ISSN: 0975-833X

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

International Journal of Current Research
Vol. 8, Issue, 02, pp.26031-26039, February, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

EVALUATION OF IN ANGIO-SUPPRESSIVE ACTIVITY OF THE *BIOPHYTUM SENSITIVUM* (BARK) EXTRACTS BY CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

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ARTICLE INFO

Article History:
Received 16th November, 2015
Received in revised form
22nd December, 2015
Accepted 20th January, 2016
Published online 14th February, 2016

Key words:

Angio-Suppressive, *B.sensitivum* (bark), Chick Chorioallantoic Membrane (CAM), Tertiary Vitelline Veins (TVV).

ABSTRACT

Angiogenesis is the formation of new blood vessels from pre-existing vessels, capillaries and post-capillary venules. It plays an important role in many normal physiological processes such as normal tissue growth, embryonic development, wound healing and menstruation. Many natural inhibitors block the process of angiogenesis which may leads to inhibition of tumor growth. The chick chorioallantoic membrane (CAM) assay is well vascularized in vivo model used to study angiogenesis, anti-angiogenesis and teratogenic effect of individual compounds and complex plant extracts. In the present investigation angio-suppressive activity of B. sensitivum (bark) in acetone extract is evaluated by chick chorioallantoic membrane (CAM) assay. The fertilized eggs of Gallug gallus (murghi) was incubated at 38^0C with relative humidity. The embryos of 48, 72 and 96hrs CAM were exposed to 1mg/ml acetone extract of B. sensitivum(bark) and were further incubated upto 144hrs and CAM was studied. The present investigations showed angio-suppressive effect of B.sensitivum (bark) on the number and area of tertiary vitelline veins (TVV).

Citation: **Manisha Mhaske and [2]Ghanshyam Gonjari, 2016**. "Evaluation of in angio-suppressive activity of the *biophytum sensitivum* (bark) extracts by chorioallantoic membrane (cam) assay", *International Journal of Current Research,* 8, (02), 26031-26039.

INTRODUCTION

Background of study

Cancer now affects as many as 24 million people worldwide, and results in over seven million of people die each year (Jemal *et al*., 2011). It is a complex multifractional disease and still very hard to treat. More than half of all people live with being diagnosed with cancer but cancer treatment is still far from perfect. The conventional and chemical compound used in cancer treatment damaging lipids, proteins and also DNA, brings mutation and causing severe side effects that can affect a person's quality of life (Ellnaim *et al*., 200368). However, many of the natural plant products and their phytochemical derivatives is a prime target to afflictions such as growth of solid tumours, arthritis and inflammations. Therefore, there is a necessity to explore their uses and to conduct antiangiogenic studies to ascertain their chemo-therapeutic properties. Hence, this review sheds light on angiogenic inhibitors and plant derived antiangiogenic compounds for cancer. Traditional Indian Medicine (TIM) is an important source of potentially useful new compounds for the development of chemotherapeutic agents. The importance of TM has increasing in all regions of the developing countries as- In Canada 70% of the population have used complementary medicine. The 158 millions of the adult population in the United States use complementary medicines and according to the USA Commission it was spent US $17 billion for Alternative and Complementary medicines.

Circulatory system is formed by process of vasculogenesis, it involves denovo vascularisation of blood vessels and endothelial cells from embryonic mesoderm. While angiogenesis is the neo-vascularisation of blood vasculature from pre-existing blood vessels (Griffioen and G. Molema, 2000). It is a tightly regulated process that involves signaling and extracellular matrix which induces the migration, proliferation and tube formation of endothelial cells leading tumor neovascularisation (Folkman and Klagsbrun, 1987). Tumor rapidly grow by continual supply of nutrients and oxygen from blood vessels, and then proliferate at another site forming metastasis of cancer (Folkman, 1971). Cancer becomes necrotic/ apoptic without vascular support and nutrient supply (Holmgren *et al*., 1995), (Parangi *et al*., 1996). Therefore, angiogenesis Playing a critical role in the progression of cancer.

