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Evaluation of Anti-Angiogenic Effect of Naturally Occurring Compound from Ficus.Bengalensis on Regeneration & Development of Zebra-Fish Fin

Uday P kundap [α], Dr. Sadhana Sathaye [σ], Rachana D Sarawade [ρ], Dr. Ketan Patel [ω] & Pankaj D Jain [¥]

Abstract- Angiogenesis is the process that leads to the formation of new blood vessels or neovascularisation. Angiogenesis inhibitors are designed to prevent the formation of new blood vessels. The main aim of this study is to evaluate Anthocyanin obtained from medicinal plant *Ficus.bengalensis* for Anti-angiogenesis & to standardize a method for the study of angiogenesis. A variety of animal models have been described to provide more quantitative analysis of *in vivo* angiogenesis and to characterize pro- and anti Angiogenic molecules. However, it is still necessary to establish a quantitative, reproducible and specific method for studies of angiogenesis factors and inhibitors. *In-vivo* animal models were used, Anti-Angiogenic effects on Zebrafish fin regeneration. Parameters observed were Regenerative angiogenesis & variation in Zebrafish fin. In regenerative angiogenesis, test drug shows inhibition of fin regeneration as compared to Control Group. We demonstrate that the zebrafish is a viable model for screening small molecules that can inhibit angiogenesis. corroborating to the validity of the standardized method, Studies support and accelerate the application of Zebrafish & model that predict the anti-Angiogenic potential value of compound.

Keywords: anthocyanin, regenerative angiogenesis, vegf (vascular endothelial growth factor), zebrafish embryos (larvae).

I. Introduction

Angiogenesis, the process that leads to the formation of new blood vessels or neovascularisation, which is highly important during development but is largely not observed in the adult, except physiological exceptions in which angiogenesis occurs under tight regulation found in the female reproductive system and during wound healing. (Malin Dollinger et al.) In pathological situations, however, angiogenesis may be turned on, which contribute to the onset and progression of most severe human pathologies characterized by high mortality, including cancer, diabetes, obesity and retinopathies. Thus, angiogenesis is one of the largest and fastest evolving areas of research today, the knowledge of the molecular mechanisms that regulate neovascularisation continues to emerge, and there is increasing hope for the new discoveries that will lead to newer therapies targeting angiogenesis. (Uday P Kundap et al 2013) Angiogenesis is the physiological process through which new blood vessels develop from pre-existing vessels. This is distinct from vasculogenesis, which is the *de novo* formation of endothelial cells from mesoderm cell precursors. Anti-angiogenesis is a form of targeted therapy that uses drugs or other substances to stop tumors from making new blood vessels. Without a blood supply, tumors can't grow. Anti-angiogenesis research began more than 35 years ago with the work of the late Judah Folkman, MD. (Bagchi. D et al 2004, Han-Chung Wul et al) Anthocyanin are the flavonoid compounds that produce plant colours ranging from orange and red to various shades of blue and purple. Anthocyanin are members of the flavonoid group of phytochemicals, which is a group predominant in teas, honey, wines, fruits, vegetables, nuts, olive oil, cocoa and cereals. The flavonoids are thought to be perhaps the most important single group of phenolic in food. (Gael McGill et al.) Adult zebrafish have a remarkable regenerative capability. Many tissues which may not be regenerated in mammals are quickly regenerated in zebrafish. Among these are the heart, retina, maxillary barbell and fins importantly, as they regenerate, new blood and lymph vessels grow into the regenerating tissue – which enables studies on regenerative angiogenesis. One commonly used assay in the adult zebrafish, based on this principle is the regenerating tail fin. After amputation, the tail fin will re-grow and after approximately 1 month, the fin is back to its original size.

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The zebrafish embryo has become an important vertebrate model for assessing drug effects. It is well suited for studies in genetics, embryology, development, and cell biology. (L. D. Jensen1, et al) Zebrafish embryos exhibit unique characteristics, including ease of maintenance and drug administration, short reproductive cycle, and transparency that permits visual assessment of developing cells and organs. Because of these advantages, zebrafish bioassays are cheaper and faster than mouse assays, and are suitable for large-scale drug screening. The main aim of this study is to evaluate Anthocyanin obtained from medicinal plant Ficus.bengalensis for Anti-angiogenesis & to standardize a method for the study of angiogenesis. A variety of animal models have been described to provide more quantitative analysis of in vivo angiogenesis and to characterize pro- and anti Angiogenic molecules. However, it is still necessary to establish a quantitative, reproducible and specific.

II. Material & Methods

a) Collection of plant

The dried stem bark of Plant *Ficus.bengalensis Linn*. Was collected from Uran region of Navi-Mumbai Maharashtra India & were authenticated from Agarkar Research Institute, G. G. Agarkar Road, Pune, Sample deposited on 13/9/2012 & voucher number allotted is S/B-110.

b) Extraction of Anthocyanin

This unit describes methods for extraction, isolation, and purification of anthocyanin pigments from plant tissues. The choice of extraction method should maximize pigment recovery with a minimal amount of adjuncts and minimal degradation or alteration of the natural state.

