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RESEARCH ARTICLE

ANTI-ANGIOGENIC AND PRO-APOPTOTIC ACTIVITY OF AQUEOUS EXTRACT OF *LEUCAS ASPERA* IN EHRLICH ASCITES CARCINOMA MODEL *IN VIVO*

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ABSTRACT

Angiogenesis is the creation of new blood vessels. The process of angiogenesis involves the migration, development, and differentiation of endothelial cells, which contour the inside wall of the blood vessels. The development of antiangiogenic agents to block new blood vessel growth will repress metastasis and encourage apoptosis of tumor. The present object focuses on phyto-constituents and antiangiogenic activity of the *Leucas aspera* leaves extract. Aqueous extract showed potent inhibitory activities against EAC cells procreation in in-vivo; this evidence shows that leaf extract of *leucas aspera* also has been showed anti- angiogenic activities. Aqueous extract steadily decreases the body weight & tumour volume when compare to control. Further, inhibition of blood vessels in intraperitoneum cavity of mice treated with *Leucas aspera,* which proves antiangiogenic activity. Chorioallantoic membrane assay (CAM) provides further evidence towards angiogenic inhibitory activity. Our coming up proposal is focusing in the direction is to enhancement of clincher based research and chemical characterisation of these compounds could further enhance the efficacy of this plant- based medicine in angiotherapy.

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INTRODUCTION

New growth in the vascular network is important since the proliferation, as well as metastatic spread, of cancer cells depends on a correct supply of oxygen and nutrients and the removal of waste products. New blood and lymphatic vessels form through processes called angiogenesis and lymphangiogenesis, Tumor growth and metastasis depend on angiogenesis and lymphangiogenesis triggered by chemical signals from tumor cells in a phase of rapid growth (Folkman 1971). Angiogenesis, an event which describes the development of new vasculature from the pre vessel is dragging lots of attention in scientific research field due to its role in various physiological and pathological processes (Folkman, 1990). Angiogenic switch plays key role in the development of vasculature and in cancer cells the angiogenic switch is 'on' where the interrupted balance between pro-angiogenic factors enables cancer cells to acquire angiogenic phenotype (Folkman, 2003, Carmeliet and Jain, 2000) which stimulate the sprouting or intussusceptive angiogenesis the pre-existing vasculature (Hlushchuk *et al*., 2008) switch leads to over expression of many pro proteins, mainly vascular endothelial growth factor (Saeki *et al*., 1997), platelet factor PD-ECGF (Saeki *et al*. 1997, Toi *et al*.,1995) basic fibroblast growth factor bFGF, growth factor (TGF)-α, (TGF) (PLGF), hepatocyte growth factor, tumor necrosis factor (TNF-α) (Nishida *et al*., 2006, Hillen, 2007) IL-18 (part *et al*., 2005) IL-8 (Brat *et al*., 2005) but VEGF is been considered to play a fundamental role in regulation of angiogenesis (Ferrara 2001).

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Anti-angiogenic agents target the blood vessels mainly by inhibiting the action of angiogenesis inducers such as VEGF (Folkman, 1974). It's been understood that the Anti-angiogenic therapies are comparatively well tolerated than traditional cytotoxic chemotherapic target specificity. It's been understood that the antiangiogenic therapies are comparatively well tolerated than traditional cytotoxicity chemotherapy target specificity (Lacouture, 2009). Plants being integral part of human diet, also serving the mankind in maintaining their better health. In the last few decades, many excellent natural products with anti-angiogenic properties have been investigated. Some compounds like Acylphloroglucinols (Hypericum perforatum L., Hypericaceae) (Ades *et al*, 1992), Xanthohumol (Humulus lupulus L., Cannabaceae), (Schmidt, S *et al*, 2012) ( )-Epigallocatechin-3-O-gallate (Camellia sinensis) (Xu, F *et al*, 2013). It inhibits the activation of HIF-1α, NF-κB and VEGF expression, thereby suppressing tumor angiogenesis and cancer progression (Li, X *et al*, Sakamoto *et al* 2013). In search of new effective medicinal plant for inhibition of tumor angiogenesis, *Leucas aspera* is a species within the *Leucas* genus and the *Lamiaceae* family. Although the species has many different common names depending on the region in which it is located, it is most commonly known as *Thumbai* is distributed throughout India from the Himalayas down to Ceylon (Rai.V *et al* 2005, Nadkarni KM *et al* 1976). It has been proven to possess various pharmacological activities like antifungal, antioxidant, antimicrobial, antinociceptive and cytotoxicity activity. Preliminary chemical examination of Leucas aspera revealed presence of triterpenoids in entire plant. Whole plant is reported to contain oleanolic acid, ursolic acid and 3-sitosterol. Aerial parts are reported to contain nicotine, sterols, and alkaloids. Reducing sugars, glycoside (M. S. Prajapati, *et al* 2010). We identified potent activities in *leucas aspera* as a plant of potential ability in fighting against tumour angiogenesis. *Leucas aspera* is well known for its significant medicinal values. In the current report effort has been made to evaluate the anti-angiogenic activity of aqueous leaf extract of *leucas aspera* in Ehrlich Ascites Carcimoma (EAC) model.

