ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 11, pp.42136-42142, November, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

COMPLEX OLIGOSACCHARIDE INTERACTING *LAGENARIA SICERARIA* SAP AGGLUTININ INTIMIDATES THE MICROVESSEL DENSITY (MVD) OF DALTON'S LYMPHOMA IN MURINE SYSTEM

[1,2]Vigneshwaran Vellingiri, [1]Prabhu Thirusangu, [3]Krishna, V., [2]Siddanakoppalu N. Pramod and [1,*]Prabhakar, B. T.

[1]Molecular Biomedicine Laboratory, Postgraduate Department of Studies and Research in Biotechnology, Sahyadri Science College (Autonomous), Kuvempu University, Shivamogga-577203, Karnataka, India
[2]Laboratory for Immunomodulation and Inflammation Biology, Department of Studies and Research in Biochemistry, Sahyadri Science College (Autonomous), Kuvempu University, Shivamogga-577203, Karnataka, India
[3]Postgraduate Department of Studies and Research in Biotechnology & Bioinformatics, Kuvempu University, Shankaraghatta, Shivamogga-577203, Karnataka, India

ARTICLE INFO

Article History:
Received 15th August, 2016
Received in revised form
10th September, 2016
Accepted 21st October, 2016
Published online 30th November, 2016

Key words:

Lagenaria siceraria,
Latex lectin,
Bottle Gourd,
Anticancer,
Daltons lymphoma Ascites (DLA).

ABSTRACT

Lagenaria siceraria (Bottle gourd) was described in ancient Indian text ayurveda for varied health benefits. We have previously reported that the crude latex sap from *L. siceraria* with potent lectin activity significantly regresses the tumor progression by targeting angiogenesis and inducing cell death. In this present investigation the semipurified latex lectins from *L. siceraria* latex were investigated for antiproliferative activity in mice models. The *L. siceraria* latex sap agglutinin (LSA) were isolated by ammonium sulphate precipitation. LSA agglutinated trypsinizeed chick and human erythrocytes irrespective of blood type. Heamagglutination was inhibited by glycoproteins comprising of complex oligosaccharides and not with simple carbohydrates. LSA exerted significant cytotoxicity towards murine DLA cells *in-vitro*. Further the *in-vivo* anti-proliferative of LSA elucidated a clear regression of Dalton's lymphoma ascites tumor in mice. Hispathological and immunohistochemistry studies inferred LSA abrogated microvessel density (MVD) leading to the tumor inhibition and prolonged survival. This is further evident from the decreased VEGF levels in ascites. In conclusion, this study reports that the semipurified LSA exerts potent antiproliferative activity activity targeting tumor angiogenesis. This investigation highlighting the bioactivity of semipurified LSA for the first time, has potential therapeutic implications for malignant pathologies.

Copyright©2016, Sadiqa Shafiq et al. *This is an open access article distributez under the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.*

Citation: Vigneshwaran Vellingiri, Prabhu Thirusangu, Krishna, V., Siddanakoppalu N. Pramod and Prabhakar B.T. 2016. "Complex oligosaccharide interacting lagenaria siceraria sap agglutinin intimidates the microvessel density (MVD) of Dalton's lymphoma in murine system", *International Journal of Current Research*, 8, (11), 42136-42142.

INTRODUCTION

Malignant neoplasia is highly deleterious pathology based on multiple etiologies, multiple cell targets, and distinct developmental stages, which contributes to enhanced disease complexity and therapeutic outcome (Hanahan and Weinberg, 2000). This pathological perplexity has tugged the continuing demand for development of novel antiproliferative drugs, drug combinations, and chemotherapy strategies by methodical and scientific exploration of enormous pool of synthetic, biological, and natural products (Wang *et al*., 2008).

****Corresponding author: Prabhakar B.T.***
Molecular Biomedicine Laboratory, Postgraduate Department of Studies and Research in Biotechnology, Sahyadri Science College (Autonomous), Kuvempu University, Shivamogga-577203, Karnataka, India.

However, due to the severe side-effects of conventional chemotherapies, considerable attention has been drawn in identifying plant based medicines, particularly those included in our diet, which are more compatible to interfere with carcinogenic or mutagenic processes (Jedrychowski *et al*., 2009, Nagendraprabhu *et al*., 2011). Of particular note substantiative to this, over 50% of anticancer drugs approved by United States Food and Drug Administration since 1960 were originated from natural resources, especially from terrestrial plants (Wang *et al*., 2008). Lectins, the diverse class of carbohydrate binding proteins are extensively dispersed in nature with an ample amount in plants. Plant lectins possess analogous biological activities and chemical properties. They originate in vegetative organs like roots, leaves, rhizomes,