Basic Protocol 1 describes the extraction of Anthocyanin with acetone and their partition with chloroform. Basic Protocol 2 describes a simple, fast, and effective method for purification of Anthocyanin from polyphenol compounds, sugars, and organic acids using solid-phase adsorption. This produces a uniform composite sample with a high surface area, which allows for efficient pigment extraction. (Oszmianski and Lee, 1990, Sheikh Anis, et al. 2012)

Basic Protocol – 1

Acetone Extraction & Chloroform Partition of Anthocyanin: In this method, acetone extracts the Anthocyanin from the plant material and chloroforms partitioning further isolates and partially purifies the pigments. The addition of chloroform results in phase separation between the aqueous portion (which contains the anthocyanin, phenolics, sugars, organic acids, and other water-soluble compounds) and the bulk phase (which contains the immiscible organic solvents, lipids, carotenoids, chlorophyll pigments, and other nonpolar compounds). This method has the advantage of producing an extract with no lipophilic contaminants. The absence of a concentration step minimizes the risk of acid-dependent pigment degradation. (Oszmianski and Lee, 1990)

Materials: Powdered plant material,Frozen Acetone 70% (v/v) aqueous acetone or aqueous acidified acetone: 70% aqueous acetone with 0.01% HCl, Chloroform, Acidified water: 0.01% (v/v) HCl in deionized, distilled water,Waring Blender with stainless steel container (Waring) or general-purpose homogenizer, Whatman no. 1 filter paper, Buchner funnel, Separatory funnel, 500-ml boiling flask

Rotary evaporator with vacuum pump or water aspirator, 40°C

Basic protocol – 2

Anthocyanin Purification: Purification of anthocyanin-containing extracts is often necessary, as the solvent systems commonly used for extraction are not specific for anthocyanin. Considerable amounts of accompanying materials may be extracted and concentrated in the coloured extracts, which can influence the stability and/or analysis of these pigments (Jackman and Smith, 1996). Anthocyanin purification using solid-phase extraction (Figure) permits the removal of several interfering compounds present in the crude extracts. Mini-columns containing silica gel 60 chains bonded on silica retain hydrophobic organic compounds (e.g., anthocyanin, phenolics), while allowing matrix interferences such as sugars and acids to pass through to waste. Washing the retained pigments with ethyl acetate will further remove phenolic compounds other than anthocyanin. (Oszmianski and Lee, 1990)

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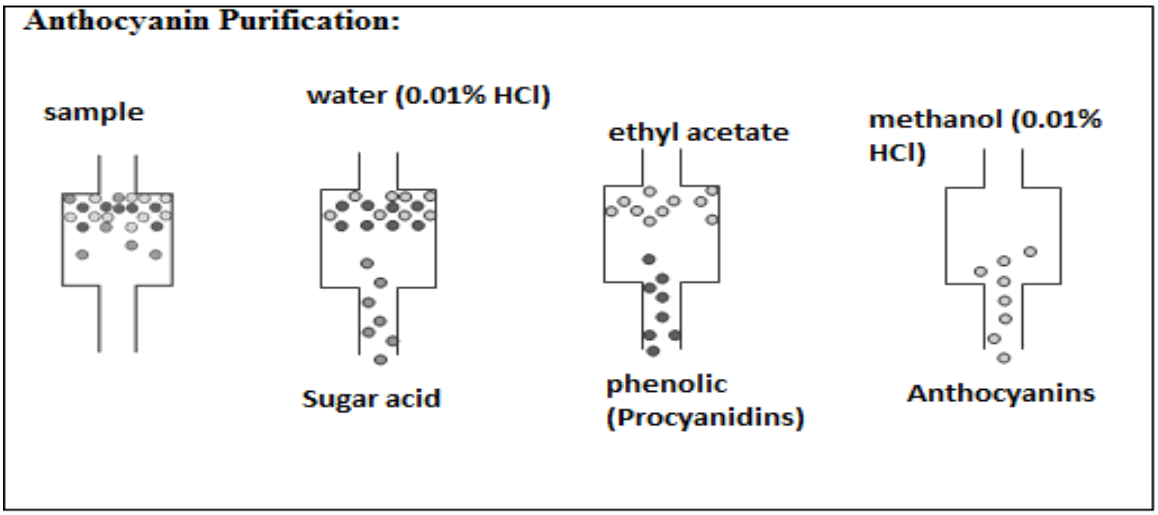

Figure 1 : Anthocyanin Purification

Materials: Methanol, Acidified water: 0.01% (v/v) HCl in deionized, distilled water, Aqueous anthocyanin extract (see Basic Protocol 1), Ethyl acetate, Silica gel 60, Acidified methanol: 0.01% (v/v) HCl in methanol, 50- to 100-ml boiling flask, Rotary evaporator with vacuum pump or water aspirator, 40°C, Freeze-resistant container (optional).

