Original Article

Inhibition of *in vivo* angiogenesis by *Anacardium occidentale* L. involves repression of the cytokine VEGF gene expression

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ABSTRACT: Lethal tumor growth and progression cannot occur without angiogenesis, which facilitates cancer cell proliferation, survival, and dissemination. Among the many growth factors and cytokines engaged in angiogenesis, the cytokine vascular endothelial growth factor (VEGF) is regarded as the most potent and specific. Angiogenesis inhibitors are recognized as potentially useful agents for treating angiogenesis-associated diseases and VEGF represents a promising and well-studied target for antiangiogenic agents. In this study, we have tested the crude ethanolic extract of the leaves of *Anacardium occidentale* Linn, on Ehrlich ascites tumor cells (EAT) *in vivo* and *in vitro*. *Anacardium occidentale* extract (AOE) was able to suppress VEGF-induced angiogenesis *in vivo* in the chorioallantoic membrane, rat cornea, and tumor-induced angiogenesis in the peritoneum of EAT-bearing mice. The extract inhibited cell proliferation of different tumor cells such as EAT, BeWo, and MCF-7 *in vitro* in a dose-dependent manner and it reduced the VEGF level in the ascites of treated mice. A decrease in the microvessel density count and CD31 antigen staining of treated mice peritoneum provide further evidence of its antiangiogenic activity. Our results from Northern blot analysis and ELISA demonstrate that AOE can downregulate endogenous VEGF gene expression at the mRNA and protein level. Furthermore, results of our gene analysis of VEGF-promoter luciferase reporter indicated that this effect is mediated by transcriptional repression of VEGF promoter activity in EAT cells treated with AOE. Taken together, the data suggest that the VEGF system of angiogenesis is the molecular target for the antiangiogenic action of AOE.

Keywords: Angiogenesis, Ehrlich ascites tumor cells, VEGF, *Anacardium occidentale*

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1. Introduction

Tumor angiogenesis is a critical component of tumor growth and metastasis, and the targeting of the vascular supply of tumors is an intense field of interest, with many promising preclinical trials highlighting the potential effectiveness of this form of therapy (*1*). Increased vascularity may allow not only an increase in tumor growth but also greater enhancement of hematogenous tumor embolization. Thus, inhibiting tumor angiogenesis may halt tumor growth and decrease the metastatic potential of tumors. Generated from a variety of tumors, the cytokine vascular endothelial growth factor (VEGF) is the most important angiogenic factor associated closely with induction and maintenance of the neovasculature in tumors (*2*), so the inhibition of VEGF expression by tumor cells is known to have an impact on angiogenesis-dependent tumor growth and metastasis.

A balance between angiogenic and anti-angiogenic factors has given rise to a significant interest in the use of exogenous anti-angiogenic agents for the treatment of solid tumors, and research has demonstrated that anti angiogenic treatment retards tumor growth (*3*). Although new chemotherapeutic drugs of both synthetic and natural origin are occasionally discovered, there is no satisfactory cure for a disease like cancer. Thus, an important step is to screen antineoplastic compounds from plants either in the form of crude extract or as a component isolated from them (*4*). Anticancer agents from medicinal plants appear to be satisfactory 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.

Oriental herbal medicine has been used since ancient times to treat malignancies. Systematic characterization of active phytochemicals in medicinal herbs and their mechanisms of action are important for providing the rationale for their efficacy and for transforming herbal practices into evidence-based medicine. Several studies have shown that extracts from a number of herbal medicines or mixtures have anticancer potential *in vitro*, *in vivo*, or both (*5-8*). For example, alcohol extracts of

Ganoderma lucidum can induce apoptosis in MCF-7 human breast cancer cells (*6*). An aqueous extract of *Paeoniae lactiflora* can inhibit growth of Hep G_2 and Hep 3B hepatoma cells (*8*) whereas aqueous extracts of Bu-Zhong-Yi-Qi Tang (a mixture of 10 herbs) have also suppressed growth of hepatoma cells (*7*). The water soluble ingredients of Sho-saiko-To (a mixture of 7 herbs) inhibit proliferation of KIN-1 human hepatoma cells and KMC-1 cholangiocarcinoma cells (*9*). Finally, PESPES (a mixture of 8 herbs) was developed for clinical treatment of prostate cancer and has been shown to inhibit growth of colon cancer cells (*10*). The crude methanolic extracts obtained from *Hypericum caprifoliatu* and *Hypericum myrianthum* have shown an antiproliferative effect on HT-29 human colon carcinoma cells and H-460 non-small cell lung carcinoma (*11*). A crude aqueous *Sutherlandia frutescens* whole plant extract has been found to induce cytotoxicity in neoplastic cells (cervical carcinoma) and CHO (Chinese Hamster Ovary cells) cell lines (*12*). The antiangiogenic and pro-apoptotic effect of the hexane fraction of *Tinospora cordifolia* on Ehrlich ascites tumor (EAT) cells has also been investigated (*13*).

