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Marked antitumor activity of cat's whiskers tea (*Orthosiphon stamineus*) extract in orthotopic model of human colon tumor in nude mice

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Abstract

Orthosiphon stamineus is used to treat kidney ailments including angiogenesis-dependent diseases. *O. stamineus* has shown to possess strong anti-angiogenic activity. In present study, an orthotopic nude mouse model of colon cancer was employed to study the factors that influence suppression of tumor by standardized 50% ethanol extract of *O. stamineus* leaves (EOS). Human colorectal cancer cells (HCT116) were surgically injected into the cecal wall of mice. Two different oral doses (100 and 200 mg/kg/day) were given for 5 weeks. EOS suppressed 61.62±3.7% and 82.8±1.5% tumor growth at 100 and 200 mg/kg, respectively. Tumor histology revealed significant reduction in vascularization. Anti-angiogenic efficacy of EOS was investigated in human endothelial cells (HUVEC). *In vitro,* EOS inhibited migration and tube formation of HUVECs. HPLC data showed high content of rosmarinic acid in EOS. This work supports previous anti-tumor works on the plant in which suppression of VEGFR phosphorylation is thought to be involved.

Keywords: *Orthosiphon stamineus*; Orthotopic colon tumor model; antiangiogenesis; VEGFR-2 tyrosine kinase; HPLC analysis; bioactive markers.

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Introduction

Colorectal cancer is the third most commonly diagnosed malignant neoplasm worldwide (Shike et al. 1990). Angiogenesis is essential for tumor development, and tumor vasculature is considered an optimal target for anti-cancer strategies, thus it is a vital necessity to discover or develop antiangiogenic agents as the treatment for malignancy or as the adjunct to standard chemotherapeutic regimens. Prevention of the supply of new blood vessels results in reduced tumor size and metastases (Fayette et al. 2005). Angiogenic inhibitors have been developed and successfully tested in various preclinical tumor models (Brekken et al. 2000; Kuba et al. 2000) and some of these drugs already entered phase I clinical trials (Fox et al. 2001; Gasparini 2001; Jain 2001; Klement 2001; Ferrara et al. 2004).

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 herbal tea, well known as cat's whisker's tea or Java tea. The herb is used as a folk medicine for the treatment of a variety of angiogenesis related diseases including rheumatism, tumorous, diabetic blindness and psoriasis (Perry 1980; Jaganath and Ng 2000). *O. stamineus* contains more than 20 phenolic compounds, including two flavonol glycosides, nine lipophilic flavones, nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid (Sumaryono et al., 1991) and nitric oxide inhibitory isopimarane-diterpenes (Awale et al., 2003). Scientific studies have found that the leaves exhibit dynamic pharmacological properties such as, antioxidant, anti-inflammatory and anti-bacterial properties (Masuda et al. 1992; Tezuka et al. 2000; Ho et al. 2010). A number of safety assessment studies have been reported that *O. stamineus* is deprived of chronic toxicity, genotoxicity, nephrotoxicity and hepatotoxicity (Chin et al. 2008; Muhammad et al. 2011; Kannappan et al. 2010; Chin et al. 2009). Phytochemical studies reported bioactive pentacyclic compounds (Tezuka et al., 2000) with more than 20 phenolic compounds, two flavonol glycosides, nine lipophilic flavones, nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid (Yam et al. 2010; Sumaryono et al. 1991), and nitric oxide inhibitory isopimarane-diterpenes (Awale et al. 2003). Recently, we have shown that 50% ethanol extract of *O. stamineus* leaves (EOS) inhibits angiogenesis in rat aorta and growth of colorectal tumor implanted ectopically in nude mice (Sahib et al. 2009; Ahamed et al. 2012). The present work is an

attempt to prove scientifically that *O. stamineus* could be the potential alternative remedy for angiogenic dependent tumors if the antiangiogenic property of EOS is investigated and systematically proven using a suitable animal model of cancer which mimics the exact nature of human disease. Accordingly, the aim of this study is to evaluate the anticancer activity of EOS against colorectal carcinoma xenografted orthotopically in nu/nu athymic nude mice and, to investigate and characterize the anti-angiogenic efficacy of EOS in human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Chemicals for HPLC analysis

HPLC grade methanol, acetonitrile, tetrahydrofuran and ortho-phosphoric acid were obtained from Merck, Germany. Reference standards such as, betulinic acid, oleanolic acid and ursolic acid purchased from Sigma Aldrich (Germany) and sinensetin, 3'hydroxy-5, 6, 7, 4'-tetramethoxyflavone and rosmarinic acid were procured from Indo Fine Chemical Company (Hillsborough, USA). Deionised water for HPLC was prepared using ultra pure water purifier system (Elgastat, Bucks, UK). All other chemicals and solvents (analytical grade) were purchased from Merck Germany.

