International Journal Of

Recent Scientific Research

ISSN: 0976-3031
Volume: 7(6) June -2016

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

Kaythegowdanadoddi Srinivasa Balaji., Malavalli Guruswamy Dileepkumar., Chakkere Shivamadhu Madhu., Basavaraju Puttalingaiah., Kagepura Thammaiah Chandrashekara and Shankar Jayarama

THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 6, pp. 12024-12028, June, 2016

International Journal of Recent Scientific Research

Research Article

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

Kaythegowdanadoddi Srinivasa Balaji[1]., Malavalli Guruswamy Dileepkumar[1]., Chakkere Shivamadhu Madhu[2]., Basavaraju Puttalingaiah[3]., Kagepura Thammaiah Chandrashekara[4] and Shankar Jayarama[1]*

[1]Department of Biotechnology, Teresian College (Affiliated to University of Mysore), Siddartha Nagar, Mysore, India
[2]Department of Biochemistry, Yuvaraja's College, University of Mysore, Mysore, India
[3]Bhoomigen seeds Pvt. Ltd., Bangalore, India
[4]Institute of Excellence, University of Mysore, Manasagangorti, Mysore, India

ARTICLE INFO

Article History:

Received 05th March, 2016
Received in revised form 08th April, 2016
Accepted 10th May, 2016
Published online 28st June, 2016

Key Words:

Cadabafruticosa, Anti-angiogenesis, EAC, Geimsa, CAM.

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-angionenic 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 dug designing.

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 pre existing vascular vesseles 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

*Corresponding author: **Kaythegowdanadoddi Srinivasa Balaji***
Department of Biotechnology, Teresian College (Affiliated to University of Mysore), Siddartha Nagar, Mysore, India

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 Ethno traditional 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-amyloidogenic, immunomodulatory, anti-inflammatory, 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 andMandya 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 obtainedfrom Department of Biotechnology and Zoology, University of Mysore (Mysore, India), Ehrlich AscitesCarcinoma (EAC) cells also called mouse mammary carcinoma cells are maintained in our laboratory and areroutinely used for *in-vivo* transplantation. Agarose, Tryphan blue, Giemsa stain, Ehidiumbromide wereobtained from Hi-media research laboratory. All other chemicals and reagents were of highest gradecommercially 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 37^0 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 (5×10^6) EAC cells were injected to mice intraperitonealregion 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 extractregress 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 neovasculature by taking photograph using Sony digital camera.

Giemsa Staining

The validity of apoptosis was confirmed through the use of light microscopy in which cells wereassessed for apoptotic morphology using wright giemsa stain [Jayarama *et al*.2015, Roopashree, R. *et al* 2015 and JyotiBala 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.

Chorioallatoic 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 12^{th}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 14^{th}day and inspected for changes in the vascular density in the area under the cover slip and photographed using sony digital camera.

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

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 compare to treated mice.

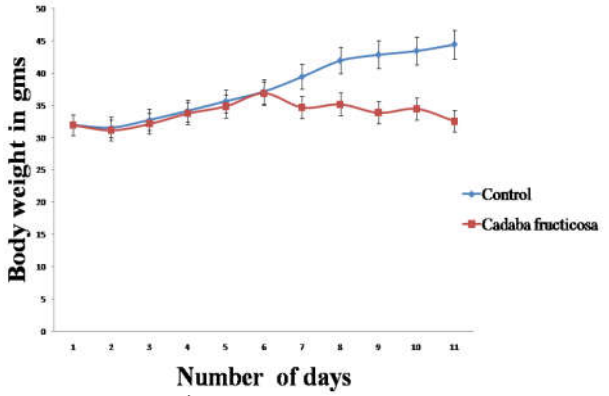

Fig.1.EAC cells (5×10^6cells/mouse, i.p) were injected intomice and body weight of the mice was recorded to follow tumorgrowth.Every alternate day ***Cadabafruticosa*** extract is administered from the 6thday onwards (100 mg/kg body weight). Minimum of 5 mice were used for the experiments andhas to be repeat two more time for statistical significance.

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 trypan 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.

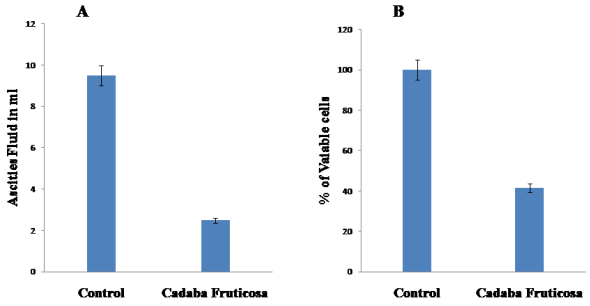

Fig.2.EAC bearing mice treated with *Cadabafruticosa*extract are sacrificed after giving each dose cells along with ascites fluid were harvested. The number ofcells per mouse was determined by counting the cells usinghemocytometer (Fig. 2A) and ascites volume was recorded (Fig. 2B).

