Evaluation of Antiangiogenic and Antiproliferative Potential in Ethanolic Extract of *Dioscoria bulbifera* L.

Kaveri K, Yashaswini B, Sheela M L and Bharathi P.Salimath*

Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore-570006, Karnataka, India.
*For Correspondence – salimathuom@gmail.com

Abstract

Historically, plant products have enjoyed a rich use for their medicinal properties in herbal medicine. Plant compounds with multiple anticancer characteristics are essential to be developed as anticancer drugs. In the same context, we have made an attempt to screen seven crude ethanolic extracts of medicinally vital plants for their antitumor activity using Ehrlich Ascites Tumor (EAT) model. *Dioscoria bulbifera* L. is a plant used as traditional medicine in mainland China, having antitumor effects in its extracts and/or ingredients. And since, our preliminary results indicated that, *D. bulbifera* rhizomes extract (DBRE) had the best antitumor, antiproliferative and antiangiogenic potential amongst other plants; it was chosen for further *in vitro* and *in vivo* studies. The peritoneum of mice treated with DBRE showed significant reduction in peritoneal angiogenesis, which was further confirmed by inhibition of neovascularization in rat cornea and chick chorioallantoic membrane (CAM) *in vivo*. Additionally, we noted attenuated micro vessel density (MVD) count and endothelial cell proliferation in the histological section of DBRE treated mice peritoneum. Quantitation of VEGF in the DBRE treated ascitic fluid of EAT mice showed significant reduction in VEGF secretion when compared to untreated controls. DBRE also exhibited excellent antiproliferative effects against EAT, choriocarcinoma, breast cancer cells, glioblastoma, endothelial cells *in vitro* in a dose dependent manner. Further, antiangiogenic activity of DBRE in the tube formation assay strengthened the presumption that *D. bulbifera* may be a potential supplementary source for cancer therapy.

Key Words: Angiogenesis, VEGF, *D. bulbifera*, Anti-proliferation, Matrigel, Rat cornea, CAM

Introduction

Angiogenesis, or neovascularization, is the process of generating new blood vessels derived as extensions from the existing vasculature (1). It is an elementary step in a variety of physiological and pathological conditions including wound healing, embryonic development, chronic inflammation, and tumor progression and metastasis (2–5). Complex and diverse cellular actions are implicated in angiogenesis, such as extracellular matrix degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes (6). The angiogenic process is strongly controlled by a wide variety of positive or negative regulators, which are composed of growth factors, cytokines, lipid metabolites, and cryptic fragments of hemostatic proteins (6), and many of these
factors are initially characterized in other biological activities. Among these molecules, vascular endothelial growth factor (VEGF), a soluble angiogenic factor produced by many tumor and normal cells, plays a key role in regulating normal and abnormal angiogenesis and inhibition of VEGF expression by tumor cells is known to have an impact on angiogenesis dependent tumor growth and metastasis (7).

Antiangiogenic agents from medicinal plants appear to be suitable for the control of diseases and prolonging the life of the patient. There has been a continuous search for compounds to use in the prevention or treatment of cancer, and especially for agents with reduced toxicity. Several studies have shown that extracts from a number of herbal medicines or mixtures have already displayed their anticancer/antiangiogenic potential in vitro/in vivo or both (8, 9, 10, 11, 12, 13, 14, 15). Plant-based compounds, such as, resveratrol, catechins genistein, curcumin, in addition to others, such as diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaiacin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, and eugenol have already proved their anticancer abilities (16). Also, numerous antiangiogenic drugs are in different phases of clinical trials presently (17).

