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Original Article

STUDY OF *CAMELLIA SINENSIS* AND *BOSWELLIA SERRATA* IN RESPECT TO ANTIANGIOGENIC EFFECT

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ABSTRACT

Objective: The process of angiogenesis is finely balanced by Proangiogenic factors and antiangiogenic factors. Any imbalance between these factors may result in a group of diseases with varied morphologic and pathologic changes, called angiogenesis dependent diseases. One has to arrest the growth of unwanted blood vessels like in metastasis of tumour, arthritis, retinopathies, etc. As the angiogenesis is simultaneously controlled by several factors, one needs to target the whole process through a holistic approach i.e. targeting more than one mechanism at a time. Objective of our study was to evaluate antiangiogenic potentials of *Camellia sinensis* and *Boswellia serrata* in whole animal models along with their safety individually as well as in combination.

Methods: These extracts were evaluated for their antiangiogenic effect using three models – Chicken Chorioallentoic membrane assay, Rat Subcutaneous air sac and Mesenteric window angiogenesis.

Results: The results of toxicity study showed that aqueous extracts of *Camellia sinensis* and *Boswellia serrata* are safe on oral administration in acute as well as repeated dose toxicity studies. It also showed antiangiogenic effects of individual extracts as well as their combinations. The study suggests that there may be synergistic effect in these extracts particularly in mesenteric window model.

Conclusion: From the study it can be concluded that these extracts can be clinically evaluated on angiogenesis dependent diseases.

Keywords: *Boswellia serrata*, *Camellia sinensis*, Safety, Angiogenesis, CAM, Angiogenesis dependent disease, Arthritis, Metastasis.

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 it is 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 development of fetus, 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]. 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, restenosis, 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 categorised 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 vascularisation 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 signaling systems have some limitations in their use. Some clinical experiences have found toxicities for these agents. Basically angiogenesis related signaling 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

Mast cell secretagogue Compound 48/80 was purchased from Sigma Life Sciences, Bangalore, India, Brown Leghorn Chicken eggs were purchased from Simran Hatcheries, Dhule, *Boswellia serrata* extract was purchased from Natural Remedies, Bangalore, while *Camellia sinensis* leaves were purchased from local market and got authenticated by Department of Botany, P. R. Ghogare College of Science, Dhule. All other 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 the School of Pharmacy and Technology Management, Shirpur. Standard diet for animals was purchased from Amrut Agrovet, Sangli.

Extraction of *Camellia sinensis*

Dried leaves of *Camellia sinensis* were extracted by hot maceration method [17] with little modification. In brief, weighed quantity of CS leaves macerated in 1:40 proportion in water at 80^0C for 2 hours. Resultant solution was filtered through muslin cloth and filtrate was dried at 60^0C to constant weight. The extract thus obtained was stored in refrigerator till further use. The extract was termed as (GT)

Phytochemical investigations

Both extracts were subjected for qualitative phytochemical investigation and extraction specific parameters, like ash value, acid insoluble ash, and Loss on drying.

Standardization of *Camellia sinensis* extract

a. Total phenolics in the *Camellia sinensis* extract were calculated using Folin - Ciocalteu Assay [18]. Extract (1 mL) was added to a flask containing distilled water (9 mL). Then Folin-Ciocalteu's phenol reagent (1 mL) was added and the mixture was mixed thoroughly. After 5 min, 7% sodium carbonate (10 mL) was added. The mixture was diluted to 25 mL with the addition of distilled water (4 mL) and allowed to stand at room temperature for 90 min. Absorbance was measured at 760 nm using a Perkin Elmer lambda 510 spectrophotometer.

Total Phenolics are expressed as mg Gallic acid equivalents (GAE)/g samples. The same procedure was repeated to all standard Gallic acid solutions (0–1000 mg, 0.1 ml-1) and standard curve was obtained.

b. Total flavonoid content was determined using Aluminium Chloride colourimetric assay [18]. Extract (1 mL) was added to distilled water (4 mL) in a flask. Then, 5% $NaNO_2$ (0.3 mL) was added. After 5 min, 10% $AlCl_3$ (0.3 mL) was added and after 6 min, 1 M NaOH (2 mL) was added. The mixture was diluted to 10 mL with distilled water. The absorbance of the solution was measured at 510 nm using a Perkin Elmer lambda 510 spectrophotometer. The results are expressed as mg catechin equivalents (CE)/g samples.

Standardization of *Boswellia serrata* extract (BA)

Standardization of Boswellia serrata extract was done using HPLC method of estimation of boswellic acids. Briefly a sample of Boswellia serrata extract was prepared by dissolving 40mg Boswellia serrata 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 serrata 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 [19]. Following toxic class method, both extracts were evaluated to 2000 mg/Kg dose. No any signs of toxicity were observed even at this dose. So the extracts were classed as unclassified. Therefore in further studies dose of 250 mg/Kg and 125 mg/Kg are used.