The cashew, *Anacardium occidentale* Linn., is a multipurpose tree that provides numerous resources and products. The bark and leaves of the tree are used medicinally and the cashew nut has international appeal and market value as a food. Clinical studies have documented this tree's action as an antiseptic, antidysenteric, antibacterial, antiulcerative, antidiabetic, cough suppressant, decongestant, diuretic, febrifuge, refrigerant, and astringent (*14*). The active principles are thought to be tannins, anacardic acid, and cardol. Research has shown that these chemicals curb the darkening effect of aging by inhibiting tyrosinase activity and that they are toxic to certain cancer cells. Anacardol and anacardic acid have shown some activity against Walker carcinosarcoma. Anacardic acid isolated from the nut shell liquid of *A. occidentale* L. has been found to have antibacterial activity against *Streptococcus mutans* ATCC 25175 (*15*). The hydroethanolic extract of *A. occidentale* leaves has been shown to have an antiulcerogenic effect (*14*). *Semecarpus anacardium* Linn of the family *Anacardiaceae* has been tested for its antitumor activity against mammary carcinoma in animals (*16*). The mechanism of antitumor activity of *Semecarpus anacardium* seems to be through the suppression of hypoxic and angiogenic factors (*17*). *Semecarpus anacardium* nut oil has been shown to have an apoptotic effect on the following human tumor cell lines: acute myeloblastic leukemia (HL-60), chronic myelogenic leukaemia (K-562), breast adenocarcinoma (MCF-7), and cervical epithelial carcinoma (HeLa) (*18*). The current study attempts to identify whether the leaf extract of *Anacardium occidentale* L. inhibits tumor growth *in vivo*. This is the first report of the antiproliferative effect of *Anacardium occidentale* L. extract (AOE) on different tumor cell lines *in vitro* and the antiangiogenic effect of AOE *in vivo* through propagation of ascetic transplantable tumors like EAT that grow as cell suspensions in the intraperitoneal cavity of mice.

2. Materials and Methods

Swiss albino mice (6-8 weeks old) 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. BeWo (Choriocarcinoma), MCF-7 (Breast cancer) and HEK 293 (Human embryonic kidney) cell lines were from the National Center for Cell Science, Pune, India. [^{3}H]thymidine and α-[^{32}P]ATP were 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-hydroxyl ethylmethacrylate were from Sigma Aldrich, USA. Fertilized eggs were from a government poultry farm in Bangalore, India. Anti-CD31 antibody was from Santa Cruz Biotechnology, CA, USA. A mammalian transfection kit and β-gactosidase assay kits were from Stratagene, USA. A luciferase reporter assay kit was from BD Bioscience, USA. An RNeasy kit was procured from Qiagen, USA. All other reagents were of the highest analytical grade.

2.1. *Plant material*

The leaves of *Anacardium occidentale L* were collected from the campus of the University of Mysore, Manasagangotri, Mysore, Karnataka, India, in April 2007 and identified by a taxonomist. Identification was confirmed by depositing the voucher specimens in the Herbarium of the Department of Botany, University of Mysore, Mysore (voucher specimen number: UOM. BOT.0133) and by comparing them with available voucher specimens. The leaves were dried in the shade and powdered. The dried leaf material (1 g) was extracted exhaustively with 100 mL of 50% ethanol at room temperature for a period of seven days. Ethanol removal was done by evaporation in order to obtain crude ethanolic AOE of the leaves at a concentration of 1 mg/0.05 mL. The sample was further diluted in saline to obtain the required concentrations for each assay.

2.2. *In vitro culture of EAT, BeWo, MCF-7, and HEK 293 cells*

EAT, MCF-7, and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin. BeWo cells were cultured in DMEM/Ham's nutrient mixture F-12 medium with 10% FBS, 100 units/mL penicillin,

100 µg/mL streptomycin, and 250 µg/mL amphotericin. All cell lines were incubated in a humidified atmosphere of 37°C and 5% CO_2. When cells reached confluency, they were passaged by trypsinizing with trypsin/EDTA and were then used for the experiments.

2.3. *Tumor and normal cell proliferation assay*

A proliferation assay was carried out as described previously (*19*) in tumor and normal cells. To verify the *in vitro* effect of AOE on proliferation of EAT, BeWo, MCF-7, and HEK 293 cells, 50,000 cells/well were seeded in 12-well plates in their respective media and grown in 5% CO_2 at 37°C for 2 days. The sample was filter sterilized and diluted with cell culture medium (1 µg/µL). On the 3rd day, [^{3}H]thymidine (1 µCi/mL medium) was added and AOE was tested at the concentrations of 0.0, 1.0, 5.0, 10.0, and 50 µg/mL. 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. Each concentration of AOE was then plotted against the percentage cell survival. A dose-response curve was thus generated and the IC_{50}, *i.e*. the concentration of the extract required to inhibit cell growth by 50%, was determined.

2.4. *In vivo angiogenesis assays*

2.4.1. *Chorioallantoic membrane (CAM) assay*

Chorioallantoic membrane (CAM) assay was carried out in accordance with the method described previously (*20*). 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 (50 ng per egg) or AOE (100 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 coverslip and photographed at 40 × magnification.

2.4.2. *Corneal micropocket assay/Rat cornea assay*

A corneal micropocket assay was performed in accordance with the method described previously (*21*). 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 AOE (100 µg/pellet). Aliquots of 10 µL of 12% Hydron/EtOH alone (Group 1), with cytokine VEGF (Group 2), and with VEGF and AOE (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 non-specific inflammation and localization of the pellets. On day 7, the rats were anesthetized and the corneas were photographed using a CCD camera (40 ×).

2.4.3. *In vivo growth of EAT cells and peritoneal angiogenesis assay*

EAT cells or mouse mammary carcinoma cells (5×10^6) 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 AOE extract inhibited tumor growth and angiogenesis mediated by EAT cells *in vivo*. Leaf extract 133 mg/kg body weight was injected into the peritoneum of the EAT-bearing mice every day after the 6th day of transplantation. The body weight of the mice was monitored from the 1st day till the 12th day. The animals were sacrificed on the 7th to 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 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.

