concentrations of EOS for 24 h. B: Control; C: EOS (25 $\mu\text{g/mL}$); D: EOS (12.5 $\mu\text{g/mL}$); E: Suramin (10 $\mu\text{g/mL}$). F: Because of the successful migration of endothelial cells in untreated group, the wound is almost closed after 18 h; whereas in EOS-treated group, the wound remained open even after 18 h incubation. EOS (12.5 $\mu\text{g/mL}$) caused significant inhibition of endothelial cell migration. At a concentration of 25 $\mu\text{g/mL}$, EOS caused dislodgement of monolayer of endothelial cells (indicated by the arrows) with almost complete inhibition of migration.

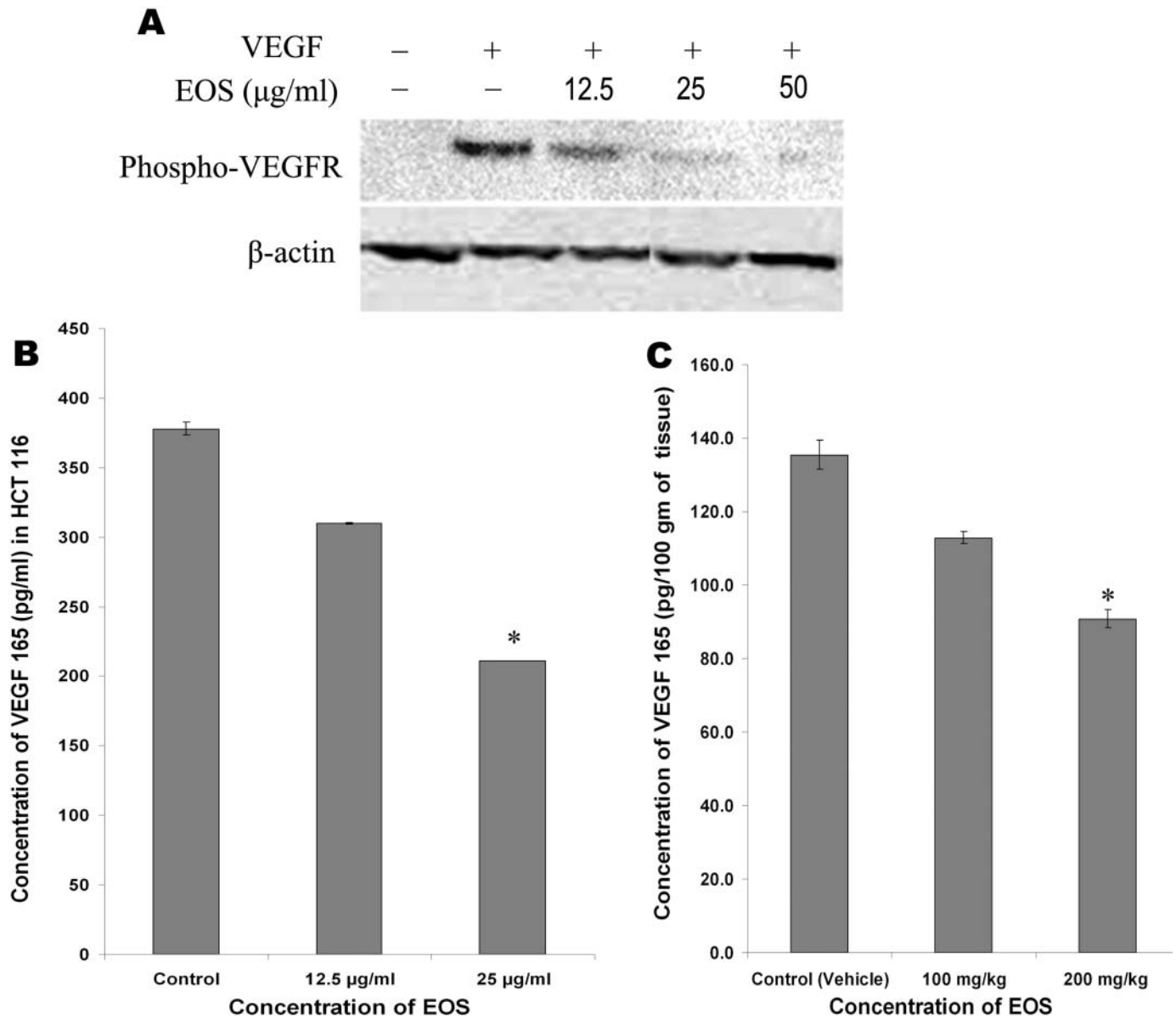


FIG. 5. A: Phosphorylation of VEGFR-2 was assessed by Western blot with antiphospho-VEGFR-2 antibody. The results showed that ethanol extract of *O. stamineus* leaves (EOS) inhibited phosphorylation of VEGFR-2 in a dose-dependent manner. B: In vitro determination of vascular endothelial growth factor (VEGF) 165 level in HCT 116 cells: The level of VEGF in untreated cells was found to be 378 ± 5 pg/ml, whereas the treated cells showed 310 ± 0.82 and 211 ± 0.26 pg/ml at 12.5 and 25 μg/ml concentration of EOS, respectively ($n = 6$, values are in mean \pm SEM). * $P < 0.05$. C: In vivo determination of VEGF level in colon tumor tissue homogenates: Tumor tissues harvested from placebo control group showed 135.5 ± 4 pg VEGF/100 g of tissue homogenate, whereas the tumor tissues harvested from the animals treated with 100 and 200 mg/kg EOS showed 112.9 ± 2 and 90.9 ± 3 pg VEGF/100 g of tissue homogenate, respectively ($n = 10$, values are in mean \pm SEM). * = $P < 0.05$.

approach has also been confirmed (29). Nevertheless, diterpenes isolated from *O. stamineus* have been shown to demonstrate antiproliferative activity against highly liver metastatic murine colon 26-L5 carcinoma cells (30,31). Further studies on these diterpenes revealed their efficacies to inhibit the tumour promoter induced inflammation of 12-O-tetradecanoylphorbol-13-acetate in mouse model (32). Furthermore, EOS significantly inhibited the key aspects of angiogenesis in endothelial cells,

