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RESEARCH ARTICLE

ANTI-ANGIOGENIC ACTIVITY OF AQUEOUS EXTRACT OF *H. INTEGRIFOLIA* IN EHRLICH ASCITES CARCINOMA MODEL

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ABSTRACT

Angiogenesis plays an important role in tumor formation and proliferation. The development of anti-angiogenic agents to block new blood vessel growth will inhibit metastasis and induce apoptosis of the cancer cells. Many natural health products that inhibit angiogenesis also manifest other anticancer activities. The present article focuses on phyto constituents bark extract of *H. intergefolia* have a high degree of anti-angiogenic activity. The aqueous leaf extract inhibits the Ehrlich ascites tumor cell proliferation by *in-vivo*. The antiangionenic activity of *H. intergefolia* 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 *H. intergefolia* indicated that the plant extract decreased VEGF production and the cytokine induced neovascularization. Our preliminary results suggest anti-angiogenic activity of *H. intergefolia.* Further evidence-based research and chemical optimization of these compounds could further enhance the effectiveness of these plant-based medicines in angiotherapy.

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INTRODUCTION

Blood vessels fulfill the oxygen and nutrients demand of a cell and plays critical role in the development and survival of a tissue right from the embryonic development to adulthood (Carmeliet, 2005). Angiogenesis, an event which describes the development of new vasculature from the pre-existing blood vessel is dragging lot of attention in scientific research field due to its role in various physiological and pathological processes (Folkman, 1990). Unlike normal cells continuously growing tumor cells are highly dependent on blood vessels which support their development and metastasis (Zetter, 1988). Angiogenic switch plays key role in the development of vaculature and in cancer cells the angiogenic switch is 'on' where the interrupted balance between pro-angiogenic and anti-angiogenic factors enables cancer cells to acquire angiogenic phenotype (Folkman, 2003, Carmeliet and Jain, 2000) which stimulate the sprouting or intussusceptive angiogenesis from the pre-existing vasculature (Hlushchuk *et al*., 2008). The switch leads to over expression of many pro-angiogenic proteins, mainly vascular endothelial growth factor VEGF (Saeki *et al*., 1997), platelet-derived endothelial cell growth factor PD-ECGF (Saeki *et al*. 1997, Toi *et al*., 1995), basic fibroblast growth factor bFGF, angiopoietin, transforming growth factor (TGF)-α, (TGF)-β, Placental growth factor (PLGF), hepatocyte growth factor, tumor necrosis factor-alpha (TNF-α) (Nishida *et al*., 2006, Hillen, 2007) IL-18 (Park *et al*., 2001), IL-8 (Brat *et al*., 2005) but VEGF is been considered to play a fundamental role in regulation of angiogenesis (Ferrara, 2001). 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 antiangiogenic therapies are comparatively well tolerated than traditional cytotoxic chemotherapeutic agents due to their target specificity (Lacouture, 2009). Plants being integral part of human diet, also serving the mankind in maintaining their better health. Several plant derived phytochemicals such as stilbene glycoside (Hussain *et al*., 2009) triptolide (Zhu *et al*., 2010), torilin (Kim *et al*., 2000), genistein (Yu *et al*., 2012), wogonin (Wu *et al*., 2008) have successfully displayed their potentiality in inhibiting tumor angiogenesis. In search of new

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effective medicinal plant for inhibition of tumor angiogenesis, we discovered *Holoptelea Integrifolia* as a plant of potential ability in fighting against tumor angiogenesis. *Holoptelea Integrifolia* belongs to the family Ulmaceae, commonly known as Indian Elm, distributed all over tropical and temperate regions of Northern hemisphere including India. (Sandhar *et al*., 2011, Sharma and Singh, 2012). *H. integrifolia* is well known for its significant medicinal values. The major phytoconstituents of the *H. integrifolia* are sterols, saponins, terpenoids, 1, 4-naphthalenedione, Holoptelin-A and B, 2-aminnaphthaquinone, amyrin, Hexacosanol, octacosanol, Friedelin, epifriedelinol, betulin and Betulinic acid (Kumar *et al*., 2012, Srivastava *et al*., 2013). Stem bark, leaves, seeds and fruit pulp are the Different parts of plant used in Traditional system of medicine to treat edema, rheumatic swelling, leprosy, dyspepsia, piles, eye inflammation, hemorrhoids, jaundice, gastritis, menstrual disorder (Ahmed *et al*., 2013; Saxena *et al*., 2013).