All the above facts led us to pursue the search for novel bioactive lead structures with antiangiogenic activity for which seven medicinal plants were selected for further study which are known medicinal plants having anticancer activity. However, their antiangiogenic and antiproliferative effects have not been investigated before. The plants that have been selected for the study include Dioscorea bulbifera L. (Dioscoreaceae), Acorus calamus (Araceae), Annona squamosa (Annonaceae), Streblus asper (Moraceae), Bauhinia variegata (Caesalpinaceae), Thespesia populnea (Malvaceae) and Erythrina suberosa (Fabaceae). The plant D. bulbifera L. is found to possess anti bacterial (18) and antitumor activity (19). The rhizome of Acorus calamus is reported to possess insecticidal properties (20). It is reported that the fruits of Annona squamosa are used in folk medicine as a remedy to treat several microbial diseases (21). Streblus asper has been reported to possess anticancer activity (22). It is used traditionally in leprosy, piles, diarrhea, dysentery, elephantiasis (23). B. variegata has been shown to have anti-inflammatory (24) and antitumor effect (25). The plant T. populnea has been investigated for antibacterial (26), anti-inflammatory (27) and for the treatment of Alzheimer’s disease (28). E. suberosa has been reported to be used for the treatment of dysentery and ulcer (29).

In the present study, an attempt has been made to screen the ethanolic extracts of these seven medicinal plants using in vivo and in vitro assays such as peritoneal angiogenesis assay, rat cornea assay, CAM assay, H and E and CD-31 immunostaining, VEGF quantitation (ELISA), tube formation assay and antiproliferation assay. The results of this study are expected to provide better understanding of the antiangiogenic and antiproliferative potential of the therapeutic plant extracts.

Materials and Methods

The shade dried plant materials (10g each) were extracted exhaustively with 100ml of 50% ethanol at room temperature for a period of seven days. Ethanol was evaporated in order to obtain crude ethanolic extracts of the plants at a concentration of 1mg/ml. The rhizomes of D. bulbifera were collected from Western Ghats, Shimoga, India, in March 2008. The shade dried rhizomes of A. calamus were purchased from Herb Shop in Mysore, India. The plant A. squamosa (except root), the leaves of S.asper, the bark of B. variegata, fruits of T. populnea
and leaves of *E. suberosa* were collected from the campus of University of Mysore, Manasagangotri, Mysore, India in June 2008. Identification of the plant material was confirmed by depositing the voucher specimens in the Herbarium of the Department of Studies in Botany, University of Mysore, Mysore.

Swiss albino mice (6-8 weeks old) and Male Wister rats were obtained from the animal house, Department of Zoology, University of Mysore, Mysore, India. EAT (mouse mammary carcinoma) cells are maintained in our laboratory and are routinely used for *in vivo* transplantation. HUVECs were procured from Cambrex Biosciences, Walkersville, USA. BeWo (Choriocarcinoma) cells, MCF-7 (Breast cancer) cells, U-87 (Brain tumor) cells and HEK 293 (Un-transformed Human embryonic kidney) cell lines were from the National Center for Cell Science, Pune, India.

Endothelial growth medium (EGM-2) was procured from Cambrex Biosciences, Walkersville, USA [3H] thymidine was from the Baba Atomic Research Center, Mumbai, India. DMEM, FBS and penicillin-streptomycin were from Invitrogen, USA. DMEM/Ham’s nutrient mixture F-12 and poly-2-hydroxyethylmethacrylate were from Sigma Aldrich, USA. Fertilized eggs were from a government poultry farm in Bangalore, India. Matrigel was from Becton Dickinson Labware, Bedford, MA. All other reagents were of the highest analytical grade.

**In vitro culture of EAT, BeWo, MCF-7, U-87, Endothelial and HEK-293 cells**: BeWo (Choriocarcinoma) cells were cultured in DMEM Ham’s F-12 medium with 10% FBS, 1% Penicillin-Streptomycin and Gentamycin. EAT, MCF-7, U-87 (Glioblastoma) and HEK 293 cells were maintained in DMEM with 10% FBS and 1% Penicillin-Streptomycin. Endothelial cells were cultured in EGM-2 medium with 2% FBS, 0.04% hydrocortisone, 0.1% long R3-human Insulin like growth factor (IGF-1), 0.1% ascorbic acid, 0.4% human fibroblast growth factor (bFGF), 0.1% VEGF, 0.05% gentamycin and 0.05% amphotericin-B according to the manufacturer’s protocol. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. When the cells reached confluency, they were passaged by trypsinization using 0.025% trypsin/0.01% EDTA. For the experiments, cells from passages 2-5 were used.