2.4.4. *Mouse survivability assay*

A mouse survival assay was performed on EAT mice treated with AOE (133 mg/kg body weight) or not every day after the 6th day of transplantation. About 20 EAT-

bearing animals were used in this study; 10 served as a control and the remaining 10 were treated with AOE. The mice were weighed every day starting after the 1st day of tumor transplantation and weighing continued for the duration of their life span. Mice were assessed for mortality twice daily, in the early morning and late afternoon. Mice were euthanized upon reaching the criteria for morbidity. Deaths occurring overnight were recorded the next morning.

2.5. *Immunohistological analysis (H and E staining) for microvessel density scoring*

To determine whether AOE inhibits microvessel density, the effect of the extract 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 40 × magnification.

2.6. *CD31 immunostaining for proliferating endothelial cells in peritoneal blood vessels*

The effect of the extract on proliferating blood vessel endothelial cells was determined by staining the paraffin sections with anti-CD31 antibody as described previously (*22*). Peritoneum sections were processed as per the protocol supplied by the manufacturer. In brief, sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol, and washed in distilled water. Antigen retrieval was done by heating the sections at 95°C for 15 min. The sections were treated with 3% H_2O_2 in PBS to block endogenous peroxidase activity. They were blocked in blocking serum for 30 min to reduce non-specific binding and were incubated with anti-CD31 (PECAM-1) antibodies overnight at 4°C. Following PBS washing, slides were incubated with secondary antibody (biotinylated rabbit anti-mouse IgG) for 1h at room temperature followed by ABC reagent for 45 min. Antigen and antibody complex was detected using a substrate (DAB, 100 µL/section) for 5 min. 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 successively for 2 min each in 50% ethanol, 80% ethanol, and absolute alcohol. After a xylene wash, the slides were mounted using Entellan mountant solution and the sections were evaluated using a DIAPLAN light microscope and photographed (40 ×).

2.7. *Northern blot analysis*

Total RNA was extracted from untreated EAT cells and EAT cells treated with AOE (1 mg/mL of cells) at regular time intervals starting at 0-4 h using an RNeasy kit according to the instructions from the manufacturer. Total RNA (20 µg) was separated by 1.2% agarose-formaldehyde gel electrophoresis and blotted onto a nylon membrane that was baked and hybridized with α [^{32}P]-dATP-labeled $VEGF_{165}$. The hybridized blot was processed and transferred to IP, and the image was scanned with a phosphoimage analyzer. After scanning, the blots were stripped and reprobed for expression of GAPDH as an internal control using labeled GAPDH cDNA.

2.8. *VEGF-enzyme linked immunosorbent assay (VEGF-ELISA)*

VEGF-ELISA was done using ascites fluid collected from treated or untreated mice as described previously (*23*). In brief, 100 µL of ascites sample from AOE-treated or untreated mice were coated onto 96-well microplates using a coating buffer at 4°C overnight. Wells were washed and blocked with blocking buffer (5% skimmed milk powder in PBS) for 2 h at 37°C, followed by incubation with anti-$VEGF_{165}$ antibodies (1:1,000). Recombinant anti-mouse $VEGF_{165}$ was used to set up the standard curve. After incubation for 2 h, the wells were washed before they were treated with 100 µL/well of goat anti-rabbit IgG conjugated to alkaline phosphatase (1:2,000). Incubation was continued for another 2 h at room temperature, and plates were washed prior to addition of 100 µL of the substrate *p*-nitro-phenyl phosphate (*p*-NPP). After incubation for 30 min at room temperature, the reaction was terminated by adding 0.1 N NaOH and the absorbance at 405 nm was measured with a Medispec ELISA reader.

2.9. *Transient transfection and luciferase assay*

To determine the effect of AOE on tumor or normal cells, EAT and HEK 293 cells were respectively transfected with 2 µg of VEGF promoter-luciferase reporter constructs containing the 5' flanking region (–2068 bp) of the human VEGF gene promoter coupled to the promoterless luciferase reporter gene vector pCDNA3 and 2 µg of the β-galactosidase expression vector β-Gal. Transient transfection assays were performed using a calcium phosphate transfection kit according to the manufacturer's instructions. In brief, 2×10^5 cells were seeded in 6-well plates and cultured to 60-70% confluency. The transfected cells were cultured further for 20 h followed by incubation with or without AOE (0.0, 1.0, 5.0, 10.0, and 50 µg/mL). Cells were washed once with PBS and lysed with reporter lysis buffer. Luciferase (Luc) activity of

the cell extract was determined using the luciferase assay system. β-Galactosidase (β-Gal) activity was determined by measuring hydrolysis of *O*-nitrophenyl β-D-galactopyranoside using 50 µL of cell extract at 37°C for 2 h. Absorbance was measured at A_{405}. Luciferase activity was determined using 50 µL of cell extract. The reaction was initiated by injection of 100 µL of luciferase assay substrate. Relative Luc activity (defined as VEGF reporter activity) was calculated as Luc (relative light units per 50 µL cell extract)/β-Gal activity (A_{405} per 50 µL cell extract per 2 h).

2.10. *Plant extraction*

To further verify the chemical nature of the specific fraction, the dried plant powder of *A. occidentale* was extracted sequentially from non-polar to polar solvents, namely petroleum ether-hexane-benzene-chloroform-ethylacetate-acetone-methanol and ethanol. The solvents were evaporated with a rotary evaporator and all of the fractions (100 µg/dose) were tested for antiangiogenic activity *in vivo* in the EAT model.

