ANTI-TUMOR AND ANTI-ANGIOGENIC ACTIVITY OF CADABA FRUTICOSA LEAF EXTRACTS IN MURINE CARCINOMA MODEL

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INTRODUCTION

Formation of ascites is observed in patients with advanced cancer, especially ovarian cancer. Once accumulation of ascites occurs, the prognosis is poor. Abdominal paracentesis performed to relieve patient’s symptoms. But reaccumulation of ascites occurs very quickly, while sufficient amount of drugs cannot be administered due to poor condition of the patients. Thus, the development of new effective approaches to treating cancer patients bearing ascites tumor is urgently needed. As an attempt in this direction, we have focused on angiogenesis in ascites tumor model [S. L. Parsons et al 1996, A. Kashani et al., 2008]. Angiogenesis is the formation of new blood vessels out of the pre-existing vascular vessels and involves a sequence of events that are of key importance in broad array of physiological and pathological processes. Under physiological condition, angiogenesis is restricted to processes such as embryogenesis, wound healing and in menstrual cycle.

Angiogenesis also occurs in pathological processes such as inflammation [T. Griga et al 1998], rheumatoid arthritis [M. Nagashima et al 1995] and tumor development [J. Folkman et al 1995]. Tumor growth is angiogenesis dependent [J. Folkman et al 1971 and J. Folkman et al 2000] and tumor can establish their own blood supply by processes very similar to normal angiogenesis. The induction of new blood vessel growth by a tumor is mediate through the action of many proangiogenic factors that include FGF-1 and FGF-2, VEGF, TNF, angiogenin, TGF-beta, PDGF, angiopoietin and pleiotropin.

The several factors described, there is increasing evidence that VEGF is the prime regulator of normal and tumor angiogenesis, VEGF has been found to be the major permeability inducing factor responsible for fluid accumulation in the ovarian hyperstimulation syndrome and large amount of VEGF have been found in variety of effusions accompanying

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ABSTRACT

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. We evaluate the tumor inhibitory activity of cadabafruticosa leaf extracts on EAC cells administered mice targeting apoptosis and angiogenesis, which is physiological process involving the growth of new blood vessel from pre-existing one. However, it is also plays a major role in the transition of tumors from a dormant to a malignant stage. The medicinal plant we screened and identified as Cadabafruticosa, which has potent anti-angiogenic and pro-apoptotic activity and expressed direct cytotoxicity to EAC cells by inducing apoptosis as evidenced by decrease in tumor volume, viable cell count and body weight of EAC bearing mice. In treated mice group also found that membrane blebbing, DNA fragmentation, a characteristic features, of cell undergoing apoptosis. Significant anti-angiogenic activity was confirmed by its inhibition of angiogenesis in in-vivo peritoneal lining and chorioallantoic membrane assay. Our preliminary results suggest that the Cadabafruticosa leaf extract may be a potent pro-apoptotic and anti-angiogenic agent which may exploit to treat cancer disease as well as in the field of pharmaceutical drug designing.

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pathological disorders like edema formation in brain, synovial fluid and malignant ascites [D. Marme et al 1996].

It is thus considered important to screen antineoplastic compounds from plants either in the form of crude extract or as a component isolated from them. Anticancer agents from medicinal plants appear to be satisfactory for the control of the diseases and prolong the life of the patient. There has been a continuing search for compounds useful in the prevention or treatment of cancer, especially for agents with reduced toxicity. Although a few natural and synthetic compounds have been characterized as potential inhibitors of tumor angiogenesis and have found entry into clinical trials [W. Auerbach et al 1994].

Intense efforts have been made to identify potent tumor inhibitors with increased selectivity. 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. Ethnobotanical use of plant-derived natural products plays a significant role in the discovery and development of potential medicinal agents. *Cadabafruticosa* is a plant well known for its medicinal value in Indian Ayurveda. *Cadabafruticosa* possesses potential immunomodulatory activity and has therapeutic potential for the prevention of autoimmune diseases [Mohamed Al-Fatimi et al 2007]. *Cadabafruticosa* plant having a anti-pyretic, anti-inflammatory, immunomodulatory, anti-inflammation, antioxidant and anti-analgesic, anti-diabetic and hypoglycaemic properties [Mythreyi, R, Sasikala. E et al 2008 and Viqaruddin Ahmad et al 1971]. We herein report for the first time that the aqueous extract of *Cadabafruticosa* leaf extract effectively inhibits growth of Ehrlich ascites Carcinoma cells in-vivo and acts as inhibitor of VEGF induced angiogenesis.

**MATERIALS AND METHODS**

*Cadabafruticosa* plants were collected from 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 Carcinoma (EAC) cells also called mouse mammary carcinoma cells are maintained in our laboratory and aerobically used for in-vivo transplantation. Agarose, Trypan blue, Giemsa stain, Ehidium bromide were obtained from Hi-media research laboratory. All other chemicals and reagents were of highest grade commercially available.

