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Piper longum inhibits VEGF and proinflammatory cytokines and tumor-induced angiogenesis in C57BL/6 mice

E.S. Sunila, G. Kuttan *

Department of Immunology, Amala Cancer Research Centre, Amala Nagar, Thrissur 680 555, Kerala, India

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Abstract

The antiangiogenic activity of *Piper longum* was studied using in vivo as well as in vitro models. In vivo, antiangiogenic activity was studied using B16F-10 melanoma cell-induced capillary formation in C57BL/6 mice. Intraperitoneal administration of the extract (10 mg/dose/animal) significantly inhibited (50.6%) the number of tumor-directed capillaries induced by injecting B16F-10 melanoma cells on the ventral side of C57BL/6 mice. The cytokine profile in the serum of these animals showed a drastically increased level of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, GM-CSF and the direct endothelial cell proliferating agent, VEGF. Administration of the methanolic extract of *P. longum* could differentially regulate the level of these cytokines. The level of IL-2 and tissue inhibitor of metalloprotease-1 (TIMP-1) was increased significantly when the angiogenesis-induced animals were treated with the extract. The extract of *P. longum* at non-toxic concentrations (10 μg/ml, 5 μg/ml, 1 μg/ml) inhibited the VEGF-induced vessel sprouting in rat aortic ring assay. Moreover, *P. longum* was able to inhibit the VEGF-induced proliferation, cell migration and capillary-like tube formation of primary cultured human endothelial cells. Hence, the observed antiangiogenic activity of the plant *P. longum* is related to the regulation of these cytokines and growth factors in angiogenesis-induced animals.

Keywords: Angiogenesis; Cytokine profile; *Piper longum*; Tube formation

1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing endothelium, is a fundamental step in a variety of physiological and pathophysiological conditions including wound healing, embryonic development, chronic inflammation, and tumor progression and metastasis. Angiogenesis is also indicated in the pathogenesis of variety of disorders: proliferative retinopathies, age-related mascular degeneration, tumors, rheumatoid arthritis and psoriasis [1–5]. Complex and cellular actions are implicated in angiogenesis, such as extracellular matrix degradation, proliferation and migration of endothelial cells to form tubes [6]. The angiogenic process is tightly controlled by a variety of positive or negative regulators, which are composed of growth factors, cytokines, lipid metabolites and cryptic fragments of haemostatic proteins [6] and many of these factors are initially characterized in other biological activities. Altered levels of proinflammatory and proangiogenic factors are observed in various forms of cancer [7] including melanoma [8]. The proinflammatory cytokines such as IL-1β, IL-6, TNF-

* Corresponding author. Tel.: +91 487 2307950; fax: +91 487 2307868.

E-mail address: amalaresearch@rediffmail.com (G. Kuttan).

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α, GM-CSF act as autocrine growth factors for tumor cells. These cytokines could be prometastatic or pro-angiogenic and their deregulated expression directly correlates with the metastatic potential of several human carcinoma [9]. Cytokines have an important role in tissue repair and tumor progression. Among these molecules, vascular endothelial growth factor produced by many tumor and normal cells, plays a key role in regulating normal and abnormal angiogenesis. VEGF is an important growth factor proven to be specific and critical for a mitogen for vascular endothelial cells derived from arteries, veins and lymphatics but it is devoid of consistent and appreciable mitogenic activity for other cell types [10]. It binds to endothelial cell surface receptors and activates various functions of the cell including angiogenesis [11,12]. Since angiogenesis have an important role in the promotion of several diseases such as tumor progression, rheumatoid arthritis and proliferative retinopathies, the antiangiogenic therapy is a promising diversion in the treatment of the above disorders. Thus, the identification of the agents, which could inhibit the tumor-specific angiogenesis, plays an important role in the prevention of metastasis in which angiogenesis do an important role.

Piper longum Linn. popularly known as "Pippali" belonging to the family Pipperacea, an important medicinal plant is used in traditional medicine in Asia and Pacific islands especially in Indian medicine [13]. *P. longum* is a component of medicines which is reported as good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut-related pain and arthritic conditions [14]. Other reported beneficial effects of *P. longum* include analgesic and diuretic effects, relaxation of muscle tension and alleviation of anxiety [15]. In our early studies, *P. longum* has also been shown to possess immunomodulatory and antitumor activity [16]. The present study is to evaluate the antiangiogenic effect of *P. longum* by in vitro as well as in vivo methods and differential regulation of proinflammatory cytokines in B16F-10 melanoma cells injected animals.