The bark is bitter, astringent, thermogenic, anti-inflammatory, digestive, carminative, laxative, depurative, anthelmintic, repulsive and urinary astringent (Kare *et al*. 2011). Stem bark and seed used against ringworms, eczema, leucoderma and other skin diseases (Sharma *et al*., 1992, Maheshwari, 1990). Other pharmacological properties of the plant are antimicrobial activity (Sharma *et al*. 2009, Vinod *et al*., 2010), wound healing potentiality (Srinivas Reddy *et al*., 2008), antitumor (Guo *et al*., 2013, Lakshmi *et al*., 2010) and anti-inflammatory activity (Srinivas *et al*., 2009, Kalpana, 2010). Plant also exhibit antidiabetic activity (Sharma *et al*., 2010), antiobese activity (Bombhole, 1985). In the current report effort has been made to evaluate the anti-angiogenic activity of aqueous bark extract of *H. integrifolia* in murine Ehrlich Ascites Carcimoma (EAC) model.

MATERIALS AND METHODS

H. integrifolia (HI) plant bark samples were collected from rural parts of Mysore and Mandya districts of Karnataka. The herbarium of the specimen was made and maintained in the Botany, 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 were 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.

Preparation HI plant extract

Preparation of aqueous extract of HI bark was followed with the method previously reported (Kalyani *et al*., 2013) Thus, the bark of HI were dried at 50º C and crushed in a blender and the crude powder was extracted with sterile distilled water at 100º C for 3 hours. The aqueous extract was evaporated at 60º C under pressure. Finally the extract was dissolved in DMSO to make a stock solution (100mg/ml).

Determination of phytochemicals in *H. integrifolia* bark extract

Phytochemical screening of *H. integrifolia* bark extract for, alkaloids, flavonoids, terpenoids, saponins, glycosides and tannins was conducted using standard protocols as reported earlier (Ayoola, 2008, Wadood, 2013).

In-vivo EAC cell growth and HI extract treatment

In-vivo culture of EAC cells, treatment with HI extract and isolation of EAC cells from peritoneum cavity was done as reported earlier (Jayarama *et al*., 2013). In brief EAC cells (5×10^6 cells /mouse) were injected intraperitoneally (i.p) into 8-10 week old swiss albino mice and weight of the animals were monitored every day. Six days after inoculation HI extract (100 mg/kg body weight/i.p, in 0.1% DMSO) was injected intraperitoneally into the EAC bearing mice every alternate day and the mice were sacrificed on the 12^{th} day. Untreated and HI extract treated EAC bearing mice were sacrificed, EAC cells along with the ascites fluid were harvested and used for further experiments. Inner lining of the peritoneal cavity was examined for vasculature by taking photograph using Sony digital camera.

Giemsa Staining

The apoptosis morphology of the EAC cells 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 HI treated EAC bearing cells were dropped slowly on to a glass slide. Slide was air dried, and then fixed with methanol, Giemsa stained 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

The *in-vivo* CAM angiogenesis assay was performed as reported previously (Jayarama *et al*., 2013). In brief fertilized eggs were incubated at 37^0C in a humidified and sterile atmosphere for 9 days and a window was opened on the eggshell exposing the CAM. The HI extract was placed on sterile discs, which were allowed to dry under sterile conditions. A loaded and air-dried HI 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 11^{th} day. The windows were opened on the 11^{th} day and inspected for changes in the microvessel density in the area below the cover slip and photographed using the Sony digital camera.

VEGF-ELISA

The level of the cytokine VEGF secreted by EAC cells into peritoneal ascites was measured by ELISA as described earlier (Belakavadi and Salimath, 2005)

Histopathological analysis (H&E staining)

Peritoneum from mice with and without HI extract treatment was fixed in formalin. Sections (5µm) were made from paraffin embedded peritoneum and stained with H&E. Histological examination of the slides were then performed by two investigators, one of which was blinded to treatment. Microvessel counts were derived by averaging the number of vessels with clearly defined lumens or linear shape seen in 10 high power field (HPF) of high-density areas and low-density areas. Final MVD is the mean score obtained from the areas counted using Radical light microscope, India, attached to CCD camera.