Repeated Dose Toxicity studies of extracts

Repeated dose toxicity of both the extracts was carried out at 250mg/Kg, 500mg/Kg and 1000 mg/Kg dose to evaluate any adverse effect of chronic use following OECD 408 guidelines [20]. Adult Wistar albino rats weighing between 170 to 200g were selected as test animals. 5 male and 5 female rats were used for each group. Animals were treated for 90 days with a daily dosing and observed for biological, hematologic, and behavioral parameters as per OECD guidelines.

In Vivo models of Angiogenesis

Chicken Chorioallentoic Membrane Assay

Chorioallantoic membrane (CAM) assay was performed following method described by Nguyen and his colleagues, with little modification [21]. Fertile Brown Leghorn chicken eggs were obtained from a local hatchery. 70% ethanol was sprayed on these eggs for disinfecting their surface. They were kept in humidified incubator at 37.5^0C ($\pm 1.5^0$C). Eggs were rotated 4 to 6 times a day to ensure uniform vssel development. On embryonic day 4 a window was cut on the shell over the embryo to expose chorioallentoic membrane (CAM). Eggs were sealed again with cellophane film and incubated for further two days. On the 6^{th} embryonic day 10 microliter of either 0.9% sodium chloride (Normal saline) solution or solutions of extracts (10mg/ml) were placed on intact CAM. These eggs were again incubated for further 24 hours. After this period, i.e. on 7^{th} embryonic day the eggs were observed for number of primary, secondary and tertiary vessels formed, nature of blood vessels and area of the CAM covered.

For statistics one way ANOVA followed by Dunnett's multiple comparison tests was followed.

Rat Subcutaneous Air Sac Angiogenesis

Angiogenesis was induced in rat in the subcutaneous space to result an inflated Air Sac following method described by Lichtenberg J, with little modification [22].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 aqueous suspensions of extracts for 12 days at 125 mg/Kg and 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

Mesenteric window angiogenesis was performed as described by Norrby [23]. 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 extracts for 14 days at 125 mg/Kg or 250 mg/Kg twice daily 1 hour before administration of Mast Cell

Secretagogue compound. Extracts were suspended in 1% Carboxymethyl Cellulose solution. After 14 days the animals were sacrificed and mesentery was removed carefully to view 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

1. Extraction specific parameters

The extraction yield for *Camellia sinensis* extract was found to be 35%W/W. Moisture content in the dried extract was calculated to be 4.04% W/W, total ash was found to be 1.7%, while acid insoluble ash was 0.3%. For Boswellia serrata extract moisture content was found to be 0.59%W/W, Total ash was 1.8%W/W, while acid insoluble ash was found to be 0.2%.

2. Phytochemical investigation

Table 1: Preliminary Phytochemical investigation of extracts

Test	*Camellia sinensis* Extract	Boswellia serrata Extract
Tests for alkaloid		
Wagner's test	-	-
Test for carbohydrate		
Molish Test	-	+
Test for glycosides		
Borntrager's test (Anthraquinone)	-	-
Killer Kiliani test (Cardiac glycosides)	-	-
Froth test (Saponin glycosides)	-	-
Test for flavonoids		
Shinoda test	+	+
Test for tannins		
Ferric Chloride test	+	-
Test for proteins		
Biuret test	-	-
Hydrolysis test	-	-
Test for steroids / terpinoids		
Salkowaski test	-	-

(+ indicates the presence of phytochemical while – indicates absence.)

3. Standardisation of *Camellia sinensis* extract

Total phenolics in the *Camellia sinensis* extract were calculated using Folin - Ciocalteu Assay. Phenolic content in the extract was calculated on the basis of Gallic acid equivalent per unit dry weight of sample. It was found that sample extract of *Camellia sinensis* contain 82.03 mg Gallic acid equivalents /g of dry extract. Fig. 1 represents the calibration curve and the line equation for estimation of total phenolics.

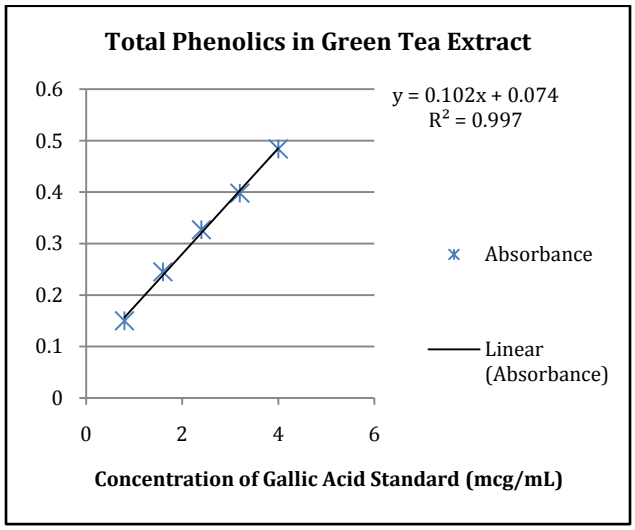

Fig. 1: graph of total phenolic content of *Camellia sinensis* extract

Total flavonoid content was determined using Aluminium Chloride colourimetric assay. It was found to contain 20.24 mg catechin equivalent /g of dry extract.