2. Materials and methods

2.1. Animals

Four- to six-week-old male C57BL/6 mice weighing 20–25 g were purchased from National Institute of Nutrition, Hyderabad, India. The animals were housed in well-ventilated cages in air-controlled rooms. They were fed with normal mouse chow (Sai Durga Feeds, Bangalore, India) and water ad libitum. All the animal experiments were carried out according to the rules of Animal Ethics Committee, Government of India.

2.2. Cells

B16F-10, a highly metastatic mice melanoma cell line, was procured from National Centre for Cell Sciences, Pune, India and maintained in culture using DMEM (HIMedia, Mumbai) with 10% FCS (Biological Industries, Israel) and antibiotics.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously [17] and used in passages 3–6. The cells were grown in 199 medium supplemented with 20% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 ng/ml growth factors like VEGF and FGF at 37 °C in 5% CO_2 atmosphere.

2.3. ELISA kits

Highly specific quantitative 'Sandwich' ELISA kits for mouse IL-1β, IL-6, TNF-α, GM-CSF and IL-2 were purchased from Pierce Biotechnology, USA and the ELISA kit for VEGF and TIMP-1 was purchased from R&D System, USA. The sensitivity of IL-1β and IL-2 is 3 pg/ml. IL-6, TNF-α and GM-CSF have the sensitivity of 7 pg/ml, 9 pg/ml and 5 pg/ml, respectively. For VEGF and TIMP-1, the sensitivity is 3 pg/ml. The quantikine mRNA probes and calibrator kit for the detection of mouse VEGF was purchased from R&D system USA.

2.4. Extract preparation

2.4.1. Source

Authenticated *P. longum* was obtained from Amala Ayurvedic Centre.

2.4.2. Preparation of alcoholic extract

100 g dried fruit powder was stirred overnight in 70% ethanol (1 L), centrifuged at 7275×*g* for 10 min at 4 °C and supernatant was collected. Methanol was removed in vacuum and the yield obtained was 26%. The extract was resuspended in PBS (pH 7.2). Phytochemical analysis of the extract showed the presence of polyphenolic alkaloid.

2.5. Determination of the effect of *P. longum* in in vivo angiogenesis

Angiogenesis was induced in three groups of C57BL/6 mice (6 mice/group) by injecting B16F-10 melanoma cells (10^6 cells/animal) intradermally on the shaven ventral skin surface of each mouse [18]. Group I animals received phosphate-buffered saline (PBS) and served as vehicle control. Group II was treated with reference compound TNP-470 at a concentration 30 mg/kg body weight as recommended by

NCI. Group III animals were treated with *P. longum* extract at a concentration of 10 mg/dose/animal for five consecutive days.

After 24 h and 9 days of tumor challenge, blood was collected in a sterile manner from the caudal vein of each mouse. Serum was separated and used for the estimation of various cytokines using ELISA kits according to the manufacturer's instructions [19].

After 9 days, all the animals were sacrificed, ventral skin cut removed, washed in PBS and the number of tumor-directed capillaries per cm^2 around the tumor was counted using a dissection microscope [20].

2.6. Determination of the effect of P. longum in in vitro angiogenesis

As described previously [21], aortas were harvested from Sprague–Dawley rats 6 weeks of age. 96-well flat bottom titre plate was coated with collagen, after gelling, the ~1-mm-long aortic rings were placed in the wells and sealed in the place with an overlay of 50 μl of collagen. VEGF with or without condition medium and *P. longum* extract was added to the wells in a final volume of 200 μl of 199 medium with 10% FCS. As control, medium alone was assayed. On day 6, the rings were analyzed by phase-contrast microscopy for microvessel outgrowth.

2.7. Quantitation of gene-specific mRNA of VEGF in B16F-10 melanoma cells

Quantikine mRNA is a novel method, which can be used to quantitate gene-specific mRNA at lower levels. Briefly, B16F-10 cells (1×10^6) were plated in 30-mm petridish in DMEM with 10% FCS at 37 °C in 5% CO_2. Cells were then treated with *P. longum* (10 μg/ml) for 4 h. After the incubation, the cells were washed and mRNA preparations were made according to the manufacture's procedure. mRNAs were hybridized with gene-specific biotin-labeled detection probes and digoxigenin alkaline-labeled detection probes in a microplate. Hybridization solution was then transferred to a streptavidin-coated microplate and the mRNA probe hybrid was captured. Following wash to remove the unbound conjugate, a substrate solution was added. An amplifier solution was then added and the developed colour was measured spectrophotometrically at 490 nm.

