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ANTIANGIOGENIC ACTIVITY OF AQUEOUS EXTRACT OF *CAMELLIA SINENSIS*.

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ABSTRACT

Leaves of *Camellia sinensis* (CS) have been used since ancient time and are believed to have a variety of beneficial effects on human body. Recent human studies have shown that green tea 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 *camellia sinensis* 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 *C. sinensis* is useful as an herbal medicine for angiogenesis dependent diseases.

KEY WORDS: Green tea, angiogenesis, CAM, mesenteric window, subcutaneous air pouch, Antiangiogenic.

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INTRODUCTION

Angiogenesis - the processes of formation of new blood vessels is one of the developmental processes during embryogenesis. It is to a very limited extent in postnatal life [1]. The process of new blood vessel development is completely down regulated in adults except female reproductive system [2]. Researches in last few years have made the process more clear and understandable. It is demonstrated that the processes of angiogenesis is a highly controlled processes through the synthesis of proangiogenic and antiangiogenic factors [3]. Dysregulation of this processes may lead to metastasis of tumours, arthritis, vascular malformations, psoriasis, warts, nasal polyps, endometriosis, ovarian cysts etc[4]. Such pathologic angiogenesis also exist within eye[5]. It is one of the major causes of vision loss in diabetic retinopathy, age related macular degeneration, etc [6]. Angiogenesis is found to get modified by certain endogenous or exogenous substances, factors that affect angiogenesis include some mechanical as well as some chemical factors. These factors include

a) Angiogenesis stimulating agents

b) Angiostatics

Angiogenesis is a complex process involving an extensive interplay between cells, soluble factors and extracellular components. Along with cells, they include about 15 matrix metalloprotinases, and about 30 endogenous soluble factors in body [7].

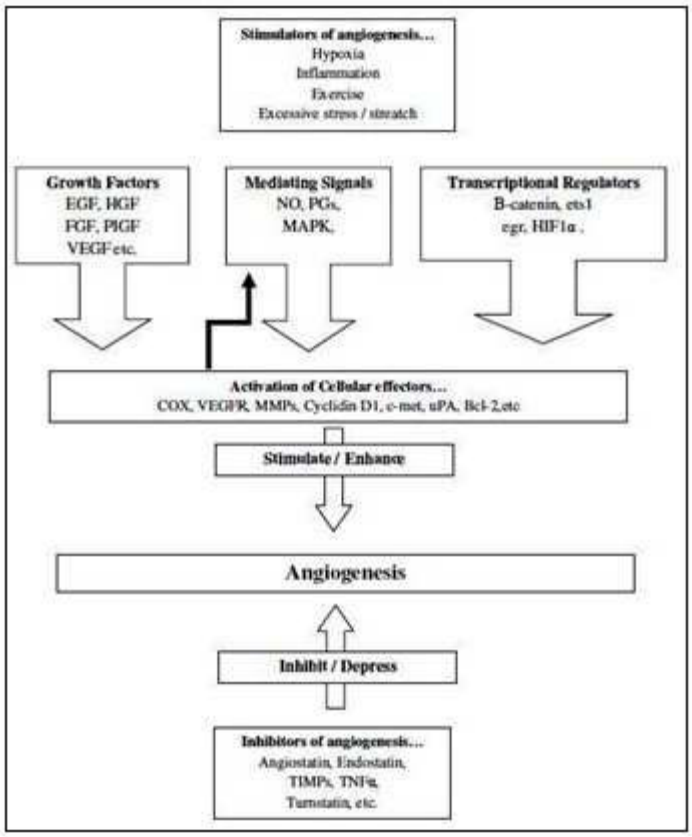

Figure 1
Key players in the angiogenesis signal cascade.