Cell Culture and Reagents

Endothelial Cell Medium (ECM) supplied with endothelial cell growth supplements (ECGS) was purchased from ScienCell, USA. RPMI 1640, Dulbecco's Modified Eagle Medium (DMEM), trypsin and heat inactivated foetal bovine serum (HIFBS) were obtained from GIBCO, UK. Phosphate buffered saline (PBS), penicillin/streptomycin (PS) solution, MTT reagent, suramin, vincristine and gentamicin were purchased from Sigma-Aldrich, Germany. Fibrinogen was obtained from Calbiochem, USA and Matrigel matrix (10 mg/mL) was acquired from BD Bioscience, USA. All chemicals used in this study were of analytical grade.
Human umbilical vein endothelial cell line (HUVEC) was provided by ScienCell, USA. HUVECs were maintained in ECM medium supplemented with 5% HIFBS, 1% PS and 1% ECGS. Human colorectal tumor cell (HCT 116) and normal colon fibroblast cell (CCD-18Co) were sourced from American type culture collection (Rockvill, MD, USA). HCT 116 cell lines were maintained in RPMI 1640 containing 10% HIFBS and 1% PS. CCD-18Co cells were cultured in DMEM supplemented with 10% HIFBS and 1% PS. NCI NuNu mice were obtained from Jackson Laboratory, USA. Animals were maintained in a sterile condition with the aid of IVC (Individually Ventilated Cages).

Plant collection and authentication

The flowering twigs and the leaves of *O. stamineus* were collected from a contract farming facility in the state of Penang, Malaysia. The specimen was labeled and annotated with date of collection. A voucher specimen number (11009) was deposited at the herbarium of School of Biology, Universiti Sains Malaysia (USM).

Preparation of extract

The 50 % ethanol extract of the *O. stamineus* leaves (EOS) was prepared by maceration process as previously described (Ahamed et al. 2012). The powdered material (100 g) of the air dried leaves was extracted with 1 L of 50 % ethanol at 40 °C for 24 h with continuous stirring. The extract was filtered through filter paper (Whatman No. 1) with a Buchner filter under vacuum and concentrated to dryness in a rotary evaporator (Buchi, Switzerland) and stored at −20 °C until further use. The yield of the extract obtained was 7.4 g (approximately 7.4 percent).

High Performance Liquid Chromatography (HPLC) analysis of EOS

Operational parameters of HPLC

The high performance liquid chromatography (HPLC) was performed using an Agilent Technologies Series 1100 system equipped with degasser, an auto sampler, a column heater, quaternary pump and UV detector. The Column (Nucleosil C18, 250 mm x 4.6 mm, 5 µm) was maintained at 25 °C and 20 µl volume of injected sample were eluted by an isocratic mobile phase comprising of methanol: tetrahydrofuran: water (0.1 % H3PO4); (55:5:40 v/v) at a flow rate of 0.7 ml/min, separation time of 30 min and the detection was performed at 330 nm. The following standard reference compounds were used as bioactive markers: rosmarinic acid, orthosiphol A, 3'hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF), and 5, 6, 7, 4', 5'-pentamethoxyflavone (sinensetin) for flavonoids, and diterpenes Fig 1. The isocratic mobile phase comprised of acetonitrile: water (0.1% H_3PO_4) at a ratio of 86:14 v/v, flow rate of 1 ml/min, separation time of 30 min and the detection was performed at 210 nm for the betulinic acid, oleanolic acid and ursolic acid (triterpenoids) Fig 1. 20 µl each of the sample and the standards were injected. ChemStation A.08.03 performed analytical data acquisition. The standard calibration curves were established by plotting the peak areas against different concentrations. Reference standards were identified by comparison of their retention time values and spiked with the samples. The external standard method was used for quantification of the bioactive markers in the sample of the extract. All the biomarkers were determined by HPLC fingerprint analysis as reported before (Ahamed et al. 2012; Akowuah et al. 2004; Chen et al. 2003).

