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Antiangiogenic activity of *Andrographis paniculata* extract and andrographolide

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Abstract

Inhibition of angiogenesis is currently perceived as one of the promising strategies in the treatment of cancer. In this study we analyzed the antiangiogenic activity of *Andrographis paniculata* extract (APE) and its major component andrographolide (ANDLE) using both *in vitro* and *in vivo* models. Intraperitoneal administration of APE and ANDLE significantly inhibited the B16F-10 melanoma cell line induced capillary formation in C57BL/6 mice. Analysis of serum cytokine profile showed a drastic elevation in the proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF and the most potent angiogenic factor VEGF in angiogenesis induced animals. Treatment of APE and ANDLE significantly reduced this elevated levels. Moreover, VEGF mRNA level in B16F-10 cell line showed a reduced level of expression in the presence of APE and ANDLE. Serum NO level which was increased in B16F-10 melanoma injected control animals was also found to be significantly lowered by the administration of APE and ANDLE. Antiangiogenic factors such as TIMP-1 and IL-2 level was elevated in APE and ANDLE treated angiogenesis induced animals. In the rat aortic ring assay APE and ANDLE inhibited the microvessel outgrowth at non toxic concentrations. Taken together our results demonstrate that APE and ANDLE inhibit the tumor specific angiogenesis by regulating the production of various pro and antiangiogenic factors such as proinflammatory cytokine, nitric oxide, VEGF, IL-2 and TIMP-1.

Keywords: *Andrographis paniculata*; Angiogenesis; Proinflammatory cytokines; Nitric oxide; VEGF

1. Introduction

It is well established that tumor growth beyond the size of 1–2 mm whether at the primary or metastatic site is angiogenesis dependent [1,2]. Angiogenesis, growth of new capillaries form preexisting vasculature is a fundamental process required for tissue differentiation in embryogenesis while it is rare in adults except during the female reproductive cycle and wound healing [3,4]. Angiogenesis is a tightly controlled multistep process in which pro and antiangiogenic factors are in equilibrium to neutralize one another. Imbalance of this equilibrium due to either upregulation of proangiogenic or down regulation of antiangiogenic mediators induces angiogenesis which include several growth factors and cytokines [5]. The growth of new capillaries is often triggered in conditions of pathological cellular proliferation, ischemia or

Abbreviations: APE, *Andrographis paniculata* extract; ANDLE, andrographolide; IL-1β, interleukine-1β; IL-2, interleukine-2; TNF-α, Tumor Necrosis Factor-α; GM-CSF, Ganulocyte Monocyte-Colony stimulating Factor; VEGF, Vascular endothelial growth factor; TIMP-1, Tissue inhibitors of metalloproteinase-1; MTT assay, 3-[4,5-dimethylthiazol-2-yl]-2,5diphenol tetrazolium bromide assay.

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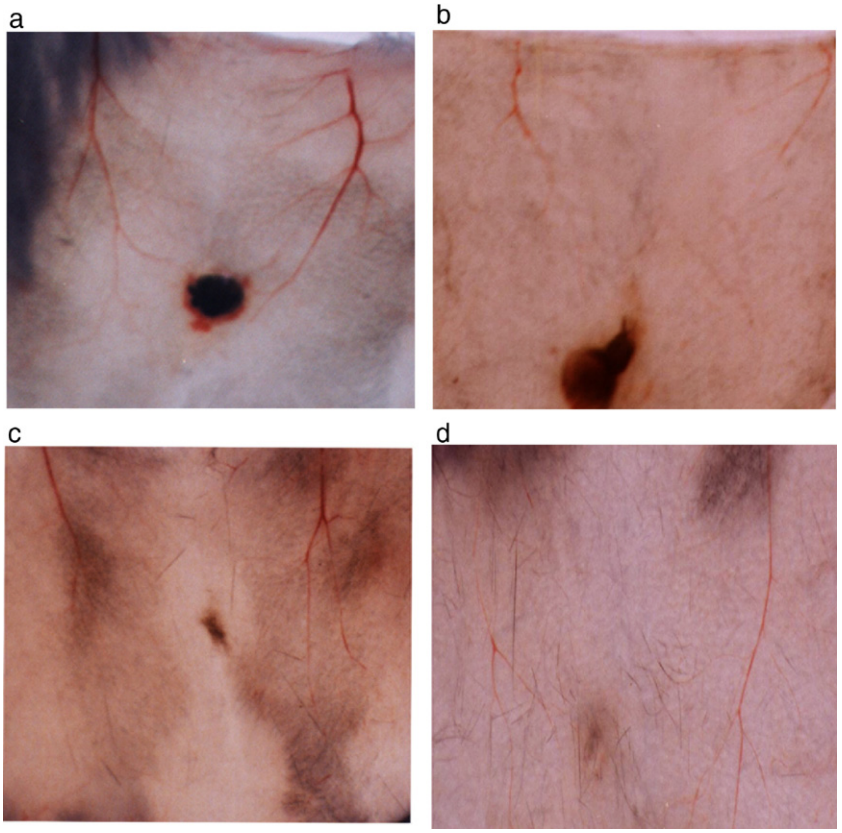


Fig. 1. Effect of APE and ANDLE on *in vivo* angiogenesis. a) Vehicle control b) APE treated (10 mg/dose/animal) c) ANDLE treated (500 μg dose/animal) d) TNP 470 (standard). Angiogenesis was induced in C57BL/6 mice by injecting B16F-10 melanoma cells (10^6 cell/animal) intradermally on ventral skin surface of each mouse and treated with APE and ANDLE started simultaneously with tumor challenge for 5 consecutive days. All animals were sacrificed after 9 days, Ventral skin cut removed and the number of tumor directed capillaries per cm^2 around the tumor was counted using a dissection microscope.

chronic inflammation [6,7]. Unlike the situation in physiological condition, blood vessel growth unabated in cancer and other pathologies and tumor angiogenesis sustains the progression of cancers.