(EGF – Epidermal Growth Factors, HGF – Hepatocyte Growth Factors, FGF – Fibroblast Growth Factor, PIGF – Placental Growth Factor, VEGF – Vascular Endothelial Growth Factor,

NO – Nitrous Oxide, PGs – Prostaglandins, MAPK –Mitogen Activated Protein Kinase, ets and egr aremembers of ETs family responsible for transcription activation or inhibition , HIF1α – Hypoxia Inducible Factor, COX – Cyclo-oxigenase enzyme, VEGFR – Vascular Endothelial Growth Factor Receptor, c-met - , uPA- Urokinase Plasminogen Activator, Bcl-2-is a regulator protein regulating apoptosis, TIMP – Tissue Inhibitor of Metalloprotinase, TNFα – Tissue Necrosis Factor Alpha.) Antiangiogenic drugs targeting only VEGF can cause hypertension and proteinurea. These effects can be correlated to VEGF inhibition on vasculature[8]. As angiogenesis can be induced through any of the ways of cascade in different disease states, one should deal with a system approach to treat angiogenesis dependent diseases rather than restricting to target based approach. In our study we evaluated antiangiogenic activity of aqueous extract of *camellia sinensis* (CS) using in vivo as well as ex ovo techniques.

MATERIALS AND METHODS

1. Chemicals, reagents, and animals

Compound 48/80 was purchased from Sigma Life Sciences, Bangalore, India, Brown Leghorn Chicken eggs were purchased from Simran Hatcheries, Dhule, green tea leaves were purchased from local market and got authenticated. The voucher sample of plant specimen is deposited in the laboratory. All other chemicals used for the study were of analytical grade, purchased from Qualigens, Mumbai, India. Animals were procured from animal house of School of Pharmacy and Technology Management. The protocols for animal studies were approved by IAEC, of the School of Pharmacy and Technology Management, Shirpur.

2. Preparation of extract

Dried leaves of *Camellia sinensis* were extracted by hot maceration method[9], 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.

3. *Acute Toxicity studies*

Extract was evaluated for acute toxicity following toxic class method. The study was performed according to OECD 423 guidelines[10].

4. Repeated dose toxicity studies

Extract was further evaluated for repeated dose toxicity studies as per OECD 408 guidelines for oral toxicity [11].

5. Mesenteric window angiogenesis

Mesenteric window angiogenesis was performed following method described by norrby *et.*al with little modification[12]. In brief Wistar albino rats were divided in four groups of six animals each and treated similarly except drugs. One group didn't receive any drug treatment and acted as a Negative control. Angiogenesis was induced by i.p. injection of the mast cell secretagogue compound 48/80 twice daily for 4.5 days to animals in remaining three groups. Animals were treated with oral administration of normal saline or CS extract 125mg/Kg or CS extract 250 mg/Kg twice a day for 14 days. Test compounds or saline are administered 1 hour before each injection of compound 48/80. After 14 days animals were sacrificed and mesentery was removed carefully to view at least four windows per animal. Angiogenesis is quantified by microscopically counting the number of vessel profiles per unit length of the mesenteric window. Mesenteric window specimens were spread, fixed on objective slides and stained with hematoxyline and eosin to measure the relative vascularized area. Statistics was applied using one way ANOVA followed by Dunnett's test.

6. Subcutaneous air pouch method

Subcutaneous air pouch angiogenesis was induced to Wistar Albino rats following method described by Lichtenberg *et al* with little modification[13]. Briefly, Under anesthesia 10–15

ml of air was introduced dorsally to Wistar Albino rats weighing 150–180 g by subcutaneous injections using a 25 gauge needle to produce an air sac located approximately 4–5 cm behind the head of the animal. The air sacs are re-inflated every alternate day. After approximately 10 days, when a sufficient lining of cells has been established. The animals are treated for 10 days with various doses of test compounds orally. After 10 days treatment, the animals were sacrificed. The overlying skin of the air sac was removed to expose the transparent membrane. The extent of vascular proliferation is scored in situ:

1+: slight background vascularization;
2+: few new vessels;
3+: many new vessels;
4+: very intense formation of new vessels.

The membranes are examined microscopically after staining with haematoxylin and eosin.
Statistics was applied using one way ANOVA followed by Dunnett's test.

7. Chorioallentoic membrane assay

Chorioallantoic membrane (CAM) assay was performed following method described by Nguyen and his colleagues, with little modification[14, 15]. 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 to 4 days of incubation, the 7-day-old eggs were observed. Observations were made on a self-made lamp and the position of embryo head was circled. 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 sample was applied to the CAM surface. The chick embryo was then returned to the incubator. Three days later CAM was cut out from eggs and the number of vessels was observed and vessels radially converging toward the center were then counted under a microscope. The percent inhibition of tertiary vessels were calculated using the equation:
Where VN is Number of Vessels

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

Statistics was applied using one way ANOVA followed by Dunnett's test.