bulbs, tubers, corms, stems, bark, flowers, fruits, phloem sap, latex, and nectar (Peumans & Van Damme, 1995, Hivrale & Ingale, 2013). Among this, plant latex, exudates and resins contain various secondary metabolites and proteins, often in concentrations that are much higher than other parts (Agrawal & Konno, 2009). Recent evidences indicate that the latex of most species contains a diversity of biologically active lectins having pharmacological attribute. Specifically, in recent years, a great number of lectins with *in vivo* and *in vitro* antiproliferative properties against cancer cells have been isolated and characterized (Glaucia *et al*., 2012). Thus there is an increased tendency to use the latex lectins in combating cancer due to the cytotoxic-, apoptosis- and autophagy-inducing efficacy (Liu *et al*., 2010). *Lagenaria siceraria* fruit, popularly known as Bottle gourd is mentioned in ancient Indian texts like ayurveda for its varied health benefits including cardioprotective, cardiotonic and diuretic properties (Sayyed *et al*., 2010). Bottle gourd belongs to the Cucurbitaceae family which has nearly 100 genera and over 750 species (Puri *et al*., 2011). Our group has recently reported that the latex sap from the "Old world plant" *Lagenaria siceraria* with potent lectins activity displaying immunostimulatory characteristics with significant antiproliferative activity targeting angiogenesis and apoptosis (Vigneshwaran *et al*., 2016). In this present investigation we for the first time, sought to investigate the *in-vivo* antiproliferative actity of the partially isolated latex agglutinins targeting the tumor associated angiogenesis and MVD.

MATERIALS AND METHODS

Chemicals and reagents

Ficoll histopaque, Fetuin, Anti-VEGF were obtained from Sigma–Aldrich, USA. DMEM medium, antibiotic-antimicotic solution, Fetal Bovine Serum (FBS) from Invitrogen, USA. Anti CD-31 antibody, Immunostaining kit from Leica Biosystems, Germany. Ovalbumin, thiobarbituric acid (TBA), ammonium sulphate and all the carbohydrates were from HiMedia Laboratories, India. Cell culture plastic wares were from eppendorf. All other chemicals used in this experiment were of analytical grade. Protein marker is from New England Biolabs. Photographs were taken using Canon power shot Sx500 IS camera. Bottle gourd was procured from the local farm in Shivamogga, Karnataka.

Partial isolation of *Lagenaria siceraria* Latex agglutinins (LSA)

Preparation of *Lagenaria siceraria* Latex buffer extracts

Lagenaria siceraria latex extracts were collected as per our previous reports (Vigneshwaran *et al*., 2016). Briefly, the fresh latex was collected by making a multiple orifice in the epicarp of the fruit using a sterile needle. The oozed out latex sap was mixed with 10 mM PBS (pH- 7.4) in the proportion of 1:10 (10%, *v/v*) and immediately kept in the magnetic stirrer at 4°C to avoid coagulation. After homogenizing for 2-3 h, the suspension was centrifuged at 3000 rpm, 4°C for 15 min to remove debris or the coagulated substances.

Ammonium Sulphate Precipitation and Dialysis

The processed clear latex extract was treated with ammonium sulfate at 20% saturation at 4ºC. The resultant precipitate was collected by centrifugation at 3000 rpm for 30 min. The pellet was resuspended in 10 mM PBS pH 7.2 containing 0.9% NaCl. The protein sample with heamagglutinating activity was desalted and concentrated using a 10 kDa cutoff dialysis membrane. Dialysis was performed against PBS at 4ºC. The resulting sample was assayed for protein content, extent of homogeneity and haemagglutination activity. The obtained clear protein solution (LSA - *Lagenaria siceraria* latex sap Agglutinin) was used for all the further experiments.

SDS-PAGE

Crude *Lagenaria siceraria* latex extracts and precipitated LSA samples were electrophoresed under reducing 12% SDS-PAGE analysis, using Bio-Rad mini electrophoresis unit. The gels were then subjected to Coomassie brilliant blue and silver staining in accordance with standard protocols. Post protein band visualization the gels were documented using Bio-Rad Gel Documentation^{TM} XR + Imaging system. Bradford assay was used to determine the protein concentration.

Heamagglutination assay (HA) and HA inhibition assay

Heamagglutination (HA) and HA inhibition test were done as per the methods described earlier (Pramod *et al*., 2015). Briefly, a 2% suspension of trypsinized RBC's human (Type A, B, AB & O) and Chicken (0.2 ml) was added to an equal volume of serially diluted protein solution in an agglutination plate, gently mixed and incubated at 37°C for 1 h to visualize the agglutination. Peripheral venous blood was obtained after the informed consent from all healthy human participants in the age range of 20-30 years. HA inhibition tests was performed by preincubating LSA with serially diluted sugars (Carbohydrates in 0.1 mL PBS). An equal volume of 2% trypsin-treated chick erythrocyte suspension was then added to the solution and the agglutination was visualized after 1 h; Minimal concentration of sugars required to inhibit agglutination was evaluated by their ability to completely inhibit HA activity.

Leucoagglutination assay

Human peripheral blood lymphocytes (PBL's) were separated using Ficoll-Hypaque and density gradient centrifugation (Vigneshwaran *et al*., 2016). Briefly, ten milliliters of heparinized human venous blood was layered carefully on Ficoll-Hypaque (density=1.077 g/ml) contained in polystyrene tube. After centrifuging the tubes at 250 x g at 25°C for 20 min, below the plasma layer, the separated buffy coat containing lymphocytes were aspirated, washed and re-suspended in PBS. The cells were then added to an equal volume of LSL extracts in an agglutination plate, gently mixed and incubated at 37°C for 1 h and visualized under microscope.

Cell culture and *in-vitro* treatment

Murine Dalton's Ascites Lymphoma (DLA) cells was procured from Indian Institute of Science (IISc), Bengaluru, India, DLA was maintained in DMEM with 10% FBS in 5% CO_2 at 37°C. The antiproliferative potential of LSA (0, 3, 5, 10, 25, 50µg in PBS) for 48 h was evaluated by cytotoxicity assays such as MTT, trypan blue dye exclusion and LDH leak assay with an appropriate vehicle and positive control (Al-Ghorbani *et al*., 2015).