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According to Sturk *et al*. (2005) angiogenesis is a multi-stepped process such as- 1) Endothelial cell activation by growth factors (including VEGF, bFGF) 2) degradation of capillary wall by (matrix metalloproteinases) 3) networking of blood vessels 4) migration of endothelial cells into the extracellular matrix towards the angiogenic stimulus 5) reorganization of endothelial cells to form tubules within a central lumen and 6) an astomos of blood vessels. Angiogenesis is playing an important role in ischemic chronic wounds in healing (Kerbel, 2005), coronary artery disease (Schneider *et al*., 2000) and to fight cancer and malignancies (Folkman, 1987). Whereas excessive angiogenesis leads to pathological disorders such as breast cancer (Skobe *et al*., 2001), colorectal cancer (Furudoi *et al*., 2002) and neck squamous cell carcinoma (charoenrat *et al*., 2001). The cancerous cells are genetically stable, do not mutate, hence more sensitive to apoptic effects of the cytotoxic agents. While endothelial cells in tumor bed shows high proliferation rate and becomes more susceptible to cytotoxic agents. Therefore it becomes compelling target for antiangiogenesis treatment (Folkman, 2003). The conventional treatment such as chemotherapy, radiation therapy and surgery for treatment of solid malignant tumors and cancer causing severe side effects and that can affect a person's quality of life (Brenner *et al*., 2004), (Long, 2003) and (Kim *et al*., 2002). Consequently antiangiogenic agents prohibit the neovascularization of cancer tissue as well as growth of the tumor, and hence it is beneficial in the treatment of cancer (Fernandez *et al*., 2004). Whereas medicinal herbs multi-constituents can be promising more clinical evaluation and application as adjuvants to produce synergistic effects in conventional anticancer drugs, with less side effect.

Morphology and Phytochemistry

The plant B.sensitivum belongs to family Oxalidaceae commonly known as 'Lajwanti' in Marathi, growing throughout the tropical regions of South Asia, Africa and Madagascar. The B.sensitivum (L.) DC. (Oxalidaceae) is a small annual plant, with sensitive leaves similar to the 'touch – me-not' plant as it inward curling of its leaves in response to touch stimuli (Teja Sri *et al*., 2013). The flower of this plant are called as 'Dasapushpam' as sacred of ten flower in the kerala state of Maharashtra (Varghese *et al*., 2010). The phytochemical analysis showed presence of flavonoids such as amentoflavone, luteolin, iso-orientin (Ravishankara *et al*., 2003), Caffeoylquinic acid, linalool oxide 3′, 8″- biapigenin (Jachak *et al*., 1996), proanthocyanidins (Bucar *et al*., 1997), phenolic compounds such as lutalyl oxide (Bucar *et al*., 1998), Hyluronidase and triterpenoids.

Review of relevant literature

The leaves of B.sensitivum commonly known as "Nagbeli," in folk medicine and used in a treatment of diabetes mellitus (Puri Baral, 1997) and (Pant and Joshi, 1993). The Biophytum sensitivum plant parts has several medicinal properties like treatment of asthma and phthisis (Pullaiah, 2002), immunomodulatory (Guruvayoorappan and Kuttan, 2008). hypochole-sterolemic (Puri, 2003), antioxidant activity (Guruvayoorappan and Kuttan, 2006) and cell mediated immune response (Guruvayoorappan, 2007). The leaves extracts of B. sensitivum in methanol, chloroform and acetone showed significant antibacterial activity against Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumonia, Klebsiella pneumoniae, Salmonella typhi (Natarajan *et al*., 2010). While the aqueous extract of B.sensitivum leaves significantly showed antitumor activity against the transplantable murine tumor (Bhaskar and and Rajalakshmi, 2010).

Chick Chorioallantoic Membrane (CAM) assay

The chick chorioallantoic membrane (CAM) assay were used in study of anti-angiogenesis (Min Liu *et al*., 2013). It is an attractive alternative, successful and feasible model widely used in the study of angiogenesis (Richardson and Singh, 2003) and demonstration of antiangiogenic activity (Auerbach *et al*., 2003). It is a relatively simple, inexpensive, good reproducible, commonly used in study of induction of angiogenesis and anti-angiogenesis drugs by tumor cells (Presta *et al*., 1999) and (Brooks *et al*., 1994). It is commonly referred to as "Hen's Egg Tests (HET) (Lupke, 1986),(Schoffl *et al*., 1992). It has significant application in the study of tumor cell invasion and metastasis (Tufan and Satiroglu-Tufan, 2005), (Deryugina and Quigley, 2008), (Ossowski, 1998) and (Zhai *et al*., 2007), prostate cancer (Wittig-Blaich *et al*., 2011) and (Kobayashi *et al*., 1998), ovarian cancer metastasis (Ween *et al*., 2011), (Lokman *et al*., 2011) and to assess ovarian cancer invasion and metastasis (Chang *et al*., 2011). Keeping in view of tremendous work the present study aimed to evaluate the anti-angiogenic activity of B.sensitivum(bark) in acetone extract on chick chorioallantoic membrane (CAM) assay. Whereas phytochemical constituents of B.sensitivum(bark) is a significant to exploit novel anti-cancer drugs in future.