MATERIALS AND METHODS

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 were maintained in our laboratory and are routinely used for in-vivo transplantation. Agarose, Trypan blue, Giemsa stain, Ethidium bromide were obtained from Hi-media research laboratory. All other chemicals and reagents were of highest grade commercially available.

Plant extract

The plant of the *leucas aspera* was collected from rural area of Malavalli (tq), Mandya (dist), and India. The leaves were dried and powdered; 10 gm powder was extracted with 200 mL of distilled water, (3 times) for 3 h under reflux. After extractions, the extracts were combined and filtered with Whatman filter paper No.1, and then were concentrated in vacuum to dryness and considered as aqueous extract of *leucas aspera.* All extracts were stored at 4 ºC until use.

In-vivo EAC cell growth and *leucas aspera* extract treatment

In-vivo culture of EAC cells, treatment with *leucas aspera* extract and isolation of EAC cells from peritoneum cavity was done as reported earlier (Jayarama *et al*., 2013). In brief EAC cells (10^6 cells) were injected intraperitoneally into 8-10 week old Swiss albino mice and weight of the animals were monitored every day. Six days after *Leucas aspera* extract (100 mg/kg body weight/i.p, in 0.1% DMSO) was given to intraperitoneally of the EAC bearing mice for every alternate day and body weight was recorded up to 12th day. Control and treated EAC bearing mice were sacrificed; EAC cells along with the ascites fluid were collected. Quilting of the peritoneal cavity was examined for vasculature was photographed by using Nikon camera.

Isolation of EAC cells from mice

After the 3 doses of aqueous extract (100mg/mL) treatment on alternate days, mice were sacrificed and Ascites fluid of both control and treated mice was collected in sterile tubes and volume was recorded. The collected cells were used for further studies as described below.

DNA isolation and Agarose gel electrophoresis

EAT ($5X10^6$ cell/mL) were treated with the indicated concentration of test extract for different time period. After the supernatant was removed by centrifugation (1500 rpm, 4 ºC), the cells were washed with 1 mL of PBS and was precipitated by centrifugation at 3000 rpm for 10 min at 4 ºC. Cells were lysed in a lysis buffer containing 50 mM Tris–HCl, pH 8.0 and 0.5% SDS and incubated for 30 min at 37ºC. The cell lysate was subjected to 8 M potassium acetate precipitation and left for 1 h at 4ºC. The supernatant was subjected to phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by chloroform extraction. DNA was precipitated by adding 1:2 volumes of ice-cold ethanol. The precipitated DNA was digested with 20 µg/mL RNase at 37 ºC for 30 min. The DNA (1µg) was resolved on 1.2% Agarose gel in TAE buffer and documented using Bio Bee Gel Doc system.

Giemsa Staining

The apoptotic and cell morphology features of control & treated cells were evaluated by using Giemsa staining. Both the cells were fixed into clean sterile glass slide and airs dry it. Dried slide was fixed by using fixative (methanol) followed by Giemsa and dipped in distilled water. Finally the slides were observed under oil immersion light microscopy. The chromatin and nuclear condensation cells were identified as apoptotic studies.

Chorioallantoic membrane (CAM) assay

The in-vivo CAM angiogenesis assay was performed as reported previously (Jayarama *et al*., 2013). In brief fertilized eggs were incubated at 37 ºC. In a humidified and sterile atmosphere for 9 days and a window was opened on the eggshell exposing the CAM. The *Leucas aspera* extract was

placed on sterile discs, which were allowed to dry under sterile conditions. A loaded and air-dried *Leucas aspera* extract smeared discs and control (0.9% saline) disc were placed on the CAM. Windows were sealed and the eggs were returned to the incubator until 11th day. The windows were opened on the 11th day and inspected for changes in the micro vessel density in the area below the cover slip and photographed using the Sony digital camera.