MTT assay

The effect of DLA cell proliferation following the treatment with LSA was examined by MTT assay, as described previously (Al-Ghorbani *et al*., 2015). Cells treated with or without LSA was incubated for 48 h. MTT reagent (5 mg/ml) was added, and the change in color due to the proliferating cells was evaluated. Concentration of LSA showing a 50% reduction in cell viability (IC_{50}) was assessed.

Trypan blue dye exclusion assay

The effect of LSA on the viability of DLA cells were investigated using trypan blue dye exclusion assay as per the previous methods (Al-Ghorbani *et al*., 2015). DLA cells treated with or without LSA were harvested and resuspended in 0.4% trypan blue, and the viable cells were quantified using a hemocytometer. The IC_{50} value was estimated after 48 h of treatment.

LDH leak assay

Lactate dehydrogenase (LDH) leak assay was performed to assess LDH release following the treatment of DLA cells with LSA after 48 h of incubation, as described previously (Al-Ghorbani *et al*., 2015). Briefly, the cells were lysed using 0.1% Triton X-100 in PBS. The amount of LDH released in both the culture media and the cell lysate was measured at 490 nm using an ELISA reader (Robotronics, India). The percentage of LDH release was calculated as LDH release in media/(LDH release in media + intracellular LDH release) x 100.

In-vivo Anti-tumor activity

Animal and ethical statement

Swiss albino mice aged 5-6 weeks, weighing 25-30 g were housed under standard laboratory conditions and fed with commercial rodent meal and water ad libitum (Goudarshivananavar *et al*, 2014). All the animal experimentations were approved by the Institutional Animal Ethics Committee (IAEC), National College of Pharmacy, Shimoga, India, in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for laboratory animal facility (NCP/IAEC/CL/101/05/2012-13).

Animal tumor development and treatment

DLA cells were cultured *in vivo* by intraperitoneal *(i.p)* transplantation to develop ascites tumor as per our previous published methods (Thirusangu *et al*., 2016a). After the development of optimal tumor growth on 4^{th} day, the mice were divided into Group A and Group B (n=3/group). Three doses of LSA were administered on alternative days which were injected *i.p.* to Group A mice at a concentration of 20 mg/kg bodyweight. Group B was maintained as an appropriate vehicle (10 mM PBS) control. The survivability of the animals (n = 10 each) was determined separately.

Lipid Peroxidation assay

The effect of LSL in minimizing the tumor induced lipid peroxidation was examined by measuring the Thiobarbituric acid reactive substances (TBARS) in the control and treated tumor bearing animals as per the previously described methods (Vigneshwaran *et al*., 2016).

Peritoneal microvessel density (MVD) assessement

Peritoneal microvessel density was quantified by H&E staining as described previously (Al-Ghorbani *et al*., 2015, Vijay Avin *et al*., 2014a). Shortly, the formaldehyde-fixed peritoneum of the control and treated were processed for H&E staining. Then quantification of MVD was performed using light optical microscopy in the areas of peritoneum containing the highest number of capillaries.

Immunohistochemical (IHC) analysis

Immunostaining using anti-CD31 antibody was done to evaluate MVD as per the manufacturer's instructions. The slides were observed in light microscope and the changes in intensity of the antibody staining were evaluated (Thirusangu *et al*., 2016a).

VEGF-ELISA

The level of VEGF was estimated in ascites of DLA bearing mice treated by ELISA as per described protocols (Thirusangu *et al*., 2016b). On note, 100 µl of ascites from the control and LSA treated were coated in a coating buffer at 4°C overnight. Subsequently, wells were incubated with anti-$VEGF_{165}$antibodies (Sigma Aldrich, USA), followed by incubation with secondary antibodies tagged to alkaline phosphatase. VEGF-A was quantified by measuring absorbance at 405nm by using pNPP as substrate.

Statistical analysis

Values were expressed as mean ± standard error (SEM). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Student's t-test ($*p < 0.05$) and ($**p < 0.01$).

RESULTS

Isolated *Lagenaria siceraria* latex agglutinin (LSA) displays broad specific cell agglutinating activity

The agglutinating lectin fractions from *Lagenaria siceraria* Latex was partially isolated using ammonium sulphate salt precipitation technique. Reducing SDS PAGE analysis stained with Coomassie brilliant blue and silver staining depicted approximately ~80% homogeneity of the protein (Fig. 1A and B). The molecular mass of the protein was found to be apparently ~20 to 25 kDa (Fig. 1A and B). LSA was evaluated for the heamagglutination activity which agglutinated chick (Fig. 2A) and human erythrocytes irrespective of blood type (Data not shown). Leucoagglutination assay displayed a clear agglutination of the cells mediated by LSA in the microscopic observation (Fig. 2B). The lowest concentration for LSA to give a visible heamagglutination was found to be ~3 µg in human RBC suspension. LSA heamagglutination was inhibited by glycoproteins like fetuin and ovalbumin which indicates its specificity for the complex oligosaccharides (Table 1).

