Indian Journal of Natural Products and Resources
Vol. 9(2), June 2018, pp 126-131

Angiogenic effect of indigenous herbal extracts: *Bombax Ceiba* and *Erythrina variegata*

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Received: 09 June 2017; Revised 21 March 2018

Angiogenesis is the process of formation of new vasculature; an important process in various pathological conditions. The study is focused on the screening of herbal plants targeting angiogenesis. The ethanolic extract of plant *Bombax ceiba* (EEBC) and *Erythrina variegata* (EEEV) were used. The experimental methods included regenerative angiogenesis assay in adult zebrafish and developmental angiogenesis assay in zebrafish embryos. For the regenerative angiogenesis assay, the regeneration of amputated fin length was evaluated. The adult zebrafishes were divided into 5 treatment groups of 8 fishes in each group. In developmental angiogenesis assay, various phenotype changes in embryos were observed. The embryos were divided into 5 groups with 12 embryos in each group. For both assays grouping was; group I vehicle control (DMSO), group II high dose of EEBC, group III low dose of EEBC, group IV high dose of EEEV and group V low dose of EEEV. The results of both the assays suggest that EEBC showed significant (p <0.05) pro-angiogenic activity while EEEV showed significant (p <0.05) anti-angiogenic activity. Therefore EEBC can prove beneficial in diseases related to insufficient angiogenesis like in management of wound healing while, EEEV in diseases related to excessive angiogenesis like management of cancer.

Keywords: Angiogenesis, *Bombax ceiba*, *Erythrina variegata*, Zebrafish, Zebrafish embryos.

IPC code; Int. cl. (2015.01)−A61K, 36/00, 36/48

Introduction:

Bombax ceiba is one of the important medicinal plants and it belongs to the family Bombaceae. It is known by different names such as silk cotton tree, red cotton tree, Indian kapok tree, *shalmali*, *semal*, and *shimul* etc. The plant is found in the tropical and subtropical region in Asia especially in India, Sri Lanka, Pakistan, Malaysia, Myanmar and in Bangladesh. It has many medicinal uses in the traditional system of medicine such as *Ayurveda, Siddha,* and *Unani*[1,2]. According to *Ayurveda*, *B. ceiba* has many medicinal properties like a stimulant, astringent, hemostatic, aphrodisiac, diuretic, antidiarrheal, cardiotonic, emetic, demulcent and antipyretic etc[3,4]. Different parts of the plant are used for different purpose. A paste of leaves and flowers of this tree is applied as an external application for skin irritation. Seed oil is used for manufacturing of soaps and lubrication substances. Seeds are applied on the skin in smallpox and chicken pox. Leaves are used as a laxative and hematinic. The bark of the plant is used for wound healing[5]. *Erythrina variegata* is also known as the 'Indian coral tree' in Asia or 'tropical coral' in the Pacifics and it belongs to the family Fabaceae. It is an important multipurpose tree species and thrives well in the arid and semiarid region. It is found in many tropical and subtropical regions. *E. variegata* has been used in folk medicine for treatment of insomnia, malarial fever, helminths, venereal disease, asthma and toothache[6-8]. The alkaloid erythroidine abundant in *E. variegata* was used as a muscle relaxant[9]. The juice of *E. variegata* stimulates lactation and is useful in dysmenorrhea. The root extract possesses anti-microbial activity. The bark of the plant is used as a laxative, diuretic, expectorant, liver tonic and anti-rheumatoid. A warm poultice of the leaves is applied externally to relieve rheumatic joint pain. The leaf extract is also used as a nervine sedative, collyrium in ophthalmia, anti-asthmatic, anti-epileptic, nematicidal, antiseptic and as an astringent[10,11].