2.8. Endothelial cell proliferation assay

[^{3}H]-Thymidine incorporation assay was carried out as described previously [22]. Briefly, HUVECs were seeded at a density of 5×10^4 cells/well in gelatin-coated 96-well plates. After 24 h, various concentrations (10 μg/ml, 5 μg/ml, 1 μg/ml) of *P. longum* extract was added and stimulated by the addition of 2 ng/ml VEGF for 30 h, followed by the addition of 1 μCi/well of [^{3}H] thymidine for 16–18 h. After two washes with ice-cold PBS, high molecular weight DNA was precipitated using 10% trichloroacetic acid at 4 °C for 15 min. ^{3}H radioactivity was solubilized in 0.2 N NaOH and determined by Rack Beta liquid scintillation counter.

2.9. Endothelial cell migration assay

Human endothelial cell motility assay was carried as described previously [23]. Briefly, 96-well titre plate was coated with type-I collagen and incubated overnight at 37 °C. HUVECs were seeded into the coated wells at a density of 2×10^5 cells/well and incubated for 24 h. Scrape the monolayer cells to make a clear area with a narrow tip and wash with serum free medium. Various concentrations (10 μg/ml, 5 μg/ml, 1 μg/ml) of *P. longum* extract followed by VEGF (20 ng/ml) were added into the wells and incubated for 24 h. After incubation, the cells were fixed and stained using crystal violet and photographed (10×).

2.10. Tube formation assay

Tube formation assay was performed as described previously [24]. Briefly, 30 μl of growth factor reduced ice-cold matrigel was pipetted into a 96-well flat bottom titre plate and kept for 30 min at 37 °C. HUVECs were seeded into the layer of matrigel at a density of 1×10^3 cells/well and followed by the addition of 2 ng/ml VEGF and FGF. Various concentrations of (10 μg/ml, 5 μg/ml, 1 μg/ml) *P. longum* extract was added into the wells and incubated for 48 h at 37 °C in the presence of 5% CO_2 atmosphere. After incubation the cells were fixed, stained using Diff Quick stain and photographed (20×).

2.11. Statistical evaluation

The experiments were repeated thrice and all data are presented as mean ± S.D. Statistical analysis was performed by Student's *t*-test. *P*-values less than 0.05 were considered to be significant.

3. Results

3.1. Effect of P. longum on angiogenesis (in vivo)

The ability of *P. longum* to inhibit in vivo tumor-induced angiogenesis was examined by injecting B16F-10 melanoma (10^6 cells/animal) cells intradermally on the shaven ventral side of animals. The number of tumor-directed capillaries was significantly reduced in the *P. longum* extract-treated group. Control animals had an average number of 32.4 ± 3.2 capillaries around the tumor (Fig. 1a) whereas the extract-treated animals had only 15.5 ± 1.8 capillaries (Fig. 1c). The reference compound TNP-470-treated animals had only 3.5 ± 1.0 capillaries (Fig. 1b) showing 88.8% inhibition in neovessel formation when administered subcutaneously to the animals as recommended by NCI (Table 1).

