Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2175

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **3 (6):** 183-191 (2015)

International Journal of Pure & Applied Bioscience

Research Article

OPEN ACCESS

Effects of the *Biophytum sensitivum* (L.) DC leaf extracts on anti-angiogenic properties by chorioallantoic membrane (CAM) assay

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ABSTRACT

Biophytum sensitivum (Linn) DC (Oxalidaceae) is a medicinal plant used as antitumor, radioprotective, antibacterial, antidiabetic and ayurvedic medicine. Antiangiogenic property of acetone extract of B. sensitivum (leaf) is evaluated by chick chorioallntoic membrane (CAM) assay in vivo. Treatment doses of leaf extracts were administered at 48, 72 and 96hrs of incubation by window method and observed for angiogenesis at 144hrs of development. The results by comparing with DNS control and normal embryo showed prominent antiangiogenic effect with significant reduction in number and area of tertiary vitelline veins. The inhibition of angiogenesis was highly significant at 48hrs. The role of metabolites acting by obstructing signaling of angiogenic agents from epithelial cells or may be inducing apoptosis is discussed for it's anti-angiotherapantic use.

Key words: *anti-angiogenesis, Biophytum sensitivum, chick chorioallntoic membrane (CAM) assay.*

INTRODUCTION

Angiogenesis is a multi-stepped process of sprouting blood vasculature by endothelial cell migration, proliferation and tube formation. It plays an important role in pathological processes such as wound healing, placentation, embryogenesis, inflammatory disorders and metastasis of tumor growth[13,22,48]. The process of angiogenesis is an important strategy for tumor growth and metastasis[9,14,35]. Several pro-angiogenic inducer factors such as fibroblast growth factor (FGF)[34,50], Vascular permeability factor/Vascular endothelial growth factor (VPF/VEGF)[10,44] and angiopoietins[53] have played an important role in regulation of angiogenenesis process and generation of cancer cells. Whereas factors such as angiostatin, endostatin and thrombospondin inhibit the process of angiogenesis[15]. Any imbalance in the angiogenesis leading to numerous complex diseases such as chronic inflammatory disorders, cancer and some pregnancy related diseases such as intrauterine growth restriction and preclampsia[4,7,28], antitumor[5], cell mediated immune response[19], anti-cancer effect[16] and lowered blood sugar on nicotinamide induced rats and streptozotocin[43].

Traditionally many herbs and phytochemicals have been recognized as a rich source of angiogenesis both in vitro and *in-vivo* eg. *Benincasa hispida*[33], *Withania somnifera* root[36] and *Gastrodia elata* Blume[1]. *Biophytum sensitivum* DC. (Oxalidaceae) is a small, sensitive, annual herbs growing throughout the tropical regions of South Asia, Africa and Madagascar[54].

Cite this article: Mhaske, M. and Gonjari, G., Effects of the *Biophytum sensitivum* (L.) DC leaf extracts on anti-angiogenic properties by chorioallantoic membrane (CAM) assay, *Int. J. Pure App. Biosci.* **3(6):** 183-191 (2015). doi: http://dx.doi.org/10.18782/2320-7051.2175

Natarajan and Srinivasan[39] have reported that acetone extract of dried *B. sensitivum* (leaf) showed anti-bacterial activity by chorioallantoic membrane (CAM) assay. According to Vijayan *et al*.[55] *B. sensitivum* (leaf) in acetone extract inhibited the growth of fungal pathogens and having excellent larvicidal and pupicidal potential activity against *Aedes aegypti* strain[51]. While the ethanolic extract of *B. sensitivum* exhibited maximum antifertility activity[26]. The ethanolic extract of *B. sensitivum* DC. significantly showed the peptic anti-ulcer activity induced by aspirin in wistar albino rats[3] and anti-bacterial activity[39].

The chick embryo CAM developed as a successful, feasible and sensitive model to investigate physiological and pathological circumstances and in vivo research on angiogenesis and anti-angiogenesis[41,47].

As no any report available on anti-angiogenic activity, the present study was carried out to evaluate the anti-angiogenic activity of *B. sensitivum* (leaf) in acetone extract by chicken chorioallantoic membrane (CAM) assay.

Objective of study

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

Statistical analysis

The data was expressed as Mean ± SEM and the statistical significance between groups was analyzed[11] using student 't' test. The values of $p > 0.05$, $p > 0.01$, $p > 0.001$ were considered as significant.