Among various inducers of angiogenesis, the vascular endothelial growth factor (VEGF) is the best characterized and probably the most important molecule with biological effect that includes regulation of hemopoietic stem cell development, extracellular remodeling and inflammatory cytokine generation [8,9]. The role of VEGF on human melanoma metastasis to the lymph node is well proved [10]. As a central mediator of angiogenesis VEGF has emerged as an important target for angiogenic therapy.

Inflammation is a response of vascularized tissue to sublethal injury [11] and considered as one of the major contributors of carcinogenesis. The proinflammatory cytokines such as IL-1β, IL-6, TNF-α, GM-CSF act as autocrine growth factors for tumor cells. The existence of angiogenesis and inflammation was observed in many conditions. Inflammatory cells in the stromal area of many tumors may produce proangiogenic cytokines and growth factors [4]. Tumor Necrosis Factor-α (TNF-α), a cytokine produced early in the inflammatory cascade,

Table 1
Effect of APE and ANDLE on tumor directed capillary formation

Treatment	No. of tumor directed capillaries	% of inhibition
Control	27.33±1.37	
APE	17.5±1.38*	35.96
ANDLE	18.83±1.06*	31.1
TNP 470	3.33±1.46*	87.81

Angiogenesis was induced by injecting 10^6 B16F-10 melanoma cells intradermally.
All the animals were sacrificed 9 days after tumor challenge and number of tumor directed microvessels per cm^3 around the tumor was counted using dissection microscope.
The values are mean±SD. Significance was determined by comparing treated with control.
*$P<0.001$.

and has been shown to promote carcinogenesis in murine skin tumors [12]. When produced chronically it may act as a tumor promoter, contributing to the tissue remodeling and stromal development necessary for tumor growth and spread [13].

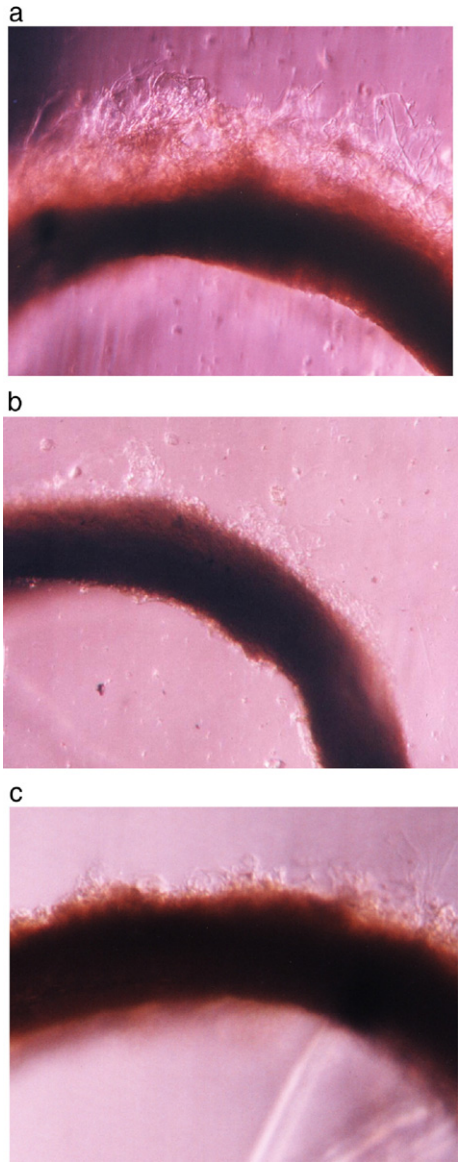


Fig. 2. Rat aortic ring assay showing the effect of APE and ANDLE on *in vitro* Angiogenesis. a) Rat aortic ring in the presence of conditioned medium of untreated B16F-10 melanoma cells. b) Rat aortic ring in the presence of untreated B16 F-10 melanoma cells and APE (10 μg/ml). c) Rat aortic ring in the presence of conditioned medium of untreated B16 F-10 melanoma cells and ANDLE (0.25 μg/ml). Aorta from 8 to 10 week old male Sprague Dawley rat was excised and cut into ~ 1-mm long pieces and placed in a collagen coated 24 well plate. The rings were incubated in the presence and absence of APE (10 μg/ml) and ANDLE (0.25 μg/ml) for 6 days and then analysed by phase contrast microscope for microvessel growth.

Elevated expression of proinflammatory cytokine and proangiogenic factors (IL-1β, IL-6, IL-8, GM-CSF and VEGF) in cultured squamosa carcinoma cell line *in vitro* and in tumor specimen suggest that it may promote tumor growth by inducing angiogenesis [14]. Existence of normal dynamic equilibrium between Matrix mellatoproteinases (MMPs) and Tissue inhibitor of Metalloproteinase (TIMP) is disrupted by suppression of TIMP-1 production as seen in many cancers, which enhance their metastatic spread [15].