2.11. *Statistical analysis*

Effects of various groups on various biological outcomes were statistically evaluated using analysis of variance and by use of a Student's *t*-test; levels of significance were evaluated with the *p* value. All experiments were repeated at least three times to ensure reproducibility. The results are expressed as means ± SE, with $p < 0.05$ considered to be statistically significant.

3. Results

3.1. *AOE inhibits in vitro proliferation of tumor cells*

EAT, BeWo, MCF-7, and untransformed HEK 293 tumor cells were used to verify if AOE inhibits the proliferation of tumor or normal cells *in vitro*. AOE inhibited proliferation of different tumor cell lines in a dose-dependent manner. As shown in Figures 1 A, B, C and D, a maximum of 80%, 85%, 70%, and 20% inhibition of proliferation was seen in EAT, BeWo, MCF-7, and HEK 293 cells, respectively. When compared to the effect of AOE on tumor cells, little or no effect was seen with untransformed normal HEK 293 cells. The IC_{50} of the extracts are shown in Table 1. The IC_{50} of AOE on EAT, BeWo, and MCF-7 cells was at concentrations between 1-20 µg/mL. AOE had a very similar inhibitory effect on all three cell lines. The IC_{50} shows 50% inhibition of growth of cells at a given concentration. Here, a higher IC_{50} value means a less toxic extract. The IC_{50} of AOE on HEK -293 cells was significantly greater than that on other cell lines, indicating that AOE was less toxic to normal cells than to cancer cell lines.

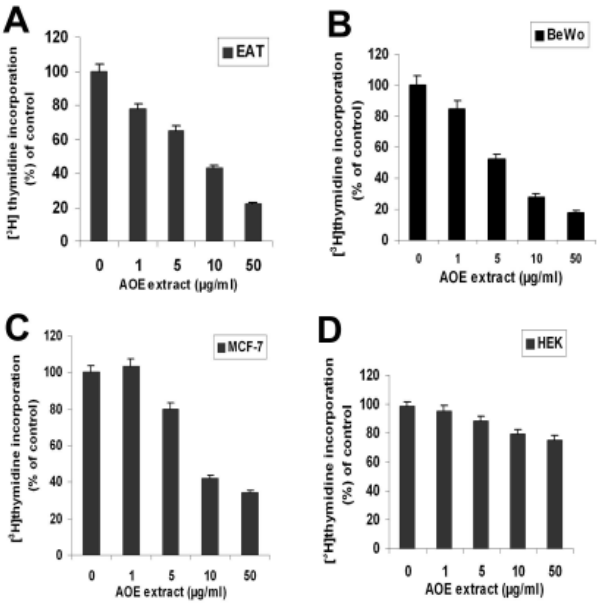

Figure 1. Effect of AOE on proliferation of normal and tumor cell lines. EAT (A), BeWo (B), MCF-7 (C), and HEK 293 cells (D); cells (50,000/well) were treated with AOE or left untreated in the presence of [^{3}H] thymidine (1 µCi/mL). After 48 h of incubation, the incorporated [^{3}H] thymidine into the cells was quantified by scintillation counting. All data are presented as the mean from three different experiments with triplicates and means of ± S.E.M.

Table 1. Antiproliferative activity (IC_{50} value, µg/mL) of the crude ethanolic extract of *Anacardium occidentale* L

Sample	EAT	BeWo	MCF-7	HEK-293
AOE	8.11469	5.54608	15.85254	597.36224

3.2. *Angioinhibitory effect of AOE*

The CAM assay and rat cornea assay are commonly used for *in vivo* validation of the angioinhibitory activity of antiangiogenic molecules. Results shown in Figures 2A and B indicate that AOE has a direct effect on inhibition of angiogenesis in an *in vivo* model system. When compared to the extensive angiogenesis seen in VEGF-treated CAM and rat cornea, angiogenesis at the site of the application of AOE was significantly reduced.

3.3. *In vivo treatment of AOE inhibits growth of EAT cells and extends the survival period*

The result in Figure 3A indicates that control EAT-bearing mice had a gradual increase in body weight of about 8-10 gms when 5×10^6 cells were injected on day zero. When compared to the body weight of control EAT-bearing mice on day 12, the body weight of the treated mice decreased significantly by about 50%, indicating the effect of the extract in preventing the growth of tumor cells. In a fully grown ascites tumor, a volume of 8-9 mL of ascites is usually generated during the tumor growth period of 12 days. In AOE-treated mice, the volume of ascites was about 1-2 mL (Figure 3B). The number of viable cells in full-grown EAT-bearing mice is about 48 $\times 10^6$/mouse while this number was reduced in AOE-

