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Anti-Angiogenic Activity of *Caesalpenia Bonducella* Leaf Extracts in Ehrlich Ascites Tumor Cells *In-Vivo*

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Abstract. Angiogenesis is the physiological process involving the growth of new blood vessel from pre-existing vessels. However, it is also a fundamental step in the transition of tumors from a dormant to a malignant stage. It has been suggested that blocking of angiogenesis and the action of the cytokine VEGF could be possible in cancer therapy. Medicinal plants continue to play a central role in the healthcare system of large proportions of the world's population, particularly true in developing countries like India. One such medicinal plant we screened and identified is *Caesalpenia bonducella,* which has potent anti-angiogenic activity. The aqueous leaf extract inhibits the Ehrlich ascites tumor cell proliferation by *in-vivo*. The anti-angionenic activity of *C. bonducella* was confirmed by its inhibition of angiogenesis in *in-vivo* assays, peritoneal and chorioallantoic membrane assay. Reduction in the levels of the cytokine VEGF and microvessel density count in the peritoneum of mice treated with *C. bonducella* indicated that the plant extract decreased VEGF production and the cytokine induced neovascularization. Our preliminary results suggest that the *C. bonducella* extract may be a potential anti-angiogenic agent which may exploit to treat cancer disease.

Keywords: *C. bonducella,* Anti-angiogenic, VEGF, EAT cells.

1. Introduction

Angiogenesis, the growth of new capillary blood vessels is important in normal processes such as development of the embryo, formation of the corpus luteum and wound healing. It is also a component in the pathological processes such as chronic inflammation, certain immune responses and neoplasia [1–3]. Furthermore, angiogenesis is a property of most solid tumors and is necessary for their continued growth [4]. Vascularized tumors spread locally and distantly through metastases; therefore, one of the main challenges in cancer research is to understand the mechanisms by which tumors at a given stage stimulate the development and growth of new microvessels. There are many inducers of blood vessel formation, which include cytokines such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin-2. Inhibitors of angiogenesis include heparin, angiostatin and endostatin. Thus, the balance of these inducers and inhibitors would result in endothelial cell quiescence or angiogenesis [5]. Because the expression of VEGF has been implicated in tumor angiogenesis, pharmacologic intervention that affects the VEGF expression may influence progression and prognosis of cancer patients [6]. Although a few natural and synthetic compounds have been characterized as potential inhibitors of tumor angiogenesis and have found entry into clinical trials [7], intense efforts have been made to identify potent angiogenic inhibitors with increased selectivity.

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Since antiquity, plants have been used to treat many ailments. However, it was not until the 1800s that pure compounds were isolated from plants, paving the way for modern pharmaceuticals Ethnotraditional use of plant-derived natural products plays a significant role in the discovery and development of potential medicinal agents. *Caesalpenia bonducella* (Gujagu) is a plant well known for its medicinal value in Indian Ayurveda. *Caesalpenia bonducella* possesses potential immunomodulatory activity and has therapeutic potential for the prevention of autoimmune diseases [8]. *C. bonducella* seed extracts has anti-pyretic and anti-analgesic properties [9]. The different extracts of *C. bonducella* shows anthelmintic activity, anti-amyloidogenic activity, immunomodulatory, analgesic, antipyretic, anti-inflammatory, antioxidant activity, ant diabetic and hypoglycemic activity, and also used as nootrophic or memory enhancer [10, 11].We herein report for the first time that the aqueous extract of *C. bonducella* effectively inhibits growth of Ehrlich ascites tumor cells *in-vivo* and acts as inhibitor of VEGF induced angiogenesis.

2. Materials and Methods

C. bonducella plants were collected from Western Ghats of India and also rural parts of Mysore and Mandya districts of Karnataka. The herbarium of the specimen was made and maintained in the P.G. Department of Biotechnology, Teresian College, Mysore. Swiss albino mice (8-10 week old) were obtained from Department of Biotechnology and Zoology, University of Mysore (Mysore, India), Ehrlich Ascites tumor (EAT) cells also called mouse mammary carcinoma cells are maintained in our laboratory and are routinely used for *in-vivo* transplantation. Agarose, Tryphan blue, Giemsa stain, Ehidium bromide were obtained from Hi-media research laboratory. All other chemicals and reagents were of highest grade commercially available.