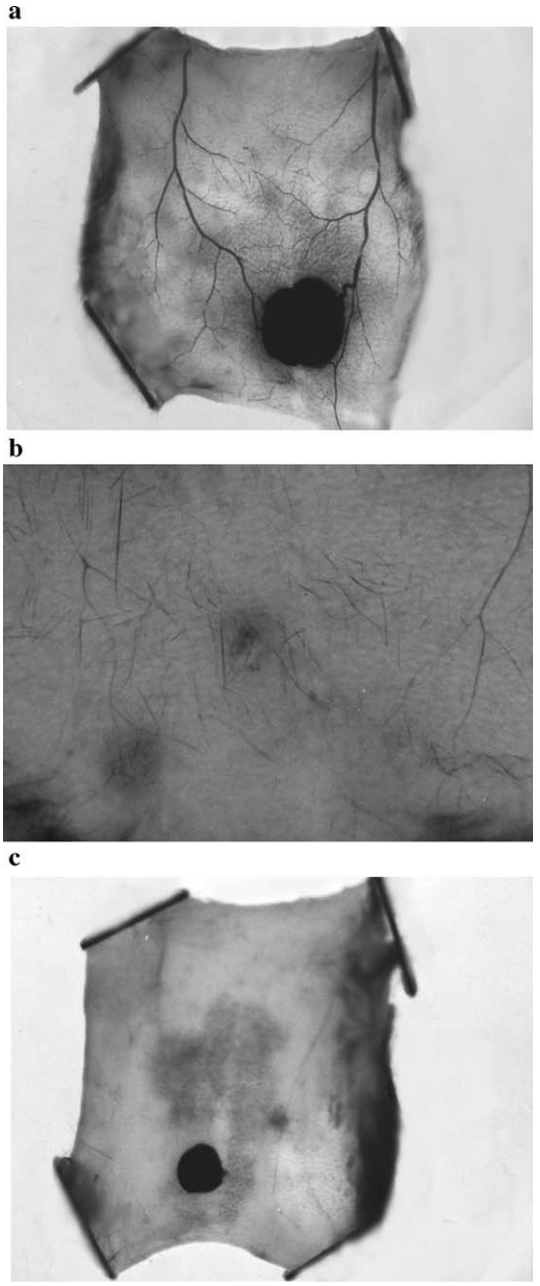

Fig. 1. Effect of *P. longum* on in vivo angiogenesis. (a) Vehicle control. (b) TNP 470 (standard). (c) *P. longum* treated at a concentration of 10 mg/dose/animal. Angiogenesis was induced in C57BL/6 mice by injecting B16F-10 melanoma cells (10^6 cells/animal) intradermally on the shaven ventral skin surface of each mouse and treated with *P. longum* (10 mg/dose/animal) of the extract starting simultaneously with the tumor challenge for 5 days. After 9 days, all the animals were sacrificed, ventral skin cut removed and the number of tumor-directed capillaries per cm^2 around the tumor was counted using a dissection microscope.

3.2. Effect of P. longum on proinflammatory cytokines of angiogenesis-induced animals

Proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in the serum of angiogenesis-induced animals were showing a varying pattern during the period of study. In control animals, the level of IL-1β in the serum was doubled 24 h after tumor cell inoculation (30 ± 4.5 pg/ml) compared to normal (16 ± 3.5 pg/ml) and maintained the same level even after 9 days. Even though extract of *P. longum* did not affect this initial elevation of this cytokine at 24 h, but it effectively reduced to 21.7 ± 3.8 pg/ml by day 9 after tumor induction (Table 2).

TNF-α level (630 ± 8.5 pg/ml) was drastically elevated in the serum of control animals after 9th day of tumor induction compared to the normal level of TNF-α (20 ± 3.2 pg/ml). Administration of *P. longum* could effectively downregulate the elevated level of TNF-α to 185.9 ± 9.2 pg/ml by 9th day of tumor induction. Similarly administration of *P. longum* could lower the elevated level of IL-6 (69.4 ± 5.8 pg/ml), compared to the control animals (320 ± 9.5 pg/ml) after 9th day of tumor induction. The normal level of IL-6 is 35 ± 6.5 pg/ml (Table 2).

3.3. Effect of P. longum on the colony stimulating factor and vascular endothelial cell growth factor levels in B16F-10 cell injected animals

The levels of growth factors such as GM-CSF and VEGF were determined using ELISA kits. In control animals, the level of GM-CSF (30 ± 3.2 pg/ml) and VEGF (150 ± 12 pg/ml) in the serum was elevated after 9th day of tumor induction compared to the normal levels of GM-CSF (18 ± 3.1 pg/ml) and VEGF (16 ± 8 pg/ml). Administration of *P. longum* could effectively lower the elevated level of GM-CSF (20.1 ± 2.5 pg/ml) and VEGF (79 ± 12.4 pg/ml) after 9th day of tumor induction.

3.4. Effect of P. longum on the IL-2 and TIMP-1 profile of angiogenesis-induced animals

The lowered levels of IL-2 in the control animals (16 ± 2.1 pg/ml) 24 h after angiogenesis induction was nor-

Table 1
Effect of *Piper longum* on tumor directed capillary formation

Treatment	No. of tumor directed capillaries/cm^2	% of inhibition
Control	31.4 ± 3.2	–
Piper longum	15.5 ± 1.8*	50.6
TNP 470	3.5 ± 1.5	88.8

The values are mean ± S.D.
Angiogenesis was induced by injecting 10^6 B16F-10 melanoma cells intraperitoneally. All the animals were sacrificed 9 days after tumor challenge and number of tumor-directed microvessels per cm^2 around the tumor was counted using a dissection microscope.