c) Animals

Animals were obtained from the Animal House of H(S)NCB's facility for Breeding & Experimentation, Dr. L.H. Hiranandani college of Pharmacy, opposite to Ulhasnagar station, CHM Campus, Ulhasnagar-03, maintained in an animal holding room (Protocol Registration no. 879/ac/05/CPCSEA). The experimental protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Efforts were made to minimize animal suffering and to reduce the number of animals used.The experiments were performed after the approval by the Institutional Animal Ethics Committee (IAEC) and were carried out in accordance with the current guidelines for the care of laboratory animals. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Zebrafish: Danio rerio (wild type) were obtained from local suppliers and maintained at 280C on a 14 hours light/10 hour dark cycle in 40 L glass tanks with four females and eight males in separate tanks. PH of breeding water was maintain at 6.6 - 6.8, temperature of 78-82 0 F. Embryos were collected by natural spawning with 2:1 male to female ratio and staged according to Kimmel et al. Embryo stage is denoted as hours post-fertilization (hpf). (Amy L Rubinstein.2003, Christian Lawrence. 2000).

d) In-vivo Model Method

Anti-Angiogenic effects on Zebrafish fin regeneration assay: (John N et al, Kimmel CB 1995 et al)

i. *Setting up the tank:* For Setting up the tank 5 .5 gallon tank was taken, 25 watt submersible heater, air stone supplied with air from a vibrator pump and enough marbles to place on the bottom of the tank to a depth of 2 .5 inches deep across the whole tank bed. For conditioning, feeder with variety of frozen and flake food 3-5 times, PH was set at 6.6 with a water temperature of 780 F. (Christian Lawrence. 2007)

ii. *Amputation of Zebrafish Fin:* Eight Zebrafish were transfer into each beaker. Tip of zebrafish fin was cut to initiate angiogenesis in the model. Percent Regeneration of the Zebrafish was calculated at 7th day to determine the anti-Angiogenic activity.

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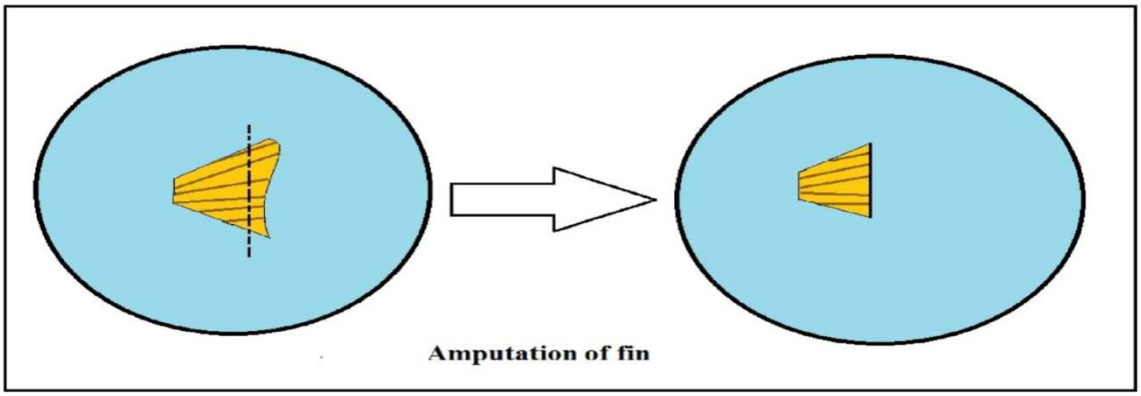

Figure 2 : Amputation of Zebrafish fin

e) Drug treatment

i. *Tank A:* 600ml of water with fish feed (Three times a day)

ii. *Tank B:* Vehicle control group: 200µl methanol & 200µl PEG-400; Dose added i: e 80µl in 600ml water.

iii. *Tank C:* Standard drug: 1ngm of Std. drug in 200µl of PEG-400. 1000ng = 1µgm; Dose added i: e 80µl in 600ml of water = 0.5ppm concentration of drug in 600ml water (paclitaxel), 1000ηgm of Paclitaxel = 0.5ppm = in 600ml water

iv. *Tank D:* Test drug: 1mg of drug in 200µl methanol & 200µl PEG-400; Dose added i: e 80µl in 600ml water = 3.3ppm concentration of drug in 600ml water. 1mg of test drug = 3.3ppm = in 600ml water.

III. Results

a) Anti-Angiogenic effects on Zebrafish (Danio rerio) fin regeneration assay

Adult Zebrafish have a remarkable regenerative capability; many tissues which may not be regenerated in mammals are quickly regenerated in Zebrafish. As they regenerate, new blood & lymph vessels grow into the regenerating tissue - which enables studies on regenerative angiogenesis.