Preparation of standard solutions and sample for HPLC analysis

For the HPLC analysis, 100 mg EOS was dissolved in 25 ml mixture of methanol: water (1:1) and the contents sonicated for 10-15 min. Then the contents were transferred to 25 ml volumetric flask and the volume was made up to 25 ml. All samples were filtered through a 0.45 µm filter (Whatman). Similarly all reference compounds were weighed (about 5 mg) and each dissolved in 5 ml of methanol and then filtered through a 0.45 µm filter (Whatman). The stock solution was used for further dilution. The samples were kept in refrigerator at -20 ^{0}C prior to analysis.

In vivo human colorectal tumor xenograft in athymic mouse model
Anti-tumor activity of EOS

HCT 116 cells (10^6 in 200 µl media) were implanted surgically into the cecal wall of nude mice. (4-6 weeks old, 20-22 g). 18 mice randomly divided into three groups of six mice each. After the complete healing of the incision wound, the treatment with the extract was initiated in about six days post cell inoculation, group I received 0.1 ml distilled water (control) and groups II and III 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 five weeks. Body weights were recorded every 7 days. After 35 days, the mice were sacrificed, and the tumors were removed. Tumor volume and size was determined. The present work was approved by the USM Animal Ethical Committee (Reference Number USM / Animal Ethics Approval / 2011 / (72) (337).

Cytotoxicity assay

Cytotoxicity of EOS was evaluated using MTT assay against HCT 116 and CCD-18Co cell lines. The assay was carried out using the method described by Mosmann (1983) with minor modification, following 48 h of incubation. The assay plates were read using a micro-titer plate reader (Hitachi U-2000, Japan) at 570 nm absorbance. 1% DMSO was used as a negative control.

In vitro antiangiogenic activity of EOS

HUVEC proliferation assay

HUVECs were maintained in ECM containing 5% HIFBS and 1% PS, 1% ECGS. The cells were seeded in 96-well plates at a density of 2×10^4 cells/well in 100 µl growth media and kept overnight to facilitate attachment. The cells were exposed to EOS (100 to 3.625 µg/mL) for 48 h. After incubation, the viability of HUVECs was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolim bromide) assay (Mosmann 1983). 20 µl of MTT solution (5 mg/mL in PBS) was added to each well. After 4 h incubation, the mixed media and MTT solution were carefully discarded and then the crystallized dye was solubilized with DMSO. Vincristine was used as the reference standard. The amount of blue dye formed was determined by measuring the absorbance at 570 nm.

Migration assay

The assay was performed according to Liang et al. (2007) with minor modifications. Briefly, a wound was created using 200 µl micropipette tip on a confluent monolayer of HUVECs in 6 well plate. The detached cells were removed by washing twice with PBS. 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. 10 fields for each concentration were captured and minimum 10 readings of distance for each field were measured.

Tube formation assay

HUVECs were harvested and seeded in ECM medium (5% HIFBS) containing VEGF (100 ng/ml) onto 4-well culture plates coated with 150 µl Matrigel (5 mg/ml). The cells were treated with various concentrations of EOS and incubated at 37 ºC for 24 h (He et al. 2010). Suramin was used as a positive control at 10 µg/ml in the growth medium. After treatment, the media was discarded, and the cells were washed twice with Hank's balanced salt solution and stained with Calcein AM (8 µg/mL) for 45 min at 37 °C, under 5% CO_2. The dye was discarded, and the cells were washed twice to remove excess dye. The cells were imaged under an inverted florescence microscope at low magnification. The web junctions, defined as intersections of three or more tubes, were counted in each microscopic field (Bsndyopadhyay et al., 2002). The percentage of inhibition was represented as the mean ± S.D.

Statistical analysis

Statistical analysis involved use of the Statistical Package for Social Sciences (SPSS). Data are given as the mean ± S.E. and statistics were performed using one-way analysis of variance (ANOVA). Significant differences between the control and treatment groups.