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Angiogenesis is the process of formation of blood vessels from pre-existing blood vessels; mediated by the endothelial cells that line the blood vessels. Endothelial cells in the process of angiogenesis undergo complex sequence events that include the secretion of metalloproteases and other matrix-degrading enzymes, cell migration into the newly created space, endothelial cell division and proliferation, and vessel formation[12]. These are well-regulated processes involving a number of stimulators such as fibroblast growth factor, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietins, activators of integrins and inhibitors such as thrombospondin, angiostatin, and endostatin; thus plays important role in the normal physiology of angiogenesis[13,14]. Angiogenesis is an important step in various pathological conditions like tumour progression because angiogenesis provides nutrients that maintain the viability of diseased tissue. Angiogenesis associated with a tumour allows a tumour to maintain its growth and also facilitates metastatic spreading by establishing a connection to the existing vasculature. In addition to angiogenesis, it has become clear that inflammation is a key component in cancer insurgence that can promote tumour angiogenesis. The difference between angiogenesis in normal condition and angiogenesis in diseased condition is blood vessels formed in diseased tissue are highly disorganized and their walls have numerous openings; and because of this tumor vessels are not able to mature through the recruitment of smooth muscle cells and pericytes, leading to the formation of leaky vessels in the tumor[15,16]. In the case of congestive heart failure, chronic wound and non-union fracture the angiogenesis is insufficient where the growth of blood vessels and circulation of blood is not proper. It leads to tissue death and delayed wound healing followed by organ amputation. To overcome this problem the angiogenesis process is needed to be improved[17].

The plant *B. ceiba* is reported with wound healing activity[5] while *E. variegata is* reported with anti-cancer activity[9], indicating them with the probable modulatory action of angiogenesis. Thus the effect on angiogenesis of this plant can prove as the rationale behind their reported activities as either in wound healing or anti-cancer action. In the present study, the plants selected were *B. ceiba* and *E. variegata* to evaluate their potential effects on angiogenesis process.

Material and Methods

Plant material

The plant materials of *B. ceiba* bark and *E. variegata* leaves were collected from Rajesh chemicals, Mumbai. Both the powder materials were authenticated from Taxonomist Dr. H. M. Pandit (Formerly Head and Associate Professor of Botany, Department of Botany, Guru Nanak Khalsa College, Mumbai). Voucher specimens number namely vdj p 1060432 (Bark powder of *B. ceiba* L.) and vdj p 1060429 (Leaf powder of *E. variegata* L.) were deposited in Department of Pharmacology at Bharti Vidyapeeth's College of Pharmacy, Navi Mumbai. The powdered plant material was macerated for 8 hours using the solvent petroleum ether for defatting of the material. The crude extract was obtained by placing 50 g of powder in the soxhlet extractor[18] using ethanol at 40 °C for 48 hours. The crude extract of the plant was further evaporated in the rotavac evaporator to get free-flowing powder. The typical yield obtained for a crude ethanolic extract of *B. ceiba* was 21.61 % w/w and for ethanolic extract of *E. variegata* was 17.9 % w/w.

Experimental animal

The adult and wild-type of zebrafishes were procured from a local supplier. The fishes were kept in the tanks and supplied with atmospheric air and with spirulina granules, dried earthworms and tetramine flakes 3 times daily. Sodium thiosulphate was added to dechlorinate the tank water. The pH of the water was maintained to neutral and 12 hours light/dark cycle in specialized zebrafish tanks was maintained. Fishes were acclimatized for 15 days prior to the study.

The zebrafish embryos were procured from a local supplier and kept in embryo medium. The composition of embryo medium is sodium chloride (0.29 % w/v), potassium chloride (0.013 % w/v), calcium chloride (0.049 % w/v), magnesium sulphate (0.081 % w/v) in distilled water.

Experimental procedure

Regenerative angiogenesis assay

The zebrafishes were divided into 5 treatment groups; each group was having 8 fishes. Group, I was treated with vehicle control (25 µL/ g of DMSO per g of zebra fish), Group II and III were treated with 0.43 and 0.2 mg of EEBC per g of zebrafish respectively while group IV and V was treated with 0.38 and 0.19 mg of EEEV per g of zebrafish respectively. The

one-tenth of LD_{50} was used for main study [19]. On the 1st day of study, fishes were anaesthetized by 2-phenoxy ethanol and the caudal fin was amputated up to 50 % of its fin length. The dose was administered by oral route using hamilton syringe of capacity 10 µL on every alternate day to the 7th day of study. Post amputed images were taken on the 3rd day and 7th day by Motic digital microscope with Image plus 2.0 software (Model: DMWB1-222ASC) and length of the regenerated fin was measured and calculated[20].