Nitric oxide (NO) is an intracellular and intercellular signaling molecule generated from L-arginine, catalysed by a family of enzymes Nitric oxide synthases (NOS), which are either constitutive (cNOS) or inducible (iNOS). A wide variety of biological effects are attributed to this molecule including neovascularization [16,17]. Vasodilation by smooth muscles relaxation, mediated by NO is a prerequisite for endothelial cell to enter the angiogenic cascade [18] and has reported that increased NOS activity correlates positively with increase of vascular density and tumor growth [19].

Natural compounds contains a range of complex organic chemicals that may have synergistic effect on various physiological process in normal and disease state by interacting multiple metabolic pathways [20]. Many naturally occurring compounds have been reported as the inhibitors of tumor specific angiogenesis [21,22]. One of the first isolated antiangiogenic agents is a phytochemical [20].

Andrographis paniculata, a medicinal herb is reported to have antidiabetic [23] and antithrombotic [24] effects. It belongs to the family Acanthaceae and is a rich source of many bioactive compounds. We have already reported its antioxidant and antiinflammory activity [25]. Major active component of this plant andrographolide has shown to possess pharmacological properties which encompass antihepatotoxic [26] and antiviral effects [27]. It is recently been reported that andrographolide inhibits NF-*k*B binding to DNA and thus reducing the expression of proinflammatory proteins such as cycloxygenase-2 (COX-2) [28]. On the bases of these reports we analysed the tumor specific antiangiogenic activity of ethanolic extract of *A. paniculata* (APE) and andrographolide (ANDLE).

2. Materials and methods

2.1. Animals

C57BL/6 mice (4–6 weeks old, 20–25 g body wt.) were purchased from the National Institute of Nutrition, Hyderabad, India. The animals were fed with mouse chow (Sai Durga

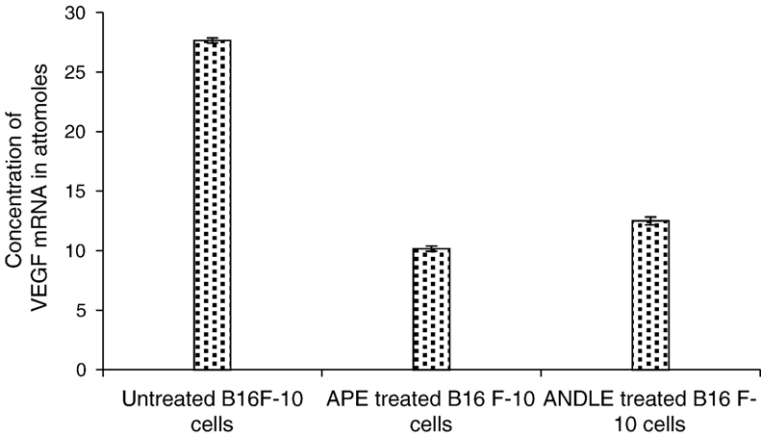


Fig. 3. Effect of APE and ANDLE on VEGF mRNA level in B16F-10 melanoma cells.

Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were conducted according to the rules and regulations of Animals Ethics Committee, Govt. of India.

2.2. Cell lines

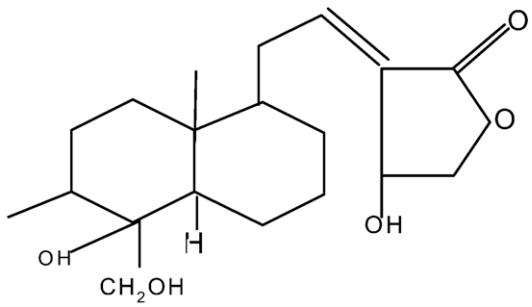
B16 F-10 melanoma cell line was obtained from National Centre for Cell Sciences, Pune, India and maintained in Dulbeco's Modified Egles Medium (DMEM) (Hi Media, Mumbai, India) supplemented with 10% Fetal Calf Serum (FCS) and antibiotics.

2.3. ELISA Kit

Highly specific quantitative sand witch ELISA Kit for mouse IL-1β, IL-6, TNF-α, GM-CSF and IL-2 were purchased from Pierce Biotechnology, USA and ELISA Kit for VEGF and TIMP-1 was purchased from R & D system, USA.

2.4. Preparation of the plant extract and isolation of andrographolide

Air dried whole plant was powdered and extracted using 70% ethanol by stirring overnight at 4 °C. Supernatant was collected after centrifuging at 10,000 rpm at 4 °C for 10 min. Ethanol was evaporated. The yield of the extract was 14%. Phytochemical analysis of the extract showed the presence of terpenoids and flavanoids. Andrographolide was extracted from the dried plant powder according to method of Handa and Sharma [29]. Briefly, the whole plant material was dried, powdered and extracted at room temperature successively with 4 × 40 l petroleum ether (60–80 °C), Chloroform and methanol in soxhlet extraction assembly. The methanolic extract was concentrated and activated charcoal (400 g) was added to it. After 24 h, charcoal was filtered off and the filtrate was concentrated under reduced pressure and left overnight for crystallization. The crystals were collected by filtration and purified by recrystallization. The isolated andrographolide was compared with an authentic sample by thin layer chromatography. The purity of the isolated compound was determined by melting point (230 °C), UV absorption (maximum at 225 nm), ^{13}CNMR and Mass spectrum.