Table 2
Effect of *Piper longum* on pro-inflammatory cytokine profile of angiogenesis-induced animals

Cytokines (pg/ml)	Normal		Control		*Piper longum*	
	Day 1	Day 9	Day 1	Day 9	Day 1	Day 9
IL-1β	16 ± 3.5	–	30 ± 4.5	30 ± 3.7	27.5 ± 4.1	21.7 ± 3.8
IL-6	35 ± 6.5	–	35.3 ± 5.6	320 ± 9.5	39.4 ± 4.6	69.4 ± 5.8
TNF-α	20 ± 3.2	–	180 ± 6.3	630 ± 8.5	178.4 ± 6.7	185.9 ± 9.2
GM-CSF	18 ± 8.31	–	70 ± 5.6	30 ± 3.2	39.8 ± 5.9	20.1 ± 2.5
VEGF	16 ± 8	–	62 ± 9	150 ± 12	58 ± 11.3	79 ± 12.4
IL-2	23 ± 3.2	–	16 ± 2.1	20 ± 2.5	21.8 ± 3.9	33 ± 5.1
TIMP	600 ± 36	–	350 ± 19	360 ± 22	943 ± 28	1002 ± 37

All the values are mean ± S.D.
Blood was collected from the angiogenesis-induced animals (see Table 1) at the indicated time points after tumor challenge. Serum was separated by centrifugation and the cytokine level was estimated by ELISA method.

malized by the treatment with *P. longum* (21.8 ± 3.9 pg/ml). The IL-2 level in the extract-treated animals on day 9 was found significantly enhanced (33.8 ± 5.1 pg/ml) compared to the control (20 ± 2.5 pg/ml) as well as normal (23 ± 3.2 pg/ml) animals.

The tissue inhibitor of metalloprotease level in the serum of normal mice was 600 ± 36 pg/ml, which was reduced by the induction of tumor cell to 350 ± 19 pg/ml in the untreated control animals. But *P. longum* (943 ± 28) treatment could elevate the levels of this MMP inhibitor. Blood sample on 9th day also showed a similar TIMP-1 profile in angiogenesis-induced animals. In control, it was only 360 ± 22 pg/ml and *P. longum* administration (1002 ± 37 pg/ml) could maintain the initial elevation thereby negatively contributing to the formation of neovessels.

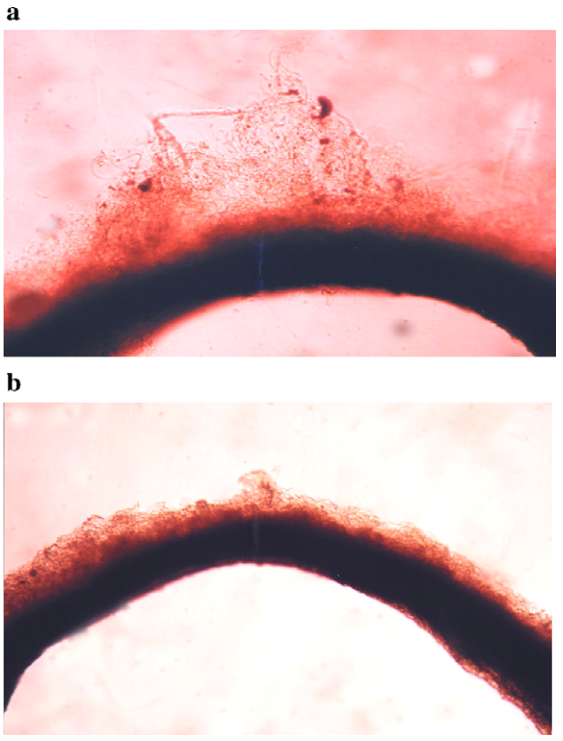

Fig. 2. Rat aortic ring assay showing inhibitory effect of *P. longum* on in vitro angiogenesis. (a) Rat aortic ring in the presence of conditioned medium of B16F-10 melanoma cells alone. (b) Rat aortic ring in the presence of conditioned medium of B16F-10 melanoma cells and extract of *P. longum* (10 μg/ml). Dorsal aorta from a freshly sacrificed Wistar rat was cut into ~1-mm-long pieces and placed in a collagen-coated 96-well plate. The rings were incubated for 24 h at 37 °C in complete medium and then exchanged for conditioned media from the B16F-10 melanoma cells along with *P. longum* extract (10 μg/ml). The rings were further incubated for 6 days and then analyzed by phase-contrast microscopy for microvessel outgrowth.

3.5. Effect of P. longum on the vessel sprouting from rat aorta

The sprouting vessels from aortic rings were investigated to determine whether *P. longum* inhibits VEGF-induced angiogenesis ex vivo. VEGF (20 ng/ml) significantly stimulated vessel sprouting in conditioned medium alone (Fig. 2). The presence of *P. longum* (10 μg/ml) resulted in a significant reduction of vessel sprouting induced by VEGF as well as conditioned medium, and its inhibitory activity was dose dependent.

3.6. Effect of P. longum on the VEGF mRNA expression

The level of VEGF mRNA expression was highly elevated in the untreated B16F-10 melanoma cells (27.65 ± 0.21 attomoles). Treatment with *P. longum* for 4 h could significantly reduce the elevated level of VEGF to 11.1 ± 0.32 attomoles* (*$P < 0.001$).