MATERIAL AND METHODS

Plant material and preparation of extracts:

The properly identified fresh leaves of *B. sensitivum* were collected from the local area of Satara district, Maharashtra, India. The fresh leaves were chopped into smaller pieces and air dried for week in shady places. The dried pieces were grinded into fine powder. The leaves of experimental material were soaked in acetone extract and filtered through Whatman filter paper no.1. The material was evaporated using speed vacuum evaporator (Buchi type). The yield extract was stored at 4^0C 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 assay:

The fertilized eggs of *Gallus gallus* (murghi) were obtained from animal hatchery, Satara. The eggs were swabbed with 70% alcohol and kept in incubator at 37-38^{0}C with relative humidity of 70-75%. The treatment doses were initiated at hrs stated as 48, 72 and 96hrs and development were till continued upto 144hrs i.e. on completion of CAM ventilation and capillary networking.

Dose selection and administration by window method :

The dose of *B. sensitivum* (leaf) extract were selected on the basis of dose that showed 100% survival without any mortality on hatching. The selected doses of *B. sensitivum* (leaf) mixed with 1ml volume of DNS and were administered on different developed 48, 72 and 96hrs incubated embryos. The extract were prepared in three groups as Sham, DNS control and treated.

The normal group of embryo was maintained as normal group. The embryo CAM of 48, 72 and 96hrs development were exposed to 1mg/ml leaf acetone extract as described in Table-1 by window method[30]. The windows were resealed using sterilized adhesive tapes and CAM were studied after 144hrs.

After 144hrs, the eggs shell were removed and embryos along with yolk were placed gently in PBS containing saline water. The embryo CAM were imaged with digital camera and kept further for imaged analysis on computer.

Quantification of angiogenesis:

Angiogenesis was quantified by tertiary vitelline capillaries for antiangiogenic response. The bifurcation points were used as initiation and termination markers. Area was measured on stereoscopic microscope and was confirmed by using graph transparencies. The observed alterations are presented in Table 2 and Fig 1 and 2.

Evaluation of CAM angiogenesis:

The morphometrical evaluation made by Melkomian *et al.*[36].

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 π = 3.14

The number of secondary and tertiary blood vessels branching points and it's morphometric evaluation were counted manually on computer image[49]. For histological evaluation paraffin block of CAM was sectioned at 5µm thickness by rotary microtome.

RESULTS AND DISCUSSION

The anti-angiogenic activity of *B. sensitivum* (leaf) in acetone extract have been determined by using chicken chorioallantoic membrane(CAM) assay (*in vivo*). The CAM assay is widely used for study of histological procedures[45], proliferation of new vessel and tumor neo-vascularization[46]. For histological evaluation all different developmental staged embryos were observed at 144hrs

Morphometric evaluation:

For anti-angiogenic evaluation branching number of primary, secondary and tertiary blood vessels were counted at 144hrs.

The number of primary, secondary and tertiary blood vessels showed constant at 48, 72 and 96hrs but slightly increase in number of blood vessels were noted in sham control embryo. While significantly increase in number of primary, secondary and tertiary blood vessels were noted in DNS control embryo. Treatment of acetone extract of *B.sensitivum* (leaf) showed marginal reduction in the number of blood vessels at all hrs of treatment but significantly reduction in the number and proliferation of blood vessels were (51.96%) observed at 48hrs treatment. While at 72 and 96hrs decrease in number of blood vessels were (48.17% and 45.11% respectively). Total area of CAM was significantly inhibited at 48 and 72hrs treatment of CAM embryo by (34.58% and 31.19% respectively). While at 96hrs total area of CAM embryo showed normalized as that of normal embryo Plate 1, Table 2 and Fig.1 and 2).

Table 1: Groups as per treatment of *B. sensitivum* (leaf) 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 *B. sensitivum* (leaf) extract on total number of blood vessels and area of CAM in chick embryos

Initiation of Treatment (hrs)	Groups	Total no. of tertiary blood vessel	Total CAM area in sq.cm
48hrs	Normal	176±4.40	26.60 ±1,54
	Sham control	175±5.02	26.30±1.73
	DNS(control)	179±5.14	28.26±2.14
	Acetone extract	86±2.10^{crz}	18.49±1.65 ^{apx}
	Normal	176±4.82	26.64±1.92

