



Evaluation of Anthocyanin for its Anti-Angiogenic Potential in Rodent Model

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ABSTRACT: Angiogenesis, the process that leads to the formation of new blood vessels or neovascularization; an important process involved in neovascular diseases and tumor. 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 antiangiogenic 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 for evaluation of anti-angiogenesis; in cotton plug Implant model in rats, Cotton plug was implanted in the upper skin of rat in control and experimental group. After 21 days, the cotton plug was analysed for haemoglobin content. It was observed that cotton plug-induced angiogenesis can be suppressed by Anthocyanin, corroborating to the validity of the standardized method. Studies support and accelerate the application of rodent model that predict the anti-angiogenic potential value of compound.

Keywords: Angiogenesis; Anthocyanin; VEGF (Vascular Endothelial Growth Factor); Inhibition of H₂O₂; VEGF receptor expression.

INTRODUCTION

Angiogenesis, the process that leads to the formation of new blood vessels or neovascularization, 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. 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 neovascularization continues to emerge, and there is increasing hope for the new discoveries that will lead to newer therapies targeting angiogenesis.² 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. The first vessels in the developing embryo form through vasculogenesis, after which angiogenesis is responsible for most, mainly, blood vessel growth during development and in disease. Angiogenesis is a normal and vital process in growth and development, as well as in wound healing and in the formation of granulation tissue. However, it is also a fundamental step in the transition of tumor from a benign state to a malignant one. The essential role of angiogenesis in tumor growth was first proposed in 1971 by Judah Folkman, who described tumor as “hot and bloody”.^{15 & 13}

Angiogenesis; Insights into Basic Mechanisms¹ virtually every cell in the body is within 100-200 μ of a capillary. This is the maximum distance over which oxygen can effectively diffuse into cells. Capillaries are the simplest and smallest units of the micro-vascular apparatus, measuring approximately 8- 20 μ in diameter. Although there are several varieties of capillaries each designed for a particular function, they are all composed of a single layer of endothelial cells invested by a basement membrane and are encircled by a pericytes or two. To give you an idea of how abundant these simple structures are, consider that they encompass a surface area of about 1000 square centimetres¹⁵. Vascular cells—in particular, endothelial cells—are deceptively indolent. They are metabolically quite active. They produce a host of cytokines, adhesion molecules, growth factors, and vasoactive peptides that have profound effects on innate and cellular immunity, organ and tissue repair, and cardiovascular, renal, and pulmonary function.

Anti-Angiogenesis; Insights into Basic Mechanisms:³ 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.

Table 1: The Plant - The Banyan Tree: Taxonomic classification of *F. bengalensis*¹³

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyte
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Hamamelidae
Order	Urticales
Family	Moraceae
Genus	Ficus
Species	<i>F. bengalensis</i>

Phytochemical constitution: Phytochemical investigation of *F. bengalensis* led to the exploration of a wide variety of constituents which are responsible for its wide range of pharmacological activities. Stem bark of *F. bengalensis* contains bengalenosides that is, glycosides or flavonoids, 5, 7 Dimethyl ether of Leucoperalgonidin-3-O- α -L-rhamnoside and 5, 3 dimethyl ether of leucocyanidin 3-O- β -Dgalactosyl cellobioside, and 5, 7, 3 trimethoxy leucodelphinidin 3-O- α -L-Rhamnoside. All these flavonoids consist of various sugars attached with OH groups of Leucoperalgonidin, Leucodelphinidin and Leucocyanidin^{14& 16}.

Anthocyanins: Anthocyanins are the flavonoid compounds that produce plant colours ranging from orange and red to various shades of blue and purple.^[5] Anthocyanins 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.

The primary players in the flavonoid group include: Anthocyanins (cyaniding, pelargonidin, petunidin).

MATERIAL AND METHODS

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.

Extraction of Anthocyanin⁹: This unit describes methods for extraction, isolation, and purification of anthocyanin pigments from plant tissues. These methods are essential laboratory operations prior to subsequent experimental work involving separation, characterization, and quantitation of the pigments. The polar character of the anthocyanin molecule allows for its solubility in many different solvents such

as alcohols, acetone, dimethyl sulfoxide, and water. 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 Anthocyanins with acetone and their partition with chloroform. Basic Protocol 2 describes a simple, fast, and effective method for purification of Anthocyanins 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 extraction¹⁶.

Basic Protocol – 1:

Acetone Extraction & Chloroform Partition of Anthocyanins^{9 & 17}: In this method, acetone extracts the anthocyanins 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.

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^[17]. Anthocyanin purification using solid-phase extraction 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.