such as migration and tube formation. Rosmarinic acid has been found to be an active caffeoyl ester, predominantly found in *O. stamineus*, which inhibits several important steps of angiogenesis including proliferation, migration, adhesion, and tube formation of endothelial cells, in addition to retinal neovascularization in a mouse model of retinopathy, which related to blocking of prooxidant-dependent VEGF expression in retina (27,33). Methoxylated flavonoids (eupatorin and sinensetin) are

important class of bioactive compounds found abundantly in *O. stamineus*. Eupatorin strongly inhibits in vitro proliferation of cancer cells (34), whereas sinensetin induces apoptosis and CYP1-mediated antiproliferation in human cancers cells (35,36).

In conclusion, the present work provides good supporting evidence that EOS inhibits the growth of colon tumor. The antitumor effect may be due to the collective contribution of phytochemicals particularly, rosmarinic acid, eupatorin, sinensetin, betulinic acid, and 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone in EOS. These results convinced that EOS exerts the antiangiogenic effect by blocking VEGF signaling pathway, and eventually the antiangiogenic property could be the principle factor for the potent antitumor property of EOS.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of the Universiti Sains Malaysia, under grant number 1001/PPSK/8120243, RU, and Malaysian Ministry of Higher Education, grant number FRGS: 203/PFARMASI/671196. The authors are also thankful to the MAKNA Research Grant No. 304/PFARMASI/650547/M122. We also acknowledge scientific contributions from Christopher Parish and his group, Cancer and Vascular Biology Centre, John Curtin School of Medical Research, Australian National University, in aiding us to establish the angiogenesis assay system.

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