Study of Ethanolic Extract of *Parkiabiglandulosa* and It's Isolate Targeting Angiogenesis

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ABSTRACT: - Angiogenesis, the sprouting of new blood vessels from pre-existing, vessels is a crucial process for tumor growth and metastasis. It is a physiological process required for wound healing, repair, embryonic development. Immediately after injury, angiogenesis is initiated by multiple molecular signals, including pro-angiogenic factors, angiopoietins, inflammation, cytokine growth factors, and cell-matrix interactions. Imbalance in this factors leads to disease condition like excessive angiogenesis in tumour. Different parts of *Parkiabiglandulosa* shows activity like anti ulcer, anti inflammatory, anti bacterial, anti fungal. Based on composition of different phytoconstituents like gallic acid, lupeol, campestrol and β-sitosterol plant was selected for the targeting of angiogenesis. β-sitosterol was isolated from crude extract by using different solvents on flash chromatography. In this study anti angiogenic effect was studied at low and high doses of EEPB (ethanolic extract *parkiabiglandulosa*), its isolate β-sitosterol and standard imatnibmesylate was studied on regenerational and developmental model of zebrafish and their embryos.

***Keywords*: Angiopoietin, Pro-angiogenic, β-sitosterol, developmental, regenerational, zebrafish.**

I. INTRODUCTION

Angiogenesis is a process of generating new capillary blood vessels i.e. neo vasculature, that occurs through sprouting of pre-existing vessels which play a crucial role during embryonic development, growth and healing. It is a complex process wherein there is extensive interaction between cells, soluble factors and extra cellular matrix (ECM) components.[1] The process of angiogensis involves various factors like pro angiogenic factor which includes vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factors (PDGF), angiopoietins, and anti angiogenic factors includes angiostatin, restin, arresten, canstatin and tumstatin etc.[2]-[6] Any imbalance in these factors leads to abnormal angiogenesis which is involved in many pathological processes including tumour growth, metastasis, age related macular degeneration, psoriasis, endometriosis, arthritis. Thus targeting angiogenesis may help to combat with such diseases. In cancer angiogenesis is major event in growth of tumour and metastasis; thus excessive proliferation of blood capillaries is crucial for supply of proper nutrients and oxygen. The cancer cells sends signal to the normal surrounding tissues which activate certain genes in tissue cells and encourages the formation of new blood vessels in tissue which have lost control of cell cycle in those affected tissues. [7]

The currently available therapy targeting angiogenesis have severe side effects like alopecia, emesis, myelosuppression etc.[1] Thus identifying the compounds with better efficacy and negligible or lesser side effects is todays need of hour. India is one of the rich sources of diverse plants. These plants can be explored for their anti-angiogenic activity or as a source of new molecule(s). *Parkiabiglandulosa* common name badminton ball tree is a large tree which provides edible seed, leaves and fruit pulp. The different parts of plant show anti-ulcer, anti-inflammatory, antifungal, and antibacterial activities. Saponins from seed bran are medicinally used for its astringent and detergent properties. Lectins of this plant have mitogenicity and antiproliferative activity.[8] Various studies have revealed that bark and leaves contain different phytoconstituents like β-sitosterol, campesterol, gallic acid and lupeol of which β-sitosterol is present in significant amount. β-sitosterol belongs to category of phytosterols and various studies are reported on anticancer actvity of these phytoconstituents.[9] In this study, anti-angiogenic activity of ethanolic extract of *Parkiabiglandulosa* (EEPB) and its isolate was evaluated by using regenerational and developmental angiogenesis model of zebrafish.

II. MATERIALS AND METHOD

A.Chemicals

Dimethyl sulfoxide (DMSO), Ethanol (70%), Calcium chloride, Sodium thiosulphate pellets were obtained from S.D. Fine chemicals. Sodium chloride, Potassium chloride, Magnesium sulphate and Methylene blue were obtained from Research Lab Fine Chem. Industries and β-sitosterol from Santa Cruz Biotech. Imatinib Mesylate from Khandelwal Laboratories Pvt. Ltd.

B. Plant

The fresh leaves of the plant *Parkiabiglandulosa* for the study were collected from VeermataJijabaiBhosleUdyan, Byculla, Mumbai. The sample drug was been identified and authenticated from Department of Botany, Guru Nanak Khalsa College Matunga Mumbai The voucher specimen (specimen #: ss 14101415) has been preserved for future reference.