LSA inhibits cancer cell proliferation *in-vitro*

Antiproliferative efficacy of LSA was examined in DLA cancer cells *in-vitro* using three independent cytotoxicity assays systems as a preliminary screening. It is evident from MTT assay that LSA inhibited the proliferative potency of DLA cells at 7.5 µg/mL concentration (Fig. 3A). Similarly trypan blue assay elucidated a similar inhibition of cell growth

at 6.7 µg/ml concentration (Fig. 3A). Cellular integrity was determined by the LDH lead assay post LSA treatment. Results revealed that LSA increased the LDH release with IC_{50} value at 5.2 µg/ml in DLA cells (Fig. 3A).

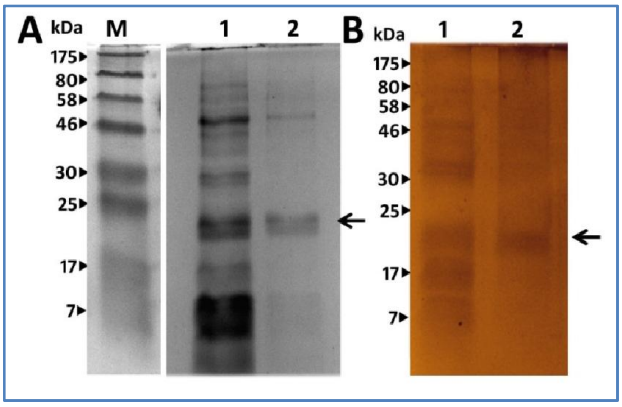

Fig. 1. Partial purification of *Lagenearia siceraria* sap agglutinins (LSA). Molecular mass determination of the partially purified LSA by SDS-PAGE under reducing conditions. Gels stained with Coomasie Brilliant Blue stain [A] and Silver stain [B]. Lane Profile: M- Marker, 1- Crude Lagenaria siceraria latex extracts, 2- Ammonium sulphate precipitated (20%) LSA. Arrow mark at lane 2 indicates partially purified protein band with apparent mass ranging from ~20 kDa to 25 kDa. Load concentration for all the samples: 20 µg/well. The gels were documented using Bio-Rad Gel Documentation™ XR + Imaging system. The results were representative of three independent experiments

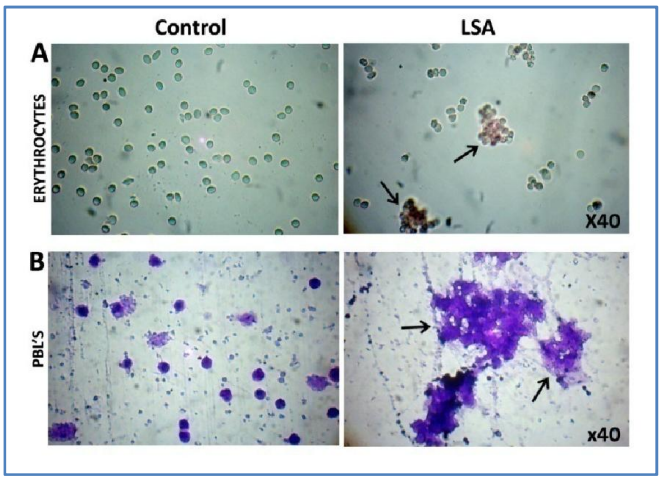

Fig. 2. Cell agglutinating activity of semipurified LSA. Heamagglutinating and leucoagglutinating activity of the partially isolated latex agglutinins were investigated. Erythrocytes and leucocytes were isolated by the methods described in the materials and methods section. [A] LSA potently agglutinates 2% trypsinized human RBC's in contrast to the control untreated with separate RBC's. Representative images are true colour microscopic photographs at 40X optical resolution. [B] LSA displays leucocyte agglutinating activity. Image depicts the cells stained with crystal violet at 40X magnification. The results were representative of three independent experiments.

LSA lectin exhibits potent antitumor effect on Dalton's lymphoma ascites tumor *in-vivo*

The *in-vivo* tumor models are the critical for the evaluation of antiproliferative efficacy. The *in-vivo* antiproliferative potential of LSA was investigated using a reliable murine DLA tumor model. Results revealed that LSA exihibited a dose dependant decrease in the tumor development as assessed by the body weight index (Fig. 3B). The reduced tumorigenic index was reflected in the decreased cell count (Fig. 3C). Ascites secretion in the LSA treated was very neglible whereas the control animal depicted abundant secretion (Fig. 3D).

Table 1. Heamagglutination Inhibition

S.No	Inhibitors	IC (µg)[a] or (mM)[b]
1.	Ovalbumin	12.5[a]
2.	Fetuin (from Bovine serum)	100[a]
3.	D-Glucose	N.I. (100)[b]
4.	D-Mannose	N.I. (100)[b]
5.	D-Galactose	N.I. (100)[b]
6.	D-Fructose	N.I. (100)[b]
7.	D-Sucrose	N.I. (100)[b]

[a]Concentration of inhibitor required to inhibit heamagglutinating activity in µg.
[b]Concentration of inhibitor required to inhibit heamagglutinating activity in mM.

The survival of untreated DLA bearing mice is maximum for 10 days, but LSA regimen has prolonged the survival duration >33 days (Fig. 3E).