**In vivo growth of EAT cells and peritoneal angiogenesis assay**: To understand the mechanism of plant extracts as antiangiogenic compounds, we tested their effect *in vivo* using EAT bearing mice. EAT cells, were maintained by i.p transplantation as described previously (30). In brief, EAT cells or mouse mammary carcinoma cells (5 × 10⁶ cells) were injected intraperitoneally into mice and growth was recorded every day until the 12th day. These cells grow in the mice peritoneum, forming an ascites tumor with massive abdominal swelling. The animals show a dramatic increase in body weight over the growth period and animals succumbed to the tumor burden 14-16 days after transplantation. To verify whether the plant extracts inhibited tumor growth and angiogenesis mediated by EAT cells *in vivo*, plant extracts (133 mg/kg body weight) were injected into the peritoneum of the EAT-bearing mice every day after the 6th day of transplantation. The animals were sacrificed on the 12th day, 2ml of saline was injected (i.p), and a small incision was made in the abdominal region to collect the tumor cells along with ascites fluid. The EAT cells and ascites fluid were harvested into a beaker and centrifuged at 3,000 rpm for 10 min. The ascites volume was measured by subtracting the volume of saline injected while harvesting the EAT cells from the
total ascites volume measured. The pelleted cells were counted by trypan blue dye exclusion method using a hemocytometer. The animals were dissected to observe the effect of the extract on peritoneal angiogenesis. All experiments were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India.

**Corneal micro pocket assay / Rat cornea assay:** This assay was performed in accordance with the method described previously (31). In brief, for the pellet preparation, hydron polymer poly-2-hydroxyethylmethacrylate was dissolved in ethanol to a final concentration of 12%. An aliquot of the Hydron/EtOH solution was added to VEGF (1 μg/pellet) with or without 10 μg/pellet of *Dioscoria bulbifera* rhizome extract (DBRE). Aliquots of 10 μl of 12% Hydron/EtOH alone (Group 1), with cytokine VEGF (Group 2), and with VEGF and DBRE (Group 3) were placed onto a teflon surface and allowed to air dry for at least 2 h. Male Wister rats weighing 300-350 gms were anesthetized with a combination of xylazine (6 mg/kg, IM) and ketamine (20 mg/kg, IM). The eyes were topically anesthetized with 0.5% proparacaine and gently proptosed and secured by clamping the upper eyelid with a non-traumatic hemostat. A corneal pocket was made by inserting a 27-gauge needle, with the pocket’s base 1 mm from the limbus. A single pellet was advanced into the lamellar pocket to the limbus using corneal forceps. The rats were observed for 24-72 h for the occurrence of nonspecific inflammation and localization of the pellets. On day 7, the rats were anesthetized and the corneas were photographed using a CCD camera (40X).

**Chorioallantoic membrane (CAM) assay:** Original chorioallantoic membrane (CAM) assay has long been a mainstay for the study of embryonic organ development. It was carried out in accordance with the method described previously (32). In brief, fertilized eggs were incubated at 37°C in a humidified and sterile atmosphere for 10 days. Under aseptic conditions, a window was made on the eggshell to check for proper development of the embryo. The window was resealed and the embryo was allowed to develop further. On the 12th day, saline, recombinant cytokine VEGF (10 μg per egg) or DBRE (50 μg per egg) was air dried on sterile glass cover slips. The window was reopened and the cover slip was inverted over the CAM. The window was closed again, and the eggs were returned to the incubator for another 2 days. The windows were opened on the 14th day and inspected for changes in the vascular density in the area under the cover slip and photographed at 40X magnification.

**H and E staining for microvessel density scoring:** To determine whether DBRE inhibits microvessel density, its effect on the angiogenic response induced by the cytokine VEGF was verified in EAT-bearing mice. EAT-bearing mice were treated regularly with the extract after the 6th day of transplantation. On the 12th day, the animals were sacrificed and the peritoneum from treated or untreated mice was fixed in 10% formalin. Sections (5 μm) were made from paraffin embedded peritoneum and stained with hematoxylin and eosin. Microvessel counts were done using a Leitz-DIAPLAN microscope with attached CCD camera and photographs were taken at 40X magnification (Table.2).