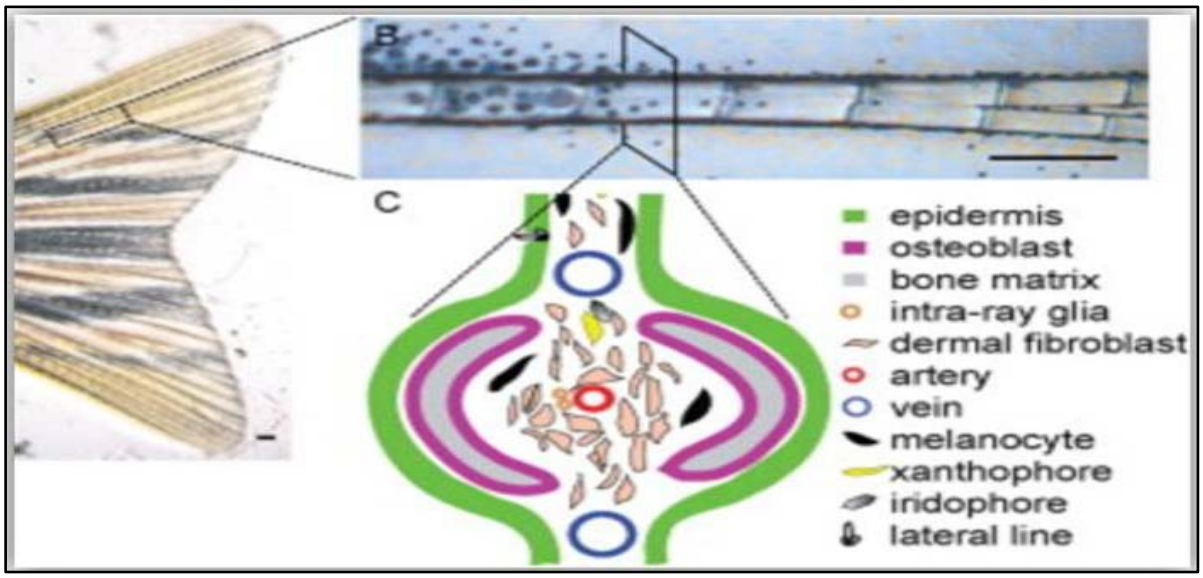

Figure : 3 a

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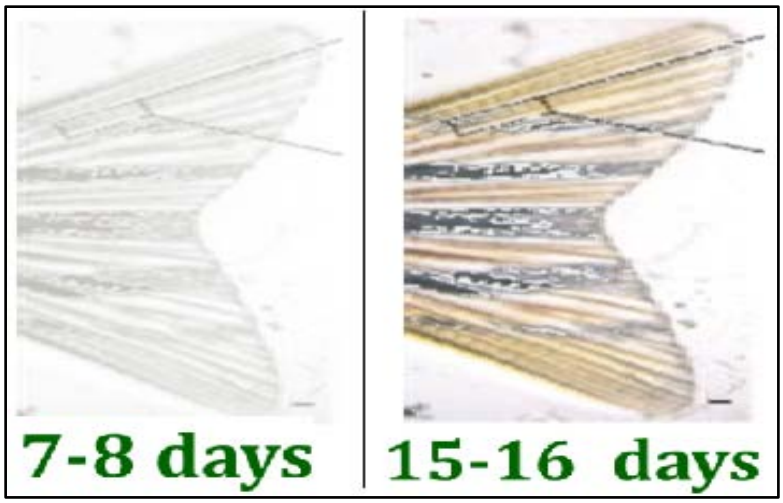

Figure : 3 b

Figure 3 a : TS of Zebrafish fin under microscope shows the presence of artery & vein, which indicates the presence of various number of blood vessels in fin,

Figure 3 b : Zebrafish fin requires approx. 7-8 days for regeneration & 15-16 days for maturation) One commonly used assay in the adult Zebrafish, based on this principle is the regenerating tail fin. After amputation, the tail fin re-grows & after approximately 1month, the fin is back to its original size. The process of Fin regeneration encompasses many of the same mechanisms as in human wound healing & regeneration & is therefore a good model of regenerative angiogenesis. The vasculature is remarkably simple & as the fin grows back various levels of vascular remodeling can be observed & therefore studied in detail. This regeneration model is probably the most commonly used, adult Zebrafish angiogenesis model, & is considered to be complementary to developmental angiogenesis.