LSA suppresses malignancy mediated peritoneal Microvessel Density (MVD)

DLA induced tumor neovasculature in mice peritoneal linings were evaluated in response to the LSA treatment. The visual observations of the peritoneum showed a drastic reduction of the microvessels in the LSA treated (Fig. 4A). In contrast control peritoneum displayed an enormous vasculature with aberrant microvessels. Further the formalin fixed peritoneal sections were subjected to histopathological evaluation using H&E staining for the quantification of Microvessel density (MVD). Results elucidated a prominent decrease in the MVD and detectable vascular channels in the peritoneum sections of the LSA treated mice with 13±1 Vessels/High Power Field (V/HPF) whereas in untreated mice it was 34±5 V/HPF (Fig. 4B). This was further revalidated by CD31 immunostaining which demonstrated a reduced vascular density with 14±2 V/HPF in LSA treated compared 40±3 V/HPF of control (Fig. 4C). LSA treatment significantly regressed the secreted VEGF levels in ascites as measured through ELISA (Fig. 5A). Biochemical assessment of liver enzyme after LSA treatment showed decreased lipid peroxidation as evaluated by measuring Thiobarbituric acid reactive substances (TBARS) (Fig. 5B).

DISCUSSION

In early 1991, Liener reported that lectin from soybean displayed potent antitumor activity which inhibited the growth of a transplanted tumor in rats (Liener, 1991, Hivrale & Ingale, 2013). Since then subsequent years witnessed an explosive growth in the use of lectins in biomedical and cancer research. Apart from the potent cytotoxicity towards cancer cells, plant lectins are also indicated for the reduction of treatment-associated side-effects as adjuvant agents during chemotherapy and radiotherapy in Europe for several years (Liu *et al*., 2010). Our group has recently demonstrated that latex sap from *Lagenaria siceraria* (bottle gourd) with potent lectin activity regressed the proliferation of tumor cells targeting angiogenesis (Vigneshwaran *et al*., 2016). In the present investigation the partially purified agglutinating protein fraction from the latex was subjected to antiproliferative

studies in murine models associated with microvessel density. Daltons Lymphoma Ascites (DLA) is the mice ascites tumor model which is a commonly used cell line to study the antiproliferative and antiangiogenic activity (Al-Ghorbani *et al*., 2015, Thirusangu *et al*., 2016a & 2016b).

Ascitic fluid plays a crucial role in DLA and is a collection of pleomorphic cells with hyperchromatic nuclei that are clumps of malignant cells. Ascites is a direct nutritional supplement that supports the tumor cell proliferation, so, inhibition of ascites secretion is considered as an important strategy to

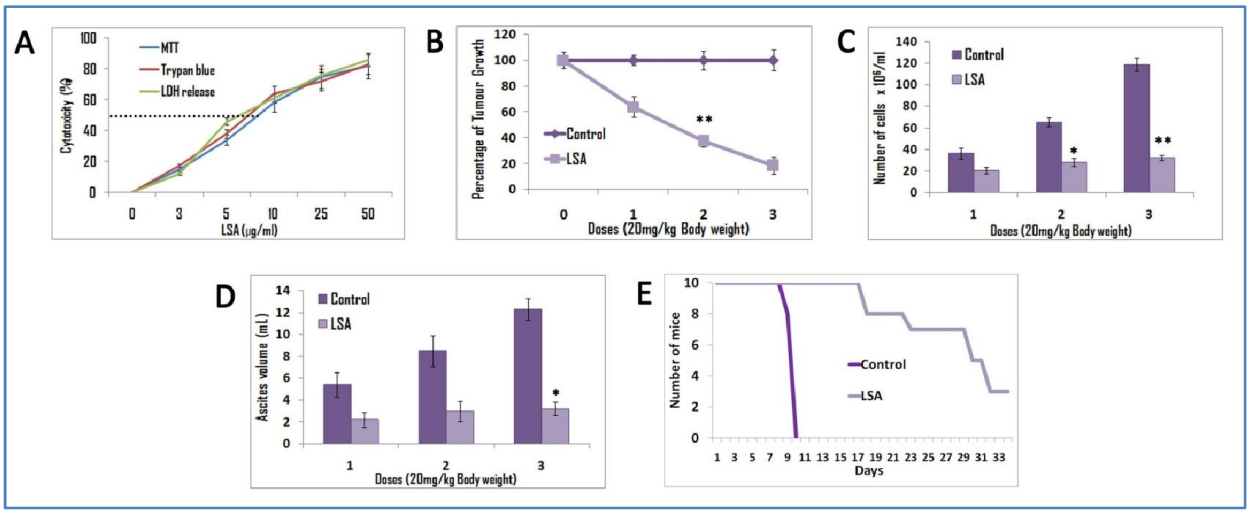

Fig. 3. LSA regresses the progression of malignant cells *in-vitro* and *in-vivo*. *In-vitro* antiproliferative assay was carried out by culturing DLA cells and treating it with LSA (0-50 µg/ml). [A] Graphs demonstrating cytotoxicity of LSA as assessed by MTT, LDH leak and Trypan blue assay. *In-vivo* antiproliferative property of LSA was investigated in Dalton's lymphoma ascites (DLA) in mice tumor model. DLA cells ($5x10^6$ cells/mouse) were injected intraperitoneally in Swiss albino mice to develop the ascites tumor. Treatment included three doses of LSA on alternative days, administered in the concentration range of 20 mg/kg *b.w.* (*i.p.*) [B] Regressed tumorigenic index in the dose dependant manner indicative of the percentage reduction in the tumor growth [C] contracted tumor cell population [D] and decrease in ascites secretion. All the parameters were evaluated in comparison to the respective control tumor bearing animal. [E] Kaplan-Meier graph showing the prolonged life span of LSA treated animals. Results are the means of three determinations, each conducted in triplicate. Statistically significant values are $^{*}p < 0.05$; $^{**}p < 0.01$