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)¹⁶.

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.

Rodents: Wister rats of either sex weighing between 100-130gm were used. Animals were kept in temperature controlled experimental room of animal house ($23^{\circ} \pm 1^{\circ}\text{C}$) and $55 \pm 10\%$ RH. They were subjected to 12:12 hours light : dark cycle for at least 7 days prior to the study treatment ensuring their acclimatization to the experimental conditions. Animals were housed in standard polypropylene cages with wire mesh top. They were fed with commercially available rodent food pellets (Supplied by Pranav agro industries ltd.) and water (supplied by Municipal Corporation of Ulhasnagar)^{5 & 7}.

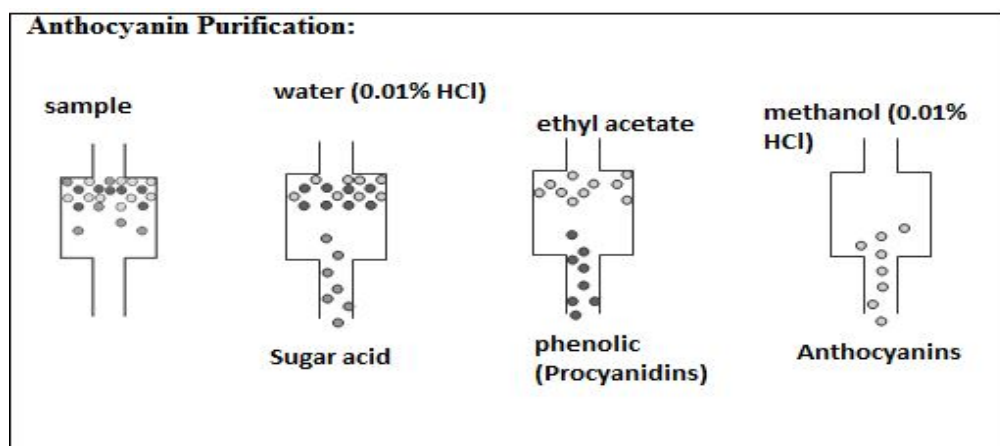


Figure 1: Anthocyanin Purification

In-vivo Model Method:

Cotton plug Implant model in rats^{3 & 4}: Mammalian model employs grafting of Cotton plug (angio-plug) under the skin of Wister rats. Within 10 to 15 days after implantation, cotton plug were infiltrated with newly formed angiogenic blood vessels originating from the pre-existing blood vessels. Anti-Angiogenesis in individual cotton plug can be measured by haemoglobin content present in cotton plug.

Procedure: Male wistar rats with an averages weight of 100-130gm are anaesthetized with ketamine (70mg/kg) xylazine (5mg/kg). The back skin was shaved & disinfected with 70% ethanol. An incision was made in the lumbar region. By a blunted forceps subcutaneous tunnels was made and a sterilized cotton pellet was place inside the tunnel in the scapular region. Within 10 to 15 days after implantation, cotton plug are infiltrated with newly formed angiogenic blood vessels originating from the pre-existing blood vessels converging towards the plug openings⁷.

Day-1: cotton plug inserted by surgery; **Day1-7:** Initiation of Angiogenesis; **Day 7-21:** Treatment with Standard & Test drug; **Day 21:** centrifuged each plug at 10,000 x g for 15 minutes in 0.01% HCl solution & analysis of acid-hematin for concentration of haemoglobin in cotton plug.

Formulation procedure:

- 1) 5ml methanol + Drug + TPGS + PEG 1600 Triturated all in mortal
- 2) Lactose was added.
- 3) Triturated in Mortal till stick paste is formed
- 4) Evaporate in rota evaporator at 70°C to remove all methanol

Evaluation: The average concentration of haemoglobin in the pellets of the control group, vehicle control group, standard group & the test group was calculated. Each cotton plug was removed from animals body and added into 5ml 0.01% HCl solution, haemoglobin react with HCl and forms a red colouring substance known as acid-hematin. The supernatant was filtered through a 0.22 micron filter (Millipore). The concentration of acid-hematin (red colour) was calculated by UV-Visible spectroscopy. The maximum absorbance of this colouring substance was at 550nm (λ max 550nm).^[4]

Drug treatment:

A = Control group: Normal control group; fed with normal animal feed and tap water through the entire experimental period (Normal control).

B = Vehicle control group; fed with normal animal feed and tap water through the entire experimental period (Vehicle control), Along with dose of vehicle/excipients used for formulation of dose.

