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Antiangiogenic Activity of *Boswellia Serrata* Extract

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ABSTRACT

Boswellia Serrata (BA) is one of the traditional medicines being used since ancient time and is believed to have a variety of beneficial effects on human body. It is more commonly used in treatment of arthritis. Recent human studies have shown that *Boswellia serrata* may contribute to reduce risk of cardiovascular diseases and cancers. However little is known about effect of whole extract on angiogenesis in animal models. In this work, we have demonstrated antiangiogenic effects of *Boswellia Serrata* extract on three different models of angiogenesis – Chicken Chorioallentoic Membrane assay (CAM), Subcutaneous air pouch in rat (SAC) and Mesenteric window angiogenesis in rat (MWA). The extract showed dose dependent reduction in blood vessel development and nature of newly formed vessels. These effects further suggest that whole extract of *Boswellia Serrata* is useful as an herbal medicine for angiogenesis dependent diseases either alone or in combination with other pharmacotherapeutic or Chemotherapeutic agents.

Keywords: Angiogenesis, Boswellia, Chorioallentoic membrane, mesenteric window angiogenesis, subcutaneous air sac,

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INTRODUCTION

Angiogenesis is the process of formation of new blood vessels from pre-existing microvasculature. It is a fundamental process in formation of new blood vessels. It is essential in reproduction, development and wound repair. In these conditions, angiogenesis turns on for a short period and then completely inhibited [1, 2]. Under physiological conditions angiogenesis is highly regulated phenomenon, because of several pro-angiogenic and anti-angiogenic factors in body[3]. In the foetal development process of angiogenesis is a very powerful process and plays a very important role. Disruption of this process leads to abortions and / or teratogenicity[4].

Angiogenesis is a complex process involving various mechanical and chemical triggers[5]. Such triggers can be grouped as pro-angiogenic and anti-angiogenic factors. In body more than 20 different pro-angiogenic and anti-angiogenic factors finely control the physiologic angiogenesis[1, 2, 3, 6,7].

In the pathologic process of angiogenesis, the balance between these pro and anti-angiogenic factors gets disturbed resulting a variety of disorders, which can be grouped as angiogenesis dependent diseases. They involve – arthritis, ischaemias, metastasis of tumour etc. In addition, numerous inflammatory, allergic, infectious, traumatic, metabolic or hormonal disorders, which are characterized by excessive vessel growth, including atherosclerosis, re-stenosis, transplant arteriopathy, warts, allergic dermatitis, scar keloids, peritoneal adhesions, synovitis, osteomyelitis, asthma, nasal polyps, choroideal and intraocular disorders, retinopathy of prematurity, diabetic retinopathy, leukomalcia, AIDS, endometriosis, uterine bleeding, ovarian cysts etc. Angiogenesis also plays a role in obesity.

Applications of angiogenesis research can be categorized in following areas…

1. Diagnostic Applications – diagnosis of angiogenesis dependent diseases.
2. Acceleration of angiogenesis in wound healing and ischaemias or infarctions in several tissues.
3. Inhibition of angiogenesis in neoplasia, arthritis, retinopathies due to corneal vascularization and such other angiogenesis dependent diseases.

Researchers are heading towards development of newer deliveries using monoclonal antibody techniques targeting various steps in tumour metastasis and also have come out with some monoclonal antibody based agents in clinical practice. Examples of such drugs include ‘Bevacizumab acting on VEGF-A, Sunitinib and vatalanib – tyrosine kinase inhibitors, Sorafenib – acting on Raf Kinase etc.[8, 9, 10] while several others under different phases of clinical or preclinical trials[11]. On the other hand researchers are trying to target tumour through different

delivery systems[12, 13, 14].

Even though having so many advantages antiangiogenic and anticancer drugs, based on targeting single receptor or signalling systems have some limitations in their use. Some clinical experiences have found toxicities for these agents. Basically angiogenesis related signalling pathways have an important role in haematopoiesis, myelopoiesis, and endothelial cell survival. Toxicities indicate that angiogenesis involves several other processes in body viz. immune system, blood flow regulation and coagulation cascade. Though anti-angiogenic agents are developed to inhibit / affect one pathway, its deleterious effects may be observed on other homeostatic mechanisms [2].