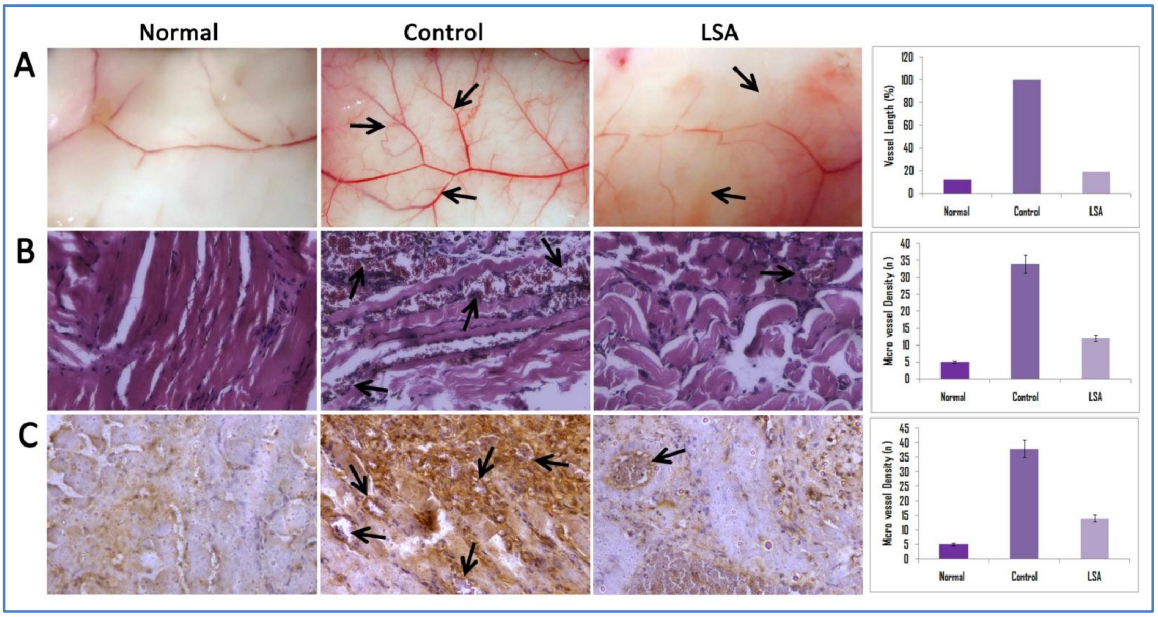

Fig. 4. LSA regresses tumor angiogenesis *in-vivo*. The inhibitory effect of LSA in the tumour induced angiogenesis was evaluated by neovasculature dependant murine ascites models in mice, treated with LSA 20 mg/kg body weight *i.p.* [A] The peritoneum of mice showing the suppressed cross sectional peritoneal vasculature and microvessel in the LSA treated. [B] Histopathological evaluation of the H&E stained peritoneum sections showing the decreased vascular count [C] IHC studies of the peritoneal sections processed with anti-CD31 antibody depicting a hyper vascularization (intensive brownish staining) in the control, whereas LSA treated exhibits repressed vasculature. H&E and IHC were captured using EVOS imaging system, Life Technologies; under 20X bright field objective. Arrows indicate the extent of vascularization. The representative MVD counts were provided adjacent to the every corresponding images. Results are the means of three independent experiments. Statistically significant values are $^{*}p < 0.05$; $^{**}p < 0.01$

It is a transplantable T-cell lymphoma of spontaneous origin, characterized by highly invasive and immunosuppressive property (Deepak *et al*., 2010). Our results indicate that LSA significantly reduced the tumor progression which is evident from the tumorigenic index and cell count (Fig. 3B and C). regress the tumor proliferation. Ascites supports the tumor cell proliferation and its secretion can lead to further aggravation of disease (Thirusangu *et al*., 2016a & 2016b). In our study, LSA treated mice showed significant reduction in the ascites secretion which was evidently linked to the significant tumor

inhibition in mice with prolonged survival (Fig. 3B-E). Tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. This is addressed by the tumor-associated neovasculature, generated by the process of angiogenesis.

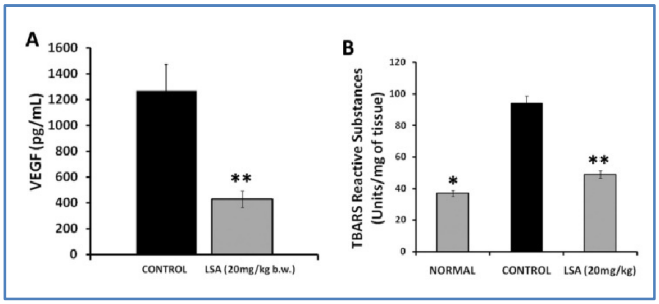

Fig. 5. LSA abrogates VEGF cytokine secretion and lipid peroxidation *in-vivo*. [A] Reduced secretion of VEGF in the ascites of the LSA treated tumor bearing mice. Untreated control shows excessive levels of VEGF as measured by ELISA. [B] Lipid peroxidation assay was done by TBARS method. LSA reduced the tumor induced lipid peroxidation in treated mice to that of control. Results are the means of three independent experiments. Statistically significant values are *p < 0.05; **p < 0.01