RESULTS

The results shown in Fig. 1 indicates that upon injection of $5x10^6$ cells into the peritoneum, and daily recorded body weight of EAC-bearing mice during a growth period of 2 weeks. Upon treatment with aqueous extract of *leucas aspera* there is decreased body weight or tumour volume suggests anti-tumour activity of the treated plant extract compared to control group. A total volume of 8ml of ascites and there was 99% cells were viable as a consequence of extensive proliferation of EAC cells *in-vivo.* However, upon treatment with *leucas aspera* aqueous extract, there is 60% regression in ascites volume and was 58.42% EAC cells were viable cells (Fig.2). Treatment of *leucas aspera* aqueous extract clearly shows the nuclear condensation cell blebbing and formation of apoptotic bodies, which are the characteristic features of cells undergoing apoptosis. Further confirmation of apoptotic activity which has confirmed by DNA fragmentation assay (Fig. 3.B). All these results clearly indicate the in- vivo anti-proliferatory effect of *leucas aspera.* When we observed peritoneal lining of treated mice there is clear demarcation in decreased new micro vasculature where as in control mice extensive neo-angiogenesis is evident (Fig.4A). The in-vivo CAM assay support the anti-angiogenic effect *leucas aspera* implanted embryonated chick eggs when compared to control (Fig.4B). Peritoneal angiogenesis and CAM assay strongly suggests the anti-angiogenic effect of aqueous extract of *leucas aspera*.

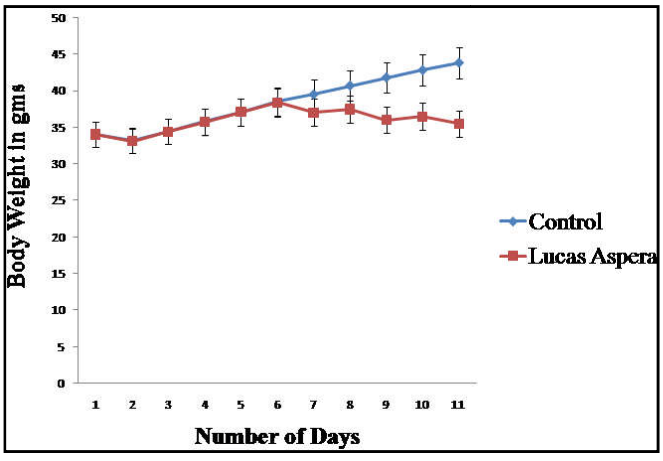

Fig. 1. Effect of *leucas aspera* extract on body weight of mice: EAT cells ($5X10^6$ cells/mouse, i.p) were injected into mice and body weight of the mice was recorded to follow tumor growth. Every alternate day *leucas aspera* extract is administered from the 6^{th} day onwards (100 mg/kg body weight).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

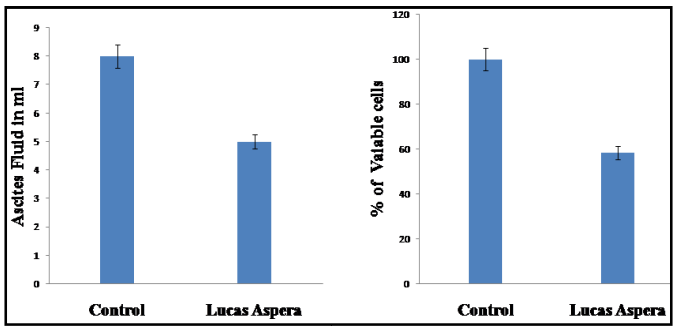

Fig.2. Ascites volume and Cytotoxicity assay for control & treated mice: The graph represents the ascites fluid volume and percentage of cell viability in EAC cells treated and untreated *leucas aspera* aqueous extract

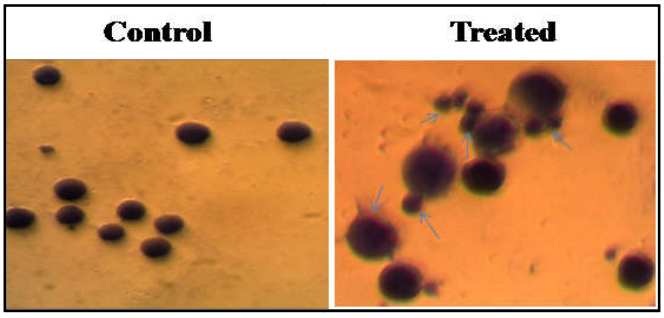

Fig. 3 A. Apoptotic morphology of EAT cells upon *leucas aspera* extract treatment: EAT cells treated with and without *leucas aspera* extract washed with PBS, fixed in methanol/acetic acid (3:1) and stained Giemsa. Both control and treated (*leucas aspera*) cells were carefully viewed under light microscope for apoptotic studies such as plasma membrane degradation, membrane blebbing, and apoptotic body formation