Objective of study

The objective of this study is to assess the effect of acetone extract of B.sensitvum (bark) on the tertiary vitelline veins (TVV) of chick chorioallantoic membrane (CAM) assay.

Significance of study

More than 300 species of herbs and plants possesses therapeutic properties and exerting beneficial pharmacological effects for the development of anticancer drugs (Jang *et al*., 1997). Many natural plant product phytochemical constituents, that may have synergistic activity to reduce the risk of metastasis and tumor progression. The present article focuses on products that have a high degree of anti-angiogenic activity, it also describes some of the many other actions of these agents that can inhibit tumour progression and reduce the risk of metastasis. Therefore, there is a necessity to explore B.sensitivum (bark) for chemotherapeutic uses and to conduct their potentially useful new compounds for development of new antiangiogenic drug in future use.

MATERIAL AND METHODS

Plant material and preparation of extracts

The properly identified fresh bark of B.sensitivum plant were collected from the local area of Satara district, Maharashtra,

India. The fresh barks were chopped into smaller pieces and air dried at (36-390C). It was washed with tap water, then shed dried for seven days in shadow. The dried barks were ground to a coarse powder with grinder. The powder (20gm) was extracted by routine methods to get acetone extracts. The extracted material was filtered with whatman filter paper no.1. The filtered material was concentrated by evaporation with high speed vacuum evaporator (Buchi type). The yield was stored at 40C under refrigerated condition till needed for. The 1mg of acetone extract was dissolved in 1ml Dextrose With Normal Saline (DNS) was purchased from Mark-Bioscience Ltd, Goa(G21730031, Exp.Dec.2015). DNS is a medicated saline used to make proper concentration of extract for treatment on CAM assay.

Chorioallantoic membrane (CAM) assay (in vivo)

The Chicken Embryo Chorioallantoic Membrane (CAM) model provides a natural in vivo model environment, to detect antiangiogenic activity of B.sensitivum(bark) extracts. Fertilized eggs of Gallus gallus(murghi) were obtained from Assistant commissioner of animal husbandry, central hatchery, Satara, Maharashtra, India. The selected fertilized eggs of Gallus gallus(murghi) were disinfected with absolute alcohol and then incubated at 380C with (65-70%) relative humidity. Extracts administration at 48, 72 and 96hrs were selected as per the development of CAM and vitelline veins.

Dose selection and administration of the extract on the chick embryo

The dose that showed 100% survivality without any mortality on hatching after treatment of B.sensitivum (bark) extract were selected. The selected doses were mixed with 1ml volume DNS (medicated saline) and administered on different developed 48, 72 and 96hrs incubated embryos. On completion of scheduled period of incubation stated above the windows were prepared in embryos under asceptic condition maintained in the Air Flow Laminar (AFL). The embryo CAM was exposed to 1mg/ml B.sensitivum(bark) in acetone extracts by window method use in (Korn, M.J. and Kramer,2009) described in Table-1.

The experiment were prepared in three groups as Sham, DNS control and treated. Normal group of embryos were maintained as normal and embryos of operative groups were operated for windows preparations and embryos of DNS control group received 1mlDNS/embryo. The windows made for administration were resealed with sterilized adhesive cello tapes and embryos were immediately retransferred into humidified incubator to continue further incubation for six days.

On completion of 144hrs, the shell were removed and embryo along with yolk were gently placed in the petridish plate containing saline water. The vasculature developing on CAM was imaged with a digital 'cannon' camera and transferred on computer for image analysis. The same analysis was confirmed with direct stereoscopic observations of well spread embryonic plates on glass slides of appropriate dimensions.

Quantification of angiogenesis

Angiogenesis was quantified by counting number of Secondary Vitelline Veins (SVV) and Tertiary Vitelline Vein (TVV) and it's associated area for antiangiogenic response. The bifurcation points were used as initiation and termination markers. The area and vasculature networking were measured on stereoscopic microscope and was confirmed by using graph transparencies. The observed alterations are presented in Table 2 and Fig 1 and 2.

