Inhibition of Tumor Growth and Angiogenesis by an Aqueous Extract of *Terminalia bellirica*

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Abstract

The fruit of *Terminalia bellirica*, possess numerous medicinal properties and is used in Indian traditional system of medicine since ancient times. In the light of above ethno-medicinal values of *T. bellirica*, in this study we investigated the antiangiogenic activity of different solvent extracts of *Terminalia bellirica* fruit pericarp (TbFP) using Ehrlich ascites tumor (EAT) model, of which the TbFP aqueous extract (TbFP-Ae) showed highly promising results. In order to grow and metastasize, the tumor cells stimulate the development of new blood vessels through a process known as angiogenesis. Vascular endothelial growth factor (VEGF) promotes majority of cancers. Our results indicate that, in the peritoneal cavity of mice, as measured by body weight, ascites formation and tumor cell number. The peritoneum of mice treated with TbFP-Ae also showed significant reduction in peritoneal angiogenesis, which was further confirmed by inhibition of neovascularization in chorioallantoic membrane (CAM) assay. Quantitation of VEGF using the ascitic fluid from TbFP-Ae treated mice showed significant reduction in VEGF secretion when compared to untreated controls. Additionally we noted the attenuation microvessel density (MVD) count in histological section of mice peritoneum. This is the first report indicating the presence of an antiangiogenic biomolecule in *T. bellirica*.

Key words: *Terminalia bellirica*, ascites tumor growth, peritoneal angiogenesis, VEGF, microvessel density.

Introduction

*Terminalia bellirica* (Gaertn) Roxb is a large deciduous tree, the fruits of which possess numerous medicinal properties and is used as laxative, astringent, rejuvenative, cardioprotective, antacid, antioxidant and antibacterial (1). *Triphala*, a botanical and an ayurvedic preparation comprises of an equal amount of three herbal fruits, *Emblica officinalis*, *Terminalia chebula* and *Terminalia bellirica* is referred as ‘Mother of all healings’. However the antitumor effect of *Terminalia bellirica* has not been paid much attention and needs to be investigated.

In order to grow and metastasize, the tumor cells should stimulate the development of new blood vessels through a process known as angiogenesis. Unlike normal blood vessels, tumor blood vessels are chaotic, irregular, and leaky, leading to an uneven delivery of nutrients and therapeutic agents to the tumor (2). The viability of tumor cells also dependent on the nutrients provided by the vasculature. Hence inhibitors of angiogenesis will starve tumor cells and block...
tumor growth (3) making this process a major target for therapeutic intervention. The principal growth factor that controls angiogenesis is (VEGF). The secretion of VEGF is found to be elevated in a majority of cancers (4,5,6) and hence VEGF is used as prognostic indicator in tumor conditions (7,8,9,10). The expression of VEGF increases angiogenesis, which in turn increases microvessel density (MVD). MVD is used as a surrogate measure of angiogenesis in pathological specimen and tumor models (11). MVD in the peritoneal sections is in vivo indication of proliferation of endothelial cells and neovascularization.

An in vitro model system like chorioallantoic membrane of chick egg is used to validate compounds for their antiangiogenic activity in non-tumor context (17). An ascites tumor growing in peritoneal cavity of mice offers a good model for validation of antiangiogenic efficacy of novel biomolecules. Ehrlich Ascites Tumor (EAT) cells are spontaneous murine mammary adenocarcinoma cells, adapted to ascites form and carried in outbred mice by serial intraperitoneal (i.p) passage. Once EAT cells are injected i.p it takes about 15 days for the tumor to develop completely. During this process the growing EAT cells secrete ascites fluid. Due to rapid growth of tumor cells and ascites burden, the animal succumbs to death within 15 days. It has been earlier reported that the vascular permeability factor (VPF) which is also known as (VEGF) is the key player in tumor angiogenesis and is secreted by (EAT) cells into the ascitic fluid (12). As a consequence, the inner lining of peritoneum shows extensive angiogenesis, which is the growth of new blood vessels.

Currently available chemotherapeutic anti-tumor drugs although effective in reducing cancer risks; lead to development of resistance in cancer Cells and patients often experience several adverse side effects (13, 14). In this context, natural compounds from plant kingdom plays a major role and form good replacement. In the light of above ethno-medicinal values of plants, in this paper we have used EAT model system in order to identify the antiangiogenic bio-molecule from Terminalia bellirica extracts. Our results indicate that the identified Terminalia bellirica fruit pericarp aqueous extract contained the antiangiogenic molecule and at molecular level the biomolecule inhibited proliferation of EAT cells, peritoneal angiogenesis, VEGF production and peritoneal microvessel MVD.