2.1. Preparation of *C. bonducella* Aqueous Extract

Preparation of aqueous extract of *C. bonducalla* was followed with the method previously reported [12]. Thus, the leaves of *C. bonducalla* were dried at 50 °C and crushed in a blender and the crude powder was extracted with sterile distilled water at 100 °C for 3 hours. The aqueous extract was evaporated at 60 °C under pressure. Finally the extract was dissolved in 100% DMSO to make a stock solution (100mg/ml).

2.2. Culture of EAT Cells In-Vivo and *C. bonducella* Extract Treatment

Ehrlich ascites tumor cells (5×10^6) were injected intraperitoneally (i.p) into mice and growth was recorded from 1st day to 12th day. During this period cells grow in the mice peritoneum forming an ascites tumor, with massive abdominal swelling. Treatment was given by injecting aqueous extract of C. bonducella dissolved in DMSO (100mg/kg body weight) from 7th day till 11th day to monitor the in-vivo effect of aqueous plant extract on EAT cell growth and proliferation. At the same time control mice bearing ascites tumor were treated with DMSO alone.

2.3. Giemsa Staining

The validity of apoptosis was confirmed through the use of light microscopy in which cells were assessed for apoptotic morphology using wright giemsa stain. Briefly cells from both control and *C. bonducella* treated EAT bearing cells were dropped slowly into slide. Slide was air dried. Then the slides were fixed with methanol, Giemsa stain and dipped in distilled water. Finally the slides were examined by high power and oil immersion light microscopy. Apoptotic cells were easily distinguishable by their reduced volume, chromatin condensation and nuclear fragmentation.

2.4. Chorioallatoic Membrane (CAM) Assay

CAM assay was essentially carried according to the detailed procedure as described by Gururaj, et. al [13]. In brief, the compounds to be tested were applied to the CAM and after 12th day the CAM was photographed.

2.5. Enzyme Linked Immunosorbent Assay for Quantitation of the Cytokine VEGF

The level of the cytokine VEGF secreted by EAT cells into peritoneal ascites was measured by ELISA as described in detail by Belakavadi and Salimath [14].

2.6. Statistical Analysis

The data were expressed as means ±S.D. The statistical analysis was performed using SPSS for Windows (SPSS, Inc.). P values less than 0.05 was considered to be significant.

3. Results

The results shown in Fig. 1 indicates that upon injection of $5x10^6$ cells into the peritoneum of mice, there is nearly 54% increase in body weight of EAT-bearing mice during a growth period of 12 days. A total volume of 10.25 ml of ascites and the total number of $17.8x10^8$ cells were routinely obtained as a consequence of extensive proliferation of EAT cells *in-vivo*. However, upon treatment with *C. bonducella* aqueous extract, there was 55% inhibition of growth of EAT cells and 60% decrease in formation of ascites fluid (Fig. 2). Treatment of *C. bonducella* aqueous extract clearly shows the externalization of phosphatidylserine residues, nuclear condensation and formation of apoptotic bodies, which is the hallmark of cells undergoing apoptosis (Fig. 3). All these results clearly indicate the *in- vivo* anti-proliferatory effect of *C. bonducella*. When compared to 54 ± 2 microvessels present in the peritoneum of EAT-bearing mice on the 12th day, the *C. bonducella* aqueous extract treated mice peritoneum showed only 7–8 microvesels. Also there is an 85% decrease in the levels of VEGF in treated animals when compared to untreated. The results as shown in Table 1 and in Fig. 4A clearly indicate that the extensive angiogenesis seen in the peritoneum of EAT-bearing mice is inhibited by *C. bonducella* extract. Our results on CAM assay further confirm the angioinhibitory effect of *C. bonducella* extract (Fig. 4B).