RESULTS

To investigate the antiangiogenic activity of *H. integrifolia* aqueous extract, mouse peritoneal angiogenesis assay and CAM assay were employed in the current study. 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 EAC-bearing mice during a growth period of 13 days. A total volume of 16 ml of ascites and there is 94.6% cells were viable as a consequence of extensive proliferation of EAC cells *in-vivo*. However, upon treatment with *H. integrifolia* aqueous extract, there was 43.7% EAC cells were viable cells and 52% decrease in formation of ascites fluid (Fig. 1).

Preliminary phytochemical analysis of aqueous extract of *H. integrifolia* bark showed the presence of steroids, terpenoids, alkaloids, glycosides, flavonoids, among these falvanoids are found to be in higher concentration (Table 1). Treatment of *H. integrifolia* 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. 2).

All these results clearly indicate the *in- vivo* anti-proliferative effect of *H. integrifolia*. Regression of extensive vasculature formation in the peritoneal region of tumor bearing mice and also in the chorioallantoic membrane control fertilized eggs upon *H. integrifolia* treatment was observed where as in both control mice peritonium and chorioallantoic membrane extensive vascular network was seen. (Fig.3). The ELISA result clearly evident to show that there is a dose dependent decrease in the secretion levels of VEGF to peritoneum of *H. integrifolia* treated mice, but in the control opposite effect is seen (Fig.4).

In addition to this the H and E staining of *H. integrifolia* treated mice peritoneal sections showed 7 ± 2 micro vessels at high power field. However there are 45 ± 5 micro vessels present in the peritoneum of control EAC-bearing mice on the 12th day is clearly observed (Table 2). All the above experimental evidences clearly suggested anti-angiogenic effect of *H. integrifolia* aqueous extract.