C. *Experimental Animals*

For the regenerative angiogenesis model, zebra fishes (*Danio rerio*) weighing between 0.5-1.0 g were procured from local supplier. Proper oxygen and nutrition supply was given and maintained with 13 hrs light and 11 hrs dark cycle. Acclimatization was done up to 15 days before start of experiment.

For developmental angiogenesis, zebrafish embryos were procured from local supplier. They were kept in embryo medium in 96 well culture plates, each plate with single embryo.

III. EXPERIMENTAL METHOD

A.Extraction

Fresh leaves of the plant were shade dried. Dried leaves were crushed and powdered. The powder was then passed through 40 mesh sieve. The powder was subjected to extraction using ethanol as a solvent in soxhlet apparatus. The crude extract that is Ethanolic Extract of EEPB was further dried and evaporated in Rotavac evaporator. β-sitosterol was isolated by optimizing mobile phase i.e. hexane:ethyl acetate (1.8:0.2) using flash chromatography.

B. Pharmacological evaluation

1*) Fin regeneration method* [10],[12]-[14]*:* The fishes were divided into 6 treatment groups with 6 fishes per group. Group I vehicle control, group II EEPB (1.704 mg/ 150 ml of water), group III EEPB (0.774 mg/ 150 ml of water), group IV β-sitosterol (1.704 mg/ 150 ml of water), group V β-sitosterol (0.774 mg/ 150 ml of water), group VI ImatinibMesylate (0.01 M/ 150 ml of water) On the day of experiment, the fishes were anesthetised by using 2- phenoxy ethanol (0.09%), then under dissection microscope the image of the whole fin was taken in order to compare it with regenerated fin. Their fin was cut up to 50% using sterile razor blade. Images were taken on 3rd and 7th day post amputation using motic microscope (4X). Length of the fin was measured by using motic image plus 2.0 to calculate fin regeneration.

2*) Developmental angiogenesis* [11], [15]-[17]For study of developmental angiogenesis zebrafish embryos were kept in 96 well microtitre plate one embryo per well in embryo medium. Embryos were treated with different concentrations; EEPB (0.2, 0.4, 0.6, 0.8, 1 mg/ml), β-sitosterol and imatnibmesylate (0.02, 0.04, 0.06, 0.08, 0.1 mM) monitored for various phenotypical changes such as tail blending, delayed hatching, abnormal yolk sac, abnormal vasculture, pericardial edema, haemorrhages and survival rate at 24 hours post fertilization (hpf), 48 hpf and 72 hpf by using motic digital microscope. Various phenotypical observations in embryos treated with EEPB, β-sitosterol and imatinibmesylate were reported.

IV. RESULTS

On day 3 length of caudal fin was significantly ($p<0.05$) less in all groups i.e. EEPB (0.774 mg/150 ml & 1.704 mg/150 ml), β-sitosterol (0.774 mg/150 ml & 1.704 mg/150 ml) and standard imatinibmesylate (0.01M/150 ml) as compared to vehicle control group. On day 7 regeneration of caudal fin was significantly ($p<0.05$) less in all groups i.e. EEPB (0.774 mg/150 ml & 1.704 mg/150 ml), β-sitosterol (0.774 mg/150 ml & 1.704 mg/150 ml) and standard imatinibmesylate (0.01M/150 ml) as compared to vehicle control group. [Table I and Fig. 2-4] In case of dorsal fin regeneration of EEPB in both low and high dose (0.774 mg/150 ml & 1.704 mg/150 ml) were it was found to be non-significant fin regeneration. Though the fin regeneration of EEPB and β-sitosterol was significantly less than vehicle control group on both day 3 and 7; it was not as less as that of standard imatinibmesylate. Further it was found that the inhibitory action on fin regeneration was dose dependent for both EEPB and β-sitosterol. The higher dose was found more effective as that of lower dose of EEPB and β-sitosterol.

TABLE I OBSERVATION IN TERMS of LENGTH (MM) OF CAUDAL FIN on DAY 3 AND DAY 7.