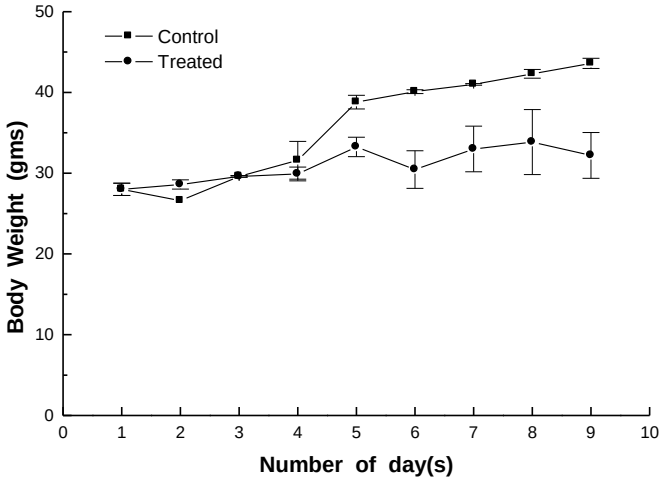

Fig. 1: Effect of *C. bonducella* extract on body weight of mice: EAT cells ($5X10^6$ cells/mouse, i.p) were injected into mice and body weight of the mice was recorded to follow tumor growth. Every alternate day *C.bonducella* extract is administered from the 6thday onwards (100 mg/kg body weight). Minimum of 5 mice were used for the experiments and has to be repeat two more time for statistical significance.

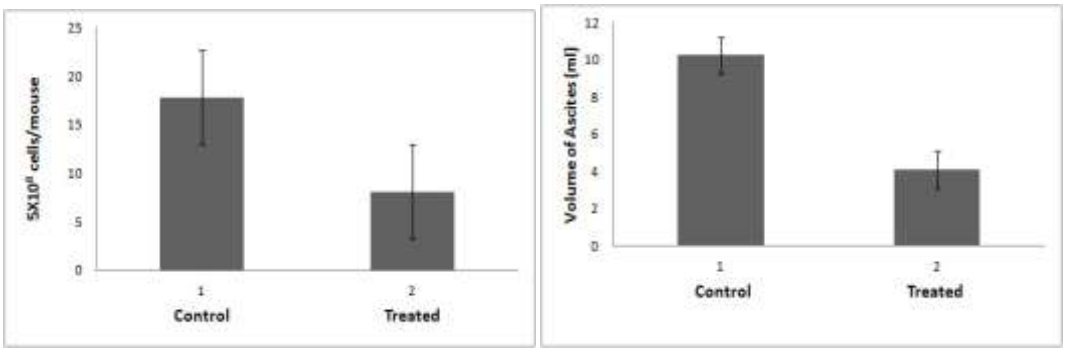

Fig. 2: Effect of *C. bonducella* extract on and EAT cell number ascites volume: EAT bearing mice treated with *C. bonducella* extract are sacrificed after giving each dose cells along with ascites fluid were harvested. The number of cells per mouse was determined by counting the cells in hemocytometer and ascites volume was recorded.

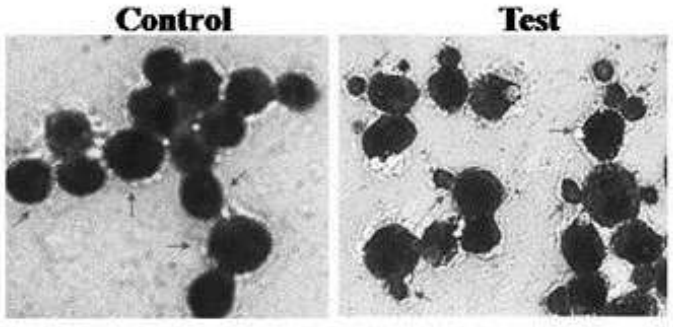

Fig. 3: Apoptotic morphology of EAT cells upon *Caesalpinia bonducella* extract treatment: EAT cells treated with and without *Caesalpinia bonducella* extract washed with PBS, fixed in methnol/acetic acid (3:1) and stained Giemsa. Both types of cells were carefully viewed under light microscope for apoptotic morphology such as plasma membrane degradation, membrane blebbing or apoptotic bodies and results were documented.