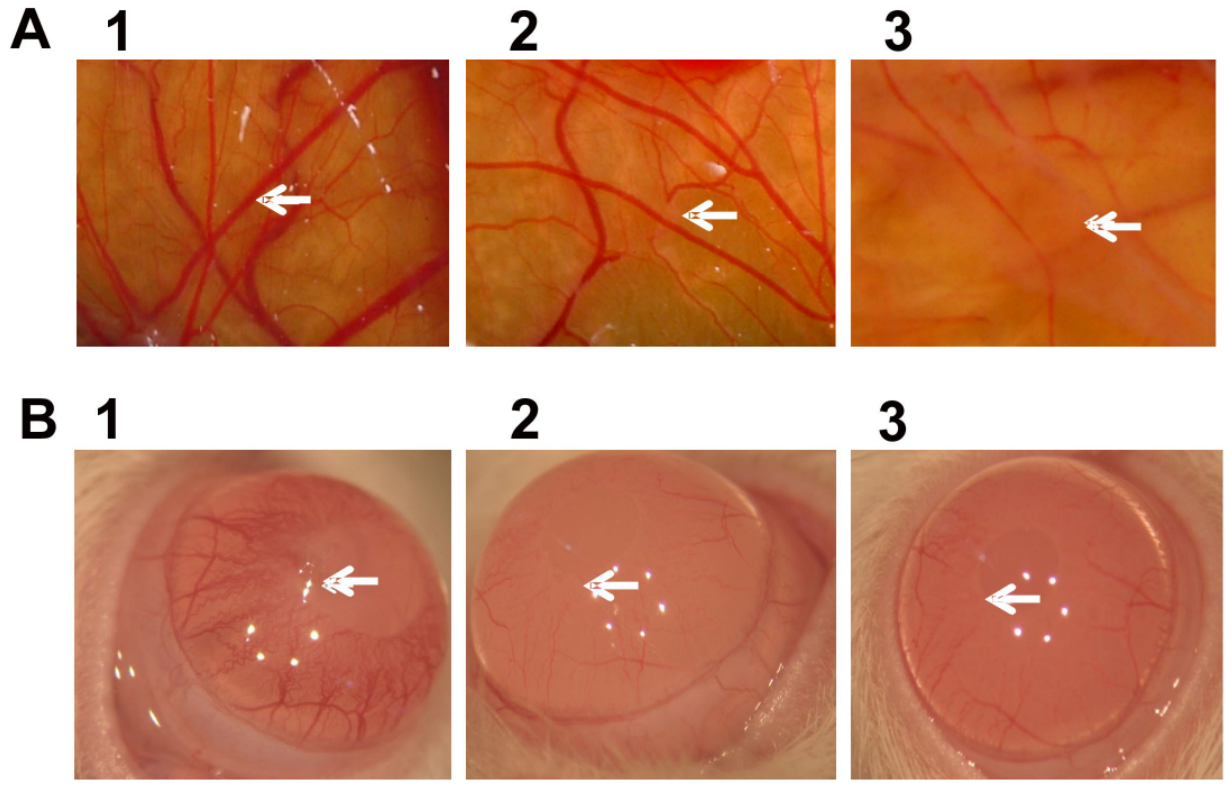

Figure 2. Effect of AOE on blood vessel regression in the chick CAM and rat cornea assays. A) VEGF alone (1) (+ control), saline (2) (– control), or VEGF + AOE (100 µg) (3) was applied to the CAM of 11-day-old chicken embryos. After 48 h of incubation, the treated area was inspected for changes in neovascularization. The arrows indicate the treated area. The data shown represent the result of an experiment that was done using a maximum of six eggs in each group. All photographs were taken at 40 × magnification. B) Photographs of VEGF-induced neovascularization observed in rat corneas: 1) hydron polymer + VEGF (1 µg) (+ control), 2) hydron polymer alone (– control), and 3) hydron polymer + VEGF + AOE (100 µg). Details of the experiments are described in the Materials and Methods. After 7 days of incubation, the corneas were photographed at 40 × magnification.

treated mice to 6×10^6/mouse (Figure 3C), indicating an 8-fold reduction when compared to the control. These results indicate the antitumor activity of AOE. In a fully grown ascites tumor *in vivo*, there is extensive peritoneal angiogenesis, as shown in Figure 3D. In AOE-treated mice, a significant decrease in peritoneal angiogenesis was observed *in vivo*.

Further, the effect of AOE on survivality of EAT-bearing animals was tested. Upon intraperitoneal transplantation of 5×10^6 cells/mice, the EAT-bearing mice survived for 15 days, with tumor cells increasing in number to 15×10^9 cells/mice. The animals succumbed to the tumor burden 15 days after tumor transplantation. AOE treatment (133 mg/kg body weight/dose, every day for 10 doses) extends the survival time of EAT-bearing mice from 15 days up to two months (data not shown).

3.4. *H & E and CD31 immunostaining*

Histological examination of the peritoneal sections of both groups revealed a relative reduction in the number of newly formed microvessels in the AOE-treated peritoneum compared to the control (Figure 4A). CD31 is used as a marker to indicate the proliferation of endothelial cells. The current results of CD31 staining indicate that there is a reduction in the number of proliferating endothelial cells in the peritoneum of AOE-treated EAT-bearing mice (Figure 4B), corroborating the results shown in the inhibition of peritoneal angiogenesis *in vivo*.

3.5. *Inhibition of VEGF mRNA levels by AOE*

In order to investigate the effect of AOE on the VEGF gene, levels of mRNA synthesis were determined in untreated EAT cells or EAT cells treated with AOE and incubated for 30 min to 4 h. As shown in Figure 5, VEGF mRNA levels decreased considerably over a period of 4 h in AOE-treated EAT cells as compared to untreated cells. The decrease in VEGF gene expression was corroborated by the reduction in the amount of VEGF protein as estimated by VEGF-ELISA in the ascites of EAT cells treated with AOE.