Developmental angiogenesis in zebrafish embryos

The embryos were divided into 5 treatment groups; each group was having 12 embryos. Each embryo was transferred in an individual well of 96 well microtiter plate. Group I was treated with vehicle control (0.03 µL DMSO), Group II and III was treated with 0.020, 0.015 ug of EEBC per µL of DMSO respectively while group IV and V was treated with 0.030, 0.020 ug of EEEV per µL of DMSO, respectively. Various changes in phenotype such as tail bending, delayed hatching, abnormal yolk sac, abnormal vasculature, pericardial edema, and haemorrhages were observed and images were taken at 24, 48 and 72 hpf by using Motic digital microscope with Image plus 2.0 software (Model: DMWB1-222ASC). The survival rate was recorded up to 96 hpf[21].

Results

Regenerative angiogenesis assay

The effect of EEBC on zebrafish caudal fin was studied up to 7 days. On day 1, the caudal fin was amputated up to 50 % and the dose was given on every alternate day to the 7th day. The images of regenerated fin were taken by motic digital microscope on day 3 and 7 (Fig. 1). The length of the regenerated caudal fin was measured and the graph was plotted. (Fig. 2 and 3)

Developmental angiogenesis assay

After the drug treatment, the embryos were observed after 24, 48 and 72 hpf using Motic digital microscope. Various phenotype changes such as tail bending, abnormal yolk sac, abnormal vasculature, haemorrhages, pericardial edema and delayed hatching were recorded at 24, 48 and 72 hpf. The survival rate was observed after 24, 48, 72 and 96 hpf (Fig. 4). The changes in phenotypes were observed after 72 hpf (Fig. 5 and 6).

Discussion

Regenerative angiogenesis assay

Zebrafish has emerged as a powerful vertebrate genetic model to study regeneration as it can regenerate one fifth to the heart ventricles, pancreas

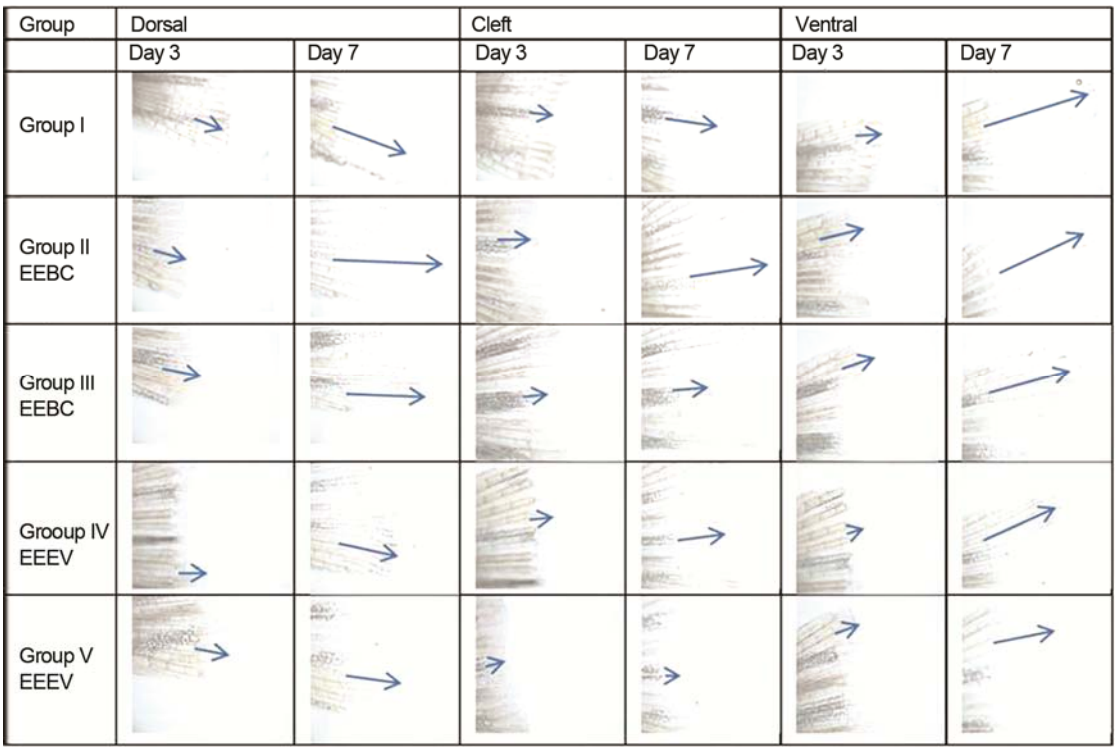

Fig. 1 — Images of the length of the regenerated fin of dorsal, ventral and cleft region on the 3rd and 7th day post amputation of EEBC and EEEV