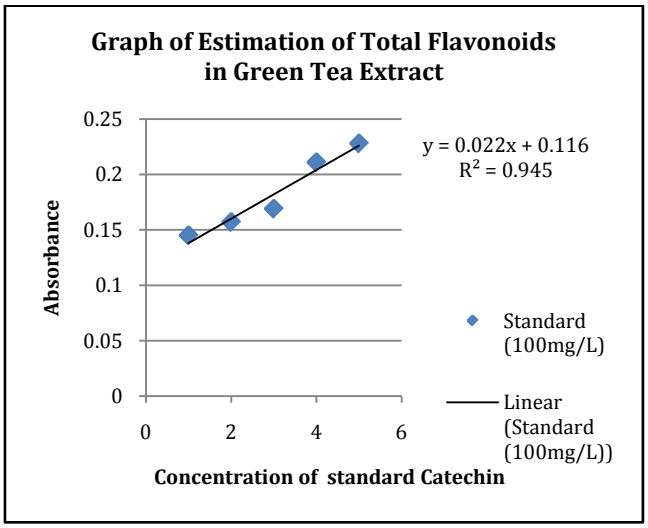

Fig. 2: graph of total flavonoid content of *Camellia sinensis* extract.

4. Standardisation of *Boswellia serrata* extract

Boswellia serrata extract was standardised for presence of Boswellic acid and alpha keto boswellic acid.

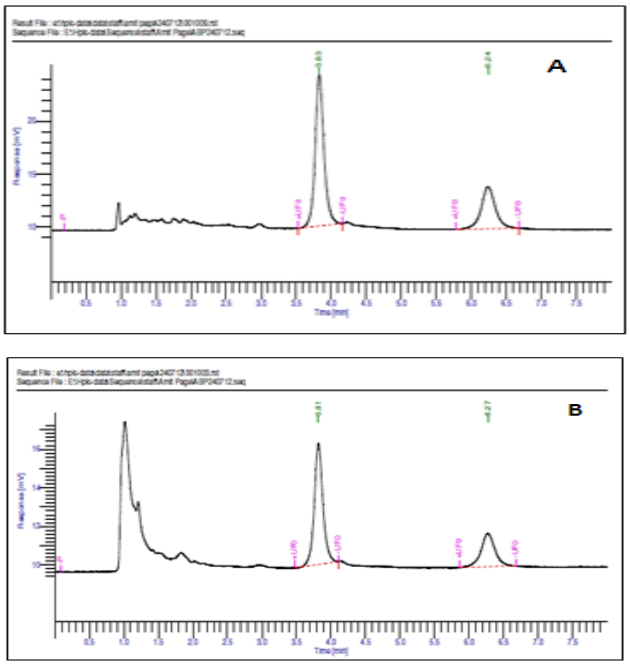

Fig. 3: HPLC chromatogram of Boswellic acid marker (A) versus extract of *Boswellia serrata* (B)

5. **Acute Toxicity studies:**

The acute toxicity studies were carried out using OECD guidelines following toxic class method. As there were no observable adverse effects even at 2000mg/Kg, extracts were characterised as "unclassified" as per OECD. Therefore doses selected were 250mg/kg and 125mg/kg.

6. **Repeated dose toxicity studies:**

Repeated dose toxicity was carried out following OECD guidelines 408. As per the observations and also from histopathologic studies, it was observed that even at the doses of 1000mg/Kg, these extracts were safe and showed no significant change in physiologic, biochemical or biological parameters, when administered alone.

The combination of 500 mg/kg of both the extracts also did not show any toxicity in these animals indicating safety in combination.

7. Effect of extracts on Chicken Chorioallentoic membrane angiogenesis (CAM):

Chorioallentoic membrane represents hypoxic model of angiogenesis. The developing embryo undergoes hypoxia to develop new blood vessels, which partly mimics angiogenesis in tumors. Administration of GT extract in the dose of 10mg/ml reduces angiogenic response to such hypoxia to about 43%, while BA was able to reduce it to 34%.

In combination whereas ½ of each extract was able to reduce the angiogenesis to 43% but in 1:1 proportion such reduction is about 54%.

