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Original article

Screening for the modulation of neovessel formation in non-tumorigenic and tumorigenic conditions using three different plants native to Western ghats of India

B.R. Vijay Avin[a], Prabhu Thirusangu[a], C.K. Ramesh[a], V. Vigneshwaran[a], M.V. Prashanth Kumar[a], Riaz Mahmood[b], B.T. Prabhakar[a,*]

[a] *Molecular Biomedicine Laboratory, Postgraduate Department of Studies and Research in Biotechnology, Sahyadri Science College (Autonomous), Kuvempu University, Shimoga 577203, Karnataka, India*
[b] *Postgraduate Department of Studies and Research in Biotechnology, Jnana Sahyadri Campus, Kuvempu University, Shimoga 577203, Karnataka, India*

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ABSTRACT

Angiogenesis is the growth of neovessels from existing vasculature. In diseases such as cancer excessive angiogenesis occurs when diseased cells produce abnormally large amounts of angiogenic factors. A wide range of plants contains compounds with angiogenesis modulating properties, which are currently known. The present study explores the screening for antiangiogenic potentials of *Argeria elliptica* Wight., *Ipomoea fistulosa* and *Leea indica* Merr which are native to Western ghats of India. The leaf materials of the plants were subjected for cold ethanolic extraction process. The crude extracts were then screened for preliminary angiogenesis assay like $rVEGF_{165}$ induced *in vivo* CAM assay, rat corneal micropocket assay and tumor induced peritoneal angiogenesis assay. The molecular basis of modulation of neovessels was verified by the expression of VEGF using RT-PCR and ELISA. The preliminary screening for the angiomodulatory effect of crude extracts revealed that *L. indica* potentially inhibited the sprouting vessels both in non-tumorigenic and tumorigenic conditions. Inhibition of VEGF expression by *L. indica* has contributed for tumor inhibitory effect. Findings suggest that, the crude ethanolic extract of *L. indica* potentially inhibits the angiogenesis by down regulating the expression of VEGF and emerged as a potent angiomodulating plant out of three plants.

1. Introduction

Angiogenesis, the process of the new blood vessel formation from the preexisting capillaries, a key event during various physiological and pathological conditions. In pathological condition like tumor microenvironment, the angiogenesis facilitate for the cancer betterment and metastasis. Targeting angiogenesis during cancer condition is the key strategy to inhibit the tumor growth [1]. Vascular endothelial growth factor (VEGF) is the cytokine secreted by the hypoxic tumor cells acts as a major player during tumor metastasis. Regulation or inhibition of VEGF may halt the tumor growth and there by metastasis. Blocking angiogenesis with antibodies of angiogenesis factors or with enzyme inhibitors is effective for treating malignancy but there is room for improvement. Plants contain many active ingredients. They are complex chemical cocktails with medicinal properties that modern pharmaceuticals cannot reproduce. A wide range of plants contains anticancer drugs like Taxol, Camptothecin and Combretastatin are also known for angiogenesis modulating properties [2,3]. In traditional Chinese medicine and ayurveda, many herbs are used in the treatment of angiogenic diseases such as cancer and rheumatoid arthritis. Thus, it is rational to explore these medicinal plants as a source of novel angiomodulators [4].

The current research work exploits the use of medicinal values of plants available in Western ghats region of Karnataka, India. Three plants namely *Impomea fistulosa* (IP), *Argeria elliptica* (AE) and *Leea indica* (LI) were selected for the screening of inhibition of neovessel formation in both non-tumorigenic and tumorigenic conditions by targeting VEGF. All the three plants were previously reported for their medicinal values against various pathological disorders including inflammation, diabetes etc [5–8] but none of the above plants were screened for anticancerous as well as antiangiogenic activity. Since these are angiogenesis mediated disorders, our study aimed to explore the efficacy of these plants against angiogenesis specifically targeting VEGF.

* Corresponding author. Tel.: +91 9632872467.
E-mail address: pbtssc@gmail.com (B.T. Prabhakar).

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2. Materials and methods

2.1. Plant material collection and processing

The leaves of IP, AE and LI were collected from the Agumbe region of Western ghats, Karnataka, during mid spring season of 2013. The plants were identified and authenticated by the Range Forest officer/Botanist of Agumbe region, Karnataka. The voucher specimen of the three plants (No. BT/PL/Ip/32, No. BT/PL/Ae/33, No. BT/PL/Li/34) were maintained in Department of Biotechnology, Sahyadri Science College, Kuvempu University, Shimoga, Karnataka, India. The leaf materials of the three plants were rinsed in distilled water and dried in room temperature for 24 h and then shade dried, powdered and sieved through 40-mesh size filter cloth and stored in well-closed container for further extraction process.

2.2. Crude extract preparation

All the powdered plant materials were subjected for cold ethanol extraction process. Exactly 1 kg of the powdered materials of IP, AE and LI were extracted with 1000 mL of ethanol for 7 days separately at room temperature with intermittent shaking. After incubation, the whole extracts were filtered through Whatman grade filter paper and were maintained in dark. The yields of the fresh ethanol extract were noted. The dried extracts thus obtained were used for future analysis.