and grows caudal fin[22]. It is useful in understanding the molecular and cellular mechanisms of regeneration, based on their regenerative ability. The caudal fin is composed of bony rays, mesenchymal tissue, blood vessels, and nerves, enclosed by the epidermis and can regenerate fully after resection. Lost caudal fin tissue in zebrafish is recovered through epimorphic regeneration i.e. blastema formation and restoration of tissue which resembles the wound healing process in humans. The adult zebrafish tail (caudal) fin has good regeneration ability making it a model of choice for studying regenerative angiogenesis in mammals[23]. In zebrafish, the process of fin regeneration includes various pathways including FGF (Fibroblast growth factor) which plays important role in blastema formation, MAP kinase etc[24,25]. Briefly regeneration of caudal fin after amputation having three regeneration stages; stage 1 is Wound healing [0-1 days post amputation (dpa)], stage 2 is formation of the regeneration blastema (1-3 dpa), a mass of high proliferation lineage-restricted mesenchymal progenitor cells; stage 3 is regenerative outgrowth and patterning of new tissue (>3 dpa)[26].

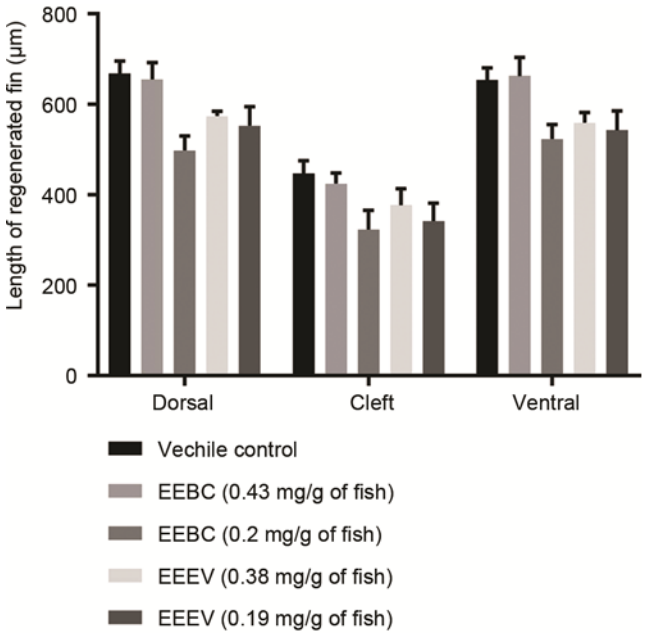

Fig. 2 — Graphical representation of the effect of EEBC and EEEV on regenerative angiogenesis assay on day 3

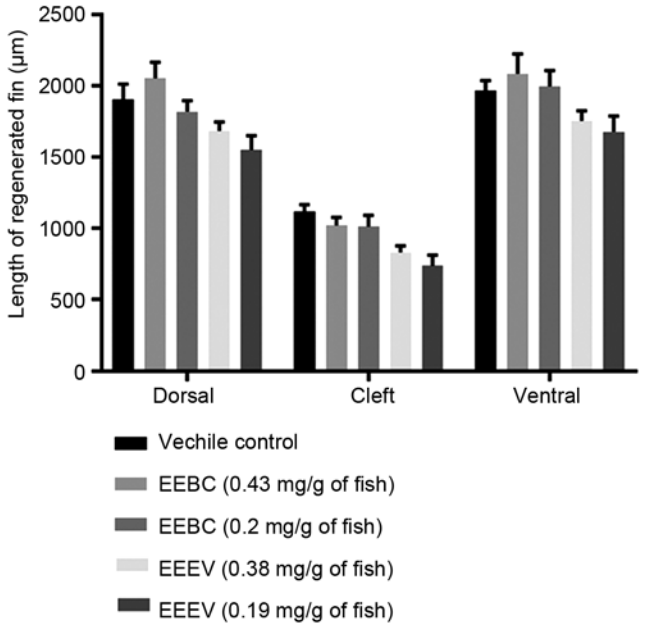

Fig. 3 — Graphical representation of the effect of EEBC and EEEV on regenerative angiogenesis assay on day 7