Results

HPLC analysis of EOS

In the present study, two different HPLC methods were applied. One for quantitative determination of flavonoids (sinensetin, eupatorin, 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone), diterpene (orthosiphol A) and polyphenol (rosmarinic acid) and the other one for quantitative determination of triterpenoids (betulinic acid, oleanolic acid and ursolic acid) present in EOS. The HPLC chromatograms of EOS (absorbance at 330 nm) along with the mixed standard of bioactive markers are shown in Fig. 1 A and B. The 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 (Akowuah et al. 2004). The result of bioactive markers (% dry weight) present in EOS is given in Table 1. Similarly, the HPLC determination of triterpenoids (betulinic acid, oleanolic acid and ursolic acid) was carried out as described by Chen et al. (2003) and Akowuah et al. (2004). The analytical plots of standard bioactive markers along with the sample are shown in Fig. 1 C and D. The results are in accordance with the previously reported (Ahamed et al. 2012).

Table 1: HPLC determination of bioactive compounds present in EOS

Biomarkers in EOS	% dry weight
Rosmarinic acid (1)	1.66±0.01
Orthosiphol A (2)	0.14±0.03
tetramethoxyflavone (3)	0.28±0.005
Sinensetin (4)	0.28±0.05
Eupatorin (5)	0.41±0.01
Betulinic acid (6)	0.02±0.01
Oleanolic acid (7)	0.03±0.01
Ursolic acid (8)	0.09±0.01

Effect of EOS on orthotopic colorectal tumor growth

To evaluate the anti-tumor effect of EOS, HCT 116 cells were injected into the cecal wall of nude mice. EOS exhibited dose dependent suppression in HCT 116 tumor growth relative to vehicle-treated control (Fig. 2). Morphology of excised tumors showed significant reduction in density of blood vessels in the tumors from treated animals compared to the tumors in control group. Fig. 2G shows the average tumor volume and size in the respective test groups. A significant antitumor activity of EOS (61.62 % at 100 mg/kg) on 35^{th} post cell inoculation day (Fig. 2G). At a dose of 200 mg EOS/kg on 35^{th} post cell inoculation day, the result was far more obvious showing potent activity of EOS (82.8 %, $P < 0.001$).

Inhibition of HUVEC proliferation and tube formation

To characterize the anti-angiogenesis activity of EOS, we first determined whether EOS inhibited endothelial cell proliferation. Result showed that EOS caused inhibition of HUVEC proliferation at higher concentrations with IC_{50} > 100 µg/ml (Fig. 3A) whereas, the standard reference vincristine exhibited potent cytotoxicity with IC_{50} 0.13 µg/ml.

To further characterize its anti-angiogenesis activity, we examined the effects of EOS on VEGF-induced tube formation by HUVEC on Matrigel, a well-established angiogenesis assay. HUVECs formed tube like networks (Figs. 3B) within 8 h, which might, in part, reflect the process of angiogenesis. The activity of EOS was more pronounced than the standard drug, suramin which showed 49.76% inhibition at a concentration of 10 µg/ml (Fig. 3C). At a concentration of 25 µg/ml (Fig. 3D), 98.26% inhibition was

observed, in which EOS absolutely abrogated endothelial tube formation, reducing the tube-like structure both in width and in length. Noteworthily, 47.22% inhibition in tube formation was observed at 12.5 µg EOS/ml concentration (Fig. 3D), which is much lower than the IC_{50} value of EOS on HUVEC proliferation. It can be seen clearly that, the endothelial cells rounded up and rendered network structures incomplete and broken in the presence of EOS.