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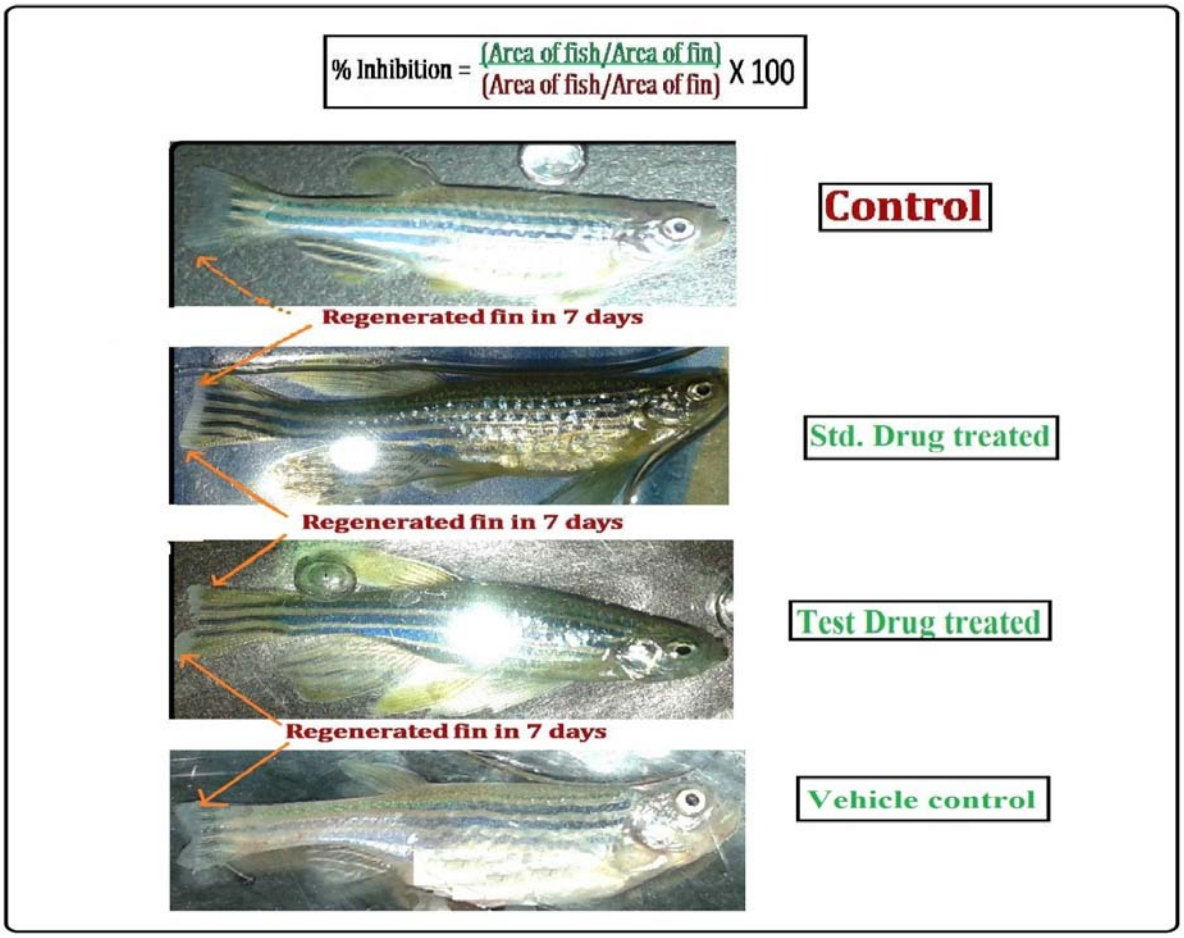

Figure 4 : Control group & vehicle control group shows the maximum regeneration of Zebrafish fin & blood vessels, Standard drug Group & test drug group shows uneven, stunted growth & irregular of regeneration Zebrafish fin).

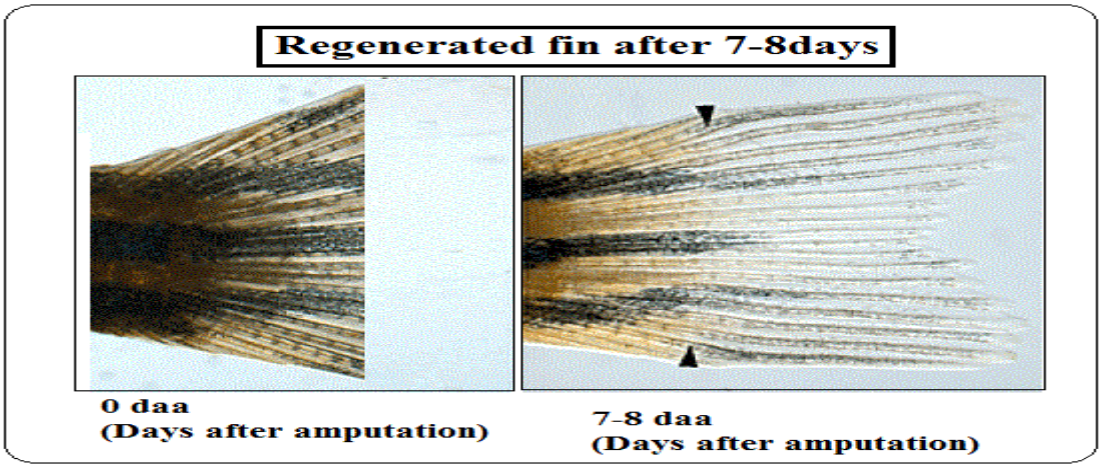

Figure 5 : Regenerated fin after 7-8days

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Microscopic view of regenerated fin