Histopathological study

After scarification of chick embryo, CAM were dissected out, fixed in 10% formaldehyde, dehydrated and embedded in paraffin wax(MP.56-580C) for histological studies. The blocks were sectioned at 4µm on rotary microtome and stained with Hematoxylene and Eosin(HE) method. Examined under microscope for histopathological changes of CAM embryo.

Evaluation of CAM angiogenesis

The author in (Melkomian *et al*., 2002), described morphometrical evaluation of CAM such as.

The total CAM area was calculated as

Area = (1/2A)X(1/2B)X π.

Where A is the longest length, B is longest width and $\pi = 3.14$. The number of secondary and tertiary blood vessels branching points and it's morphometric evaluation were counted manually on computer image. For histological evaluation paraffin block of CAM was sectioned at 5µm thickness by rotary microtome

Statistical analysis

The data was expressed as Mean ± SEM and the statistical significance between groups was analyzed by (55) using student 't' test. The values of $p<0.05$, $p<0.01$, $p<0.001$ were considered as significant.

RESULTS

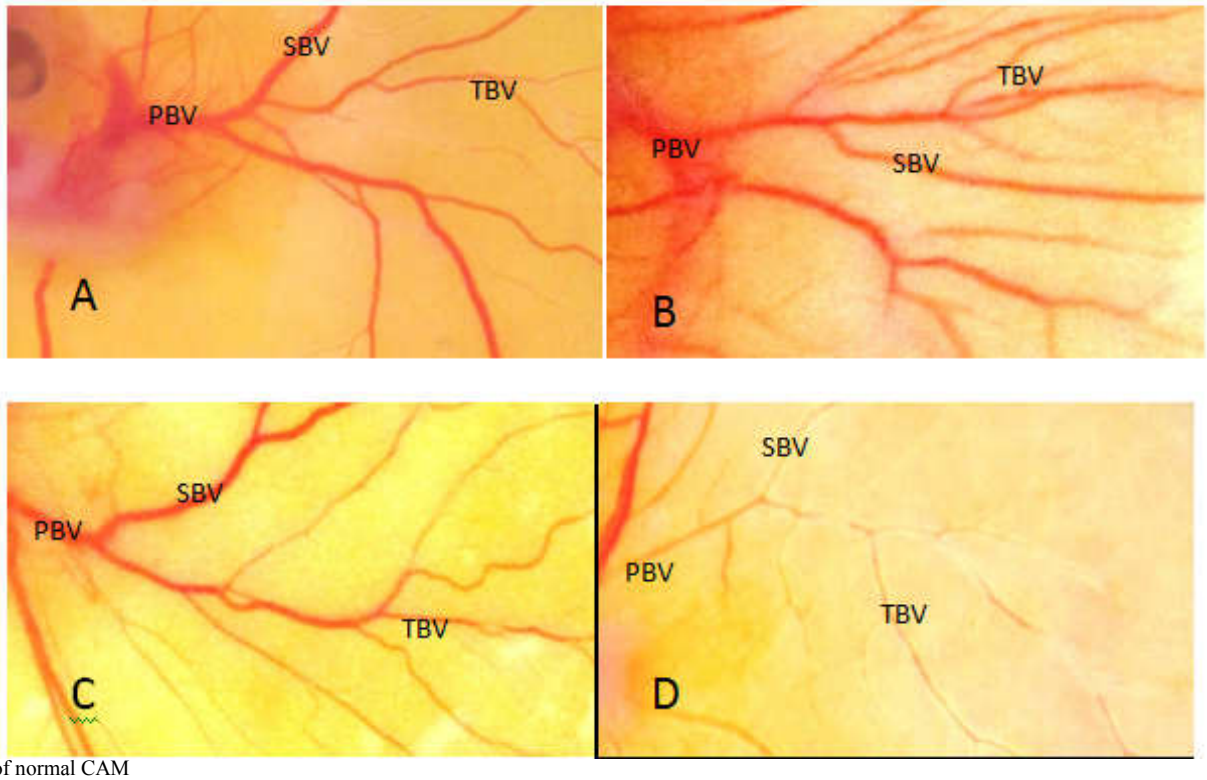
The anti-angiogenic activity of B.sensitivum (bark) in acetone extract was determined using chicken chorioallantoic membrane (CAM) assay in vitro. It is an attractive model widely used in the quamtitative evaluation of length of veins and arteries in the development of early CAM assay (Hazel, 2003). It is also used to screen anti-angiogenic activity on PBV, SBV and TBV. For histological and morphological evaluation, all different developmental staged embryos treated with acetone extract of B.sensitivum (bark) were observed at 144hrs.