**Preparation Cadabafruticosa leaf Aqueous Extract**

Preparation of aqueous extract of *Cadabafruticosa* was followed with method previously reported (Jayarama et al 2015). Thus, the leaves of *Cadabafruticosa* were dried at shaded low temperature and crushed in a blender and the crude powder was extracted with sterile distilled water using magnetic stirrer leave it for overnight. The aqueous extract was evaporated at 37°C water bath. Finally the extract was dissolved in 100% DMSO to make a stock solution (100mg/ml).

**In-vivo EAC Cells culture**

The Ehrlich Ascites Carcinoma (EAC) or mouse mammary carcinoma cells cell lines were maintained by in vivo passage in Swiss albino mice. EAC bearing Swiss albino mice were sacrificed and the tumors were withdraw under aseptic conditions. A suspension of the withdraw tumor cells was made in 0.9% sterile saline. Approximately (5 × 10⁶) EAC cells were injected to mice intraperitoneal region and growth was recorded every day until the 12th day. These cells grow in the mice peritoneum, forming an ascites tumor with massive abdominal swelling and neovascularization in the inner lining of the peritoneal wall. The animals show a dramatic increase in body weight over the growth period and animals surrender to the tumor weight 14-16 days after transplantation.

**In vivo Cadabafruticosa leaf extract treatment**

To determine whether *Cadabafruticosa* leaf aqueous extract regresses the tumor volume in vivo, *Cadabafruticosa* leaf aqueous extract (100 mg/kg body weight/ ip) were injected into the EAC-bearing mice intraperitoneally (i.p) on every alternate day starting 6 days of tumor implantation and growth of the tumor was monitored the body weight of the animals every day. Control mice were injected with 0.2 mL saline (i.p) on every alternate day. Each treatment consisted of at least five mice and each experiment was repeated thrice. Animals were sacrificed and collect the EAC cells along with ascites fluid. Inner lining of the peritoneal cavity was examined for neovascularure by taking photograph using Sony digital camera.

**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 [Jayarama et al 2015, Roopashree, R. et al 2015 and Jyoti Bala Chauhan et al 2015]. Briefly cells from both control and *Cadabafruticosa* treated EAC 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.

**Chorioallantoic Membrane (CAM) Assay**

CAM assay was carried out in accordance with the method described previously [Jayarama et al 2015]. In brief, fertilized eggs were incubated at 37°C in a humidified and sterile atmosphere for 10 days. Under aseptic conditions, a window was made on the eggshell to check for proper development of the embryo. The window was resealed and the embryo was allowed to develop further. On the 12th day, saline, recombinant cytokine VEGF or *Cadabafruticosa* leaf extract was air dried on sterile glass cover slips. The window was reopened and the cover slip was inverted over the CAM. The window was closed again, and the eggs were returned to the incubator for another 2 days. The windows were opened on the 14th day and inspected for changes in the vascular density in the area under the cover slip and photographed using sony digital camera.
RESULTS

Effect of Cadabafruticosa extract on body weight of mice

To understand the in vivo effect of cadabafruticosa leaf extract on proliferation of EAC cells, Swiss Albino mice were treated with and without cadabafruticosa leaf extract. It was found that cadabafruticosa leaf extract treated mice showed significant antiproliferative effect towards EAC cells in vivo as monitored by the body weight as shown in Fig.1. In contrast, the weights of untreated EAT bearing mice steadily increased when compared to treated mice.

Effect of Cadabafruticosa extract on and EAC cell number and ascites volume

Here we investigate the effect of Cadabafruticosa extract on and EAC cell number and ascites volume, there was more than 98% cell viable in control group mice where as it was decreased to 41% in animals were treated with cadabafruticosa leaf extract as counted by the trypsin blue dye exclusion method (Fig.2A) and Decrease in tumor cell growth also correlates with decrease in ascites volume in treated mice (Fig. 2B). The total volume of ascites in untreated mice was 9.5ml and in cadabafruticosa extract treated mice it was 2.5ml, indicates that around 85% decrease in the ascites volume. Treatment of leaf extract clearly shows the nuclear condensation and formation of apoptotic bodies, which is the hallmark of cells undergoing apoptosis when compared to control EAC cell.

Apoptotic morphology of EAC cells on Cadabafruticosa leaf extract treatment

Treatment of Cadabafruticosa aqueous extract clearly shows the externalization of phosphatidylinerine residues, nuclear condensation and formation of apoptotic bodies, which is the hallmark of cells undergoing apoptosis (Fig. 3).