**CD 31 Immunostaining for assessment of proliferating endothelial cells:** The effect of DBRE on proliferating endothelial cells was determined by staining the paraffin sections of the peritoneum of treated or untreated mice with anti CD 31 antibody. Peritoneum sections were processed as per the protocol supplied by the manufacturer (Santa Cruz Biotechnology, CA, Kaveri et al.
USA). In brief, sections were dewaxed in xylene thrice for 5 min each. The sections were rehydrated in descending concentrations of ethanol (100% ethanol for 5 min, 95% for 2 min and 80% for 2 min) and washed in distilled water. Antigen retrieval was done by heating the sections at 95°C for 15 min in a humidified atmosphere. The sections were treated with 3% H$_2$O$_2$ in PBS to block endogenous peroxidase activity. They were blocked in blocking serum for 30 min to reduce the non specific binding and were incubated with anti-CD 31 (PECAM-1) antibodies for 2 hrs. Following PBS washing, slides were incubated with secondary antibody (biotinylated rabbit anti mouse IgG) for 1 hr at room temperature. The slides were washed in PBS for 5 min and incubated with the substrate (100µl/section) followed by ABC reagent (2 ml histo buffer + 20µl Avidin solution + 20µl Biotin solution). After incubation, the slides were washed in PBS for 5 min. Antigen and antibody complex was detected using substrate (DAB, 100µl/section) for 5 min. The sections were washed thrice for 2 min in tap water and twice for 2 min in distilled water. Subsequently, the slides were counter stained with 2% hematoxylin for 5-7 min and washed again in tap water thrice for 5 min each. The slides were washed successfully for 2 min each in 50% ethanol, 80% ethanol and absolute alcohol. After xylene wash, the slides were mounted using Entellan mountant solution and the sections were scored using DIAPLAN light microscope and photographed.

Quantification of VEGF: The quantification of VEGF was carried out by enzyme linked immunosorbent assay (ELISA) and VEGF was estimated in ascitic fluid collected from both untreated and DBRE treated mice as described previously (33). In brief, 100µl of ascitic fluid from DBRE treated and untreated EAT bearing mice were coated onto 96 well microplates using coating buffer (50mM Na$_4$CO$_3$, pH 9.6) and incubated overnight at 4°C. Wells were washed and blocked using skimmed milk followed by incubation with anti-VEGF antibodies (1:1000 diluted). The wells were washed and probed with secondary antibody (1:5000 diluted) tagged to alkaline phosphatase. P-NPP was used as substrate and absorbance was measured at 405nm with medispec ELISA reader.

Tube formation assay: Tube formation of HUVECs was performed as per manufacturer’s instructions (34). Briefly, a 96 well plate was coated with 50 µl of matrigel which was allowed to solidify at 37°C for 1 h. HUVECs (1 x 10⁴cells/well) were seeded on the solidified matrigel and cultured in EGM containing DBRE (5µg) for 8 h. After 24 h of incubation at 37 °C and 5% CO$_2$, the enclosed networks of complete tubes from five randomly chosen fields were counted and photographed under an Olympus inverted microscope (CK x 40; Olympus, New York, NY) connected to CCD camera at 40x magnification.

Endothelial and Tumor cell proliferation assay: [³H] thymidine incorporation assay was carried out as described previously (35) in endothelial and tumor cells. To verify the in vitro effect of DBRE on proliferation of EAT, BeWo, MCF-7, U-87, HUVEC, and HEK 293 cells, 25,000 cells/well were seeded in 12-well plates in their respective media and grown in 5% CO$_2$ at 37°C for 2 days. DBRE was filter sterilized and diluted with cell culture medium (1 µg/µl). On the 3rd day, [³H] thymidine (1 iCi/ ml medium) and DBRE were tested at the concentrations of 25µg, 50µg, 75µg and 100µg. After 48 h, the cells were trypsinized and washed with phosphate buffered saline (PBS); high molecular weight DNA was precipitated using 10% ice-cold trichloroacetic acid. Scintillation fluid (5 ml) was added to all of the samples and radioactivity was
determined with a liquid scintillation counter. The concentrations of the samples were then plotted against the percentage cell survival.