Morphometric evaluation

The primary, secondary and tertiary blood vessels were showed constant constant number of blood vessels at 48, 72 and 96hrs, but slightly increase in number of blood vessels were noted in sham control (only window made without treated) embryo.

The branching points of PBV, SBV and TBV were counted at 144hrs for an anti-angiogenic evaluation. The embryo treated with DNS control showed significantly increase in number of blood vessels with associated area covered by them. Treatment of acetone extract of B.sensitivum (bark) showed marginal reduction in the number of blood vessels at all hrs of treatment but at 48hrs showed significantly reduction in the tertiary blood vessels number by 47.76%. While at 72 and 96hrs reduction in the proliferation of blood vessels were noted as (44.74% and 40.55% respectively).

Plate 1. Morphometric evaluation of T.S. of chick CAM showing anti-angiogenic effect by B.sensitivum (bark) extract



A- piece of normal CAM
B-piece of DNS control CAM
C-piece of sham control CAM
D-piece of B.sensitivum(bark) extract treated CAM
PBV-primary blood vessel
SBV- secondary blood vessel
TBV- tertiary blood vessel

Table 1. Groups as per treatment of B.sensitivum (bark) extracts at different developmental stages of chick embryo in hrs

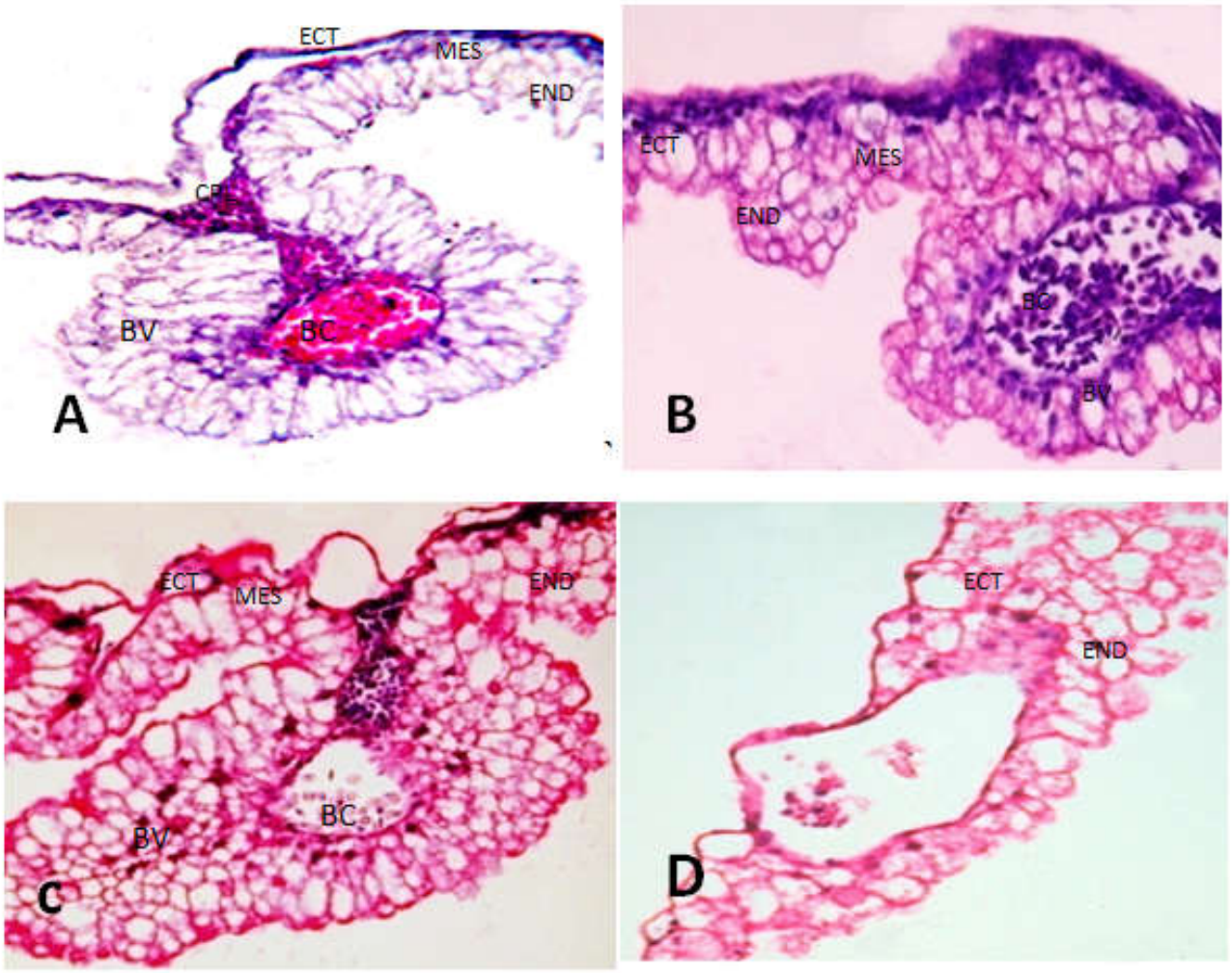
Groups as per developmental stages in hrs	Groups as per time of exposure initiations			Final development and hrs of sacrifice
	48	72	96	
Group I - 48	—	—	√	144hrs
Group II - 72	—	√	—	
Group III - 96	√	—	—	