3.7. P. longum inhibits VEGF-induced proliferation of endothelial cells

To determine antiangiogenic activity of extract of *P. longum* in vitro, its inhibitory effect on VEGF-induced proliferation of endothelial cells was evaluated. *P. longum* inhibited VEGF-induced HUVEC proliferation in a dose-dependent manner (Fig. 3). These inhibitory effects were

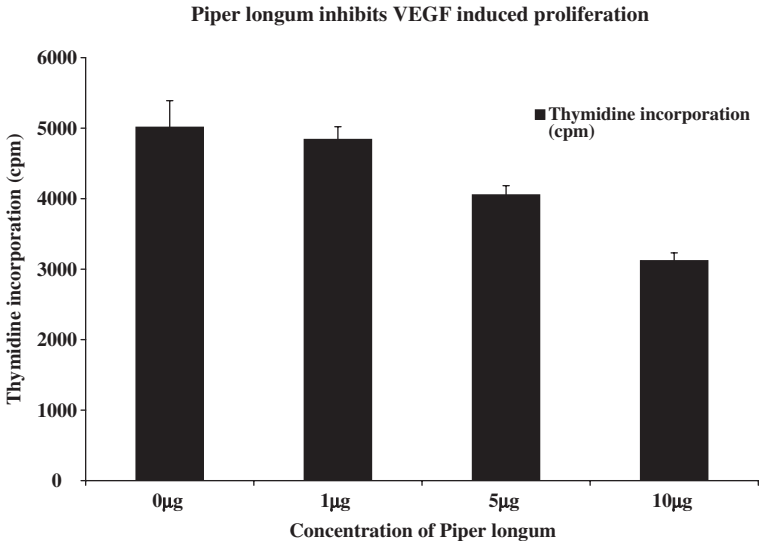

Fig. 3. Effect of *P. longum* on VEGF induced HUVEC proliferation.

not due to cytotoxicity of *P. longum* in endothelial cells. The effect of *P. longum* on DNA synthesis of HUVECs was monitored by [^{3}H] thymidine incorporation assay. VEGF (20 ng/ml) significantly increased DNA synthesis of HUVECs, and this effect was completely blocked by *P. longum*.

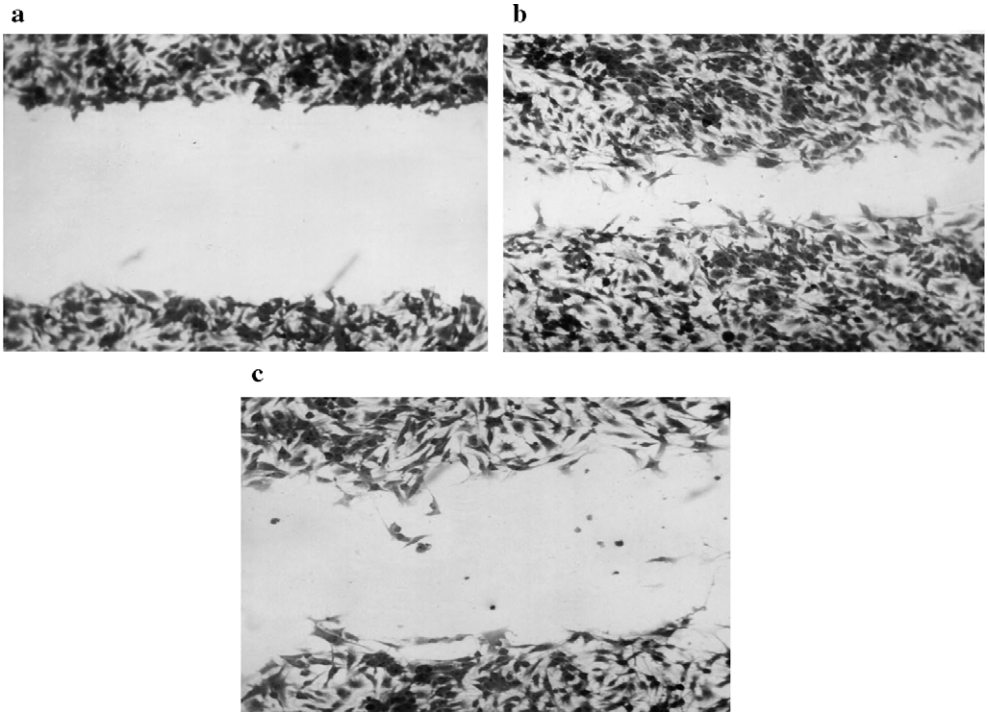

Fig. 4. Effect of *P. longum* on the inhibition of VEGF-induced migration of endothelial cells. (a) '0' h after making a clear area. (b) Control. (c) *P. longum* treated at a concentration of 10 µg/ml. The HUVECs (2×10^5 cells/well) were plated on type-I collagen coated 96-well titer plate and incubated overnight at 37 °C. A clear area was made with a narrow tip in the monolayer cells and VEGF (20 ng/ml) were added into the wells and incubated for 24 h in the presence and absence of *P. longum* extract. After incubation, the cells were fixed and stained using crystal violet and photographed (10×).