C = Standard group: Standard control group; treated with standard Paclitaxel drug formulation then fed with normal animal feed for the experimental period (Standard) 3.9mg/ kg dose

D = Test Drug group, treated with test drug formulation then fed with normal animal feed for the experimental period (Test group) 100mg/kg dose of test drug i:e 18mg – 0.5gm in 3ml distilled water.

RESULTS AND DISCUSSION

Cotton plug Implant model in rats: Mammalian model employs grafting of cotton plug (angio-Plug) under the skin of Wister rats. The average concentration of haemoglobin in the cotton plug of the control group, vehicle control group, and standard group as well as of the test group was calculated. Each cotton plug was removed from rat's body and added into 5ml 0.01% HCl solution, haemoglobin react with HCl and forms a red colouring substance known as acid-hematin. The concentration of acid-hematin (red colour) was calculated by UV-Visible spectroscopy. The maximum absorbance of this colouring substance was at 550nm (λ max 550nm).

Day-1: cotton plug inserted by surgery; **Day1-7:** Initiation of Angiogenesis; **Day 7-21:** Treatment with Standard & Test drug; **Day 21:** Dissolve each plug in 0.01% HCl solution & analysis of acid-hematin for concentration of haemoglobin in cotton plug.

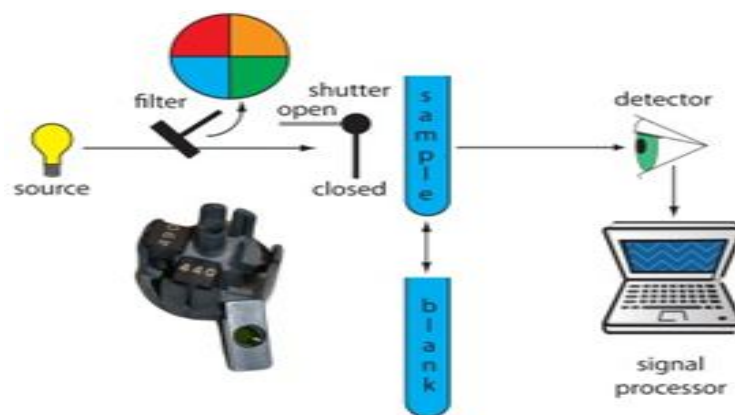


Day-1

Day-1-7

Day 7-21

Day-21



UV Analysis

Figure 2: Cotton plug implant model in rats

Standard curve of Male wistar rat haemoglobin was plotted. Absorbance of standard & test haemoglobin from cotton pellet was also measured and was extrapolated on the standard curve to find out the haemoglobin content of the sample.

Infiltration of angiogenic blood vessels in standard & test drug group is very less as compared to control & Vehicle control group. Morphological observation shows the presence of heavy blood vessel growth in control & vehicle control group, the intensity of blood vessel growth is very less in standard (3.9mg/kg) & Test drug group (200mg/kg).



Control group



Vehicle control group



Standard Drug Group



Test Drug Group

Figure 3: Images of Cotton plug (Angio Plug) after 21 Days

Table 2: Standard curve of male Wistar rats Haemoglobin

Rat Haemoglobin (ng/ml)	Reading (A550nm)	Reading2 (A550nm)	Average
5	0.2760	0.281	0.2810
10	0.3410	0.3835	0.3835
20	0.5040	0.5560	0.5560
40	0.8360	0.8675	0.8675
80	1.1000	1.1375	1.1375
160	1.3280	1.3680	1.3680