Table 2. Effect of extract of B.sensitivum(bark) on total number of blood vessels and area of CAM in chick embryos

Initiation of Treat -ment(hrs)	Groups	Total no. of tertiary blood vessel	Total CAM area in sq.cm
48hrs.	Normal	176±5.20	26.34±0.72
	Sham control	175±4.92	26.28±0.82
	DNS(control)	178±4.10	28.21±0.98
	Acetone extract	93±1.88cqz	19.34±1.02crz
72hrs.	Normal	176±5.28	26.46±1.14
	Sham control	174±3.68	26.29±1.02
	DNS(control)	190±4.34	28.94±1.22
	Acetone extract	105±4.14crz	20.89±1.18brz
96hrs.	Normal	176±4.32	26.57±1.48
	Sham control	174±5.30	26.42±1.82
	DNS(control)	185±5.40	28.98±1.92
	Acetone extract	110±3.86bqz	21.98±0.64apy

(Results expressed as mean} S.E. of 5 embryos. p-values-a<0.05, ь<0.01, c<0.001 vs. Normal embryos. p<0.05, q<0.01,r<0.001 vs. Sham control embryo. x<0.05, y<0.01,z<0.001 vs.DNS control embryo).

Plate 2. Histological evaluation of T.S. of chick CAM showing anti-angiogenic effect by B.sensitivum(bark) extract



A-Normal CAM, B-DNS control CAM, C-Sham control CAM, D- B.sensitivum(leaf) extract treated CAM BV- blood vessel, BC- blood cell, CPL- capillary plexus, ECT-ectoderm, END-endoderm, MES-mesoderm

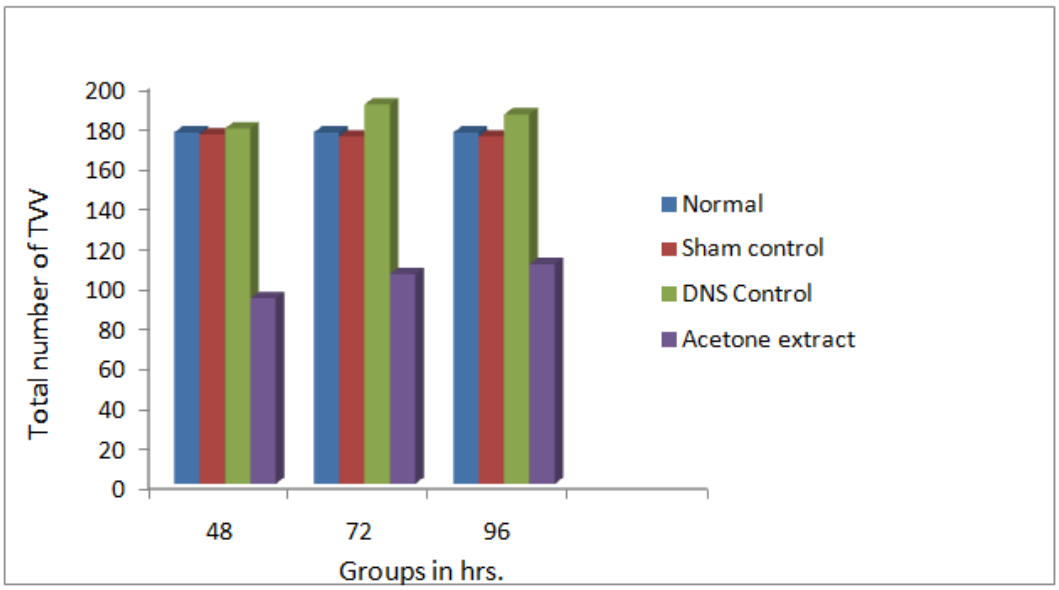


Figure 1. B.sensitivum (bark) extract influenced alterations in number of tertiary blood vessels (on 144hrs of development)