Structure of andrographolide

2.5. Administration A. paniculata extract (APE) and andrographolide (ANDLE)

Initial studies were conducted to determine the non toxic concentration of APE and ANDLE in animal as well as in *in vitro* conditions. Concentrations ranging from 250–10 μg/ml (APE) and 10–0.05 μg/ml (ANDLE) were analyzed for cell proliferation and cell viability by MTT assay. APE at concentration of 10 μg/ml and below was found to be non toxic and ANDLE is nontoxic at a concentration ranging from 0.25 to 0.05 μg/ml.

For the animal administration, *A. paniculata* extract (APE) and andrographolide (ANDLE) was dissolved in minimum volume of ethanol and resuspended in 1% gum acacia and was given intraperitoneally (i.p) at a concentration of 10 mg/dose/animal and 500 μg/dose/animal respectively.

For *in vitro* study both APE and ANDLE were dissolved in minimum volume of ethanol and resuspended in serum free

Table 2
Effect of APE and ANDLE on Serum nitrite level of angiogenesis induced animals

Treatment	Amount of NO (μmoles)		% Inhibition	
	24 h	9th day	24 h	9th day
Normal	20.76±0.41			
Control	23.54±0.50	38.95±0.82	–	–
APE	21.77±1.13*	23.21±0.64**	7.51	40.4
ANDLE	22.29±0.84*	25.33±0.36**	5.31	34.96

Angiogenesis was induced to all animals by injecting 10^6 B16F-10 melanoma cells intradermally.
Blood was collected from each animal at the indicated time points after tumor challenge. Serum was separated and the NO level was estimated by Griess reagent and % inhibition of NO production was also determined. All the values are mean±SD.
Significance was determined by comparing treated with control.
*$P<0.01$.
**$P<0.001$.

DMEM and used at a concentration of 10 μg/ml (APE) and 0.25 μg/ml (ANDLE).

2.6. Determination of the effect of APE and ANDLE on tumor specific angiogenesis in vivo

Three groups of animals were induced angiogenesis by injecting B16F-10 melanoma cells (10^6 cells/animal) intradermally on the shaven ventral surface of each mouse. Group I animals received gum acacia (1%) and served as vehicle control. Group II was treated with APE at a concentration of 10 mg/dose/animal for 5 days, while group III received ANDLE for 5 consecutive days at a dosage of 500 μg/dose/animal.

Blood was collected from caudal vein of each animal after 24 h and 9th day of tumor induction. Serum was separated and used for the estimation of various cytokines (IL-1β, TNF-α, IL-2, IL-6, GM-CSF) and growth factors (VEGF and TIMP-1) levels using ELISA Kit according to manufactures protocol. Serum TNF-α activity was also estimated by Bioassay.

2.7. Bioassay of TNF-α activity by morphological analysis of L929 cells

The L929 cells were plated in 96 well titer plate at a concentration of 5000 cells/well and incubated at 37 °C for 24 h. Serum from all group of animals control as well as treated, were added to each well containing L929 cells. The plates were then incubated at 37 °C in 5% CO_2 incubator for 48 h. After incubation, wells were fixed with 5% formaldehyde and stained with crystal violet and the cytotoxicity of L929 cells were assessed morphologically.

Nitrate level in serum was determined by Griess reaction [30]. Briefly, 100 μl sample was incubated with equal volume of Griess reagent and incubated for 10 min at room temperature and optical density was read at 540 nm. The amount of nitrate was calculated from the $NaNO_2$ standard curve.

After 9 days all the animals were sacrificed, ventral skin was carefully cut removed, washed in PBS and the number of tumor directed capillaries per cm^2 around the tumor was counted using a dissection microscope [31].

2.8. Determination of the effect of APE and ANDLE on in vitro angiogenesis

Rat aortic assay was used as the *in vitro* angiogenesis model [32]. Twenty-four well tissue culture titre plates were coated with type I collagen and allowed to set for 30 min at 37 °C in 5% CO_2 atmosphere. Aorta was excised from 8–10 week old male Sprague Dawley rats. After careful removal of fibroadipose tissues, the aorta was cut into 1 mm long cross section, placed in collagen precoated wells and covered with additional type I collagen and allowed to set. Then aortic rings were incubated in conditioned medium from B16F-10 melanoma cells (100 μl) in the presence and absence of APE and ANDLE. On day 6 the rings were analysed by phase contrast microscopy for microvessel out growth.