Apoptotic morphology of EAC cells on Cadabafruticosaleaf extract treatment

Treatment of Cadabafruticosa 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).

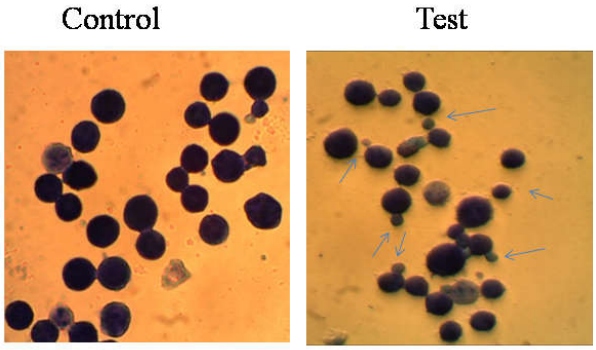

Fig.3.EAC cells treated withand without *Cadabafruticosa*extract washed with PBS, fixed in methnol/acetic acid (3:1) and stained with Giemsa. Both types of cells were carefully viewed under light microscope for apoptotic morphology such as plasma membranedegradation, membrane blebbing or apoptotic bodies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

Cadabafruticosa leaf extractinducesdegradation of DNA in EAC Cells

Biochemically, apoptosis is characterized by fragmentation of chromosomal DNA. We further verified the effect of *Cadabafruticosa* leaf extracted 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 bearing mice treated with *Cadabafruticosa* leaf extract (Fig. 5).

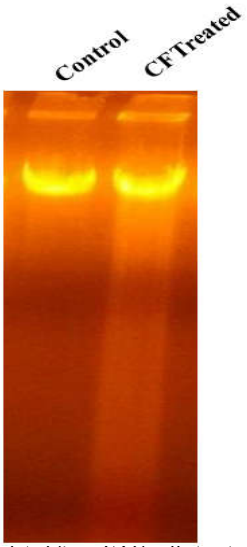

Fig. 4.DNA was isolated from EAC cells treated with and without *Cadabafruticosa* leaf extract and Extracted Total genomic DNA were resolved on 1.2% agarose gel. Apoptotic DNA fragmentation was visualized by ethidium bromide staining.

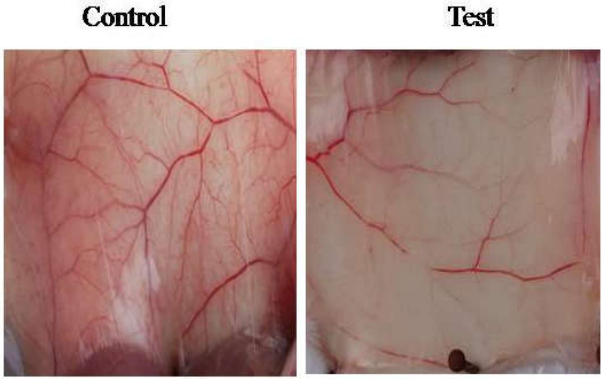

Fig.5 Extensive neovascularization in the Peritoneal lining of EAC bearing control untreated mice. Peritoneal lining of mice treated with *Cadabafruticosa* extract was inspected for angiogenesis. Inhibition of peritoneal angiognesis in *Cadabafruticosa* extract treated mice is evident.

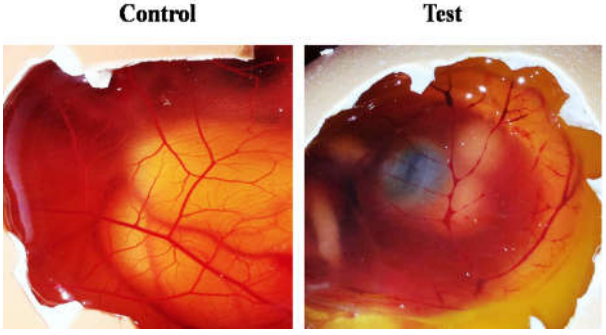

Fig. 6 Photos illustrate the formation of blood vessel branch points in either control (saline) *Cadabafruticosa* extract treated CAMs of the 12-day-old embryonatedchicken eggs. Note the significant inhibition of the formation of blood vessel branch points in the egg exposed to *Cadabafruticosa* extract.