In the current study; the zebrafishes of the vehicle control group showed normal regeneration of amputated fin. The effect of EEBC at both 0.43 and 0.2 mg dose on zebrafish tail fin regeneration showed positive result i.e. elongation of the tail fin which was significantly more than the vehicle control group; thus showing pro-angiogenic activity. This activity may be attributed to the activation of pro-angiogenic factors. While the effect of EEEV at both 0.38 and 0.19 mg doses showed negative result i.e. inhibition of regeneration of tail fin significantly (p <0.05 computed using one way ANOVA followed by Tukey's multiple comparison tests) less than the

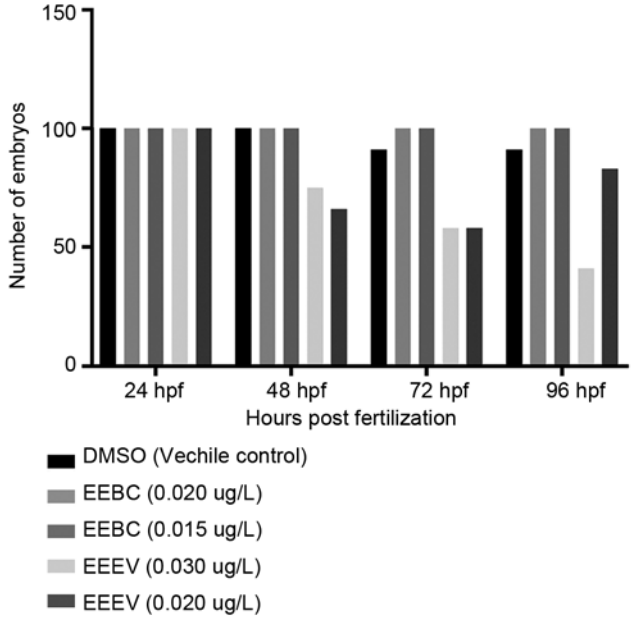

Fig. 4 — Percent survival rate at 24, 48, 72, and 96 hpf

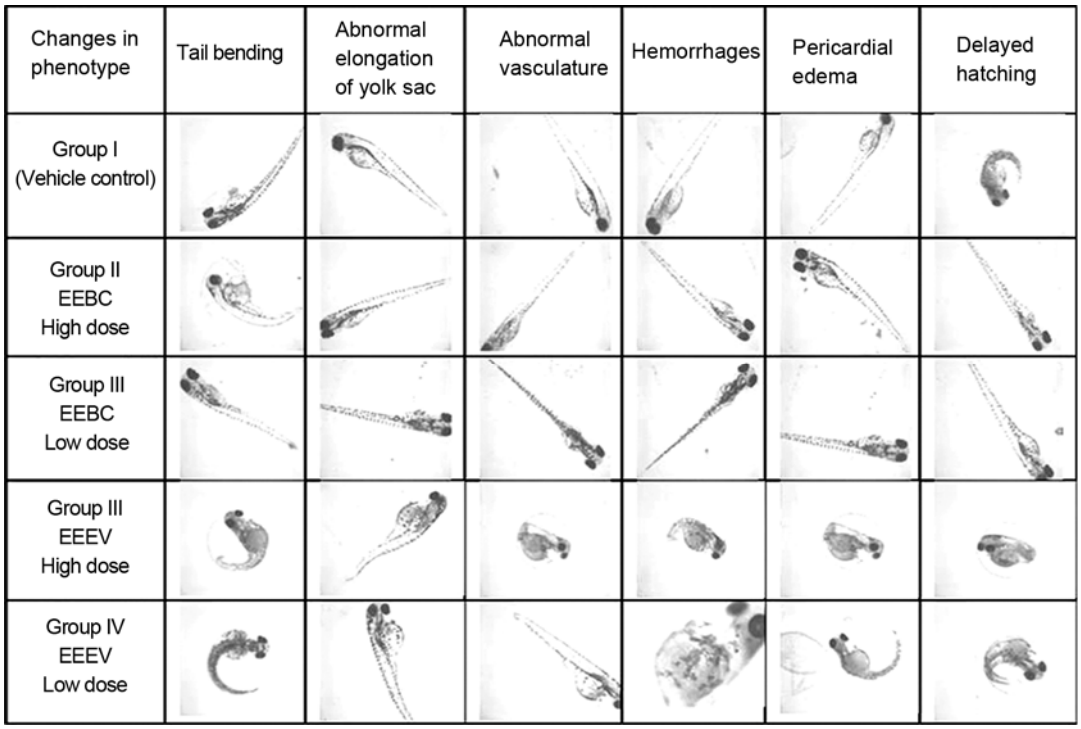

Fig. 5 — Changes in phenotype at 72 hpf

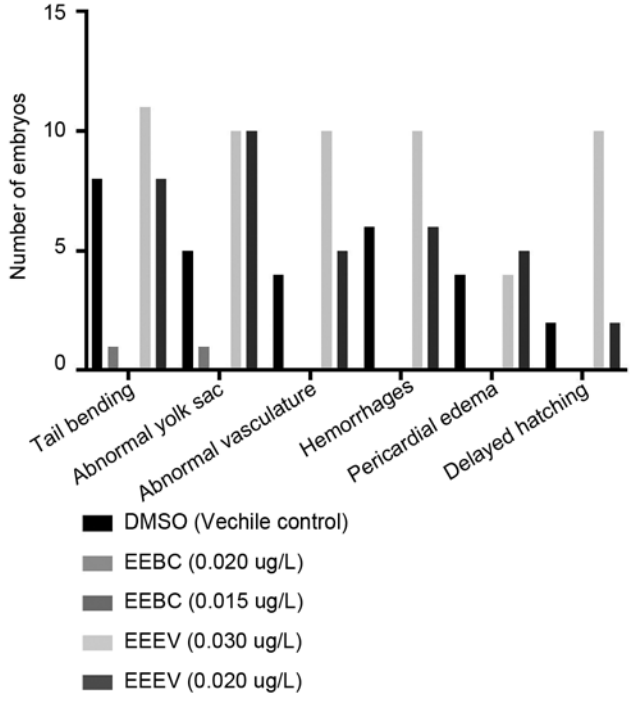

Fig. 6 — Graphical representation of phenotype changes at 72 hpf

vehicle control group; thus showing anti-angiogenic activity. Thus EEEV probably is acting via either complete inhibition or suppression of pro-angiogenic factors like PDGF, VEGF or blocking of their respective receptors[27].

Developmental angiogenesis assay

The zebrafish embryo shows a strong similarity to that of other vertebrates[28]. In zebrafish embryonal phase at the 13 somite-stages, endothelial cell precursors migrate from the lateral mesoderm and originate into the vasculature leading to the development of a single blood circulatory system at 24 hpf. Blood vessel develops during the subsequent days by angiogenesis. In the process of angiogenesis, the intersegment vessels of the trunk sprout from the dorsal