2.9. Determination of VEGF mRNA levels in B16 F-10 melanoma cells by enzyme linked immunosorbent assay (ELISA)

Effect of APE and ANDLE on VEGF mRNA level was determined by ELISA method. In this method, B16F-10

Table 3
Effect of APE and ANDLE on proinflammatory cytokine profile of angiogenesis induced animals

Cytokines	Normal	Control		APE treated		ANDLE treated	
(pg/ml)		24 h	9th day	24 h	9th day	24 h	9th day
IL-1β	16.13±1.72	31.74±1.04	32.58±1.83	30.28±1.41	18.28±0.35*	31.51±0.66	19.29±1.56*
IL-6	35.9±3.6	36.6±3.2	326.3±5.58	37.22±3.59	67.93±8.5*	37.76±2.71	78.71±7.96*
TNF-α	20±3.2	188.02±7.3	609.5±8.0	41.16±6.34	89.38±4.5*	159.93±6.5	105.54±6.6*
GM-CSF	18±3.1	65.42±1.06	28.62±1.89	29.35±1.04	19.5±0.12*	31.54±0.62	22.53±1.31*
VEGF	16.2±4.26	60.26±1.78	147±7.2	57±1.31	74.3±1.4*	59.2±4.6	83.63±7.8*

Angiogenesis was induced to all animals by injecting 10^6 B16F-10 melanoma cells intradermally.
Blood was collected from each animal at the indicated time points after tumor challenge.Serum was separated and the cytokine levels were estimated by ELISA method.
All the Values are mean±SD. Significance was determined by comparing treated with control.
*$P<0.001$.

melanoma cells (10^6 cells) were plated in a 30 mm Petri dish in DMEM with 10% FCS at 37 °C in 5% CO_2 atmosphere. The cells were then treated with APE (10 μg/ml) and ANDLE (0.25 μg/ml) for 4 h. After incubation, the cells were washed and mRNA was prepared according to manufactures instructions. The VEGF mRNA levels were calculated in attomoles.

2.10. Statistical analysis

All the data are expressed as mean±SD (standard deviation). The statistical analysis was done by one way ANOVA followed by Bonferroni test.

3. Results

3.1. Effect of APE and ANDLE on tumor specific microvessel sprouting in C57BL/6 mice

The number of tumor directed capillaries were significantly reduced by administration of APE (35.96%) (Fig. 1b) and ANDLE (31.1%)(Fig. 1c). Percentage inhibition in the capillary formation is less in ANDLE treated animals when compared to that of APE treated animals. APE treated animals had an average of 17.5±1.38 capillaries and ANDLE treated group showed an average of 18.83±1.06 capillaries while in vehicle treated control animal the number of tumor directed capillaries was 27.33±1.37 (Fig. 1a). Animals treated with TNP-470 (reference compound) showed 87.81% inhibition in the microvessel formation (Fig. 1d) with average number of 3.33±1.46 tumor directed blood capillaries (Table 1).

3.2. Effect of APE and ANDLE on rat aortic microvessel growth

As shown in Fig. 2a, conditioned medium from untreated B16F10 melanoma cells stimulated the microvessel outgrowth from rat thoracic aorta. Presence of APE at a concentration of 10 μg/ml could inhibit the microvessel sprouting from rat thoracic aorta induced by B16F10 melanoma conditioned medium (Fig. 2b). ANDLE treatment also resulted in suppression of vessel sprouting at a concentration of 0.25 μg/ml (Fig. 2c).

3.3. Effect of APE and ANDLE on VEGF mRNA expression

Untreated B16F-10 melanoma cells showed high level of VEGF mRNA expression (27.65±0.21 attomoles). Treatment with APE at a concentration of 10 μg/ml for 4 h significantly reduced this elevated level of VEGF mRNA to 10.17±0.22 attomoles while ANDLE (0.25 μg/ml) reduced the VEGF mRNA level to 12.5±0.34 attomoles (Fig. 3).

3.4. Effect of APE and ANDLE on serum nitrite levels

The serum nitrite level of control animals at 24 h after angiogenesis induction was slightly increased to 23.54±0.50 μmoles and was again elevated to 38.95±0.82 μmoles on 9th day as the neovascularization progress, compared to normal level (20.76±0.41 μmoles). Administration of APE could reduce the serum nitrite level to 21.77±1.13 μmoles at 24 h and to 23.21±0.64 μmoles on 9th day and the ANDLE treated group also showed reduction in the serum nitrite level as observed at 24 h (22.29±0.84 μmoles) and 9th day (25.33±0.36 μmoles) after angiogenesis induction (Table 2).