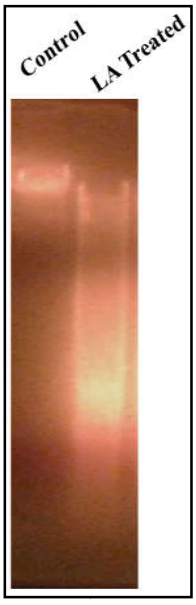

Fig. 3. B. Isolated DNA from the cells treated with leucas aspera was resolved on agarose gel for the evidence of DNA fragmentation. Further apoptotic study has been confirmed by DNA laddering assay, which is showed in Fig 3.B. EAT cells were treated with aqueous extract of *leucas aspera.* Total genomic DNA was extracted and resolved on 1.2% Agarose gel, stained with ethidium bromide and result is documented

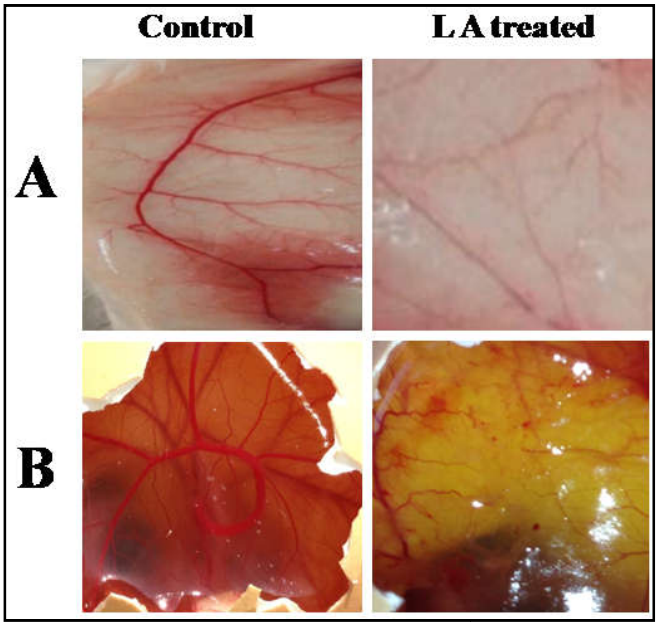

Fig.4. Aqueous Extract of *Leucas Aspera* inhibits angiogenesis *in-viv:* A) Extensive neovascularization in the peritoneal lining of EAT bearing control untreated mice. Peritoneal lining of mice treated with *leucas aspera* extract was inspected for angiogenesis. Inhibition of peritoneal angiognesis in *leucas aspera* extract treated mice is strongly evident. B) Photos illustrate the formation of blood vessel branch points in either control (saline) *leucas aspera* 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 *leucas aspera* extract

DISCUSSION

Over the recent years, more attention has been focused on the anti-angiogenic and antitumor effects from natural products. Angiogenesis is mainly depends on proper activation, proliferation, adhesion, migration and maturation of endothelial cells. Angiogenic therapy in combination with the conventional chemotherapy and radiotherapy are proved to be effective (and Waxman, 2008) in some way but besides they alter the tumor microenvironment hence leads to cancer recurrence (Kraeber-Bodéré *et al*., 2010). On the other side, herbal medicines and other plant derived natural products processes several organic chemical compounds which could be efficiently used to target many signalling pathways including angiogenesis process involved in tumor progression. Researchers have been made to evaluate anticancer efficiency of plants and their phytochemicals in the past and are still in progress. As an evident 70% of all anticancer drugs currently being used are of natural origin. Our goal is to finding a potent antiangiogenic drug, we have initiated a screening program in our laboratory designed to test a wide variety of plant extracts for angio suppressive activity. Our preliminary studies indicated that the aqueous extract from leaves of the *leucas aspera* 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 *leucas aspera* as anti-neoplastic agent. Treatment with the aqueous extract of *leucas aspera* showed induced inhibition of proliferation of tumor cells in vivo (Chauhan, 2015). Decreased ascites fluid and regression in new vasculature both in peritoneal lining and CAM assay proved its anti-angiogenic effect. Thus, our results are evident that phyto constituents of leucas aspera could be a potential source for cancer treatment, and be worthy of further cancer mechanistic studies.

Conclusion

The modernistic substantiation of this study shows the use of *Leucas aspera* extract that has an effective antiangiogenic activity. The phyto-chemical constituents of the plant could be further residential and translated course of therapy for human cancer. Further study is required to define more precisely the mechanism involved by which *Leucas aspera* extract inhibits neo-vascularisation. Our coming up proposal is focusing in the direction is to enhancement of clincher based research and chemical characterisation of these compounds could further enhance the efficacy of this plant- based medicine in angiotherapy.

Conflict of interest

The authors declare that they have no conflict of interest.

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