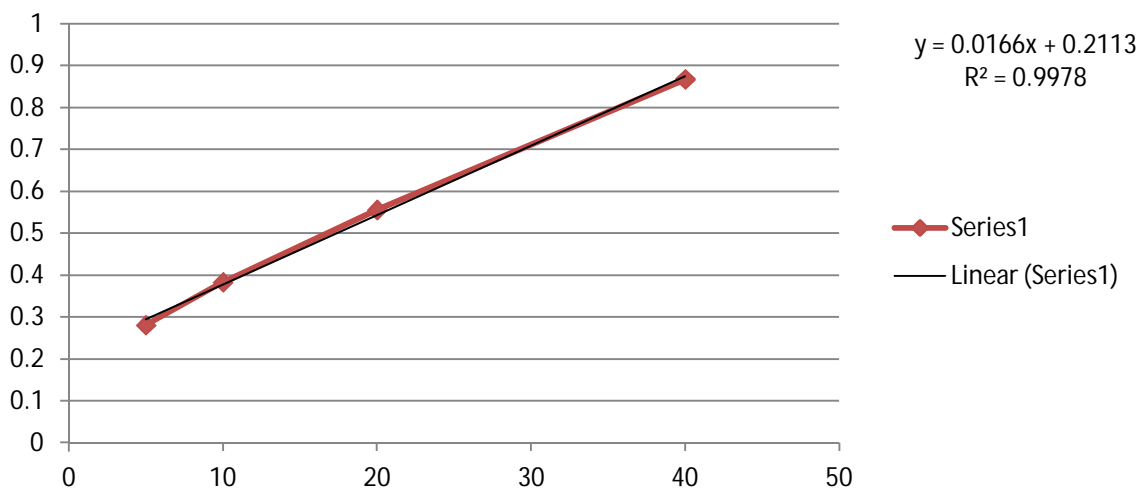


Figure 4: Standard curve of Wistar rat Haemoglobin

Table 3(a): Control group

Sr. no	Haemoglobin concentration	Average conc. (ng/ml)	% Hb conc.
R1	38.83	55.94333333	55.94%
R2	45.57		
R3	70.39		
R4	58.34		
R5	55.26		
R6	67.27		

Table 3(b): Vehicle control group

Sr. no	Haemoglobin concentration	Average conc. (ng/ml)	% Hb conc.
R1	46.83	49.27	49.27%
R2	35.57		
R3	42.39		
R4	78.34		
R5	55.26		
R6	37.27		

Table 3(c): Standard group

Sr. no	Haemoglobin concentration	Average conc. (ng/ml)	% Hb conc.
R1	21.84	21.51	21.51%
R2	16.17		
R3	9.30		
R4	22.02		
R5	34.58		
R6	25.15		

Table 3(d): Test drug group

Sr. no	Haemoglobin concentration	Average conc. (ng/ml)	% Hb conc.
R1	25.02	28.2633333	28.26%
R2	25.03		
R3	19.96		
R4	37.39		
R5	21.84		
R6	40.34		

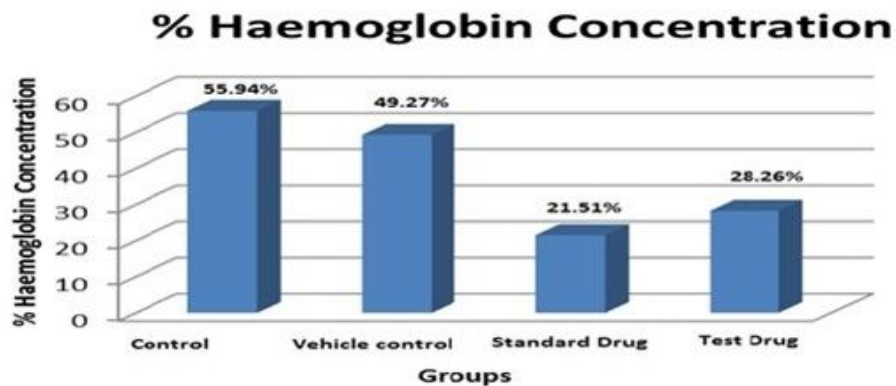


Figure 5: Haemoglobin concentration in cotton plug model

Table 4: Statistical analysis table for Haemoglobin concentration in cotton plug model

Group	Macroscopic score
Control	55.94± 4.97
Vehicle group	49.28±6.49
Standard drug (3.9mg/kg)	21.51±3.47***
Test drug(100 mg/kg)	28.26±3.46***