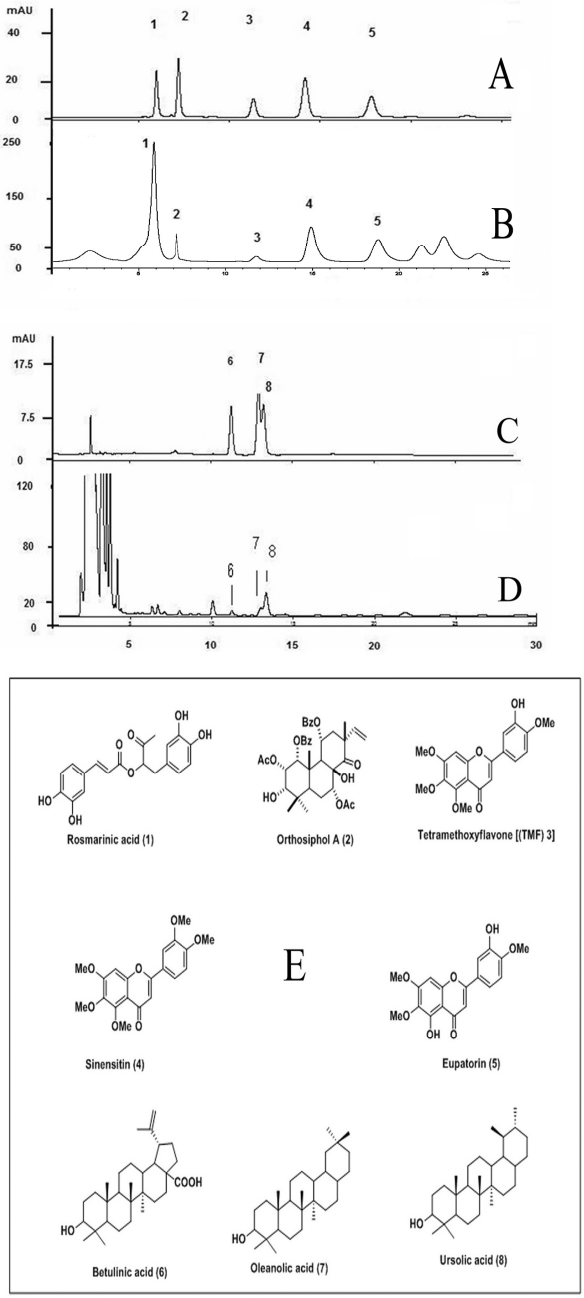

Figure 1: HPLC chromatogram and chemical structures of the biomarkers present in EOS; (A) and (C): HPLC chromatograms of standard markers, rosmarinic acid (1); orthosiphol A (2); 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (3), sinensetin (4); eupatorin (5); betulinic acid (6); oleanolic acid (7) and ursolic acid (8). (B) and (D): HPLC chromatograms of EOS showing different proportions of respective markers; (E): Chemical structures of marker compounds in EOS.