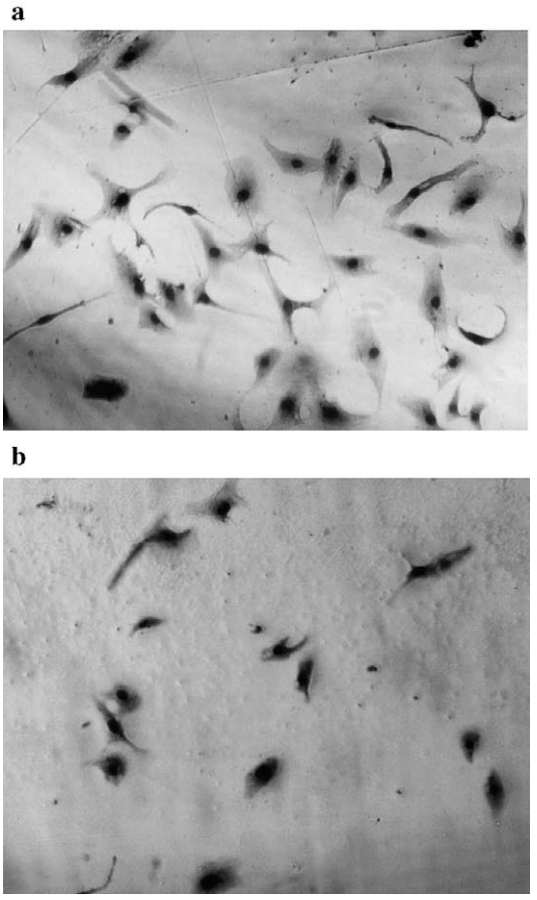

Fig. 5. Effect of *P. longum* on VEGF-induced tube formation of endothelial cells. (a) Control. (b) *P. longum* treated at a concentration of 10 μg/ml. The cells were plated on matrigel at a density of cells/well and induced to form tubes with 20 ng/ml VEGF. After incubating at 37° for 24 h, cells were treated with the presence and absence of *P. longum*. They were incubated for another 48 h and stained with Diff Quick.

3.8. P. longum inhibits VEGF-induced migration of endothelial cells

The effect of *P. longum* on the inhibition of migration was analyzed. The endothelial cell migration was drastically enhanced upon the addition of VEGF to the culture medium. *P. longum* significantly inhibited the VEGF-induced migration of endothelial cells at a concentration of 10 μg/ml (Fig. 4).

3.9. P. longum inhibits VEGF-induced tube formation of endothelial cells

The effect of *P. longum* on morphological differentiation of endothelial cells was investigated using matrigel assay. When HUVECs were placed on growth factor-reduced matrigel in the presence of VEGF, VEGF lead to the formation of elongated and tube-like structures, which were organized by much larger number of cells compared with the control. *P. longum* effectively abrogated the width and length of endothelial tubes in a concentration-dependent manner (Fig. 5). Half maximal inhibition was seen at a concentration of 10 μg/ml. These results demonstrate that *P. longum* has the ability to block VEGF-induced in vitro angiogenesis.

4. Discussion

Solid tumor growth beyond a few mm^2 in diameter requires new blood vessel growth. Without blood flow and nutrient supply, metastasis and growth of solid tumors become impossible. Since angiogenesis is essential for tumor growth, inhibition of angiogenesis has a good chance of preventing cancer from becoming malignant [25,26]. In the present study, we for the first time provide direct evidences that *P. longum* has a potent antiangiogenic activity in the in vitro and in vivo models that can support the tumor preventive action of *P. longum*. Tumor vessel count has been directly correlated with the survival of tumor cells [27] and we found that *P. longum* inhibited tumor-specific new blood vessel formation in C57BL/6 mice when they were injected with B16F-10 melanoma cells and it is notable that *P. longum* selectively inhibited tumor-induced new blood vessel formation without any visible effect on the pre-existing blood vessels.