**Materials and Methods**

Terminalia bellirica fruits were collected in and around Mysore, India and identified by Botanist. The voucher specimen of the collected plant material was deposited and voucher number UOM.BOT.4820 was obtained from the Department of Botany, University of Mysore, Mysore. Swiss albino mice were obtained from Department of Zoology, University of Mysore, Mysore with the approval of institutional animal ethics committee and experiments were conducted according to guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India. Fertilized hen's eggs were procured from the Government poultry farm, Bangalore. The chemicals and solvents were of analytical grade and purchased from Sisco Research Laboratory (SRL), Mumbai, India.

**Polarity-based fractionation of TbFP**

The pericarp of Terminalia bellirica fruits were separated, shade dried and powdered. Pulverised plant material was used for extraction
with different solvents of increasing polarity, viz., hexane, benzene, chloroform, ethyl acetate, acetone, alcohol, methanol in soxhlet extractor till exhaustion and finally with distilled water on magnetic stirrer for 24h. The solvents were evaporated using rotatory evaporator under reduced pressure of 20-22 mmHg, lyophilised, and tested for antiangiogenic activity. Extracts (33.3mg), free from solvents were dissolved in 100μl of 0.1% DMSO from which 100μl was diluted (1:1) with saline and was subsequently used for the assays.

**In vivo EAT cell growth and TbFP treatment**

Nine groups of Swiss albino mice, each group containing 5 animals was included in the study. The animals were of six to eight weeks age, weighing about 25-30g. Ehrlich ascites tumor (EAT) cells, were maintained in our laboratory by i.p transplantation as described previously (15). In brief, 5 x 10⁶ EAT cells/ mouse were injected i.p. EAT cells exhibits an exponential growth period from 6th or 7th day after tumor injection and the animal succumb to death on 12th to 14th day due to tumor burden. Each solvent extract 33.3mg was injected i.p into the tumor bearing mice, every alternate day after 5 days of tumor growth and the weight of the animals was monitored daily from the 1st day of transplantation till the 12th day of tumor growth. The mice were sacrificed on the 13th day and observed for peritoneal angiogenesis, secretion of ascites, cell number, microvessel density and secretion of VEGF.

**Ascites volume, cell number and peritoneal angiogenesis**

After sacrificing the untreated and the TbFP extracts treated EAT bearing mice, a small incision was made in the abdominal region and EAT cells along with ascites fluid were collected into a sterile polypropylene tube containing 2ml of saline and centrifuged at 3000rpm for 10min at 4°C. Volume of ascites was calculated by subtracting the volume of the saline previously added from the supernatant. The cell number was determined by tryphan blue exclusion method using hemocytometer. After collection of cells along with the fluid, the incision on the abdomen wall was extended and exposed peritoneum was examined for vascularization and photographed.

**Chorioallantoic membrane (CAM) assay**

The chorioallantoic membrane assay is a well established assay and widely used to assess angiogenesis and antiangiogenesis (16). The fertilized eggs were incubated at 37°C in a humid atmosphere for 10 days. A small window was made on the shells under aseptic condition to verify development of embryo. The window was resealed and the incubation was continued under the same conditions. On the 12th day, the window of the eggs were reopened and sterile cover slips containing air-dried saline or recombinant VEGF (50ng/egg) and TbFP solvent extracts were inverted over the CAM, resealed and returned to incubation for another 2 days. On the 14th day the windows were reopened and inspected for development of neovascularization in the area below the coverslip 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 TbFP extracts treated mice as described previously (17). In brief, 100μl of ascitic fluid from TbFP solvent extracts treated and untreated EAT bearing mice were coated onto 96 well microplates using coating buffer (50mM Na₂CO₃, pH. 9.6) and incubated overnight at 4°C, wells were washed and blocked using skimmed milk followed by incubation with anti-VEGF

Shivakumar et al
antibodies. The wells were washed and probed with secondary antibody tagged to alkaline phosphatase. P-NPP was used as substrate and absorbance was measured at 405nm with medispec ELISA reader.

**H&E staining and Microvessel density (MVD)**

The peritoneum of the mice treated with or without TbFP extracts was fixed in formalin, dehydrated with alcohol and embedded in paraffin. The 5 µm sections were taken using microtome and stained with routine hematoxylin and eosin stain. MVD was determined by ‘hotspot’ method (11) using Nikon binocular microscope. In brief 10 fields with highly vascularized areas were screened at low magnification (10x), and further magnification was changed to high-power field (HPF) (40x) and the microvessels were counted.