aorta at 20 hpf. Substantial vein vessels originate from the duct of the curvier area at 48 hpf and will form a vascular plexus across most of the dorsal-lateral aspect of the yolk sac during the next 24 hours[29]. The EEBC treated group showed 90-100 % survival rate and very fast hatching rate as well. Very less number of zebrafish embryos showed the phenotypical changes till 96 hours post fertilization. The result obtained by zebrafish embryos showed significant (p <0.05) growth of new blood vessels and also a good development of organs of a treated group of zebrafish embryos. The drug showed pro-angiogenic activity. While the EEEV treated group showed less survival rate and also showed more developmental abnormalities. The results are in accordance with the angiogenesis studies on plant *B. ceiba* and *E. variegata* conducted on the fertile white leghorn chicken eggs[30].

Conclusion

The angiogenesis assay results suggest that the plant *B. ceiba* possess anti-angiogenic activity while *E. variegata* possesses pro-angiogenic effect both in developmental and regeneration angiogenesis process. Further molecular studies can be carried out on the plant or the potential isolate(s) to understand and justify detailed mechanism of action on the angiogenesis process. Thus these plants can be a potential source for a drug of interest in the treatment of diseases related either insufficient angiogenesis such as coronary heart diseases, chronic wound, wound healing in ulcer and diabetes or in the treatment of diseases related to the excessive angiogenesis such as cancer, rheumatoid arthritis, and psoriasis.

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