Angiogenesis is induced surprisingly early during the multistage development of invasive cancers both in animal models and in humans. Once angiogenesis has been activated, tumors exhibit diverse patterns of neovascularization (Hanahan & Weinberg, 2000). Therefore blocking the angiogenesis is probably one of the most visible areas in the field of cancer therapy over the past few years (Mengfeng, 2008). Hence our study assessed the impact of tumor neovasculature in LSA mediated tumor inhibition in mice models (Fig. 4). Ascites tumor growth is accompanied by an early and dramatic enlargement in the cross-sectional areas of peritoneal microvessels. Increased mean vascular cross-sectional area was accompanied by vascular endothelial cell proliferation. Therefore, increased average cross sectional area is likely a general property of tumor microvessels, both solid and ascites (Janice *et al*., 1995). Our experimental evidences clearly exhibit that the LSA treated mice displaying a regressed peritoneal microvessels with diminished vascular cross-sectional area in contrast to the control (Fig. 4A). Therefore it is clear from this observation that LSA potently inhibited the tumor cell proliferation most evidently through the neovasculature blocade.

Microvessel Density (MVD) is the surrogate marker of tumoral neovasculature and a predominant parameter considered for quantifying intratumoral vasculature in cancer (Goddard *et al*, 2002, Al-Ghorbani *et al*, 2015). Therfore quantifying angiogenesis by MVD provides an important prognostic approach towards the disease progression and its response to drug therapy. Our current study revealed that LSA treated showed very low MVD count as evident from the microscopic evaluation of H&E stained peritoneal sections (Fig. 4B). To further affirm the LSA inhibition of tumor angiogenesis, MVD is assessed immunohistochemically in tumor biopsies by enumerating endothelial cell adhesion molecule CD31, which is reported to be significantly expressed in tumor vasculature (Al-Ghorbani *et al*, 2015). Results inferred that LSA treated showed a reduced MVD count to that of control which paralled with the H&E sections and visible angiogenic parameters of the peritoneum (Fig. 4C). Supportive to this, LSA reduced the level of Vascular endothelial growth factor (VEGF), a proangiogenic cytokine in the secreted ascites of the tumor bearing animals (Fig. 5A). The level of VEGF will be high in the secreted ascites of tumor bearing animals and it is potentially liked to the tumour cell proliferation (Vijay Avin *et al*, 2014c, Thirusangu *et al*, 2016a). Results henceforth indicate that LSA has down regulated the level of VEGF which inhibited the angiogenesis thereby halting the tumor growth. Taking together the above results, the present study elucidated the antiproliferative activity of the semipurified protein fraction of the *Lagenaria siceraria* latex. LSA suppressed the tumor cell expansion by targeting integral tumor parameters like angiogenesis that is indispensable for the malignant proliferation. These observations suggest the potential therapeutic efficacy of the LSA in targeting neoplastic diseases.

Acknowledgement

Dr. B.T. Prabhakar gratefully acknowledges the grant extended by DBT (6242-P37/RGCB/PMD/DBT/PBKR/2015), UGC (F.No.41-507/2012 (SR) and VGST (VGST/ GRD231/ CISEE/2013-14). Mr. V. Vigneshwaran kindly acknowledges the fellowship received from University Grants Commission (UGC), Government of India [F.No.41-1260/2012 (SR)]. Dr. S.N. Pramod kindly thanks the grant from UGC, Government of India [F.No.41-1260/2012 (SR)].

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author Contribution

VV performed the work and wrote the manuscript. PT assisted the work. VK, SNP, and BTP designed the work.

Abbreviations

LSA, *Lagenaria siceraria* latex sap agglutinin; DLA, Daltons lymphoma ascites; PBLs, peripheral blood lymphocytes; MVD, microvessel density; IHC, immunohistochemistry; MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; VEGF, vascular endothelial growth factor; TBARS, thiobarbituric acid reactive substances.

REFERENCES

Agrawal, A.A. Konno, K. 2009. Latex: a model for understanding mechanisms, ecology, and evolution of plant, defense against herbivory, *Annual Review of Ecology, Evolution, and Systematics* 40, 311–331.

Al-Ghorbani, M., Vigneshwaran, V., Ranganatha, V.L., Prabhakar, B.T., Khanum, S.A. 2015. Synthesis of oxadiazole–morpholine derivatives and manifestation of the repressed CD31 Microvessel Density (MVD) as tumoural angiogenic parameters in Dalton's Lymphoma. *Bioorganic Chemistry* 60, 136–146.

Deepak, P., Kumar, S., Kishore, D., Acharya, A. 2010. IL-13 from Th2-type cells suppresses induction of antigen-specific Th1 immunity in a T-cell lymphoma. *International Immunology* 22, 53–63.

Glaucia, V., Faheina-Martins., da Silveira, A. L., Cavalcanti, B. C. *et al*. 2012. Antiproliferative effects of lectins from Canavalia ensiformis and Canavalia brasiliensis in human leukemia cell lines, *Toxicology in Vitro* 26, 1161–1169.