	Caudal fin length on Day 3			Caudal fin length on Day 7		
	Dorsal	Cleft	Ventral	Dorsal	Cleft	Ventral
I	159.62 ±1.06	139.62 ±1.43	170.28±1.25	224.05±1.63	206.53 ±1.01	235.98 ±1.06
II	72.99 ±0.76*	61.33± 0.46*	79.81 ±0.58 *	94.05 ±1.37	86.01± 0.86*	97.53± 0.89*
III	80.92±0.70*	73.94± 0.56*	92.17 ±0.79 *	114.07±1.51	107.03 ±0.76*	120.53 ±0.89*
IV	59.34±0.55*	52.86± 0.50*	67.93 ±0.80 *	83.20 ±0.98 *	76.44± 1.12*	88.29± 0.80*
V	63.53±0.58*	55.69± 0.48*	69.96 ±0.86 *	81.90 ±1.55 *	80.05± 1.01*	90.36± 0.74*
VI	51.96±0.51*	43.15± 0.82*	54.11 ±0.77 *	67.92 ±1.79 *	63.46± 1.42*	74.00± 0.82*

All values are expressed as Mean ± SEM for N= 6 fishes. * indicates values were significant ($p<0.001$) when compared to control using one way ANOVA followed by Tukey's test.

Embryos were observed for period of 72 hours at (24, 48, 72 hpf.) for evaluation of effects of EEPB and β-sitosterol on various stages of embryonic development. [Table II Fig. I] Vehicle group showed less number of phenotype changes indicating normal embryonic development while the phenotype abnormalities were found to be very high in higher doses of imatinibmesylate, EEPB and β-sitosterol treated groups as compared lower doses.

TABLE II PHENOTYPE ANALYSES of TREATED EMBRYOS at 72 HOURS POST FERTILISATION

Phenotypes	EEPB (mg/ml)		β-sitosterol (mM)		ImatinibMesylate (mM)	
	0.4	0.8	0.04	0.08	0.04	0.08
Tail bending	2	7	3	8	4	10
Abnormal elongation of yolk sac	4	8	4	9	5	10
Abnormal vasculature	3	7	5	8	6	8
Haemorrhages	0	4	0	6	0	7
Pericardial edema	1	5	2	7	3	7
Delayed hatching	0	1	0	2	0	3
Total Abnormalities	10	32	14	40	18	45

Tail bending Abnormal Elongation of yolk sac Abnormal vasculature

Haemorrhages Pericardial edema Delayed hatching

Fig.1 Typical phenotype changes observed at 24,48 &72 hr.

DORSAL	DAY 3	DAY 7

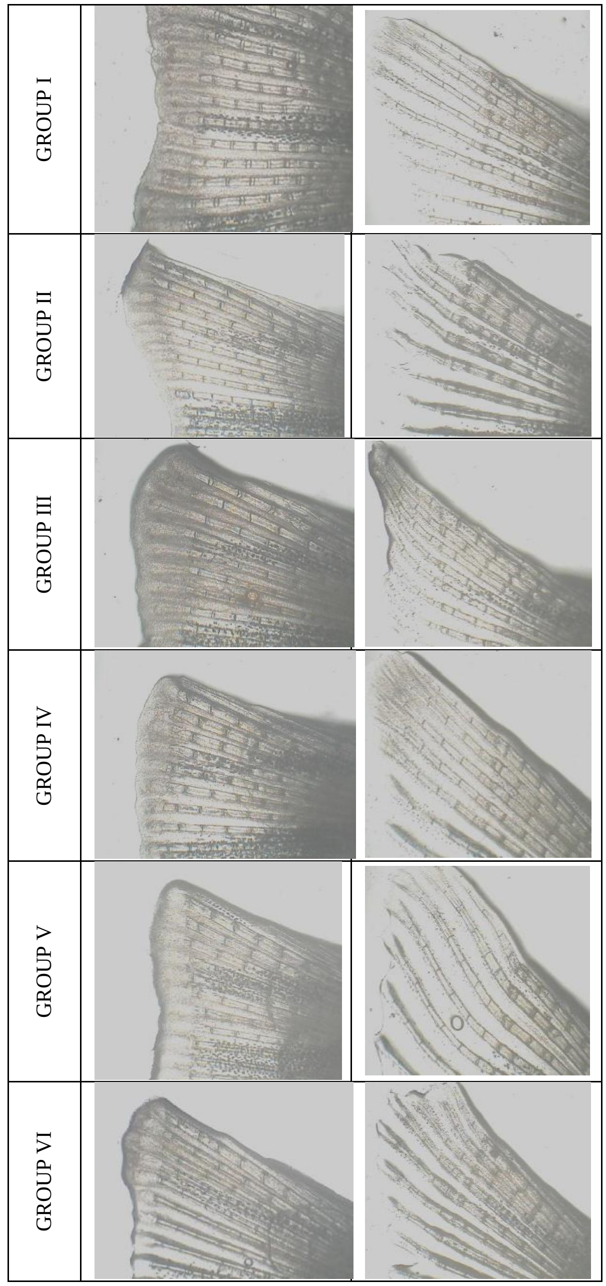
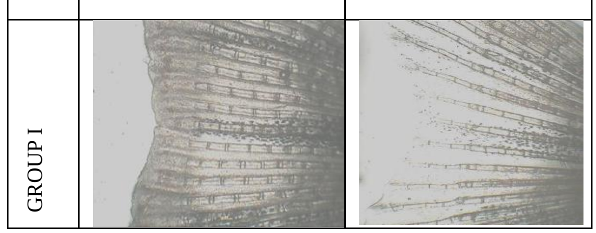