Table 2: Effects of crude drug extracts on primary, secondary and tertiary blood vessels in CAM. (n=20)

Group	Treatment Group	Primary Blood vessels	Secondary Blood Vessels	Tertiary Blood Vessels	Percent inhibition in tertiary blood vessels
1	Untreated	3.200 ±0.213	5.300 ±0.263	20.100 ±0.940	0.000
2	Saline	3.300 ±0.179	5.100 ±0.298	20.100 ±1.028	0.000
3	GT	2.900 ±0.204	5.350 ±0.221	11.300** ±0.405	43.781
4	BA	3.050 ±0.198	5.950 ±0.344	13.200** ±0.521	34.328
5	GT+BA (1/2each)	3.400 ±0.152	5.750 ±0.204	11.400** ±0.380	43.284
6	GT + BA	3.100 ±0.161	4.550 ±0.285	9.100** ±0.624	54.726

Values are expressed as mean ±SEM comparisons were made between control and test groups. Statistical significance expressed as: $^{**}p<0.01$.

8. Effect of extracts on Dorsal air sac angiogenesis in rats

When air is administered in subcutaneous area of rat, it results in inflammation of the region and formation of synovium like membrane. Angiogenesis in such membrane was found to be reduced when the animals were treated with CS extracts.

As presented in Table 3, even though GT extract at 125 mg/Kg is not able to reduce the rate and extent of angiogenesis significantly, there is significant reduction (49.39%) by 250mg/Kg dose of GT extract. Boswellia serrata 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 vascularisation in RAS by about 27%, while combination of 125mg/Kg each of these extracts is insignificant. But if combined in 250mg/Kg each, the angiogenesis in RAS can be reduced to about 50%. It indicates combination does not prove efficient than GT alone in RAS.

Table 3: Effect of crude drug extracts on formation of blood vessels in subcutaneous air sac in rats. (n=06)

Group	Treatment	No. of Blood Vessels Formed
1	Control	14.83 ±0.48
2	GT 125	13.00 ±0.37
3	GT 250	7.33 ±0.49**
4	BA 125	13.33 ±0.42
5	BA 250	10.83 ±0.87**
6	GT + BA 125	10.17 ±0.48**
7	GT + BA 250	7.17 ±0.60**

Values are expressed as mean ±SEM comparisons were made between control and test groups. Statistical significance expressed as: $^{**}p<0.01$

9. **Effect of extracts on mesenteric window angiogenesis**

Mesenteric window angiogenesis is the model of angiogenesis representing role of inflammatory mediators in angiogenesis. New blood vessels are developed in rat mesentery because of mast cell secretagauge compound 48/80. According to our studies, Boswellia serrata 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 to about 60 %. When animals treated with GT extract with control animals, it was found that GT extracts have an ability to reduce rate of formation of new blood vessels in mesentery. CS extract was found to show significant reduction (27.72%) in formation of blood vessels in mesenteric windows at dose of 250 mg/kg. Combination of these two extracts in 250mg/Kg each produces significant antiangiogenic response by causing 70%reduction in formation of new blood vessels.

Table 4: Effect of crude drug extracts on formation of blood vessels in mesenteric windows in rats. (4 windows per animal, n=6)

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**
4	GT 125	13.67±0.42
5	GT 250	11.5±0.47**
6	GT + BA 125	8.88±0.68**
7	GT + BA 250	4.67±0.55**

Values are expressed as mean ±SEM comparisons were made between control and test groups. Statistical significance expressed as: ** p<0.01.

CONCLUSION

Angiogenesis – one of the key players in angiogenesis dependent disorders is the process involving interplay of various endogenous and exogenous factors. There is an established link between angiogenesis, tissue hypoxia inflammation, oxidation of cellular components and variety of other tissue messengers and circulating mediators. Findings of this research work suggest that targeting one or two factors or receptors appear to be insufficient. Instead a system based approach is expected to fight such disorders. Both natural products, *camellia* as well as *Boswellia* are well tolerated by animals even in as high as 1000 mg/Kg dose, administered repeatedly. Antioxidant potential of *Camellia sinensis* might help reducing the angiogenesis in Choriolallentoic membrane model by about 43%, while by up to 50% in Subcutaneous air sac model. On the other hand Boswellia serrata extract is able to reduce the rate and extent of angiogenesis in mesenteric window model, which may be because of its anti-inflammatory potential. Combination of these extracts, even though fail to establish a clear cut additive effect in CAM or RSA, but the same can be seen in MWA model. These results suggest *Camellia sinensis* and Boswellia serrata have promising future in the treatment of such angiogenesis dependent disorders. These extracts can be used alone or in combination. Further evaluation of these extracts is needed in human being and in particular models of specific angiogenesis dependent disorders.

CONFLICT OF INTERESTS

Declared None

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