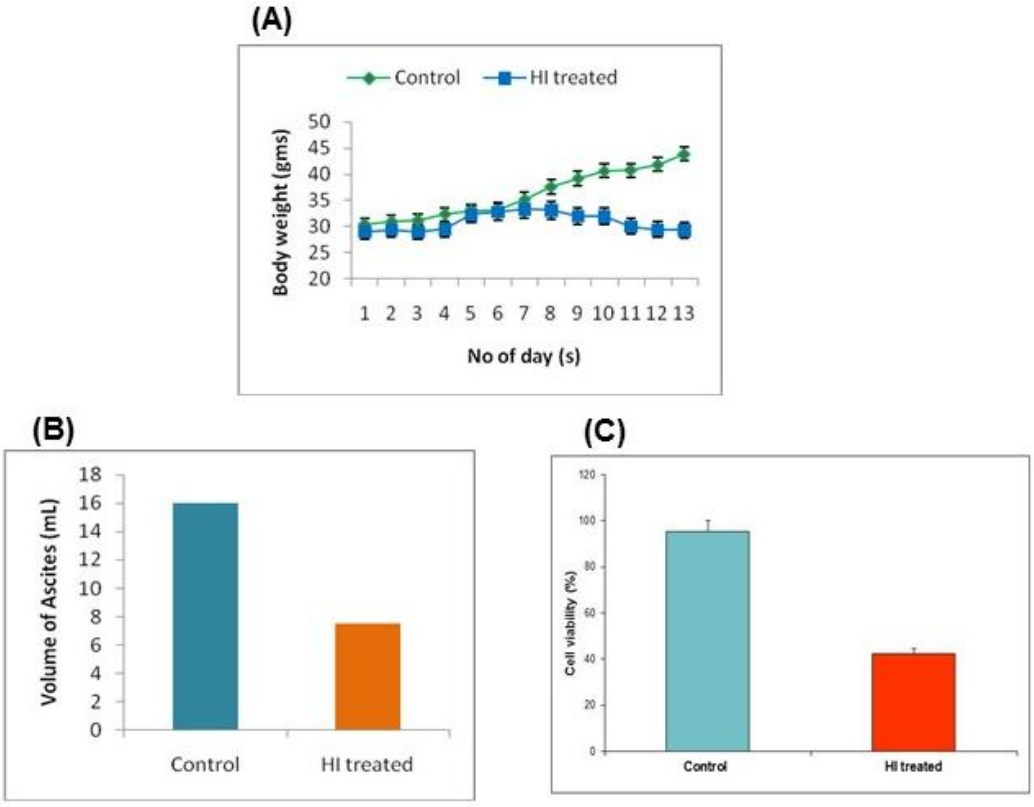

Fig. 1. In-vivo anti-tumor effect of *H. integrifolia* (HI) on EAC cells: HI extract (100mg/kg body weight) was effective in controlling EAC cell proliferation A) HI significantly reduced the mice body weight after three dose of treatment where in control mice increased body weight (up to 13gm) was observed till 13th day of transplantation. B) Measured as cites volume from both control (16mL) and treated (7.5mL) mice indicates the decreased secretion in HI treated mice. C) The percent of EAC cell viability in control (94.6%) and HI treated mice (43.7%) calculated signifies the anti-tumor effect of HI. In each group minimum 5 mice were maintained and the experiment was repeated twice for statistical significance

Table 1. Represents the preliminary phytochemical constituents of *H. integrifolia* bark aqueous extract

Phytochemicals	
Alkaloids	+
Falvonoids	-
Glycosides	+
Saponins	-
Steroids	+
Tannins	+
Triterpenoids	-

Prenence (+) Absence (-)

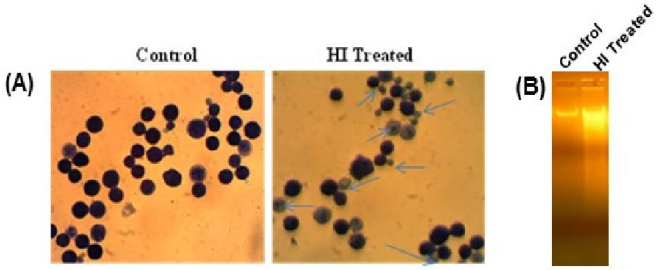

Fig. 2. Effect of HI on EAC cell morphology: HI induces apoptosis of EAC cells. A) Giemsa staining of both control and HI treated cells was shown, apoptotic features such as membrane blebbing, apoptotic body formation were apparent in HI treated cells. B) Isolated DNA from the cells treated with or without HI were resolved on agarose gel for the evidence of DNA fragmentation. Lane 1: Control DNA, Lane 2: HI treated DNA

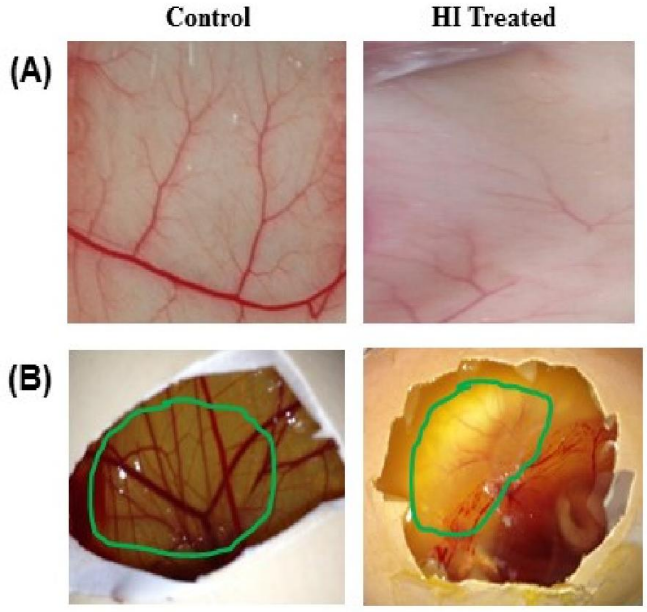

Fig. 3. Suppression of neovascularization by *H. integrifolia* extract. A. Extensive neovascularization in the peritoneal lining of EAC bearing control untreated mice. Peritoneal lining of mice treated with *H. integrifolia* extract was inspected for angiogenesis and found to be inhibition of peritoneal angiognesis in *H. integrifolia* extract treated mice is evident. B. Inhibition of angiogenesis in chick CAM assay by *H. integrifolia* extract: Photos illustrate the formation of blood vessel branch points in either control (saline) *H. integrifolia* 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 *H. integrifolia* extract