**Results**

**In vivo Effect of TbFP extracts on EAT cell growth, ascites secretion and Cell number**

The effect of different solvent extracts of TbFP on EAT cell number, ascites volume is provided in Table-1. EAT cells (5 x 10^6 cells) injected / mouse on the day of transplantation increased to an average of 1.83 x 10^8 cells/mouse at the end of the growth period in untreated animals. Animals, which received (TbFP-Ae) showed 2.8 folds reduction in the EAT cell number (0.65 x 10^6 cells/ mouse) compared with that of untreated animals. Whereas no significant reduction in EAT cell number was observed in other solvent extracts treated mice (Fig-1C). This reduction in cell number by TbFP-Ae reflected on the body weight of the animals. From Fig-1A, it is evident that the untreated and solvent extracts treated mice showed continuous increase in the body weight from the day of transplantation till 12th day. The mice that received TbFP-Ae showed an average 80.78% reduction of body weight from the 5th day. EAT cells grow as ascites tumor by accumulating large amount of ascites fluid (8.55ml), when injected intraperitoneally to mice. The in vivo effect of TbFP solvent extracts on secretion of ascites in EAT bearing mice is shown in table-1 and depicted in Fig-1B. The volume of ascites formed due to tumor induction decreased upon TbFP-Ae treatment to an extent of 75.43% (2.0±0.10ml) when compared to that of untreated EAT bearing animals (8.5±0.15ml). Other solvent extracts did not have any effect on either growth of EAT cells or formation of ascites.

![Graph showing comparison of weight in grams over number of days for different solvent extracts](image-url)
Fig. 1: Effect of different solvents extracts of *T. bellirica* on EAT cell growth, ascites volume and cell number *in vivo*. EAT cells (5x 10⁶) were injected i.p into mice and from 6th day of transplantation the mice were treated with or without solvent extracts of *T. bellirica* and body weight of both control and treated groups of the animals were monitored daily and graph was plotted. B. The volume of ascites secreted by mice treated with or without solvent extract. C. The cell number was determined by tryphan blue exclusion method. The above results are the average of 3 experiments and means of 5-animals/group.

*Shivakumar et al*
Table-1: Average EAT cell number counted using hemocytometer, ascites volume, number of microvessel density count/high power field of peritoneal section stained with haematoxylin and eosin stain and VEGF secreted in different solvent extracts treated EAT mice in vivo. (Avg- average, MVD-Microvessel density, ng ml⁻¹- nanogram/millilitre, VEGF-Vascular endothelial growth factor.

<table>
<thead>
<tr>
<th>Solvent extract treated</th>
<th>Avg. EAT cell number/mouse</th>
<th>Avg. Ascites volume secreted/mouse (ml)</th>
<th>Avg. MVD/ HPF</th>
<th>VEGF (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.83</td>
<td>8.55</td>
<td>18.09</td>
<td>1200.9</td>
</tr>
<tr>
<td>Hexane</td>
<td>1.9</td>
<td>7.45</td>
<td>15.31</td>
<td>1831.31</td>
</tr>
<tr>
<td>Benzene</td>
<td>1.73</td>
<td>7.85</td>
<td>18.32</td>
<td>1219.13</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.24</td>
<td>4.70</td>
<td>9.62</td>
<td>738.33</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>1.48</td>
<td>6.85</td>
<td>17.11</td>
<td>1190.01</td>
</tr>
<tr>
<td>Acetone</td>
<td>1.50</td>
<td>6.10</td>
<td>13.44</td>
<td>899.08</td>
</tr>
<tr>
<td>Ethanol</td>
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<td>5.65</td>
<td>12.03</td>
<td>700.31</td>
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<tr>
<td>Methanol</td>
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</tr>
<tr>
<td>Water</td>
<td>0.65</td>
<td>2.10</td>
<td>3.21</td>
<td>21.34</td>
</tr>
</tbody>
</table>

Angio-inhibitory effect of TbFP extracts

The peritoneum of untreated EAT bearing mice showed extensive angiogenesis, while TbFP-Ae treated mice showed considerable reduction in the peritoneal angiogenesis. Those mice, which received other solvent extracts, did not show any significance in reducing peritoneal angiogenesis (Fig-2A). Further, CAM assay proved that TbFP-Ae inhibited the formation of new blood vessels. The results of the CAM assay showing the inhibition of angiogenesis in comparison with that of untreated and other solvent extracts treated CAM are provided in Fig-2B.

Fig. 2A: Representative photographs of mice peritoneum a. untreated, b. Hexane, c. Benzene, d. Chloroform, e. Ethylacetate, f. Acetone, g. Ethanol, h. Methanol and i. Water extract. After 12th day the untreated and TbFP different solvent extracts mice were sacrificed and the peritoneum was observed for neovascularization. From the figure it is evident that the formation of blood vessels in the peritoneum of aqueous extract treated mice was extensively inhibited compared to the vascularization in peritoneum of untreated and other extracts treated mice.

Inhibition of tumor growth and angiogenesis
Fig. 2B: Chorioallantoic membrane (CAM) assay. a. Untreated, b. VEGF, c. Hexane, d. Benzene, e. Chloroform, f. Ethylacetate, g. acetone, h. ethanol, i. methanol and j. water extract, when blood vessels were observed under the cover slip, it was clear that the formation of new blood vessels were inhibited in the CAM treated with TbFP-Ae, compared to the untreated, VEGF and other solvent extracts treated CAM.