3.6. *AOE inhibits VEGF production in EAT cells*

In control EAT-bearing mice, over the 0-12 day tumor growth period quantification of VEGF in the ascites secreted by the growing tumor indicated that there is a gradual production and secretion of VEGF by EAT cells. These results indicate that 1,716 ng of VEGF is present in the ascites of a fully grown tumor whereas 49 ng of

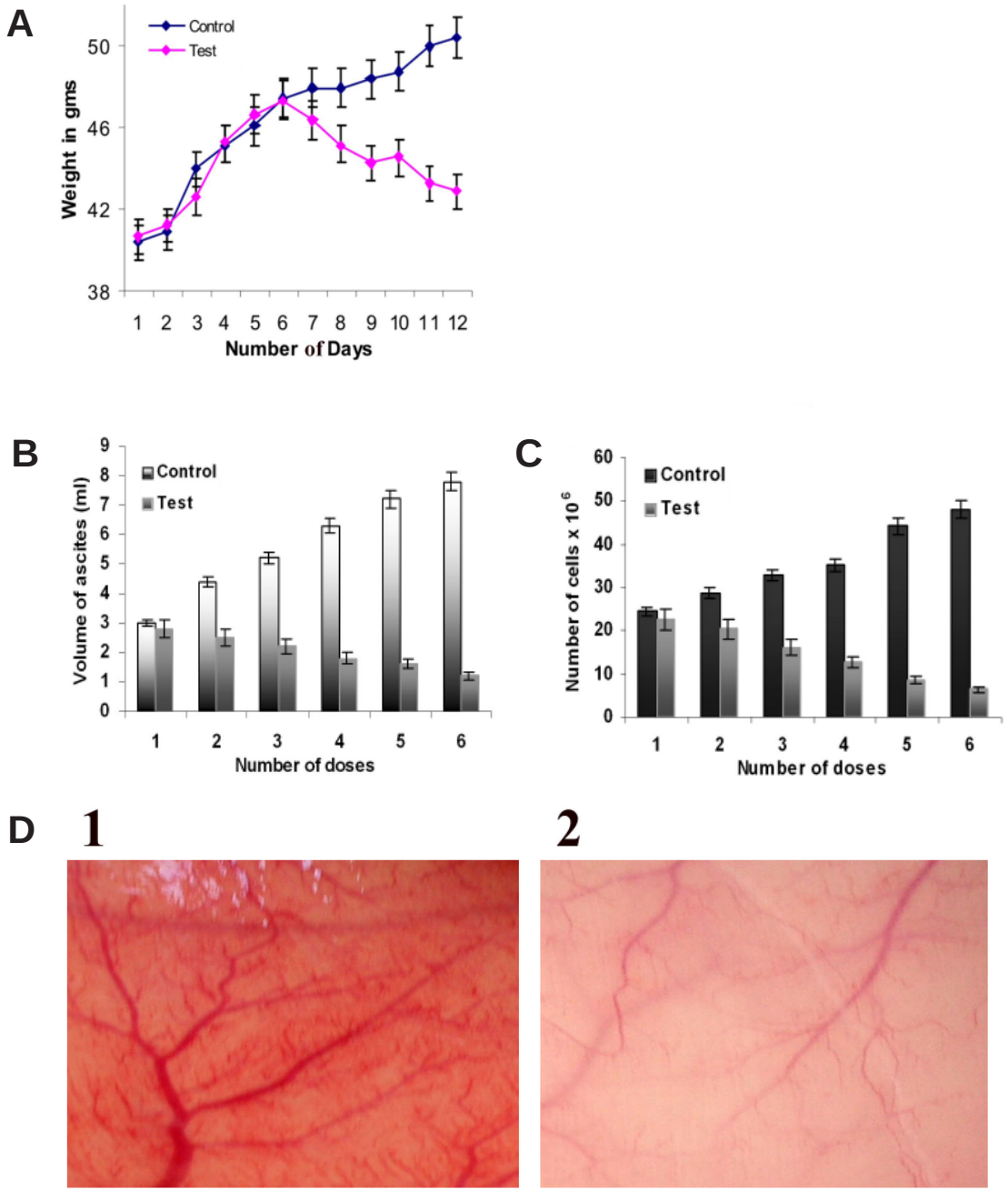

Figure 3. *In vivo* inhibition of tumor growth and angiogenesis by AOE. Body weights of EAT-bearing untreated mice or mice treated with AOE were recorded. From the 6th day onwards, AOE (133 mg/kg body weight) was administered (*i.p*) for six doses every day (A), The animals were sacrificed on the 7th-12th day. EAT cells were collected along with ascites fluid and measured (B), Cells were counted with a haemocytometer (C), The peritoneum of the animal was photographed (D), At least five mice were used in each group and the results obtained are an average of three individual experiments and means of ± S.E.M. $n = 5$ per group.

VEGF per mouse was detected in AOE-treated mice, suggesting the inhibition of VEGF secretion (Figure 6).

3.7. *Down regulation of VEGF gene expression by AOE*

To determine whether AOE modulates VEGF gene expression, the effect of AOE on VEGF promoter luciferase reporter gene analysis was tested. When compared to normal untransformed HEK 293 cells, the activity of VEGF gene expression was 50-60% higher in EAT cells than in normal cells. A dose-dependent inhibition of VEGF gene expression was seen with increasing concentrations of AOE, with a maximum of 80% inhibition with 50 µg/mL of AOE in EAT cells (Figure 7), while a maximum of 25% inhibition of VEGF gene expression was seen in HEK 293 cells.