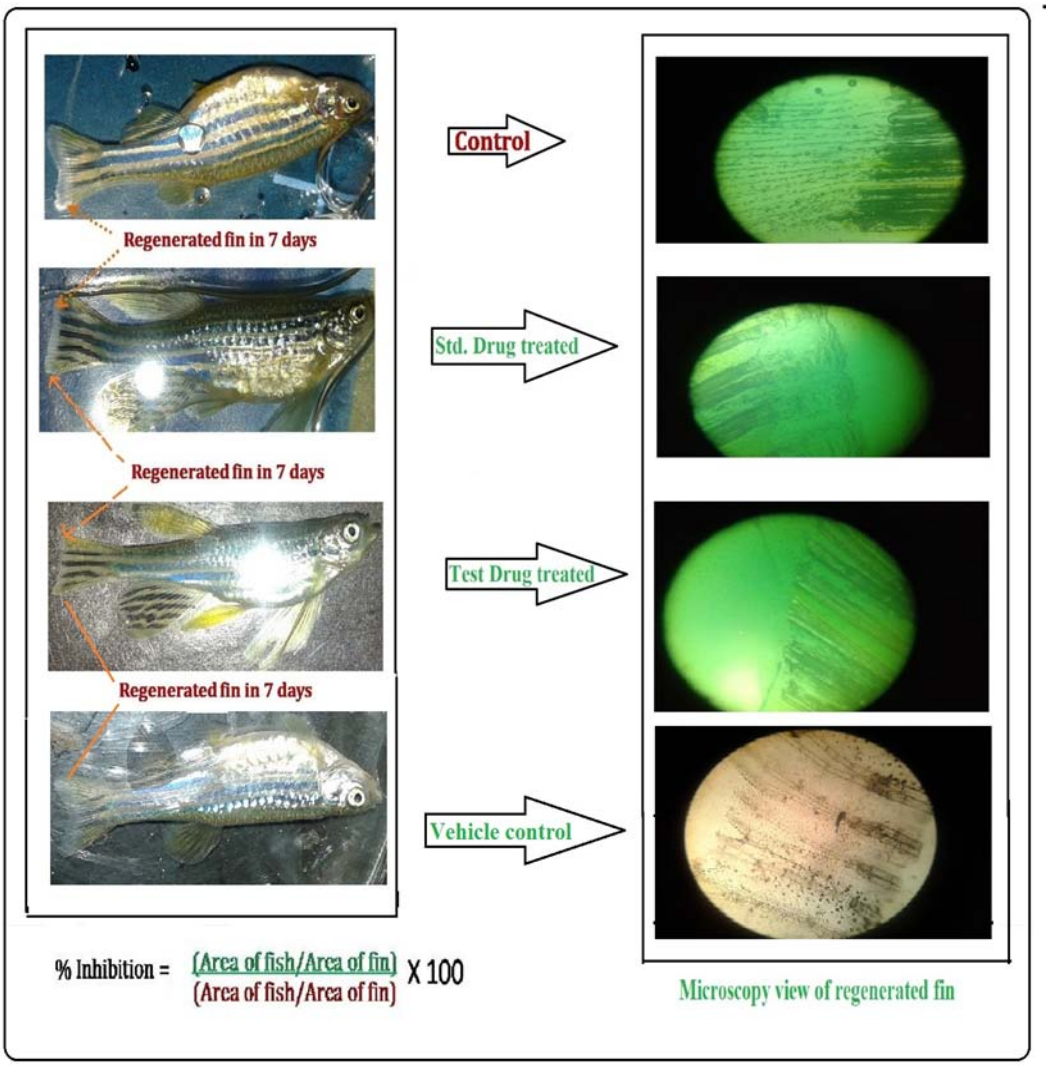

Figure 6 : (Microscopic view of Regenerated fin after 7 days: *Control group* shows the normal growth of blood vessels, *standard drug group* shows the stunted growth of blood vessels, *Test drug group* shows insufficient growth as compared to control group, *Vehicle control group* shows normal growth as control group.)

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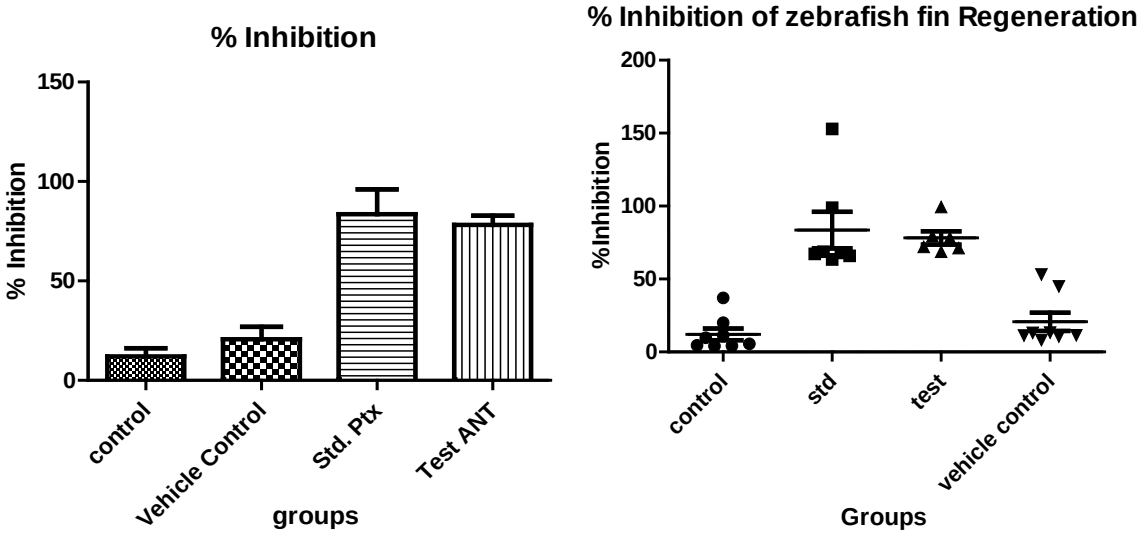
Graph for % Inhibition of Zebrafish fin regeneration assay