Effect of TbFP on production of VEGF
Quantification of VEGF over the tumor growth period of 12 days showed 1200.9 ng/ml of VEGF secreted by EAT cell bearing untreated mice. However, in the TbFP-Ae treated animals the estimated VEGF was 21.34 ng/ml. These results clearly indicate that there was a reduction of 98.34% in the secretion of VEGF levels in the ascitic fluid of mice treated with TbFP-Ae when compared to untreated animals. There was no In contrast, there was no reduction in VEGF levels in mice treated with other solvent extracts (Table–I).

Histological analysis and Microvessel density
The decreased secretion of VEGF in TbFP-Ae treated mice in turn reflected on the reduction in formation of blood vessels. The microvessel density was counted in the peritoneum section of TbFP extracts treated and untreated tumor-bearing mice. In untreated mice the average MVD/HPF was 18.09±0.02 and in TbFP-Ae treated mice it was 3.21±0.13. This accounted for the reduction of MVD by 82.2% in TbFP-Ae treated mice peritoneum. The representative photomicrograph of peritoneal sections of untreated and TbFP extracts treated mice are shown in Fig.3 and is further emphasised in Table 1.

Fig. 3: Representative photomicrographs of 5μ H&E stained mice peritoneal. Sections of control and treated with different solvent extracts of *Terminalia bellirica*. a. Untreated, b. hexane, c. benzene, d. chloroform, e. ethylacetate, f. acetone, g. alcohol, i. water extracts treated mice. Reduction of microvessels in water extract is evident from the above figure.

Shivakumar et al
Discussion

Angiogenesis is a major pathological component of a grave disease such as cancer. Antiangiogenic drugs have been shown to decrease certain tumors in animal models and induce long-term tumor dormancy. Several successful attempts have been done to explore the antiangiogenic activity from plants. By using CAM assay for validation, Wang et al. (18) reports the antiangiogenic effect of aqueous extract from twenty-four herbs. *Terminalia bellirica*, selected in this investigation is one of the plants having ethno-medicinal value and has been used in Indian traditional medicine. Polarity-based fractionation of TbFP proved that, the aqueous extract possessed highly promising antiangiogenic property in Ehrlich ascites tumor model. The EAT cell proliferation, ascites volume, tumor cell number, peritoneal angiogenesis, VEGF levels and microvessel density are biological events which offer measurable parameters to validate novel biomolecules with anti-tumor and antiangiogenic activities. This is the first report on *Terminalia bellirica* as an antiangiogenic component in mouse mammary carcinoma model. Methanol extract of stem bark *Bombax ceiba* has been shown to inhibit the tube formation in HUVEC cells (19). This being an *in vitro* assay it does not reflect on the *in vivo* antiangiogenic activity of *Bombax ceiba*. Similar to the data presented in this paper on inhibition of the development of new blood vessels in CAM assay by TbFP-Ae. Jung et al. (20) have found that the methanol extract of *Ulmus davidiana* displayed a strong inhibition of neovascularization in chick membrane. A decreased microvessel density in peritoneum of EAT bearing mice by curcumin confirmed its antiangiogenic property from studies made by Belakavadi et al., (17). The data on TbFP-Ae inhibition of tumor induced peritoneal angiogenesis clearly indicates that the extract contains a potent antiangiogenic biomolecule. In the present investigation the inhibition of EAT cell growth, *in vivo* by TbFP-Ae supports to the earlier findings that the aqueous extracts of *Acanthus ilicifolius*, *Alternanthera tenella* and *Glycyrrhiza glabra* plants inhibits growth of EAT cell (21,22,23). At molecular level, the mechanism of antiangiogenesis by TbFP-Ae involves, inhibition of the secretion of VEGF by 98.34%. Periyanayagum et al. (24) also reports that, the aqueous extract of *Justicia gendarussa* leaves inhibits angiogenesis in chorioallantoic membrane at concentrations of 25 μg, 50 μg and 100 μg. A dose of 0.1% (w/w) aqueous extracts of *Rubus suavisimus* caused 41% inhibition of angiogenesis when compared with saline treated human- tissue based fibrin-thrombin clot assay (25). The present study gains more importance as it includes both quantitative and qualitative validation of angiogenesis. Findings from this study indicate the presence of an antiangiogenic biomolecule in *Terminalia bellirica* fruit pericarp aqueous extract. This observation warrants further study to isolate and characterized the bioactive compound from this plant. As it is easily available cost effective, medicinal plant, it might form a new arsenal in antiangiogenic dependent therapy.

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Inhibition of tumor growth and angiogenesis