2.3. Preliminary phytochemical analysis

The preliminary qualitative phytochemical studies were performed to test the different chemical groups present in the leaves of *Ipomoea fistulosa* ethanolic extract (IPE), *A. elliptica* ethanolic extract (AEE), and *L. indica* ethanolic extract (LIE) as described previously [9,10].

2.4. Chorio alontoic membrane (CAM) assay

Recombinant $VEGF_{165}$ ($rVEGF_{165}$) induced *in vivo* CAM angiogenesis model was performed to study antiangiogenic effect of the crude extracts. Fertilized Giriraja Hen's eggs were procured from the local market of Shimoga, Karnataka, India. The eggs were grouped and incubated at 37 °C in a humidified and sterile atmosphere for 10 days ($n = 6$). The crude extracts (10 μg/egg) were treated on the growing CAM by making a small window on the eggshell as described earlier [11]. The windows were opened on the 12th day and inspected for changes in the microvessel density and photographed using Cannon power shot Sx500 IS camera.

2.5. Animals and ethics

Healthy Swiss Albino male mice weighing 25 ± 2.0 g were used for the culture of Ehrlich ascites carcinoma (EAC) cells and male Swiss albino whistler rat weighing 150 ± 5.0 g were used for the rat corneal micropocket assay. All the animals were grouped separately and housed in polyacrylic cages and maintained under standard conditions (25 ± 2 °C) with 12 ± 1 h dark/light cycle. Short-term acute toxicity study of all the plant extracts were carried out by injecting intraperitoneally (i.p) and LD_{50} was determined in accordance with CPCSEA guidelines. All procedures for animal experimentation were approved by the Institutional Animal Ethics Committee, National College of Pharmacy, Shimoga, Karnataka, India (NCP/IAEC/CL/101/05/2012-13).

2.6. Rat corneal assay

The rat corneal micropocket assay was performed to study the effect of the three different plant extracts. In the rat corneal micropocket assay, hydron polymer [polyhydroxyethyl-methacrylate (poly-HEMA), Sigma Aldrich, USA] was dissolved in ethanol to a final concentration of 12%. All aliquot of this mixture was then pipetted onto teflon sheet, Saline (group 1), 1 μg of $rVEGF_{165}$ (group 2), $rVEGF_{165}$ + IP 10 μg (group 3), $rVEGF_{165}$ + AE 10 μg (group 4) and $rVEGF_{165}$ + LI 10 μg (group 5) were added to each pellet and allowed to dry under a laminar flow hood at room temperature for 2 h. The pellets were incubated at 4 °C overnight. All procedures were performed under sterile condition. The corneal implantation of the pellet was done as described previously [12]. The number of blood vessels and length of the vessels were quantified after the treatment of extracts [13].

2.7. Tumor induced peritoneal angiogenesis assay

EAC cells were cultured *in vivo* and grouped separately ($n = 6$). The treatment with the crude extracts [50 mg/kg body weight intraperitoneally (i.p)] with 3 doses on every alternate day after the onset of tumor on 4th day was carried out. The suppression of angiogenesis in peritoneum of EAC bearing mice treated with or without crude extracts mice were photographed and further the total vessel length was calculated and verified for antitumor potency and survivability analysis as reported previously [14,15].

2.8. Reverse transcription-PCR

The EAC cells from LIE treated and untreated groups were harvested and the total RNA was isolated using Direct Zol RNA isolation kit (Zymo research, USA). RT was performed using oligo(dT) primers and superscript reverse transcript according to the manufacturer's recommendation (Invitrogen, USA) and amplified using VEGF-A fwd: 5′-GGGGTGTCCCATAGGGGTAT-3′ & rev: 5′-CGCCTTGGCTTGTCACATTTT-3′. Normalization of angiogenic gene expression was achieved by comparing the expression of GAPDH fwd: 5′-CGCTCATGTACCCGCTGTAT-3′ & rev: 5′-TGTCTGCCGGACTCAAAGAC-3′ for the matching sample. PCR product was resolved on 1% agarose gel and documented using Bio-rad Gel Documentation™ XR+ Imaging System.

2.9. VEGF-ELISA

VEGF-ELISA was carried out using the ascites of EAT bearing mice treated with or without crude extracts *in vivo*. In brief, 100 μL of ascites from each group was coated in a coating buffer at 4 °C overnight. Subsequently, wells were incubated with anti-$VEGF_{165}$ antibodies (Sigma Aldrich, USA), followed by incubation with secondary antibodies tagged to alkaline phosphatase and pNPP was used as substrate [11].

3. Results

3.1. Varied phytoconstituents in three different plants

Ethanol extracts of three plants were screened for the presence of phytoconstituents. The results revealed that all the extracts showed positive for the presence of alkaloids/flavonoids/phenols and tannins. Triterpenoid was found to be positive only in LIE. None of the extracts under study gave positive results for saponins. The results were summarized in Table 1.