Goddard, J.C., Sutton, C.D., Furness, P.N., Kockelbergh, R.C., OP'Byrne, K.J. 2002. A computer image analysis system for microvessel density measurement in solid tumours. Angiogenesis 5, 15–20.

Goudarshivananavar, B.C., Vigneshwaran, V., Dharmappa, K.K., Pramod, S.N. 2015. Pharmacological Potential of Tetrahydrofurano/Pyrano Quinoline and Benzo[b] furoindolyl Derivatives in Acute Inflammation, Pain and Oxidative Stress. *Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry* 13(3), 165-73.

Hanahan, D., Weinberg, R. 2000. *The hallmarks of cancer. Cell* 100, 57–70.

Hivrale, A., Ingale, A. 2013. Plant as a plenteous reserve of lectin. *Plant Signaling & Behavior* 8, e26595.

Janice, A., Nagy, Morgan, E. S., Kemp, T., Herzberg, *et al*. 1995. Pathogenesis of Ascites Tumor Growth: Angiogenesis Vascular Remodeling, and Stroma Formation in the Peritoneal Lining, *Cancer Research* 55, 376-385.

Jedrychowski, W., Maugeri, U. 2009. An apple a day may hold colorectal cancer at bay: recent evidence from a case-control study. *Reviews on Environmental Health* 24, 59–74.

Liener, I.E. 1991. From soybeans to lectins: a trail of research revisited. *Carbohydrate Research* 213, 1- 5.

Liu, B., Bian, H., Bao, J. 2010. Plant lectins: Potential antineoplastic drugs from bench to clinic, *Cancer Letters* 287, 1–12.

Mengfeng, L. (2008). Tumour angiogenesis, antiangiogenic therapy and anti-antiangiogenesis response. *Metastasis, Apoptosis and Immune Response* 91–102.

Nagendraprabhu, P., Sudhandiran, G. 2011. Astaxanthin inhibits tumor invasion by decreasing extracellular matrix production and induces apoptosis in experimental rat colon carcinogenesis by modulating the expressions of ERK-2, NFkB and COX-2, *Invest New Drugs* 29, 207–224.

Peumans, W.J., Van Damme, E.J. 1995. Lectins as plant defense proteins. Plant Physiology 109, 347-352

Pramod, S.N., Vigneshwaran, V., Venkatesh, Y.P. 2015. Immunomodulatory effects of hemagglutinating lectins from potato (solanum tuberosum) and garlic (allium sativum) on human and murine lymphocytes. *International Journal of Pharmacy and Pharmaceutical Sciences* 7, 147-153.

Puri, R., Sud, R., Khaliq, A., Kumar, M., Jain, S. 2011. Gastrointestinal toxicity due to bitter bottle gourd (*Lagenaria siceraria*)- a report of fifteen cases. *Indian Journal of Gastroenterology,* 30, 233-236.

Sayyed, N., Pradeep, D., Mohsin, Q., Sunil, P., Navin, R. 2012. Lagenaria siceraria fruit extract ameliorate fat amassment and serum TNF-α in high-fat diet-induced obese rats. *Asian Pacific Journal of Tropical Medicine*, 698-702.

Thirusangu, P., Vigneshwaran, V., Prashanth, T, Vijay Avin, B.R., Malojirao, V.H., Rakesh, H., Khanum, S.A., Mahmood, R., Prabhakar, B.T. 2016a. BP-1T, an antiangiogenic benzophenone-thiazole pharmacophore, counteracts HIF-1 signalling through p53/MDM2-mediated HIF-1α proteasomal degradation. *Angiogenesis* 19, 1-17. doi: 10.1007/s10456-016-9528-3

Thirusangu, P., Vigneshwaran, V., Ranganatha V.L., Vijay Avin, B.R., Khanum, S.A., Mahmood, R., Jayashree, K., Prabhakar, B.T. 2016b. A tumoural angiogenic gateway blocker, Benzophenone-1B represses the HIF-1α nuclear translocation and its target gene activation against neoplastic progression. *Biochemical Pharmacology* doi: 10.1016/j.bcp.2016.11.009

Vigneshwaran, V., Thirusangu, P., Madhusudana, S., Krishna, V., Pramod, S.N., Prabhakar, B.T. 2016. The latex sap of the 'Old World Plant' Lagenaria siceraria with potent lectin activity mitigates neoplastic malignancy targeting neovasculature and cell death. *International Immunopharmacology* 39, 158-71.

Vijay Avin B.R., Thirusangu, P., Ramesh, C.K., Vigneshwaran, V., Prashanth Kumar M.V., Mahmood, R., Prabhakar, B.T. 2014c. Screening for the modulation of neovessel formation in non-tumorigenic and tumorigenic conditions using three different plants native to Western ghats of India. *Biomedicine & Aging Pathology* 4, 343–348.

Vijay Avin, B.R., Prabhu, T., Ramesh, C.K., Vigneshwaran, V., Riaz, M., Jayashree, K. & Prabhakar, B.T. 2014a. New role of lupeol in reticence of angiogenesis, the cellular parameter of neoplastic progression in tumourigenesis models through altered gene expression. *Biochemical Biophysical Research Communications* 448,139-144.

Wang, J., Wang, X., Jiang, S., Lin, P., Zhang, J. *et al*. 2008. Cytotoxicity of fig fruit latex against human cancer cells, *Food and Chemical Toxicology* 46, 1025–1033.

****