Natural health products can inhibit angiogenesis along with having other anticancer mechanisms. These products have been shown to act on various molecular pathways other than angiogenesis, including epidermal growth factor receptor, the HER2/nu gene, COX-2 enzymes, NF κ-β transcription factor, the protein kinases, etc. Several herbs are traditionally being used in the treatment of cancer – some explored some still to be discovered. Explored herbs for anticancer activity still need further polishing so as to isolate the exact component / group of components responsible for its anticancer activity[15, 16]. Several Indian as well as Chinese medicinal herbs have been shown to be effective in curing ischemic diseases, arthritis, retinal angiopathies as well as other angiogenesis dependent diseases, but their functions were not scientifically tested. This work describes antiangiogenic properties of *Boswellia Serrata* (BA) extract in *in-vivo* animal models.

MATERIALS AND METHODS:

Chemicals, reagents, and animals:

Compound 48/80 (It is Mast Cell Secretagogue compound used to induce mast cell)was purchased from Sigma Life Sciences, Bangalore, India, Brown Leghorn Chicken eggs were purchased from Simran Hatcheries, Dhule. Boswellia extract was purchased from Natural Remedies, Bangalore. All the chemicals used for the study were of analytical grade, purchased from Qualigens, Mumbai, India. Animals were procured from animal house of SPTM. The protocols for animal studies were approved by IAEC, of School of Pharmacy and Technology Management, Shirpur.

Standardization of Boswellia extract :

Standardization of Boswellia extract was done using HPLC method of estimation of boswellic acids. Briefly a sample of Boswellia extract was prepared by dissolving 40mg Boswellia extract

in 10 ml solvent system being used. The resultant was filtered through disc filter to result a clear solution. This was further diluted to result 250mcg/ml solution. The marker for Boswellia extract (a combination of boswellic acid and alpha keto boswellic acid) was used as standard. The standard stock solution of 2mg/ml was prepared. It was further diluted to result 2^0 stock of 100mcg/ml. A serial dilutions of it were then prepared and the Chromatogram of the standard was recorded.

Chromatographic conditions: -

System - Perkin- Elmer Series 200 HPLC unit with Kromacil C18 column and lambda 25 spectrophotometric detector.

Solvent system: - Acetonitrile: Water (90:10) at pH 4.0 adjusted with Glacial acetic acid.

Detector: - Spectrophotometric detector at 260nm wavelength.

Runtime allowed was 08 minute.

Acute Toxicity Studies of Extracts.

Acute toxicity studies were carried out following OECD 423 guidelines. Following toxic class method and the extract was evaluated up to 2000mg/Kg dose.

Repeated Dose Toxicity studies of extracts.

Repeated dose toxicity of both the extracts was carried out at 250 mg/Kg, 500mg/Kg and 1000mg/Kg dose to evaluate any adverse effect of chronic use following OECD 408 guidelines.

***In vivo* models of Angiogenesis**

Chicken Chorioallentoic Membrane Assay (CAM)

Chorioallentoic membrane assay was performed following method described by Nguyen and his colleagues, with little modification [17,18]. Briefly, fertilized Brown leghorn chicken eggs of 3 to 4 days were received from a local hatchery. They were kept in a humidified incubator at 37^0C with the wide end up. The eggs were rotated three times a day to ensure uniform embryo development. After 3 or 4 days of incubation, the 7-day-old eggs were observed on a self-made lamp and circled the position of embryo head. Zero point five to one ml of albumin was aspirated from eggs with an 18-gauge hypodermic needle through a small hole drilled at the narrow end of the eggs, allowing the small CAM and yolk sac to drop away from the shell membrane. The shell covering the embryo air sac was punched out and removed by forceps and the shell membrane on the floor of the air sac was peeled away. At 8-days-old, 10 µL BA (10/ml) or normal saline was applied to the CAM surface. The chick embryo was then returned to the incubator. Three days later, an appropriate volume of a methanol and acetone mixture (1:1) was injected using a 33-gauge needle into an 11-day-old embryo chorioallantois. The CAM was cut out from eggs and

the number of vessels was observed and vessels radially converging toward the centre were then counted under a microscope.