Results

In vivo treatment of DBRE inhibits growth of EAT cells: Our results in Fig.1 indicate that control EAT bearing mice showed a gradual increase in body weight of about 8.5 ± 2.15 gms over 12 days growth period, when 5 × 10^6 EAT cells were injected on day zero. In the mice treated with various plant extracts from *Dioscorea bulbifera* L., *Acorus calamus*, *Annona squamosa*, *Streblus asper*, *Bauhinia variegata*, *Theespesia populnia* and *Erythrina suberosa*, a significant decrease in body weight was observed in the groups which were treated with DBRE as compared to that of the other extracts selected for screening, indicating the effect of the DBRE in preventing the growth of the tumor cells (p < 0.05). In a fully grown ascites tumor, a volume of 7.5 ± 1.71 ml of ascites was generated during the tumor growth period of 12 days. In DBRE treated mice, the volume of ascites was about 2.5 ± 1.07 ml with p < 0.01 (Table 1). The number of viable cells in full-grown EAT-bearing mice was about 805 ± 1.38 × 10^6/mouse, while this number was reduced in DBRE treated mice to 305 ± 2.06 × 10^6/mouse with statistical significance not reaching p < 0.05 (Table 1), indicating reduction when compared to the control. These results indicate the antitumor activity of DBRE. In a fully grown ascites tumor in vivo, there is extensive peritoneal angiogenesis and in DBRE treated mice, a significant decrease in peritoneal angiogenesis was observed (Figure 2a and 2b).

Angioinhibitory effect of DBRE: The rat cornea assay and CAM assay are commonly used for in vivo validation of the angioinhibitory activity of antiangiogenic molecules. Our results indicate that DBRE has a direct effect on inhibition of angiogenesis in an in vivo model system. When compared to the extensive angiogenesis seen in VEGF treated rat cornea and CAM, angiogenesis at the site of the application of DBRE was significantly reduced. DBRE at 10ìg /eye concentration, showed decreased angiogenesis in the cornea of the rat induced with VEGF (Figure 2c, 2d and 2e). In the CAM assay model, DBRE induced avascular zone formation in the developing embryos thus by inhibiting capillary development on the CAMs at 50 ìg/egg concentrations (Figure 2f, 2g and 2h).

<table>
<thead>
<tr>
<th></th>
<th>Average volume of ascites (ml)</th>
<th>Average number of cells x 10^6/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAT bearing(Control)</td>
<td>7.5 ± 1.71</td>
<td>805 ± 1.38</td>
</tr>
<tr>
<td><em>D. bulbifera</em></td>
<td>2.5 ± 1.07</td>
<td>305 ± 2.06</td>
</tr>
<tr>
<td><em>A. calamus</em></td>
<td>6.7 ± 0.95</td>
<td>675 ± 1.87</td>
</tr>
<tr>
<td><em>A. squamosa</em></td>
<td>6.9 ± 2.08</td>
<td>705 ± 1.09</td>
</tr>
<tr>
<td><em>S. asper</em></td>
<td>7.0 ± 1.09</td>
<td>735 ± 0.98</td>
</tr>
<tr>
<td><em>T. populnia</em></td>
<td>6.9 ± 0.99</td>
<td>705 ± 2.04</td>
</tr>
<tr>
<td><em>B. variegata</em></td>
<td>6.8 ± 1.54</td>
<td>700 ± 1.93</td>
</tr>
<tr>
<td><em>E. suberosa</em></td>
<td>7.0 ± 1.09</td>
<td>740 ± 1.33</td>
</tr>
</tbody>
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Table 1. Average ascites volume and EAT cell number counted using hemocytometer in different plant extracts treated EAT mice in vivo. The results are presented as mean ± standard deviation (n=3).
Inhibition of VEGF production in EAT cells by medicinal plants:
In control EAT bearing mice, over 0-12 day tumor growth period, quantitation of VEGF indicated that there is a gradual production and secretion of VEGF by the treated while in the control sections an average count of 8 was recorded.