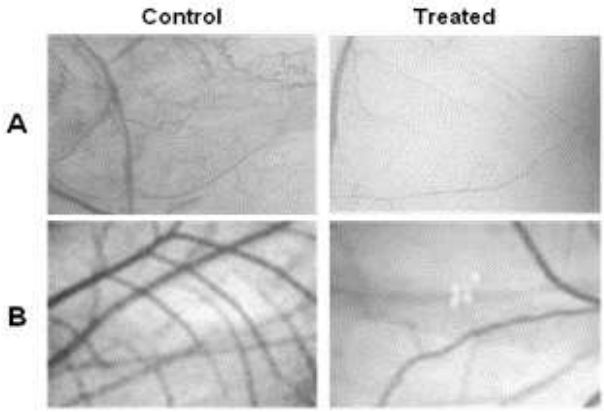

Fig. 4A: Suppression of peritoneal angiogenesis by *C. bonducella* extract. Extensive neovascularization in the Peritoneal lining of EAT bearing control untreated mice. Peritoneal lining of mice treated with *C. bonducella* extract was inspected for angiogenesis. Inhibition of peritoneal angiognesis in *C. bonducella* extract treated mice is evident; **B. Inhibition of angiogenesis in chick CAM assay by *C. bonducella* extract:** Photos illustrate the formation of blood vessel branch points in either control (saline) *C. bonducella* extract treated CAMs of the 12-day-old embryonated chicken eggs. Note the significant inhibition of the formation of blood vessel branch points in the egg exposed to *C. bonducella* extract.

Table 1. Effect of *C. bonducella* extract on in-vivo production of VEGF: Ascites from the mice treated with or without *C. bonducella* extract was collected on 12th day and VEGF was quantified by ELISA.

Samples	MVD	VEFG (ng)
Control (EAT bearing)	**54 - 56**	**2125**
Test (*C. bonducella* treated)	**7 - 8**	**235**

4. Discussion

Medicinal herbs and plants continue to play a significant role in drug discovery and development, particularly in cancer research. The overwhelming contribution of natural products to the expansion of the chemotherapeutic arsenal is evidenced by the fact that 50% of all the anticancer drugs approved worldwide between 1940 and 2006 were either natural products or natural product derived [15].The cytokine VEGF which acts in an autocrine–paracrine manner is a major mitogen for proliferation of EAT cells *in-vivo*. Angiogenesis has been shown to play a significant role in cancer growth and metastasis. Recently, anti-angiogenic drugs have been shown to eradicate certain mouse tumors and induce long term tumor dormancy and disease free survival [16]. With the goal of finding a potent antiangiogenic drug, we have initiated a screening program in our laboratory designed to test a wide variety of plant extracts for anti-angiogenic activity. Our preliminary studies indicated that the extract from the leaves of *C. bonducella* is quite potent.

Inhibition of EAT cell growth *in-vivo* with corresponding reduction in cell number, body weight and ascites volume confirms the early findings of *C. bonducella* as anti-neoplastic agent. Treatment with the aqueous extract of *C. bonducella* on EAT-bearing mice showed induced inhibition of proliferation of tumor cells *in-vivo* [17]. The bioactive compound present in the aqueous extract of *C. bonducella* has been shown to be an apoptosis-inducing component in *C. bonducella* [18]. Further characterization of active principle present in the *C. bonducella* has to be carrying out. Our results indicate that the aqueous extract of *C. bonducella* inhibits EAT cell proliferation *in-vivo* and also inhibition of neovascularization which is evidenced by CAM assay. Since there is inhibition of neovascularization by *C. bonducella* extract, it supports our view that *C. bonducella* extract may repress the expression of VEGF like factors thereby inhibiting the formation of new blood vessels and this was confirmed by ELISA. Thus, our results suggest that the extract from *C. bonducella* may be a potential supplemental source for cancer treatment, and deserves further studies. Further work is ongoing to identify the bio-active compounds and delineate the underlying mechanism, signaling cascades involved in targeting angiogenesis.

5. Acknowledgements

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