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

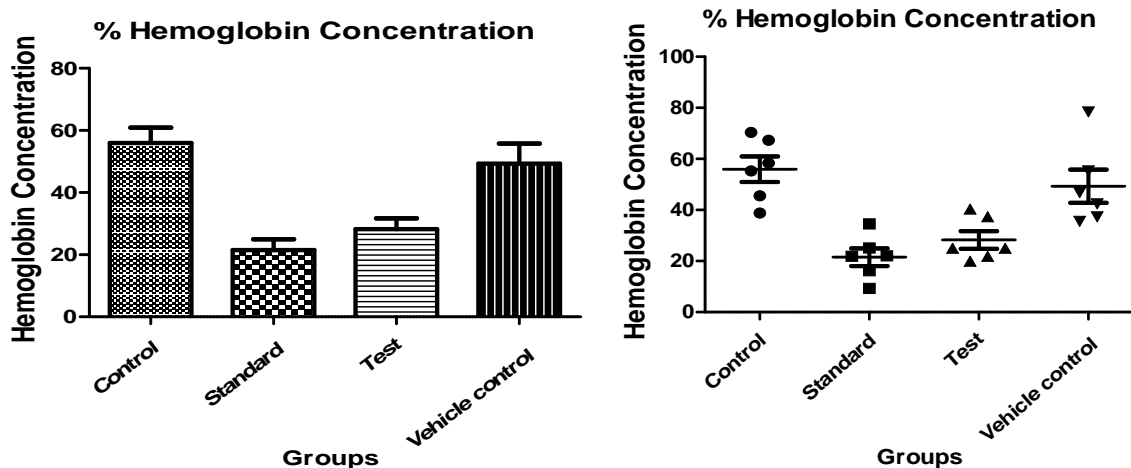


Figure 6: % Haemoglobin concentration in cotton plug model

Statistical Analysis: Haemoglobin content in sponge and in serum was tested using nonparametric Mann-Whitney test. ANOVA GraphPad Prism 5.0 software was used. ***= $P < 0.0001$ was considered statistically significant.

Anthocyanins work – The mechanisms: Angiogenesis, one of the hallmarks of cancer, vital to tumor growth and metastasis, is characterized by growth of new capillaries from pre-existing vessels. Cancer cells release vascular epithelial growth factor (VEGF), an angiogenic cytokine which stimulates blood vessel growth. Inhibition of VEGF has therefore become a primary target for anti-angiogenic strategies, and inhibitors directed against either VEGF or its receptor VEGFR-2, Anthocyanins suppress angiogenesis through the inhibition of H_2O_2 - and tumor necrosis factor alpha (TNF- α)-induced VEGF expression, as well as through the inhibition of VEGF and VEGF receptor expression. Anti-mutagenic and anti-carcinogenic activity of anthocyanins is generally ascribed to their antioxidant properties conveyed by their phenolic structure⁹. The double bonds in the ring and the hydroxyl side chains confers them potent free-radical scavenging activities (the positively charged oxygen atom in their molecule makes them more generous hydrogen donating antioxidants compared to other flavonoids), but also enables their metal chelation and protein binding properties. Apart from acting as direct free-radical scavengers, anthocyanins have been demonstrated to affect the activity of phase II enzymes well-known for their detoxifying and antioxidant properties and therefore important in cancer /preventing^{8 & 15}.

Acute Toxicity Study: LD-50 value by oral route could not be determined as no mortality was observed up to 4g / Kg dose level. No toxic reactions were observed at none of the doses employed.

Cotton plug implant model in Rat: model employs grafting of cotton plug (angio-Plug) under the skin of Wistar rats. In this model cotton plug was further implanted under the skin of wistar rats. Within 10 to 15 days after implantation, cotton plug were infiltrated with newly formed angiogenic blood vessels originating from the pre-existing blood vessels in control group & Vehicle Control group. As the new blood vessels grow inside the cotton plug the amount of haemoglobin concentration in the plug also increases. The anti-angiogenic drug block the growth of blood vessels into the plug & thus the concentration of haemoglobin were very less. Anthocyanins suppress angiogenesis through the inhibition of H_2O_2 - and tumor necrosis factor alpha (TNF- α)-induced VEGF expression, as well as through the inhibition of VEGF and VEGF receptor expression. Anti-Angiogenesis in individual cotton plug can be measured by haemoglobin content. This paper describes a quantitative method for the study of angiogenesis in mice. It offers several advantages: (i) an objective assessment of angiogenesis; (ii) reproducibility and (iii) evaluation of angiogenesis by comparison between Haemoglobin content in serum and in sponge.

Infiltration of angiogenic blood vessels in standard & test drug group is very less as compared to control & Vehicle control group. Morphological observation shows the presence of heavy blood vessel growth in control & vehicle control group, the intensity of blood vessel growth is very less in standard (3.9mg/kg) & Test drug group (100mg/kg). The test (Anthocyanin extract) & Standard (Paclitaxel) compound was evaluated for Anti-angiogenic activity after 21 days of implantation. Haemoglobin content in control group was found to be 55.94 ± 4.97 , in vehicle control 49.28 ± 6.49 , standard group (3.9mg/kg) $21.51 \pm 3.47^{***}$, Test drug (100mg/kg) $28.26 \pm 3.46^{***}$ which is significant according to statistical analysis (one-way annova). Thus, our study suggests that test drug (Anthocyanin) could be an effective drug for in vivo inhibition of angiogenesis and thus might gain significance in future therapeutics.

CONCLUSION

Design and development of small molecule therapeutics to inhibit angiogenesis has gained considerable importance in anti-angiogenesis research. We demonstrate that the Cotton plug (Angio plug) 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- α)-induced VEGF expression), inhibition of H_2O_2 - and as well as through the inhibition of VEGF and VEGF receptor expression Thus, 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.

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