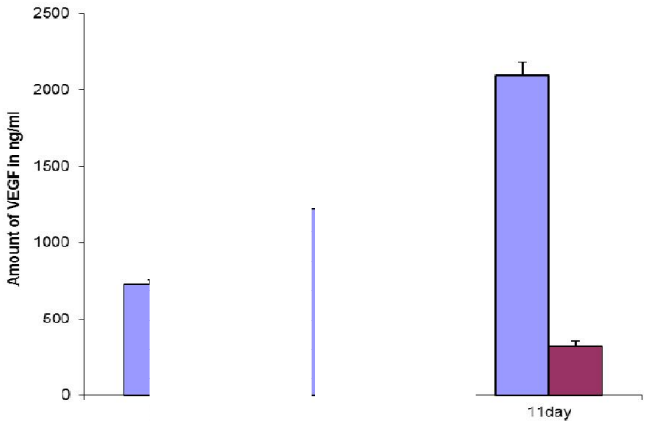

Fig. 4. VEGF quantification upon HI treatment: Ascites from mice treated with or without HI was verified for the amount of VEGF secreted by tumor cells. As it was shown in the above graph there was notable decrease in the amount of VEGF after treatment with each dose

Table 2. Showing micro vessel density (MVD) count of peritoneal sections of mice treated with and without H. integrifolia extract

Sample	MVD (HPF)
Control (untreated)	45 ± 5
H. intergefolia treated	7 ± 9

DISCUSSION

Over the recent years, more attention has been focused on the anti-angiogenic and antitumor effects of non-toxic compounds from natural products. Angiogenesis mainly depends on proper activation, proliferation, adhesion, migration and maturation of endothelial cells (Keshavarz *et al.,* 2011). Currently anti-angiogenic therapy in combination with the conventional chemotherapy and radiotherapy are proved to be effective (Ma 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 signaling pathways including angiogenesis process involved in tumor progression. Extensive researches have been made to evaluate anticancer efficiency of plants and their phytochemicals in the past and are still in progress. As an evident 74% of all anticancer drugs currently being used are of natural origin (Tan *et al.,* 2006).

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 angio suppressive activity. Our preliminary studies indicated that the aqueous extract from bark of *H. integrifolia* 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 *H. integrifolia* as anti-neoplastic agent. Treatment with the aqueous extract of *H. integrifolia* on EAC-bearing mice showed induced inhibition of proliferation of tumor cells in-vivo (Chauhan, 2015). The bioactive compound present in the aqueous extract of H. integrifolia has been shown to be an apoptosis-inducing component in H. integrifolia (Lakshmi, 2010, Soujanya, 2011). Our results indicate that the aqueous

extract of H. integrifolia inhibits EAC cell proliferation *in-vivo* and also inhibition of neovascularization which is evidenced by CAM assay (Negrão *et al.,* 2013 and Pesca *et al.*, 2013). Since there is inhibition of neovascularization by *H. integrifolia* extract, it supports our view that *H. integrifolia* bark extract may repress the expression of VEGF like factors thereby inhibiting the formation of new blood vessels and this was confirmed by ELISA (Nagaraj *et al.*, 2012 and Jayarama, *et al.,* 2013). Thus, our results are evident that the H. integrifolia bark extract phyto constituents may be a potential supplemental source for cancer treatment, and deserve further mechanistical studies.

Conclusion

The result of this study shows the use of *H. integrifolia* bark extract that has a potential antiangiogenic property. The phyto-chemical constituents of the plant could be further developed and translated into a therapeutic regime for treatment of human cancer where formation of peritoneal malignant ascites is a major cause of morbidity and mortality. Further study is required to define more precisely the mechanism involved by which *H. integrifolia* bark extract inhibits neo-vascularization.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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