The percent inhibition of tertiary vessels were calculated using the equation: Where VN is Number of Vessels Statistics was applied using one way ANOVA followed by Dunnett's test.

$$\% \text{ inhibition} = \frac{(\text{VN of CAM treated by normal saline} - \text{VN of CAM treated by extract})}{(\text{VN of CAM treated by normal saline})]} \times 100$$

Rat Subcutaneous Air Sac Angiogenesis (RAS): -

Angiogenesis was induced in rat in the subcutaneous space to result an inflated Air Sac following method described by Lichtenberg, with little modification [19].To adult Wistar albino rats about 10 ml of air was administered subcutaneously on the back, on alternate day in order to maintain the sac inflated. Gradually the sac became thick. The animals were divided in seven groups of six each. The animals were treated with twice a day oral administration of the Boswellia extract for 12 days at 125 mg/Kg, 250 mg/Kg. The clipped skin was dissected to expose the formed membrane on which blood vessels grow. The area was analyzed for No. of blood vessels formed and area covered by newly formed blood vessels. For statistics one way ANOVA followed by Dunnett's multiple comparison test was followed.

Rat Mesenteric Window Angiogenesis (MWA): -

Mesenteric window angiogenesis was performed as described by Norrby [20]. Wistar Albino rats were separated in 7 groups of 6 animals each and treated similarly except the drugs. Rats were administered intraperitoneally 9 doses at 12 hours interval (4.5 days) Mast cell secretagogue compound in 2mg/kg bodyweight, suspended in 0.9% normal saline solution. Animals were treated with oral administration of Boswellia extract for 14 days at 125 mg/Kg, 250 mg/Kg twice daily 1 hour before administration of Mast Cell Secretagogue compound. Extract was suspended in 1% Carboxymethyl Cellulose solution. After 14 days the animals were sacrificed and mesentery was removed carefully to view at least four windows per animal. Isolated mesenteries were washed carefully, stained with hematoxylene, and eosin. The area in each window was analyzed for No. of blood vessels formed and area covered by newly formed blood vessels. For statistics one way ANOVA followed by Dunnett's multiple comparison test was followed.

RESULTS AND DISCUSSION:

Standardization of Boswellia extract: -

Boswellia extract was standardized for presence of Boswellic acid and alpha keto boswellic acid respectively. The aqueous extract showed presence of these two acids similar to marker. Marker

selected was a combination marker so that interference (if any) between these two acids be minimized. Boswellia extract showed presence of another additional peak, which might be some other constituent of *Boswellia Serrata.* Table 1 represents chromatographic characteristics of both, marker and Boswellia extract.

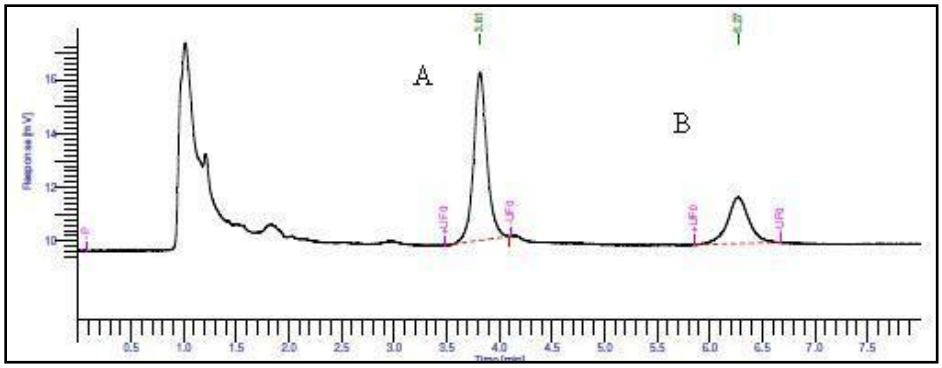

Figure.1 – HPLC chromatogram of *Boswellia Serrata Extract*. Peak 'A' α-Boswellic acid, peak 'B' 11-α-keto Boswellic acid.

Table: 1. Comparison between parameters of Chromatograms of sample and marker.