Fig. 2 Images of the length of regenerated fin of dorsal region on 3rd day and 7th day post amputation.

CLEFT	DAY 3	DAY 7



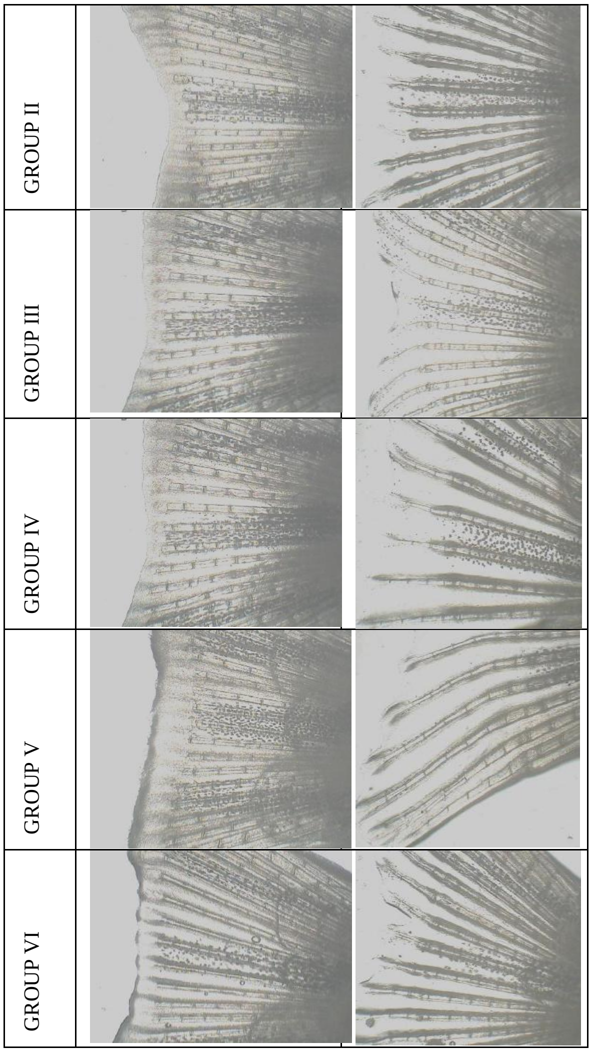
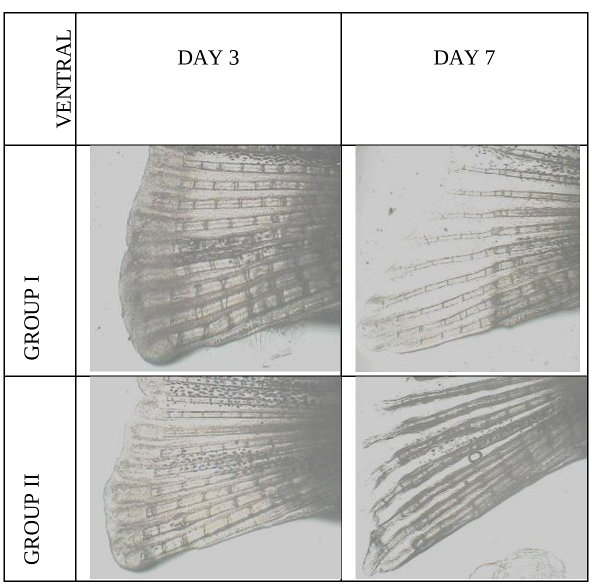