3.8. *Fractionation and identification of the specific antiangiogenic fraction of A. occidentale*

The dried leaf powder of *A. occidentale* was

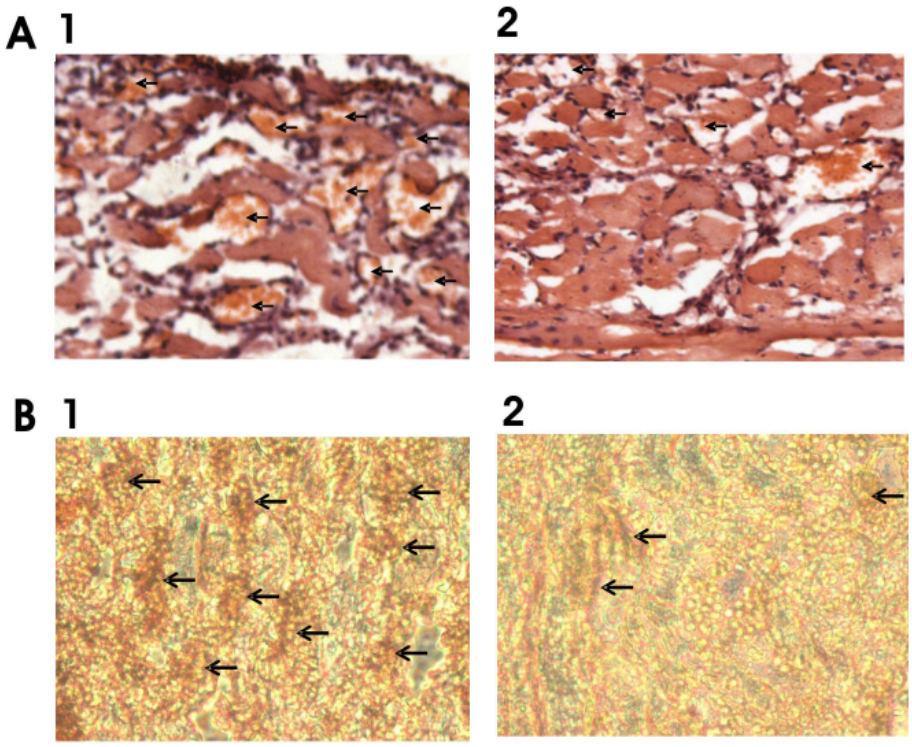

Figure 4. AOE inhibits MVD and proliferation of endothelial cells in mouse peritoneum. A) The peritoneums of control (1) as well as AOE-treated (2) EAT-bearing mice were embedded in paraffin and 5 µm sections were made using a microtome. The sections were stained with hematoxylin and eosin and observed for microvessel density (40 ×). Arrows indicate the microvessels. B) Paraffin sections (5 µm) of peritoneum of control (1) and AOE (2) mice were immunostained with anti-CD31 (PECAM) antibodies. Arrows indicate the stained activated endothelial cells.

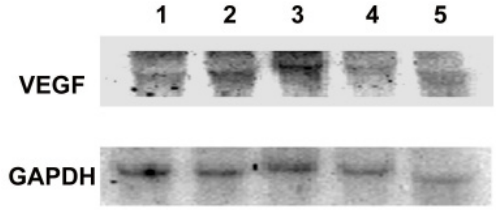

Figure 5. Effect of AOE on expression of mRNA in EAT cells. Total RNA from untreated EAT cells or EAT cells treated with AOE was isolated for varying time periods (Lane 1: Control, Lanes 2-5: 30 min – 4 h) and Northern blot analysis was performed using a $VEGF_{165}$ cDNA probe. GAPDH was used as an internal control. The experiment was performed three times.

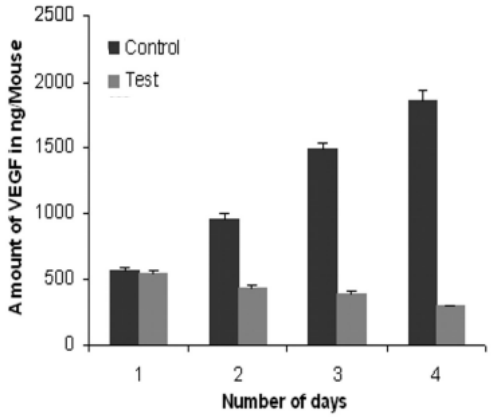

Figure 6. Effect of AOE on VEGF levels in ascites fluid *in vivo*. EAT-bearing mice were injected with AOE (133 mg/kg body weight) for four doses or left untreated, and ascites fluid was collected every day after each dose of treatment. ELISA was carried out after sacrificing the animal to quantify the VEGF in ascites fluid using anti-$VEGF_{165}$ antibodies. Strong inhibition of VEGF expression in AOE-treated mice is evident.

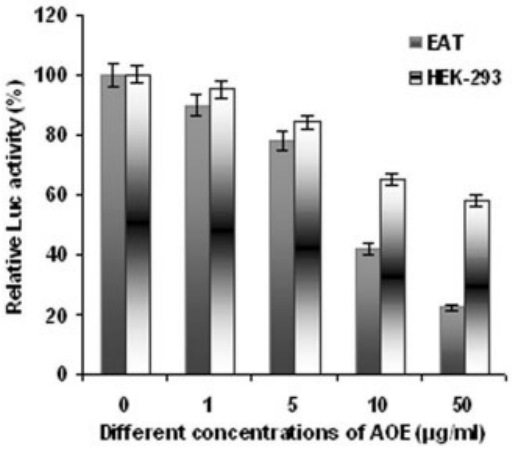

Figure 7. Effect of AOE on VEGF promoter activity. EAT and HEK 293 cells were transiently transfected with 2 µg of pLuc 2068. Forty eight hours later, cells were assayed for luciferase activity. AOE extract repressed VEGF promoter activity in a dose-dependent manner in EAT cells.

sequentially extracted with petroleum ether, hexane, benzene, chloroform, ethyl acetate, acetone, methanol, and ethanol, and all of the extracts were tested for their angioinhibitory effect using the EAT model. Results indicated a decrease in the body weight, ascites volume, cell number, and neovascularization in the peritoneum of EAT-bearing mice treated with an ethanolic fraction of *A. occidentale* (Table 2).