	Area under Curve (microVolt*S)		**% area of Peak**		**Time (Min)**	
	Peak 1	**Peak 2**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**
Marker	59203.35 ± 14412.42	26083.80 ± 6899.53	69.49 ± 0.47	30.51 ± 0.47	3.82 ± 0.02	6.28 ± 0.01
BA Extract	108062.76 ± 18539.23	48108.43 ±9873.48	69.29 ± 0.94	30.71 ± 0.94	3.89 ±0.06	6.35 ± 0.11

Values are expressed as mean ±SEM.

Toxicity studies: -

Acute toxicity studies showed no any evidence of behavioral or other changes in any animals under test. This test was carried out for the doses up to 2000mg/Kg without any toxicity in animals. So the extract was classified as category 5 or unclassified. Therefore for further studies dose of 125mg/Kg and 250 mg/Kg were selected.

Repeated dose toxicity studies, carried out for 90 days, at three dose levels of 250 mg/Kg, 500mg/Kg and 1000mg/Kg. There was no any evidence in change in behavioural pattern, hematological values, or biochemical parameters. Gross necropsy also represented no any toxicity to any animal.

Antiangiogenic effect on *In-vivo* models of angiogenesis: -

Chicken Chorioallentoic Membrane Assay: -

Antiangiogenic effect on CAM by BA Extract was performed in triplicate to confirm consistency and reproducibility in results. As presented in Table 2, When the CAM remains untreated about

20 tertiary blood vessels were observed while in the treatment group only 13 blood vessels were observed. It means the BA extract was able to reduce the rate of tertiary blood vessel development by about 34% in a dose of 10mg/ml.

Table:2. Effect of Boswellia extracts on primary, secondary and tertiary blood vessels in CAM.

Treatment Group	Primary Blood vessels	Secondary Blood Vessels	Tertiary Blood Vessels	Percent Inhibition
Untreated	3.2 ± 0.213	5.3 ± 0.263	20.1 ± 0.940	0.000
Saline	3.3 ± 0.179	5.1 ± 0.298	20.1± 1.028	0.000
BA	3.1 ± 0.198	5.9 ± 0.344	13.2 ± 0.521**	34.328

Values are expressed as mean ±SEM. Statistical significance expressed as: **p<0.01

Rat Subcutaneous Air Sac Angiogenesis

Acute toxicity studies raveled that the drug is safe even at 2000 mg/kg. So we selected 125 mg/kg and 250 mg/kg dose. When the RAS studies were performed it was found that Boswellia extract reduces the rate of new blood vessel formation in synovial membrane like structures as compared to control. As presented in Table 3, Boswellia extract at 250mg/kg dose is found to be significant in reducing formation of such blood vessels. BA was able to reduce the rate and extent of vascularization in RAS to about 73%

Table: 3. Effect of crude drug extracts on formation of blood vessels in subcutaneous air sac in rats.

Group	Treatment	No. of Blood Vessels Formed
1	Control	14.83 ±0.48
2	BA 125	13.33 ±0.42
3	BA 250	10.83 ±0.87*

Values are expressed as mean ±SEM of three experiments. Statistical significance expressed as: *p<0.05

Table:4. Effect of crude drug extracts on formation of blood vessels in mesenteric windows in rats.

Group	Treatment	No. of Blood Vessels Formed
1	Control	15.79 ±0.15
2	BA 125	12.92±0.75**
3	BA 250	6.25±0.66**

Values are expressed as mean ±SEM of three experiments. Comparisons are made between control and test groups. Statistical significance expressed as:**p<0.01

Rat Mesenteric Window Angiogenesis

Mesenteric window angiogenesis is the model of angiogenesis representing role of inflammatory

mediators in angiogenesis. According to our studies, Boswellia extract is able to reduce such kind of angiogenesis significantly. As shown in table 4, extract is able to reduce the rate of new blood vessel formation in inflammation mediated angiogenesis by about 50 %.

CONCLUSION:

Antiangiogenic effect of *Boswellia* Serrata extract was studied in three different models of angiogenesis. CAM model represent the hypoxia induced angiogenesis where BA was found to have significant effect to reduce such neovascularisation to about 34%. MWA model represents histamine induced angiogenesis, where BA was able to reduce the extent of angiogenesis by around 40%. RAS model is the model of angiogenesis representing inflammation as key player. BA in this case has also been found significant. The results obtained in this research work express use of BA in the treatment of angiogenesis dependent diseases to reduce the rate and extent in angiogenesis.

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