**H&E and CD 31 immunostaining:** Comparable reduction in the number of newly formed microvessels in the DBRE treated peritoneum (50 ìg/dose) than that of the control was observed by histological examination of the peritoneal sections of both the group. In this study, the average MVD was significantly higher in control (with an average count of 25) with vascular invasion than in DBRE treated, with an average count of 1 (Figure 3a and 3b). CD 31 is used as a marker for indicating the proliferation of endothelial cells. Our results on CD 31 staining indicated that there was reduction in the number of proliferating endothelial cells in the peritoneum of DBRE treated EAT bearing mice corroborating the results shown in the inhibition of peritoneal angiogenesis in vivo. An average count of 1 in the treated while in the control sections an average count of 8 was recorded.

**Inhibition of VEGF production in EAT cells by medicinal plants:** In control EAT bearing mice, over 0-12 day tumor growth period, quantitation of VEGF indicated that there is a gradual production and secretion of VEGF by
EAT cells. Our results indicate that 140ng of VEGF/ml with \( p < 0.01 \) to be present in the ascites of a fully grown tumor whereas in DBRE treated mice, reduction in the amount of VEGF was noted (31 ng/ml denoting \( p < 0.01 \)) suggesting the inhibition of VEGF secretion (Fig. 4).

**DBRE inhibits tube formation of HUVECs induced by VEGF:** One of the most specific tests for angiogenesis is the measurement of the ability of endothelial cells to form three-dimensional *in vitro* assay was performed to verify the effect of DBRE on the formation blood vessels by HUVECs. HUVECs in basal media could not form tubes and VEGF was used to induce tube formation. In the positive control group stimulated with VEGF (10ng), HUVECs rapidly aligned with one another and formed tube like structures resembling a capillary plexus within 8 hours. However DBRE treatment (1µg/well) prevented VEGF stimulated tube formation of HUVECs. Thus DBRE was shown to interfere with the ability of HUVECs to form *in vitro* vessel like tubes, one of the important traits of endothelial cells (Fig. 5).

**DBRE inhibits in vitro proliferation of tumor cells:** There are numerous well-established
assays for measuring cell proliferation. The most frequently used measure, the thymidine incorporation assay, will serve to introduce several of the key problems of validating *in vitro* angiogenesis assays. Inhibition of proliferation of endothelial cells and tumor cells by DBRE further supported its antiproliferative effect (Fig. 6). HUVECs and different tumor cells like EAT, BeWo, MCF-7, U-87 and untransformed HEK-293 cells were used to verify if DBRE inhibit the proliferation of tumor or normal cells *in vitro*. DBRE efficiently inhibited proliferation of endothelial cells and different tumor cell lines at a concentration range of 25-100 µg although statistical significance was not reached $p < 0.05$. However, no effect was seen in case of untransformed normal HEK-293 cells.

**Discussion**

With the advent of chemo preventive approaches for the treatment of cancer, there is widespread interest in the possibility that this approach may eventually have an effect on, and could improve the quality of life of, human cancer patients. Several natural agents with high anticancer efficacy and no or acceptable toxicity to normal tissues are suggested as possible candidates for use by cancer patients (3, 5, 6, 7, 28). Over the past years, there was a major shift in the development of cancer drugs from screening of cytotoxic drugs to the development of molecular targeted drugs. The conceptual idea is that the knowledge of the mechanism(s) of action of a drug provides a better approach to reach improved clinical results based on patient’s molecular characteristics (phytochemistry and pharmacogenomics). This was the starting point of our effort on the screening for natural products derived from plants of traditional medicinal value.

In the present study, with the aim of finding potent antiangiogenic compounds in plants, seven plants (*Dioscorea bulbifera* L., *Acorus calamus*, *Annona squamosa*, *Streblus asper*, *Bauhinia variegata*, *Thespesia populina* and *Erythrina suberosa*) were screened for their effect on proliferation of tumor cells *in vivo* and *in vitro* for the first time. Preliminary results established markedly that DBRE has potent antiproliferative and antiangiogenic effect on Ehrlich ascites tumor (EAT) cells *in vivo*. DBRE treatment in EAT bearing mice brought about a decrease in the body weight (Figure 1), ascites volume and cell number (Table 1) *in vivo*.

The growth of primary tumors and metastases depends on the degree of tumor neovascularization. Our present study provides compelling evidence that suppression of angiogenesis could be at least one of the mechanisms of the antitumor effect of DBRE.