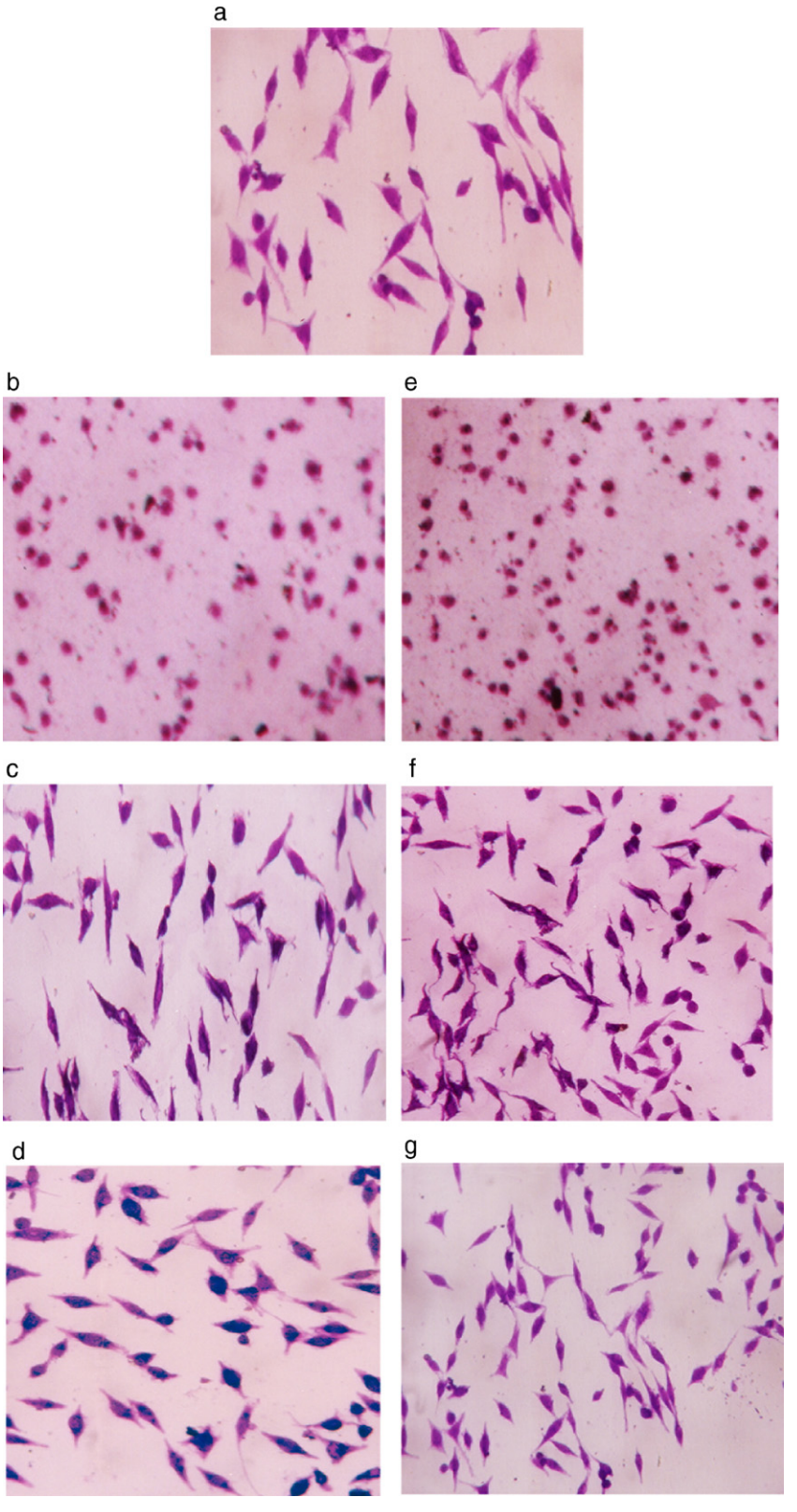
3.5. Effect of APE and ANDLE on proinflammatory cytokine (TNF-α, IL-6, IL-1β) level of angiogenesis induced animals

Alteration in serum proinflammatory cytokine level was observed in C57BL/6 mice in response to angiogenesis (Table 3). In control animals TNF-α level was drastically elevated to 188.02±7.3 pg/ml at 24 h of angiogenesis induction and to 609.5±8.0 pg/ml on 9th day when compared to normal level (20±3.2 pg/ml). Administration of APE and ANDLE was found to effectively lower the elevated TNF-α level to 141.16±6.34 pg/ml after 24 h and to 89.38±4.5 pg/ml on 9th day of angiogenesis induction. ANDLE treatment was also caused reduction in serum TNF-α level to 159.93±6.5 pg/ml and 105.54±6.6 pg/ml after 24 h and 9th day respectively. TNF-α activity in the serum was also assessed by bio-assay using TNF-α sensitive cell line L929. Serum from control animals at two time points (24 h and 9th day) produced complete cytotoxicity to the L929 cells (Fig. 4b–e) where as the serum sample from the APE and ANDLE treated animals (at 24 h and on 9th day) showed less cytotoxic to the TNF-α sensitive cell line, L929 (Fig. 4c–g) as observed from their normal morphology, which was almost same as that of normal (Fig. 4a).

The normal level of IL-1β is 16.13±1.72 pg/ml. In angiogenesis induced animals it was elevated to 31.74±1.04 pg/ml after 24 h and to 32.58±1.83 pg/ml on 9th day. Administration of APE and ANDLE could significantly lower this level to 18.28±0.35 pg/ml and 19.29±1.56 pg/ml respectively on 9th day of tumor induction.

Serum IL-6 level was slightly increased at 24 h in control (36.6±3.2 pg/ml), APE (37.22±3.59 pg/ml) and ANDLE (37.76±2.71 pg/ml) treated animals. On 9th day it was drastically elevated to 326.3±5.58 pg/ml in control