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 EACcells *in-vivo*. Angiogenesis has been shown to play a significant role in cancer growth and metastasis. Recently, antiangiogenicdrugs have been shown to eradicate certain mouse tumors and induce long term tumor dormancyand 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 ascitesvolume 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 *invivo* [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, signalingpathways involved in targeting apoptosis and angiogenesis.

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

Acknowledgements

The authors acknowledge Department of Science and Technology, Government of India (SERB/LS-886/2012) and Vision Group of Science & Technology GRD-197, Government of Karnataka, India for financial assistance. The authors would like to thank Department of Zoology, University of Mysore, Mysore for providing animal facility.

References

Parsons S.L, (1996) Malignant ascites. Br J Surg, 83: 6–14.

Kashani A, (2008) Fluid retention in cirrhosis: pathophysiology and management. QJM, 101: 71–85.

Gregoriadis G, (1995). Engineering liposomes for drug delivery: progress and problems. Trends Biotechnol, 13 (12): 527 – 537,

T. Griga, A, (1998). May, increased serum level of VEGF in patients with inflammatory bowel disease, Scand. *J. Gastroenterol*. 33(5): 504–508,

M. Nagashima, (1995) Role of VEGF in angiogenesis of rheumatoid arthritis, *J. Rhematol*, 22 (9):1624–1630,

J. Folkman, (1995). *Angiogenesis* in cancer, vascular, rheumatoid and other disease, Nat. Med, 1 27–31,

Folkman, (1971).Tumor angiogenesis: therapeutic implications, N. Engl. J. Med, 285(21):1182–1186,

J. Folkman, J.F. Holland,2000,Tumor angiogenesis, in: E. Frei III, R.C. Bast Jr., D.W. R.R. Weichselbaum (Eds.), Cancer medicine, B.C. Decker Inc, Canada, pp. 17 (3):132–152,

D. Marme, (1996). Tumor angiogenesis: the pivotal role of vascular endothelial factor, *World J. Urol*. 14 (3): 66–74,

W. Auerbach, 1994, Angiogenesis inhibition: a review. *PharmacolTher*, 63 (3):265– 311,

Mohamed Al-Fatimi, (2007). Antioxidant, antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. *J Ethnopharmacol*, 111(3):657-666,

Mythreyi.R, (2008). Antipyretic activity of leaves of Cadabafruticosa (L.) Druce. Pharmacology online.3:136-142.

ViqarUddin Ahmad, (1971). Chemical investigation of the leaves of Cadabafruticosa. *Pak. J. Sci. Ind*. Res.14: 343-338.

Shankar Jayarama, (2015) anti-angiogenic activity of aqueous extract of *h. integrifolia* inehrlich ascites carcinoma model. IJCR, 7 (7): 18241-18246.

RakeshKodagahalliSathya, (2016). 3, 5-disubstituted isoxazole derivatives: potential inhibitors of inflammation and cancer. Inflammation, 39(1); 269-280.

Rangaswamy Roopashree, (2015) Novel synthetic bisbenzimidazole that targets angiogenesis in Ehrlich ascites carcinoma bearing mice. BMC Letters, 25(12):2589-2593.

Belakavadi M, (2005). Mechanism of inhibition of ascites tumor growth in mice by curcumin is mediated 12 by NF-kB and caspase activated DNase. *Mol. Cell. Biol*, 273(1-2): 57-67.

Jyoti Bala Chauhan, (2015). antiangiogenic and proapoptotic activity of *averrhoacarambol*a fruit extract on ehrlich ascites carcinoma treated mice. IJABPT, 6(2):246-254.

Bharathi P. Salimath, (2007). Pro-apoptotic activity of imidazole derivatives mediated by up-regulation of Bax and activation of CAD in Ehrlich Ascites Tumor cells. Invest. New Drugs, 25(4):343–350.

Newmann D J, (2007) Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod, 70(3):*461-477,

Boehm T, (1997) Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature, 390*(6658):404–407,

Sriram D, (2005) Camptothecin and its analogues: a review on their chemotherapeutic potential. *Nat. Prod. Res*, 19(4):393-412,

Watanabe M, (2002). Identification of licocoumarone as an apoptosis inducing component in licorice. *Biol. Pharm. Bull*, 25 (10): 1388–1390,

****

How to cite this article:

Kaythegowdanadoddi Srinivasa Balaji *et al*.2016, Anti-Tumor and Anti-Angiogenic Activity of Cadaba Fruticosa Leaf Extracts In Murine Carcinoma Model. *Int J Recent Sci Res.* 7(6), pp. 12024-12028.

TSSN 0976-3031