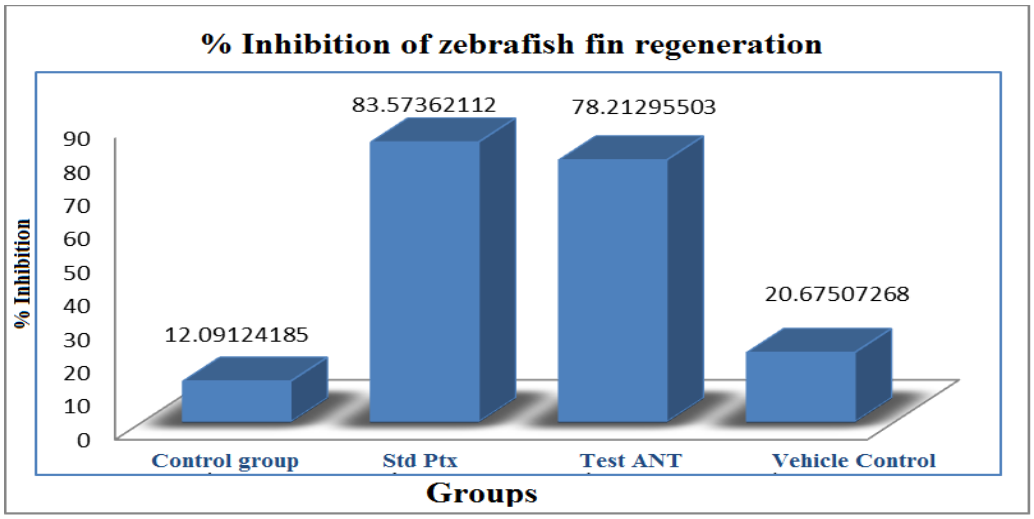


Table 2 : Statistical analysis table for Zebrafish fin regeneration assay

Group	Macroscopic score
Control	12.09± 4.045
Standard group(0.5mg/ml)	83.57±12.43***
Test group (1 mg/ml)	78.21±4.57***
Vehicle Control	20.68±6.246

Mean± S. E : *= P<0.05; **= P<0.01; ***=P<0.0001

f) Microscopic Images

Regenerated Zebrafish fin was observed under the microscope after 7-8 days of regeneration, fish from control group shows the normal & complete growth of blood vessels in fin. Vehicle control group also shows the complete & normal growth of blood capillary bloodvessels in regenerated fin. Unique observation was noticed in standard drug group, the growth of capillary blood vessels in fin was stunted, the growth of blood vessels was blocked in standard drug paclitaxal (0.5ppm conc). Fishes in the test drug shows the simillar features as standard drug group. The growth of the blood vessels was also blocked by the test compound.

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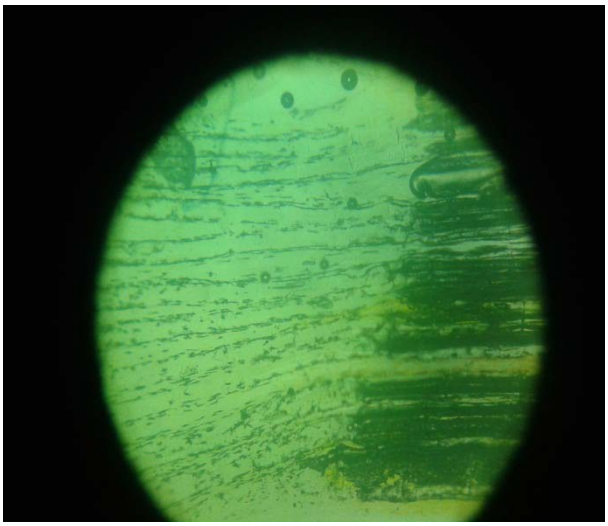
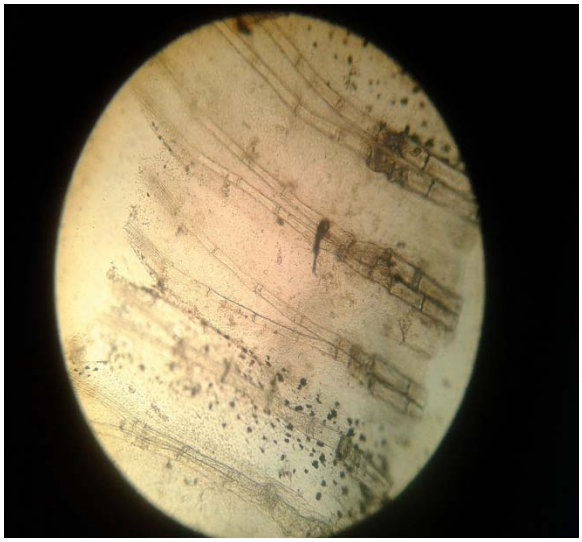
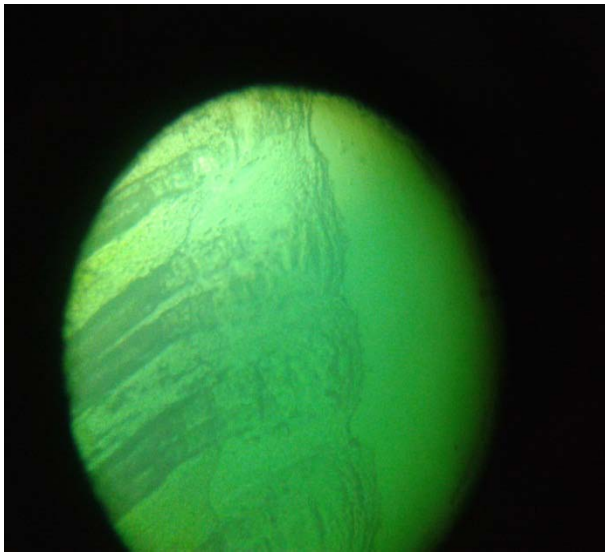