Fig. 4. Bioassay. a) Normal L929 cells. b) L929 cells incubated with serum from vehicle treated control animal after 24 h. c) L929 cells incubated with serum from APE treated animal after 24 h. d) L929 cells incubated with serum from ANDLE treated animal after 24 h. e) L929 cells incubated with serum from vehicle treated control animal after 9 days. f) L929 cells incubated with serum from APE treated animal after 9 days. g) L929 cells incubated with serum from ANDLE treated animal after 9 days. L929 cells (5000 cells/well) were plated in 96 well titre plate. After 24 h Serum form vehicle treated and APE and ANDLE treated animals at two time points (24 h and 9th day) after angiogenesis induction, was added to L929 cells and incubated at 37 °C in 5% CO_2 atmosphere for 48 h. After incubation, cells were fixed with 5% formalin and stained with crystal violet and cytotoxicity was assessed morphologically.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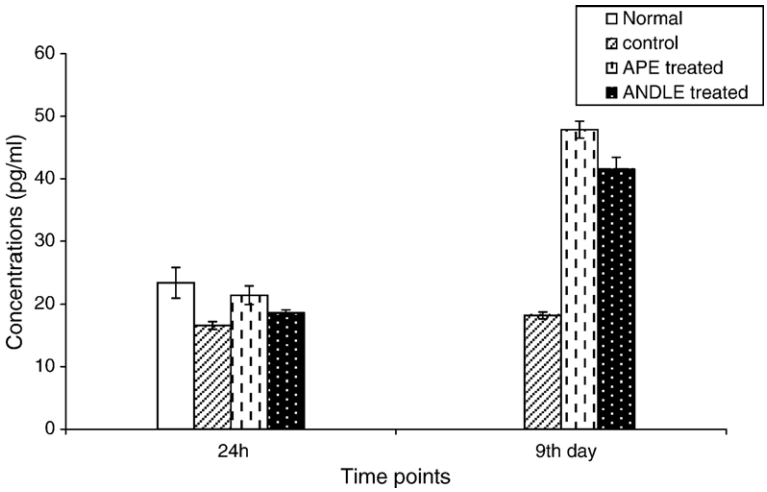

Fig. 5. Effect of APE and ANDLE on IL-2 profile in angiogenesis induced mice.

animals which was significantly reduced to 67.93±8.5 pg/ml and 78.71±7.96 pg/ml in the APE and ANDLE treated animals respectively (Table 3).

3.6. Effect of APE and ANDLE on colony stimulatory factor and VEGF level in angiogenesis induced animals

Angiogenesis induced control animals showed elevated levels of GM-CSF at 24 h (65.42±1.06 pg/ml) and 9th day (28.62±1.89 pg/ml) after tumor challenge compared to normal (18±3.1 pg/ml) (Table 3). Treatment with APE could lower this level to 29.35±1.04 pg/ml at 24 h and normalized by 9th day (19.5±0.12 pg/ml). Similarly ANDLE administration also could reduce the level to 31.54±0.62 pg/ml serum GM-CSF at 24 h and 22.53±1.31 pg/ml on 9th day respectively.

Serum VEGF level in control animal was elevated to 60.26±1.78pg/ml at 24 h and was again increased to 147±7.2pg/ml with respect to normal VEGF level (16.2±4.26 pg/ml). APE treatment considerably decreased the elevated VEGF level to 57±1.31 pg/ml (at 24 h) and to 74.3±1.4 pg/ml (on 9th day) while it was 59.2±4.6 and 83.63±7.8 pg/ml in ANDLE administered group of animals (Table 3).

3.7. Effect of APE and ANDLE on serum IL-2 and TIMP-1 level in angiogenesis induced animals

The level of IL-2 was found to be lowered in angiogenesis induced animals at 24 h after tumor induction (16.6±0.61 pg/ml) when compared to normal value (23.43±2.45 pg/ml) and it remained in the decreased level throughout the period of study (Fig. 5). When angiogenesis induced animals were treated with APE, IL-2 level was increased to 21.44±1.5 pg/ml and reached a value of 47.85±1.35 pg/ml on 9th day. Animals received ANDLE also effectively enhanced the IL-2 level, but

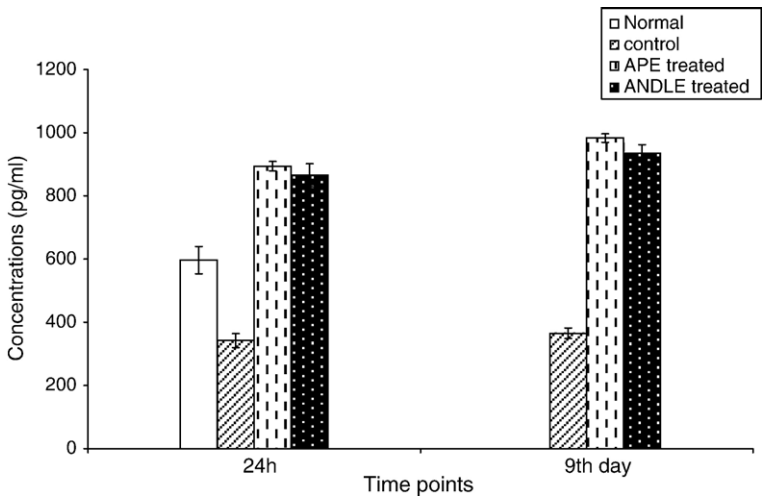

Fig. 6. Effect of APE and ANDLE on TIMP-1 prolife in angiogenesis induced mice.

less when compared to APE treated group of animals. At 24 h the level was 18.56±0.52 pg/ml and increased to 41.53± 1.35 pg/ml on 9th day.

TIMP-1, an inhibitor of MMPs, was reduced in control animals to 342±22.16 pg/ml from normal level (596±43.22 pg/ml) at 24 h and it was slightly increased by 9th day (364.04± 16.78). The APE administered group of animals showed increased production of TIMP-1 at 24 h (894±15.2 pg/ml) and 9th day (983.13±13) and a similar TIMP-1 profile was also observed in ANDLE treated group but the elevation was less compared to APE treated group. At 24 h after tumor challenge TIMP-1 level in ANDLE treated group was 856.74±36.5 pg/ml and it again increased to 935.81±26.31 pg/ml by day 9 (Fig. 6).