DNA Fragmentation Assay

EAC cells are collected from mice treated with or without Cadabafruticosa leaf extract in-vivo and DNA is isolated using phenol-chloroform method [Bharathi P. Salimath et al 2007 and Rakesh et al 2016]. In brief cells were lysed in a buffer containing 50 mMTris-HCl, pH 8.0 and 0.5% SDS, incubated for 30 min at 37°C. The cell lysate was subjected to 8 M potassium acetate precipitation and left for 1 h at 4°C. The supernatant was extracted by phenol: chloroform: isoamyl alcohol (25:24:1) and once to chloroform extraction. DNA was precipitated by adding 1:2 volumes of icecold ethanol. The precipitated DNA dissolved in 50 μl of TE buffer (pH 8.0). The DNA was digested with 20 μg/ml of RNase at 37°C for 1 h. The isolated DNA was separated by electrophoresis on 1.5% agarose gel and visualized by staining with ethidium bromide under UV transilluminator and documented using Uvp-Bio-gel Doc system.

Biochemically, apoptosis is characterized by fragmentation of chromosomal DNA. We further verified the effect of Cadabafruticosa leaf extract on DNA damage of EAC cells. As expected, Cadabafruticosa leaf extract treatment caused DNA fragmentation which then lead to the formation of DNA ladder in EAC cells (Fig. 4). This indicates that the apoptotic role of Cadabafruticosa leaf extract, EAC cells ruled out the possibility of it being necrotic to EAT cells.

Suppression of peritoneal angiogenesis by Cadabafruticosa extract

To evaluate the anti-angiogenic activity in-vivo, Cadabafruticosa leaf extract was tested mouse peritoneal angiogenesis assay. Angiogenesis is the evident in the inner peritoneal lining of EAC bearing mice and it is a reliable model for in vivo angiogenesis. Hence the peritoneal lining of Cadabafruticosa leaf extract treated mice was verified for its effect on peritoneal angiogenesis. In EAC bearing mice,
increased peritoneal angiogenesis was consistently seen when compared to the extent of peritoneal angiogenesis in tumor bearing mice receiving the *Cadabafruticosa* leaf extract treatment. A reduction in peritoneal was observed in EAC treatment. A reduction in peritoneal compared to the extent of peritoneal angiogenesis in tumor increased peritoneal angiogene.

**Inhibition of angiogenesis in chick CAM assay by *Cadabafruticosa* extract**

To evaluate the anti-angiogenic activity *in-vivo*, *Cadabafruticosa* leaf extract was tested in the CAM. In the CAM assay model *Cadabafruticosa* leaf extract Decrease the blood vessel formation in the developing embryos. Notably newly formed microvessel were regressed around the area of *Cadabafruticosa* leaf extract implanted disk (Fig. 6).

**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 [D.J. Newmann, G.M et al 2007]. The cytokine VEGF which acts in an autocrine–paracrine manner is a major mitogen for proliferation of EAC cells *in-vivo*. Angiogenesis has been shown to play a significant role in cancer growth and metastasis. Recently, antiangiogenic drugs have been shown to eradicate certain mouse tumors and induce long term tumor dormancy disease free survival [T. Boehm, et al 1997]. With the goal of finding a potent antiangiogenic drug, we have initiated ascreening 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 *Cadabafruticosa* is quite potent. Inhibition of EAC cell growth *in-vivo* with corresponding reduction in cell number, body weight and ascites volume confirms the early findings of *Cadabafruticosa* as anti-neoplastic agent. Treatment with the aqueous leaf extract of *Cadabafruticosa*on EAC-bearing mice showed induced inhibition of proliferation of tumor cells *in vivo* [D. Sriram, et al 2005]. The bioactive compound present in the aqueous extract of *Cadabafruticosa* has been shown to be anapoptosis-inducing component in *Cadabafruticosa* [M. Watanabe, et al 2002]. Further characterization of active principle present in the *Cadabafruticosa* has to be carrying out. Our results indicate that the aqueous extract of *Cadabafruticosa* inhibits EAC cell proliferation *in-vivo* and also inhibition of neovascularization which is evidenced by CAM assay. Since there is inhibition of neovascularization by *Cadabafruticosa* leaf extract, it supports our view that *Cadabafruticosa* leaf extract may repress the expression of VEGF like factors thereby inhibiting the formation of new blood vessels. Thus, our results suggest that the extract from *Cadabafruticosa* may be a potential supplemental source for cancer treatment, and deserves further studies. Further work is under pipeline to identify the bio-active compounds and delineate the underlying mechanism, signaling pathways involved in targeting angiogenesis.

**CONCLUSION**

From our data, it can be concluded that, *Cadabafruticosa* leaf extractis a very potent anti-carcinogenic agent, the active principle that brings about its effect by inhibition of angiogenesis in CAM in vivoand the tumour site of the mice intra peritoneum and the inhibition of the cell progression is by
induction of apoptosis in murine carcinoma cells. Further work is undergoing to identify the bio-active compounds and delineate the underlying mechanism, signaling pathways involved in targeting apoptosis and angiogenesis.

**Conflict of interest:** The authors declare that they have no conflict of interest.

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