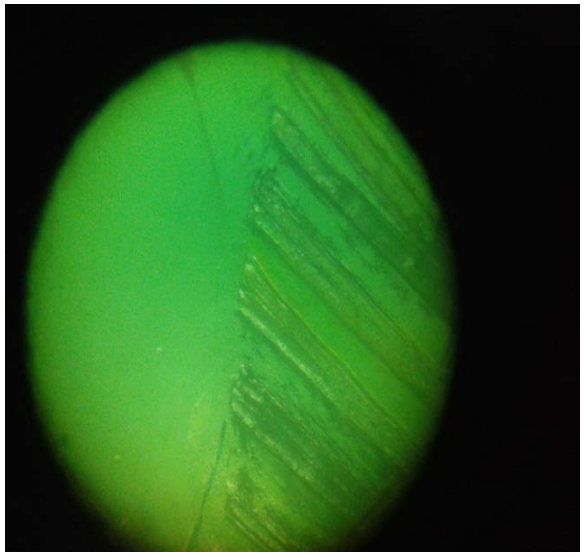
Control Group



Vehicle Control Group



Strandard Drug Group



Test Drug Group

IV. Discussion

a) *Anti-Angiogenic effects on zebrafish (Danio rerio) fin regeneration assay*

Adult zebrafish have a remarkable regenerative capability. Many tissues which may not be regenerated in mammals are quickly regenerated in zebrafish. Among these are the heart, retina, maxillary barbell and fins importantly, as they regenerate, new blood and lymph vessels grow into the regenerating tissue – which enables studies on regenerative angiogenesis. One commonly used assay in the adult zebrafish, based on this principle is the regenerating tail fin. After amputation, the tail fin will re-grow and after approximately 1 month, the fin is back to its original size. Angiogenesis process was activated in zebrafish by cutting there fin. Test compound (1mg/300ml of water) was added in the water. Comparison of test drug & Standard Drug effect was done with Control & vehicle control for regeneration of tail fin as a parameter for studying regenerative anti_angiogenesis. Control group was kept to know the normal growth of fin. (Amy L Rubinstein, 2003, Westerfield M 2000) The test (Anthocyanin extract) & Standard (Paclitaxel) compound were evaluated for Anti- Angiogenic activity after 7 days of amputation. Standard drug showed 83.57± 12.43*** Test drug showed 78.21 ± 4.57*** angiogenesis inhibition, control group 12.09 ± 4.04, Vehicle control 20.67± 6.24 of angiogenesis inhibition which is significant according to statistical analysis (one-way annova). Regenerated Zebrafish fin was observed under the microscope after 7-8 days of regeneration, fish from control group shows the normal & complete growth of blood vessels in fin. Vehicle control group also shows the complete & normal growth of capillary blood vessels in regenerated fin. Unique observation was noticed in

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standard drug group, the growth of capillary blood vessels in fin was stunted, and the growth of blood vessels was blocked in standard drug paclitaxel (0.5ppm conc). Fishes in the test drug (3.3ppm) shows the similar features as standard drug group. The growth of the blood vessels was also blocked by the test compound which shows 78.21% inhibition of angiogenesis.

v. Conclusion

Design and development of small molecule therapeutics to inhibit angiogenesis has gained considerable importance in anti-angiogenesis research. We demonstrate that the zebrafish is a viable model for screening small molecules that can inhibit angiogenesis. The mechanism of Anthocyanin action is not yet known, but hypotheses include decreased levels of tumor necrosis factor alpha (TNF-a)-induced VEGF expression), inhibition of H2O2- and as well as through the inhibition of VEGF and VEGF receptor expressionThus, our study suggests that even mill molar concentration of test drug (Anthocyanin) could be an effective drug for in vivo inhibition of angiogenesis and thus might gain significance in future therapeutics. (SimonaLucioli 2012, L. D. Jensen1,et al)

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