4. Disucssion

The angiogenic "switch" is turned on very early during tumorigenesis and may be an important step in the progression of in situ to invasive carcinoma. Inhibition of angiogenesis is currently considered to be the most promising therapeutic strategies to inhibit cancer growth, because it does not induce resistance and has little toxic effect on normal tissue [10]. In this study we analysed the antiangiogenic activity of *A. paniculata* extract (APE) and its active component andrographolide (ANDLE) by multiple assays.

Decreased number of tumor directed capillaries in B16F-10 melanoma cells injected C57BL/6 mice after the administration of APE and ANDLE indicate their potential to selectively inhibit the tumor specific capillary sprouting without damaging the pre existing vasculature. Since tumor vasculature assures the supply of necessary nutrients and CO_2, inhibition of new vessel sprouting should lead to the arrest of tumor growth and spread [33,34].

Angiogenesis and inflammation are codependent process [35]. Inflammation may promote angiogenesis by number of ways [35,36]. Epidemiological studies have revealed a strong association between chronic inflammatory condition and carcinogenesis in several human cancers [37,38]. Inflammatory breast cancer, which is fast growing, invasive, and angiogenic, over expresses several genes involved in angiogenesis [39,40]. Cytokines and angiogenic factors produced by macrophages, mast cells as well as fibroblast stimulate vessel growth [4]. In head and neck squamosa carcinoma patient's proinflamatory cytokine such as IL-6 and GM-CSF are highly expressed [14]. IL-6 is a growth factor for hematological malignancies and found to stimulate VEGF [41,13]. Here, administration of APE and ANDLE significantly reduced the IL-6 level and thus inhibit the tumor directed capillaries in the angiogenesis induced animals. IL-1β, a multifunctional, inflammatory cytokine that affects nearly all cell type often in concert with other cytokine or small mediators [42]. It promotes Lewis lung carcinoma by inducing angiogenic factor from both tumor and stromal cells [43]. The result observed in this study indicates that APE and ANDLE could also inhibit the IL-β production, which in turn inhibit neovascularization.

The major sources of Tumor Necrosis Factor (TNF-α) are macrophages and to lesser extent T lymphocyte, proliferating B cells and NK cells [12]. TNF-α exerted its regulatory effect on both iNOs and VEGF and has been found to up regulate MMP-9 expression and thereby angiogenesis [16,44]. In addition, it augments the expression of adhesion molecule on endothelial cell which may contribute their antiangiogenic activity [13]. Treatment with APE and ANDLE could inhibit the TNF-α level in angiogenesis induced animals, thus prevent the tumor directed new blood vessel formation by down regulating other angiogenic molecules. GM-CSF takes part in angiogenesis events including differentiation of angiobalst into endothelial cells and their migration and proliferation [45]. As observed in our study treatment with APE and ANDLE down regulate GM-CSF production thereby block tumor specific angiogenesis. Decreased proinflammatory cytokine (TNF-α, IL-6, IL-1β and GM-CSF) concentrations in the angiogenesis induced mice after APE and ANDLE treatment demonstrate that one mechanism by which APE and ANDLE exert their antiangiogenic activity by down regulating the inflammatory cytokine production. Similarly, serum NO level was significantly inhibited by the administration of APE and ANDLE with respect to angiogenesis induced control animals. This property of APE and ANDLE directly contribute their antiangiogenic effect.

VEGF a potent growth factor for blood vessel endothelial cells, showing pleiotropic responses that facilitate migration, proliferation of endothelial cells is stimulated by TNF-α, IL-1β, and IL-6 [46,13]. Interestingly VEGF induces NO synthase activity in endothelial cells and increase the permeability of endothelial cell. Hence, VEGF becomes a target for antiangiogenic therapy. As we observed, presence of APE and ANDLE decreased the VEGF mRNA level in B16F-10 melanoma cells is well correlate with the decreased serum VEGF level in angiogenesis induced animals after APE and ANDLE administration. Moreover, rat aortic ring assay also showed inhibition in the sprouting of new vessel from the preexisting endothelial cells strongly support the efficacy of APE and ANDLE to suppress the neovascularization.

IL-2 is of clinical value for stimulating the natural immunity by stimulating Natural Killer cell and cytotoxic T lymphocyte production [47]. Here, in the

present investigation we found that administration of APE and ANDLE significantly increased the IL-2 level which may contribute stimulation of immune system against the tumor growth.

Tissue inhibitors of Metalloproteinases (TIMP-1), natural inhibitors of MMPs which negatively regulate MMPs during extra cellular matrix turn over. Tumor invasion depends in part on the balance of MMPs with TIMP and various other proinflammatory cytokines such as TNF-α can tip the balance in favour of MMP [48]. Treatment of APE and ANDLE significantly up regulate the TIMP-1 level that lead to the inhibition of angiogenesis.

To sum up, the above results altogether prove that *A. paniculata* extract (APE) and andrographolide (ANDLE) could inhibit tumor specific angiogenesis by down regulating various proangiogenic molecules such as VEGF, NO and proinflammatory cytokines and upregulating antiangiogenic molecules like IL-2 and TIMP-1 and may be exploited to prevent tumor growth and metastasis.

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