**Fig. 6.** Effect of DBRE on proliferation of endothelial cells and tumor cells *in vitro*

EAT (A), BeWo (B), MCF-7 (C), U-87 (D), HUVEC (E) and HEK-293 (F) were plated in 12 well plates and incubated for 48h. Plant extract in concentrations 25 µg, 50 µg, 75 µg and 100 µg were added to the wells in duplicates prior to the addition of 3[H]thymidine and incubated for another 48h. The cells were trypsinized after 2 days and processed for scintillation counting. Values are presented as mean ± standard deviation (n=3).
**vivo** angiogenesis in the peritoneum of the treated EAT bearing mice (Figure 2a and 2b). DBRE inhibited VEGF induced angiogenesis in the cornea of the rat (Figure 2c, 2d and 2e). Further, the antiangiogenic activity of DBRE was confirmed with the results of CAM assay which clearly showed inhibition of neovascularization on the CAMs by inhibiting neovascularization (Figure 2f, 2g and 2h) by DBRE.

Vascular invasion and MVD studied by Hematoxylin-eosin staining of peritoneal lining section of EAT bearing mice treated and untreated with DBRE proved that angiogenesis is closely related with microvessel density of tissue and clinical aggressiveness of tumor (Figure 3a and 3b). Further evidence for the antiangiogenic potential of DBRE was seen in the result on inhibition of the extent of proliferating endothelial cells in the peritoneal lining of tumor-bearing mice which was immunostained with anti-CD-31 (PECAM) antibodies (Figure 3c and 3d).

Increased VEGF expression is closely associated with an increase in microvessel density (36). VEGF being a permeability factor plays fundamental role in the fluid accumulation and tumor growth in ascites tumor. By secreting VEGF, ascites tumor enhances the permeability of preexisting microvessel lining of peritoneal cavity to stimulate ascites formation thereby tumor progression. Inhibition of fluid accumulation, tumor growth and microvessel density by neutralization of VEGF has been demonstrated underlying the importance of VEGF in malignant ascites formation (37-39). Our results indicated that there was decrease in the VEGF secretion in DBRE treated EAT bearing mice (Figure 4). Inhibition of VEGF gene expression by DBRE should also be reflected by the levels of VEGF in the ascites secreted by the EAT cells. The current results on quantification of the VEGF in the ascites of EAT bearing mice have clearly indicated that DBRE efficiently decreases the level of VEGF in an *in vivo* model system.

Further, DBRE suppressed human endothelial cell tube formation, which is one of the hallmarks of angiogenesis indicating that DBRE inhibits endothelial cell proliferation. Endothelial cells differentiate and form capillary-like structures when seeded on matrigel. This development entails cell-matrix interaction, intercellular communication and cell mobility like *in-vivo* tumor angiogenesis. The effect of DBRE at a concentration of 1µg/well in HUVEC tube formation was studied and total numbers of tubes formed were counted. Scoring of the total number of tubes showed that DBRE caused 90% decrease in total number of tubes as compared to control (Figure 5). In this assay system, DBRE suppressed human endothelial cell tube formation indicating that it inhibits endothelial cell proliferation and consequently angiogenesis *in-vitro*.

The antiproliferative effect of the DBRE was assessed using four different tumor cell lines EAT, BeWo, MCF-7 and U-87 (Figure 6). DBRE showed strong inhibition of proliferation of all the tumor cell lines and also the HUVECs at four different concentrations (25 ìg, 50 ìg, 75 ìg, 100 ìg). Thus DBRE showed that it was the most active species. In order to test the activity of the extract on normal cells, we assessed the effect of the sample on the proliferation of non-transformed HEK-293 cells. The result indicated that the cancer cells were more susceptible to DBRE than non-transformed cells.

The present investigation represents only a preliminary screen for potent antiangiogenic and antitumor activity and points to the necessity of deeper phytochemical and biological investigations because the plant *D.bulbifera* is potentially interesting in yielding biologically active products.

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This study provides scientific evidence for the ethnobotanical use of the plant *D. bulbifera* which may help research and development of this plant for cancer. As a continuation of this work, the active compounds will be isolated and the underlying mechanism for antitumor activity will be delineated.

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