Fig. 3 Images of the length of regenerated fin of cleft region on 3rd day and 7th day post amputation.



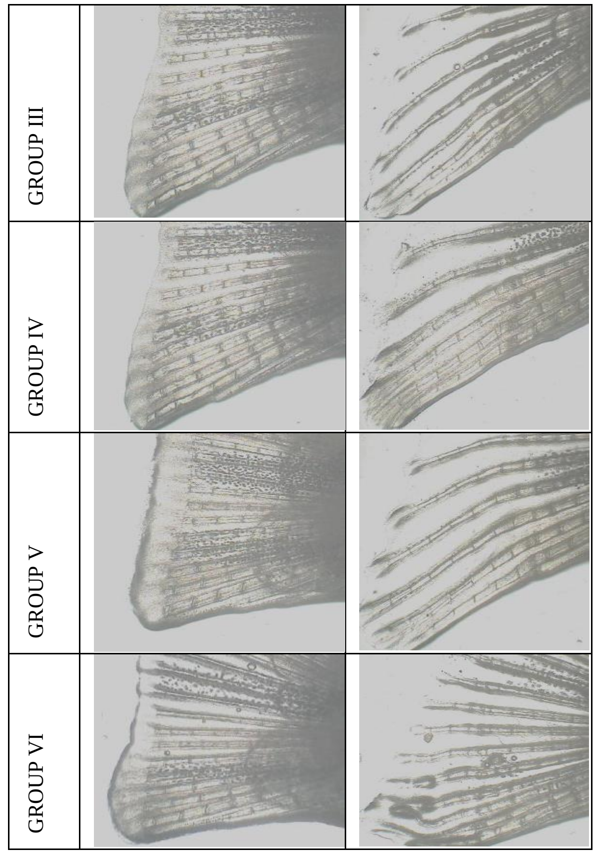


Fig. 4 Images of the length of regenerated fin of ventral region on 3rd day and 7th day post amputation.

V. DISCUSSION

In this study β-sitosterol was isolated from leaves of Parkiabiglandulosa by using flash chromatography and evaluation of angiogenesis was done for β-sitosterol and EEPB, it was compared with standard imatinibmesylate. Dose selection was done by performing toxicity studies for EEPB and β-sitosterol.

Angiogenesis is a process of generating new capillary blood vessels; it is a complex process involving extensive interplay between cells, soluble factors and ECM (extra cellular components). Zebrafish has emerged as powerful vertebrate model to study regeneration as it can regenerate such as heart, retina, maxillary barbell and fins which is not regenerated in mammals. Based on their regeneration ability it is useful in understanding the molecular and cellular mechanism of regeneration. Regeneration of tail fin is commonly used assay in adult zebrafish. 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 highly proliferation lineage – restricted mesenchymal progenitor cells; stage 3 is regenerative outgrowth and patterning of new tissue (>3 dpa).[18]-[20] Higher doses were more effective as compared to their lower doses of test and standard i.e. EEPB and β-sitosterol. Hence the inhibition of regeneration of fin was dose dependent.

Zebrafish embryos are extensively used to screen developmental changes because of its small embryo size, permeability to small molecules and ex-utero development which allows visual access to most stages throughout the development. Various phenotypes changes such as tail bending, abnormal yolk sac, abnormal vasculature, haemorrhages, pericardial edema, delayed hatching can be identified easily under microscope thus making it a useful model for angiogenesis study. The more number of abnormalities are seen as a result of stunted blood vasculature, hence indicating anti-angiogenic effect. EEPB and β-sitosterol treated groups has shown significant activity, although standard imatinibmesylate showed better anti-angiogenic activity.

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