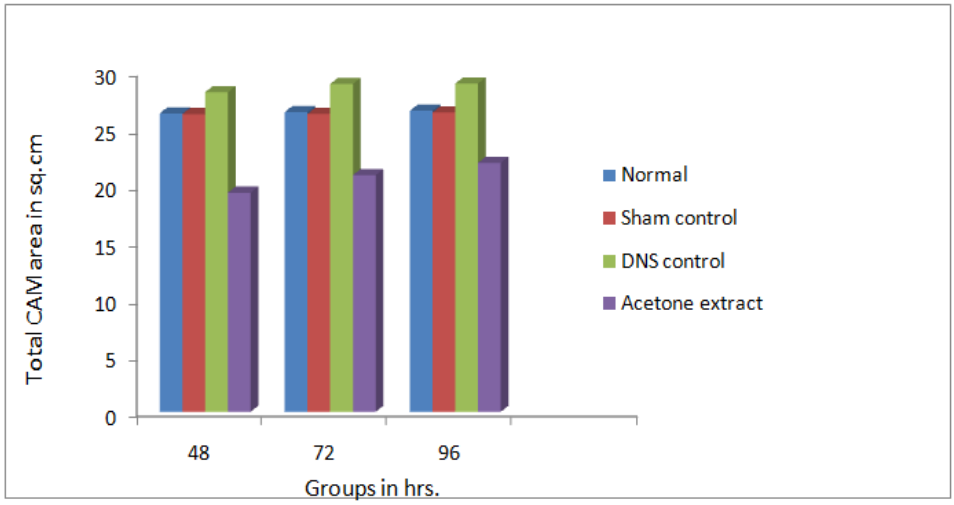


Figure 2. B.sensitivum (bark) extract influenced alterations in total area of CAM (on 144hrs of development)

Whereas total area of CAM was depleted at 48 and 72hrs by (31.45% and 27.82% respectively). Significantly decrease in number of blood vessels was noted at 48hrs treatment of B.sensitivum (bark) extract (Plate 1, Table 2 and Fig. 1 and 2). While at 96hrs total area of CAM embryo showed normalized as that of normal embryo (Plate 1, Table 2 and Fig.1and 2).

Histological evaluation

The histological evaluation of T.S. of CAM embryo was prepared as described in Material and Method (histopathological study). The normal CAM embryo at 144hrs development showed presence of three layers as ectoderm, mesoderm and endoderm. It showed large veins in the mesoderm and thin walled capillaries beneath the chorionic epithelium filled with avian RBCs (Plate 2-A). Administration of DNS on CAM, appeared well vascularized blood vessels capillary plexus(CPL) on the ectoderm along with increased in blood vasculature filled avian RBCs in the mesodermal layers(Plate 2-B). Whereas sham controlled embryo CAM showed slight decrease in the endothelial blood vessels along with mesodermal capillary plexus(Plate 2-C). Administration of B.sensitivum(bark) extract on 48hrs developed CAM, showed significantly depletion in the endothelial cell proliferation along with obliteration of blood vessels(Plate 2-D). The thickness of CAM was significantly decreased at 48hrs than 72 and 96hrs (Plate 2-D).

DISCUSSION

The sustained angiogenesis is the essential hallmark required for transformation of normal cell to a cancerous cell. The cancer cells secrets various pro-angiogenic factors such as Vascular Endothelial Growth Factor (VEGF), Basic Fibroblast Growth factor(bFGF), Epidermal growth factor(EGF), Transforming Growth Factor (TGF)-α, TGF-β, tumor necrosis factor (TNF)-α, platelet-derived endothelial growth factor(PEGF), granulocyte colony-stimulating factor(GCSF), Angiogenin, Placental Growth Factor (PGF), interleukin-8, Hepatocyte Growth Factor (HGF), that can promote the migration, proliferation and tube formation of endothelial cells. and these activities are essential steps of angiogenesis.