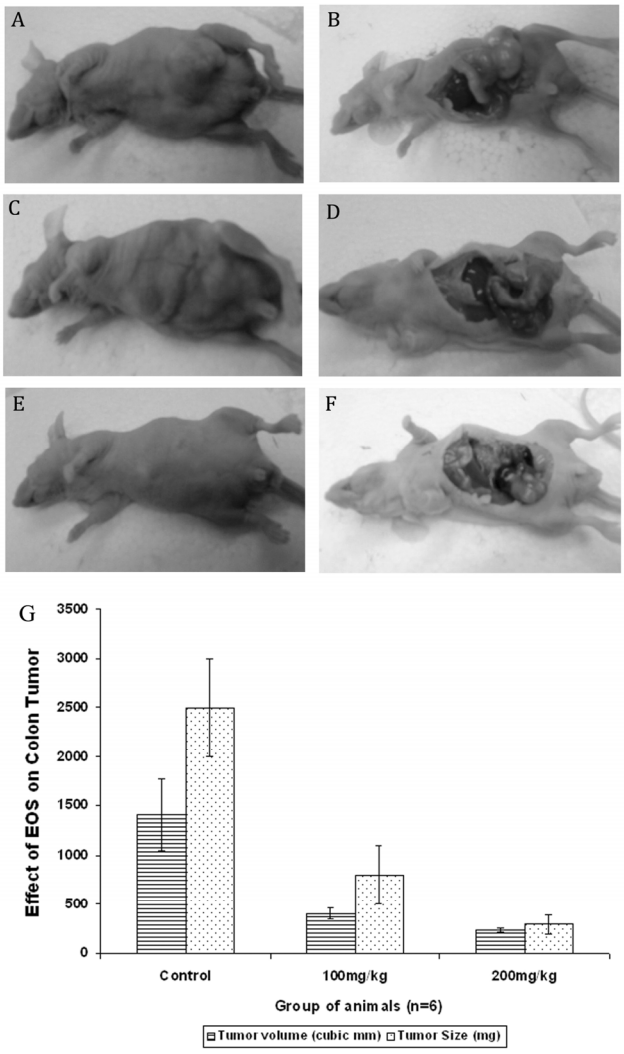

Figure 2: Figure depicts the anti-tumor activity of EOS. HCT 116 tumor implanted orthotopically in the cecal wall of nude mouse; (A) Placebo control: Animal bearing the largest size of tumor. (B) The dissection of control animal showing the uncontrolled growth of tumor. (C) Animal treated with 100 mg/kg of EOS shows significantly smaller size of tumor. (D) The dissection of animal treated with 100 mg/kg of EOS. (E) Animal treated with 200 mg/kg of EOS shows significant inhibition of HCT 116 tumor in colon. (F) The dissection of animal treated with 200 mg/kg of OS shows almost inhibition of HCT 116 tumor in cecal wall. (G) Effect of EOS on the average tumor volume and size (All values are expressed as mean ± S.E.M (n=10).

Inhibitory effect of EOS on HUVECs migration

To evaluate the inhibitory effect of EOS on endothelial cell migration process, *in vitro* wound healing assay was conducted. This 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 (Liang et al., 2007). With the help of a microtip, a scratch wound was created on the monolayer of the cells and the inhibitory effect of EOS on migration of HUVECs in the wounded area was assessed. EOS showed remarkable inhibition of HUVECs migration at 25 µg/ml by 92.6% after 12 h. At 12.5 µg/ml concentration EOS demonstrated a significant ($P < 0.05$) inhibitory effect (83.7 %) after 12 h.

Effect of EOS on proliferation of non-endothelial cells

The probable cytotoxic activity of EOS on cancer (HCT 116) and normal (CCD-18Co) cells is evaluated using MTT cell proliferation assay. Cells were exposed to different concentrations of EOS. It is observed that, even at higher concentrations, EOS failed to show significant cytotoxicity against both cells (IC_{50} value > 100 µg/ml).

Discussion

The most practical approach to alleviate the morbidity and mortality of cancer is to curb the process of carcinogenesis through the use of angiogenesis inhibitors. In tumor pathogenesis, angiogenesis is crucial and it sustains malignant cells with nutrients and oxygen. Tumor cells secrete various growth factors which trigger endothelial cells to form new capillaries. The prevention of the expansion of new blood vessel networks results in reduced tumor size and metastases.