The levels of some of the proinflammatory cytokines such as IL-1β, IL-6, TNF-α, GM-CSF evaluated in this study are also important in the process of angiogenesis. This also directly correlates with the metastatic potential of several human carcinomas like colon, pancreas, prostate and brain [28,29]. IL-6 has been suggested to be a mediator of morbidity and mortality in patients with metastatic disease [30]. IL-6 is involved in the angiogenic switch during cervical oncogenesis, by inducing VEGF via the STAT3 pathway [31]. TNF-α is among the most potent inducers of transcription factor NF-kB, which has been recognized as a major regulator of pathogen and inflammatory cytokine-inducible gene regulation [32]. Even though GM-CSF did not modulate endothelial functions related to inflammation [33], it induces endothelial cells to proliferate and migrate. In the present study, B16F-10 melanoma cells were induced intradermally to the mouse. 24 h after tumor challenge, the serum levels of the IL-1β, IL-6, TNF-α, GM-CSF got highly elevated in control animals. We found that treatment with *P. longum* could decrease the elevated level of proinflammatory cytokines in B16-F10 melanoma cells induced mice, and this may help to inhibit the angiogenesis directly or indirectly through proinflammatory cytokine mediated transcriptional regulation [31–33]. IL-2 was the first cytokine used clin-

ically for treating cancer [34] and there are reports that IL-2 promotes the proliferation and differentiation of helper T-cells, Cytotoxic T-cells and B-cells; which may augment innate or 'natural' immunity by stimulating natural killer cells [35]. We found that the level of IL-2 was significantly increased by the administration of *P. longum* extract, when compared to the control animals.

TIMP is a multifactorial inhibitor of angiogenesis, tumor growth and tumor invasion [36]. TIMP suppress the MMP activity directly and the ECM degradation due to MMP activity. In addition, it prevents cell growth as well as differentiation and it promote apoptosis [37]. It also inhibits angiogenic factor-induced endothelial cell proliferation in vitro and in vivo independent of MMP inhibition. The extract of *P. longum* could significantly increase the production TIMP-1 and could successfully shift the equilibrium towards an angiostatic condition when compared to the control animals.

Angiogenesis is composed of several processes; dissociation of pericytes from preexisting vessels, digestion of extracellular matrix with proteases, proliferation, migration and invasion of endothelial cells, tube formation, then finally remodeling occurs. VEGF is considered to be secreted from tumor cells in a paracrine fashion to induce blood vessel growth. VEGF promotes angiogenesis in three-dimensional in vitro models inducing confluent microvascular endothelial cells to invade collagen gels and form capillary-like structures [38,39]. Even though VEGF is a potent mitogenic stimulation of endothelial cells, several studies have demonstrated the ability of VEGF to function as a survival factor for endothelial cells [40]. Since VEGF is generated from a variety of tumors, it is the most important angiogenic factor associated closely with induction and maintenance of the neovasculature in human tumors [41,42]. *P. longum* treatment could inhibit the expression of VEGF mRNA level of B16F-10 melanoma cells in vitro. Supporting to this, in this study we found that treatment with *P. longum* could inhibit the level of VEGF in the serum of animals, induced with B16F-10 melanoma cells specific angiogenesis. *P. longum* almost completely suppressed VEGF-induced endothelial cell proliferation, migration and tube formation in a dose-dependent manner. The antiangiogenic activity of *P. longum* was supported by its remarkable suppression in sprouting of endothelial cells in rat aorta.

These antiangiogenic activities of *P. longum* in vivo may be explained by its inhibitory action on proliferation, migration and differentiation of endothelial cells in response to angiogenic growth factors such as VEGF. In addition to this, the increased levels of TIMP and decreased levels of proinflammatory cytokines and VEGF could help to control the survival, differentiation and proliferation of endothelial cells. It is suggested that *P. longum* may possess novel molecular properties that interfere with common angiogenic signaling pathways triggered on growth factor stimulation in endothelial cells.

The attractiveness of angiogenic therapy in cancer treatment seen in preclinical and clinical studies is their generally low toxicity, broad efficacy, and the target that the neovasculature endothelial cells are genetically stable and unlikely to develop acquired resistance. A variety of studies have suggested the inhibitory role of natural product and dietary compounds on tumor specific angiogenesis [43]. Our laboratory has reported the effect of *Tinospora cordifolia* on the inhibition of VEGF production and tumor-specific angiogenesis in mice [18]. In conclusion, our results of in vitro, ex vivo and in vivo all together states that *P. longum* may be used as a potential antiangiogenic agents and it also demonstrate a possible role of *P. longum* in preventing cancer from becoming malignant, presumably via selective curb of neovessel formation in the tumor site.

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