Targeting of angiogenesis has become a promising strategy for cancer treatment by switching off the supply of oxygen and nutrients (Cook and Figg, 2010). Anti-angiogenesis is a new complementary treatment to prevent neo-angiogenesis leading to the formation of cancer by blocking receptors of pro-angiogenic factors (Levy, 2005). Lin and Wang (2003) reported that, the phytochemical study of Biophytum sensitivum extracts indicates the presence of flavonoids, amentoflavone, terpenoids, saponins quinones and phenolic compounds. Red wine and other grape products are enriched with polyphenolic compound such as reservatrol with cancer chemopreventive effect (Jang *et al*., 1997). It also suppresss FGF-2 and VEGF-factors inducing neovascularization by in vivo method (Deryugina and Quigley, 2008) and inhibition of bovine aorta endothelial cell proliferation, migration and tube formation(in vitro) (Igura *et al*., 2001). A number of polyphenolic compounds have been shown to inhibit VEGF-induced VEGFR-2 phosphorylation required for angiogenesis (Edwards *et al*., 2014). A polyphenol present in the green tea acting as an oral angiogenesis inhibitor and also induced activation of Akt and eNOS compounds (Kim *et al*., 2007).

The amentoflavone is a biflavonoid, which inhibit the production of cytokines such as Interleukins (IL-1β and IL-6), Granulocyte colony stimulating factor (GM-CSF), IFN-γ, Tumor necrosis factor (TNF-α), and Nitric oxide (NO) by Tumor-associated macrophages (Guruvayoorappan and Kuttan, 2008) and (Guruvayoorappan and Kuttan, 2007) and also inhibit PGE2 biosynthesis associated with COX-2 and iNOS mRNA expression (Banerjee *et al*., 2002). The amentoflavone isolated from natural plant inhibit survival of metastatic tumor-bearing mice (Guruvayoorappan and Kuttan, 2007) and angiogenesis associated with placental growth factor-1 (PlGF-1) (Tarallo *et al*., 2011). The triterpenoids isolated from Betula platyphylla(TBP) inhibit tumor cell proliferation and induce apoptosis on melanoma B16 and sarcoma S180(Ikeda *et al*., 2003) and (Li *et al*., 2000). The B. sensitivum leaves in methanolic extract inhibited the tumor- formation induced in B16-F10 melanoma cells byprocesses such as inhibition of VGEF expression, significant modulation of proinflammatory cytokines as IL-1β, IL-6, TNF-α, GM-CSF, and VGEF mRNA levels (Guruvayoorappan and Kuttan, 2007).

Conclusion

Cancer is a complex multifractional disease, approximately 7 millions of people die each year with cancer disease. Sustained angiogenesis is a hallmark of cancer. More than half of all people live with being diagnosed with cancer. Many cancers can not be cured, and some are still very hard to treat. The conventional treatment causes severe side effects that can affect a person's quality of life. While phytochemicals isolated from B.sensitivum inhibit tumor by blocking proangiogenic receptors for VEGF, FGF family members and also suppressed neoangiogenesis with less side effects. Therefore, we propose that phytochemicals from B.sensitivum (bark) extracts may be considered as a powerful new anticarcinogenic drugs for the inhibition of pathological neoangiogenesis and tumor metastasis and use in the production of new anticancer drug in future.

Abbreviations

DNS: Dextrose with normal saline;
PGE2: ProstaglandinE2; NO: Nitric oxide;
COX: Cyclooxygenase
iNOS: Inducible NO synthase;
TNF-5 : Tumor necrosis factor-5 ;
ERK: Extracellular signal-related kinase
MAPK: Mitogen-activated protein kinase;
AP-1: Activator protein-1;
IL-1β : Interleukins-1β, IL-6: Interleukins-6;
(GM-CSF): Granulocyte colony stimulating factor;
IFN-γ: Intereferon- γ,
VEGF: vascular endothelial growth factor;
bFGF: basic fibroblast growth factor;
TGF-α, transforming growth factor-α;
TNF: tumor necrosis factor;
PEGF: platelet-derived endothelial growth factor;
GCSF: granulocyte colony-stimulating factor;
PGF: placental growth factor;
HGF: hepatocyte growth factor;
EGF: epidermal growth factor;
FGF: Fibroblast growth factor;
mRNA: messenger RNA;
PlGF-1: placental growth factor-1 (PlGF-1);
CAM: chorioallantoic membrane assay.

Acknowledgement

We would like to thanks for valuable guidance of Dr. G.R. Gonjari for their assistance in editing the manuscript.

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