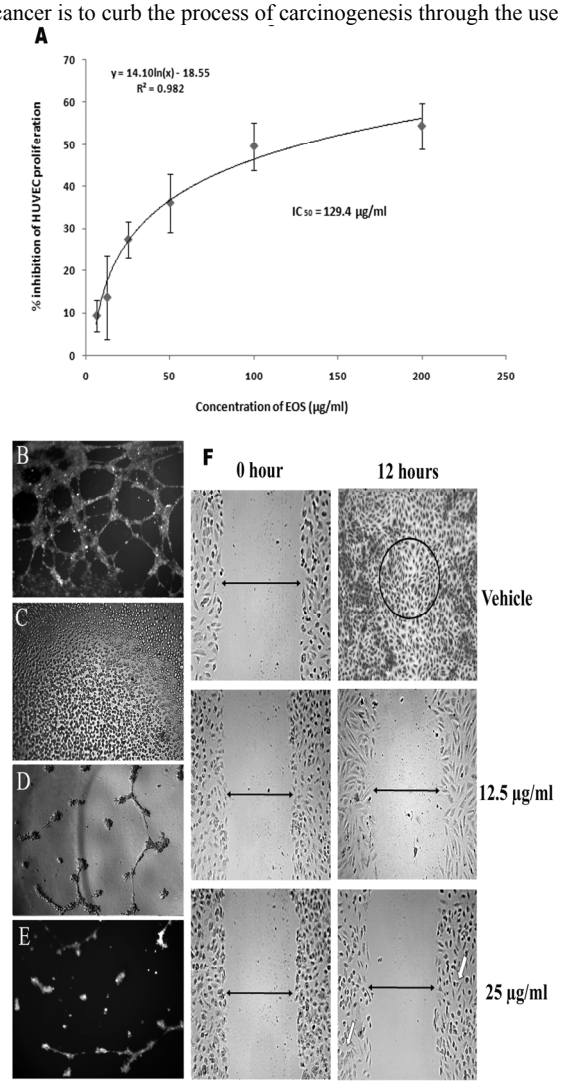

Figure 3: Effect of EOS on proliferation, tube formation and migration of HUVECs: (A): Effect of EOS on HUVEC proliferation. EOS inhibited the proliferation in dose dependent manner with IC_{50} > 100 µg/mL (n=6, values are in mean ± S.E.M). In tube formation assay, HUVECs (2 x 10^4 cells/well) were plated on Matrigel precoated 96-well plates and treated with different concentrations of EOS for 24 h. (B) Control; (C) EOS (25 µg/mL) (D) EOS (12.5 µg/mL) and (E) Suramin (10 µg/mL). (F): Due to the successful migration of endothelial cells in untreated group, the wound is almost closed after 12 h, whereas in EOS treated group, the wound remained open even after 12 h incubation. EOS (12.5 µg/mL) caused significant inhibition of endothelial cell migration. At a concentration of 25 µg/mL EOS caused dislodgement of monolayer of endothelial cells (indicated by the arrows) with almost complete inhibition of migration.

There has been an immense search for phytochemicals and micronutrients that can curb cancer development and cause tumor growth regression (Dimas et al. 2009). Most chemotherapeutic agents can destroy tumors and retard cancer growth (Sporn 1999) but may damage normal cells and tissues as well. Thus, new anticancer drugs from natural products are expected to play an important role in the development of more effective and safer strategies to inhibit the progress of cancer without causing toxicity towards the healthy cells and tissues (Sporn 1999). Cancer cells have capability to modulate signaling molecules, because of this, they are highly proliferative, migratory and matrix invasive. These properties of cancer cells can be controlled by potential anticancer agents that have strong inhibitory effects on angiogenic-related aspects or the proteins that activate pro-angiogenic cascade. Of particular interest are promising anticancer compounds from natural sources, such as commonly consumed foodstuffs, because these compounds are considered safe (Sherr 2004). Colorectal cancers are highly angiogenic and were the first tumor type to exhibit significant response to angiogenesis inhibitors (Mizukami and Chung 2007). 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 (Brekken et al. 2000).

In the present study, EOS significantly inhibited the key aspects of angiogenesis in endothelial cells, such as migration and tube formation. In our previous work, the anti-angiogenesis and anti-tumor efficacy of EOS was shown to be mediated via blocking the VEGF signaling pathway (Ahamed et al. 2012). EOS was shown to exert dual activities, on the one hand it inhibits the production of key angiogenic factor, VEGF from tumor cells and on the one hand, EOS suppresses the phosphorylation of VEGFR-2 in HUVECs (Ahamed et al. 2012). 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 (Losso 2003). This battery of compounds, each possessing their own unique anti-angiogenic property may provide a powerful cocktail that may be useful in preventing or treating neoplastic disorders. Several preclinical and clinical studies already proved that by inhibiting VEGF signaling, the angiogenesis cascade can be blocked, which further delays the tumor growth (Wedge et al. 2002).

It is found that the phytoconstituents present in *O. stamineus*, such as rosmarinic acid, eupatorin, sinensetin, betulinic acid etc., have been shown to be the bioactive compounds with remarkable inhibitory effects the proliferation of cancer cells and few of them shown to inhibit angiogenesis by suppressing the differentiation, migration, adhesion and tube formation of endothelial cells via blocking the pro-oxidant dependent VEGF expression (Huang and Zheng 2006; Kim et al. 2009; Androutsopoulos et al. 2008; Kim et al.. 2010; Androutsopoulos et al. 2009).

In conclusion, the present work supports the traditional use and the previous related works on the plant which endorse that *O. stamineus* significantly inhibits the orthotopically implanted colon tumor in immunocompromised mouse xenograft model. The antitumor efficacy could probably be due to the cumulative effect of

phytoconstituents particularly, rosmarinic acid, eupatorin, sinensetin, betulinic acid and 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone in EOS. These results convinced that VEGF-targeted inhibitory effect was largely responsible for antiangiogenic effect of the EOS on endothelial cells, and eventually the anti-angiogenic property could be the principle factor for the potent anti-tumorigenic property of EOS.

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