Table 2. Effect of different solvent fractions of *Anacardium occidentale* leaves on EAT growth *in vivo*

Solvent	Body weight		Ascites volume	Cell number	Peritoneal angiogenesis
	Day 1	Day 12			
Petroleum ether	32.6 g	40.1 g	5.0 ml	7.2×10^8/mouse	No inhibition
Hexane	33.0 g	40.6 g	5.2 ml	8.4×10^8/mouse	No inhibition
Benzene	31.9 g	41.3 g	5.5 ml	8.8×10^8/mouse	No inhibition
Chloroform	32.0 g	42.8 g	7.5 ml	8.8×10^8/mouse	No inhibition
Ethyl acetate	33.2 g	39.6 g	5.0 ml	8.8×10^8/mouse	No inhibition
Acetone	33.8 g	40.3 g	5.0 ml	6.8×10^8/mouse	No inhibition
Methanol	33.4 g	43.0 g	7.5 ml	8.96×10^8/mouse	No inhibition
Ethanol	**31.5 g**	**32.0 g**	**0.2 ml**	**0.8×10^8/mouse**	Inhibition
Tumor bearing	32.6 g	43.6 g	7.0 ml	14×10^8/mouse	Extensive angiogenesis

4. Discussion

Tumor growth and metastasis are dependent on the formation of new blood vessels. The most elegant investigation of the correlation between the onset of angiogenesis and tumor growth was carried out by Folkman *et al*. (*24*). The clinical usage of herbal medicine could have an impact on therapy for cancer. The present study was the first to provide direct evidence that an ethanolic extract of *Anacardium occidentale* L. has potent antiangiogenic activity *in vitro* and *in vivo*, corroborating the tumor-preventing action of AOE. The current authors have already reported the effect of a methanolic fraction of *Glycyrrhiza glabra* on EAT cells in screening for antiangiogenic medicinal plants (*25*). Given that angiogenesis is essential for tumor growth, the antitumor effects of AOE may correlate with its antiangiogenic activity. The antiangiogenic activities of AOE *in vivo* may be explained by its inhibitory action on proliferation of tumor cells *in vitro* in a dose-dependent manner as compared to the little effect, if at all, it has on the untransformed normal cell line. The IC_{50} of AOE on tumor cell lines was between concentrations of 1-20 µg/mL while it was significantly greater in normal cells. The lower the IC_{50} value, the more potent the extract is as an inhibitor of tumor cell growth. The current results show that there is inhibition of neovascularization by AOE in the CAM and rat cornea. Inhibition of fluid accumulation, tumor growth, and microvessel density by neutralization of VEGF has been demonstrated, underlining the importance of VEGF in malignant ascites formation (*26-28*). Since there is inhibition of neovascularization by AOE, this supports the view that AOE may repress the expression of VEGF-like factors or inhibit the secretion of such factors, thereby inhibiting the accumulation of ascites fluid and formation of new blood vessels. Further evidence for the antiangiogenic potential of AOE comes from the current results on inhibition of the extent of proliferating endothelial cells in the peritoneal lining of tumor-bearing mice. A significant decrease in peritoneal angiogenesis and levels of stained PECAM in sections of peritoneal wall confirm the antiangiogenic activity of AOE. Research has demonstrated that the density of microvessels was almost doubled in tumors from patients with metastasis (*29*). Further, the role of AOE with regard to the regulation of VEGF expression was investigated at the mRNA, protein, and gene level. This finding is in agreement with the effect AOE has at repressing endogenous VEGF expression, where VEGF is downregulated on an mRNA as well as on a protein level in a time-dependent manner. Moreover, transient transfection assays revealed that AOE downregulated VEGF promoter activity in EAT cells in a dose-dependent manner. This result suggests that transcriptional repression of the VEGF gene represents the mechanism by which AOE downregulates VEGF expression. As in normal angiogenesis, tumor angiogenesis appears to rely heavily on VEGF. In addition to producing VEGF themselves, tumors may induce the production of VEGF in their surrounding tissue; therefore, high levels of VEGF production in a wide variety of tumors and tumor-associated cells suggest that VEGF plays a pivotal role in tumor angiogenesis. Thus, an antiangiogenic agent could conceivably block the paracrine action of tumor cells and hence suppress the proliferation and survival of tumor cells. Inhibition of VEGF gene expression by AOE should also be reflected by the levels of VEGF in the ascites secreted by the EAT cells. In an EAT model, mice produced about 8-9 mL of ascites fluid over a growth period of 12-14 days; this fluid contained 230 ng of VEGF/mL (*30*). The current results on quantification of the cytokine in the ascites of EAT-bearing mice have clearly indicated that AOE efficiently decreases the level of VEGF in an *in vivo* model system. A decrease in ascites formation *in vivo* and in VEGF levels in ascites bears significant importance in terms of a clinical correlation with inhibited ascites formation in human tumors. In conclusion, the present data indicate a possible role AOE has in preventing cancer from becoming malignant, presumably *via* selective curbing of neovessel formation at the tumor site. AOE may hold potential as a pharmaceutical drug and its antiangiogenic activity may contribute to its well-documented clinical activity. Although this study investigated the antiangiogenic activity of the crude ethanolic extract of *A. occidentale*, the leaf powder of this plant has also been fractionated using different solvents based on polarity. An ethanolic fraction

produced a drastic decrease in body weight, ascites volume, cell number, and peritoneal angiogenesis on the 12th day of tumor transplantation as compared to untreated control EAT mice and EAT-bearing mice treated with other solvent fractions. Together, these data confirm that the ethanolic fraction had the maximum antiangiogenic activity. Further purification and characterization of this active compound is in progress.

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