RESULTS

1. 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 behavioral pattern, hematological values, or biochemical parameters. Gross necropsy also represented no any toxicity to any animal.

2. Antiangiogenic activity of aqueous extract of C. Sinensis on mesenteric window.New blood vessels are developed in rat mesentery because of mast cell

secretagauge compound 48/80. When animals treated with CS extract with control animals, it was found that CS 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.

Table 1
Effect of administration of CS extract on compound 48/80 induced mesenteric window angiogenesis.

Group	Treatment	No. of Blood vessels developed
1	Negative Control	0.00
2	Positive Control	15.79 ±0.15
3	CS 125	13.67 ±0.42
4	CS 250	11.5 ±0.47*

Values are expressed as mean ± SEM of observations of four windows per animal, n=06. Statistical significance is expressed as *p<0.05

3. Antiangiogenic activity of aqueous extract of C. Sinensis on subcutaneous air pouch 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 2, even though CS 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 CS extract.

Table 2
Effect of administration of CS extracts on subcutaneous air pouch angiogenesis.

Group	Treatment	No. of Blood Vessels Formed centrally /mm^2	Angiogenesis score
1	Control	14.83 ±0.48	4+
2	CS 125	13.00 ±0.37	2+
3	CS 250	7.33 ±0.49**	1+

Values are expressed as mean ± SEM of double blind observations n=06. Statistical significance is expressed as **p<0.01

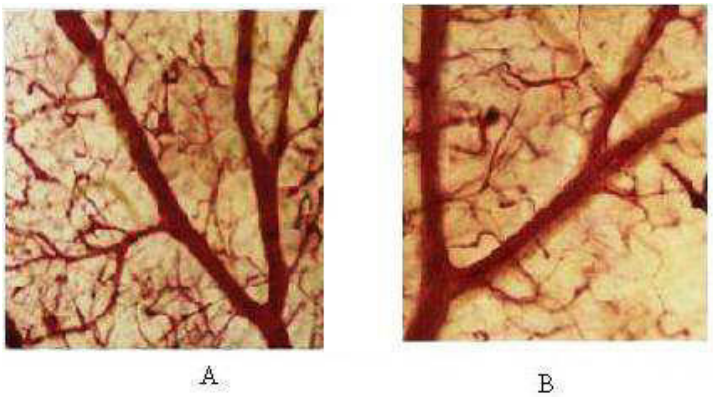

Figure 2
Effect of administration of CS extracts on subcutaneous air pouch angiogenesis. 'A' represents control membrane while 'B' represents angiogenesis in CS 250mg/Kg extract

4. Antiangiogenic activity of aqueous extract of C. Sinensis on Chorioallentoic membrane 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 CS extract in the dose of 10mg/ml reduces angiogenic response to such hypoxia to about 34%.

Table 3
Effect of administration of CS extracts on CAM angiogenesis.

	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
CS 5mg/ml	3.1 ±0.198	5.9 ±0.344	13.2 ±0.521	34.328*
CS 10mg/ml	2.9 ±0.204	5.4 ±0.221	11.3 ±0.405	43.781**

Values are expressed as mean ± SEM. Statistical significance is expressed as *p<0.05, **p<0.01

CONCLUSION

Angiogenesis is one of the major role players in prognosis of several diseases particularly arthritis, tumour metastasis, retinopathies etc. As angiogenesis is a complex process with interplay of several factors[7], ranging from endogenous factors to inducible factors, mere targeting one or two pro-angiogenic factors is insufficient. The results of the present study showed the antiangiogenic effects of aqueous extract of *Camellia sinensis* in different angiogenic situations. This implies that CS extract can be introduced as an alternative agent in the treatment of various types of angiogenesis dependent diseases. However, further investigations are still needed to evaluate its safety in particular disease and to enhance its delivery to the affected areas and also clinical trials, on larger population, are still required to determine the efficacy of *Camelia* sinensis alone as well as in combination with other pharmacotherapeutic agents.

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