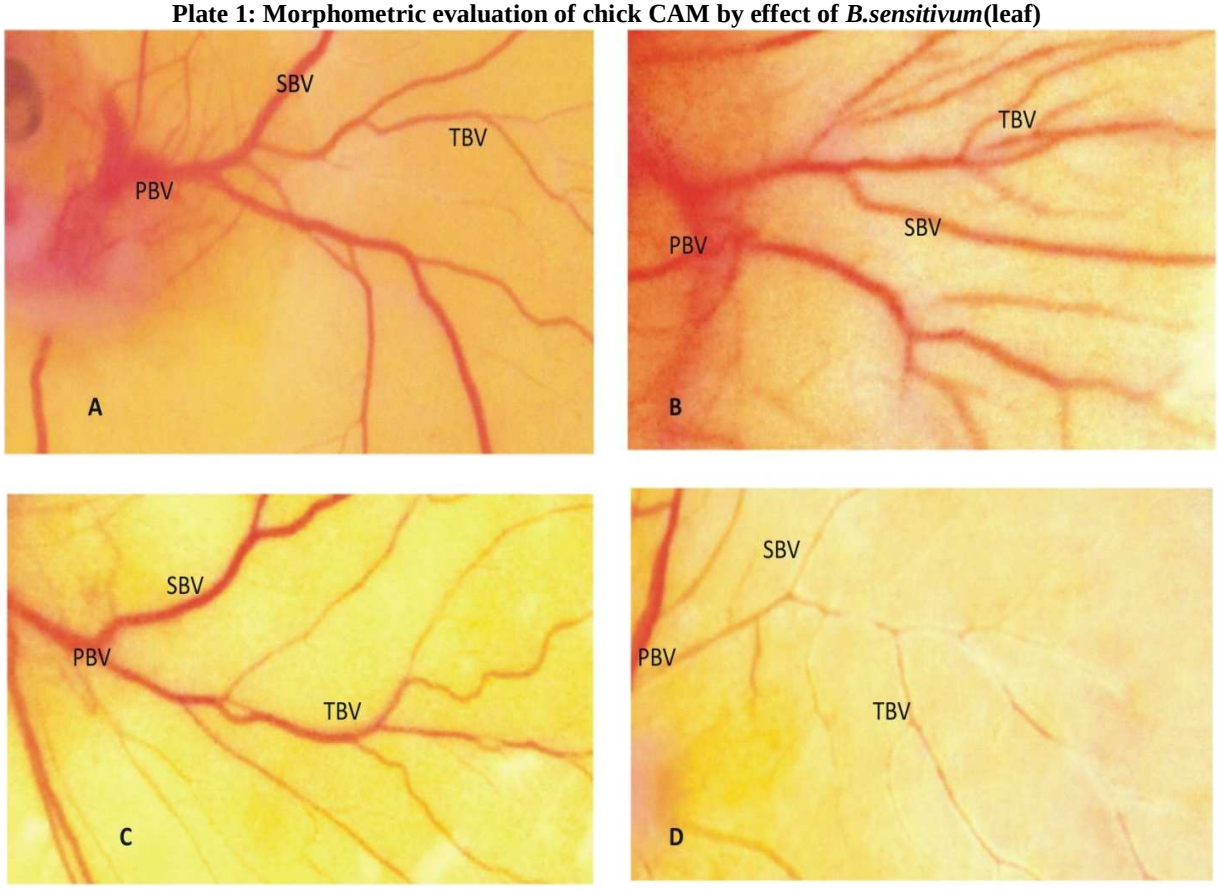
72hrs	Sham control	175±3.84	26.59±2.00
	DNS(control)	191±4.10	28.96±2.44
	Acetone extract	99±1.68^{crz}	19.93±1.86 ^{apx}
96hrs	Normal	175±5.02	26.68 ±1.06
	Sham control	174±4.10	26.62 ±1.15
	DNS(control)	184±4.82	28.99 ±1.54
	Acetone extract	101±2.78^{cqz}	20.44±2.16 ^{apy}

(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).

Histological evaluation:

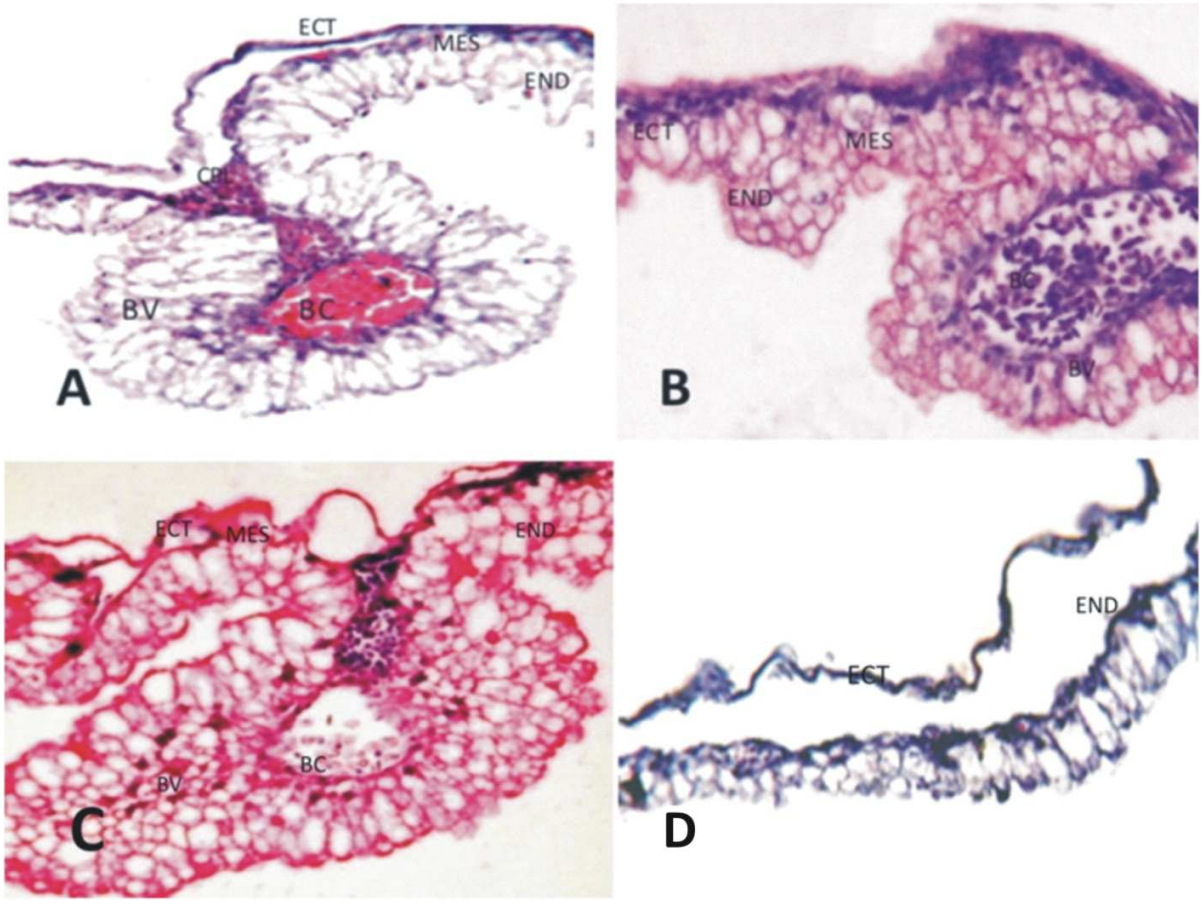
For histological evaluation T.S. of CAM embryo was histologically prepared as described in Material and Methods. At 144hrs normal CAM embryo showed presence of three layers as ectoderm, mesoderm and endoderm. A normal CAM displays large veins in the mesoderm and thin walled capillaries beneath the chorionic epithelium filled with avian RBCs (Plate 2-A). CAM treated with DNS appeared well vascularized blood vessels capillary plexus(CPL) in the ectoderm (Plate 2-B). It also showed numerous mesodermal vessels at 72hrs treatment. The pericytes and allantoic epithelium appears normal.

Plate 1: Morphometric evaluation of chick CAM by effect of *B.sensitivum*(leaf)



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

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

Fig. 1 : *B. sensitivum* (leaf) extract influenced alterations in number of tertiary blood vessels (on 144hrs of development)

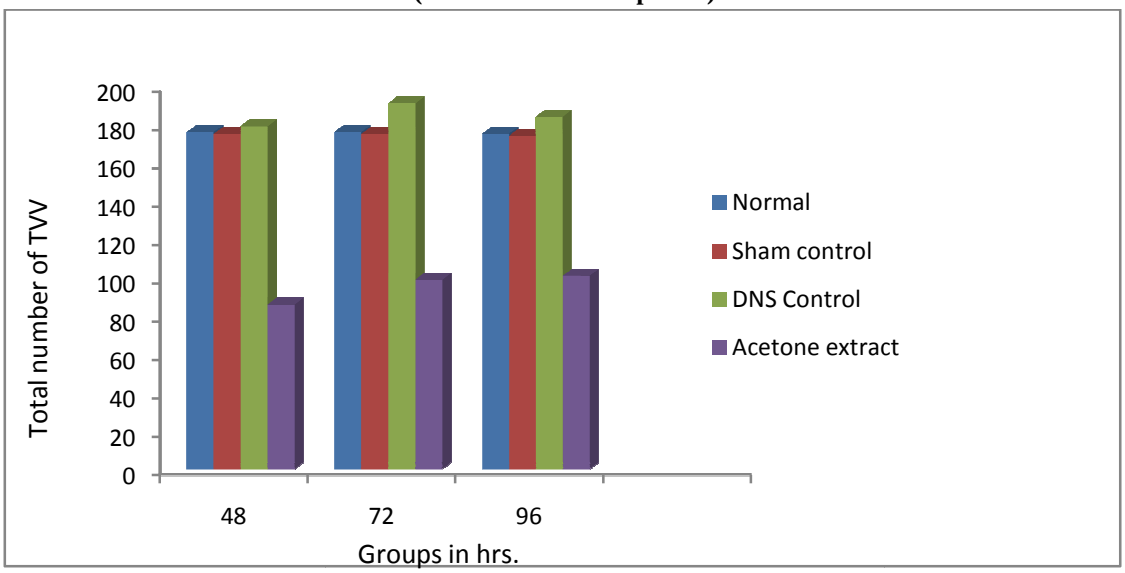
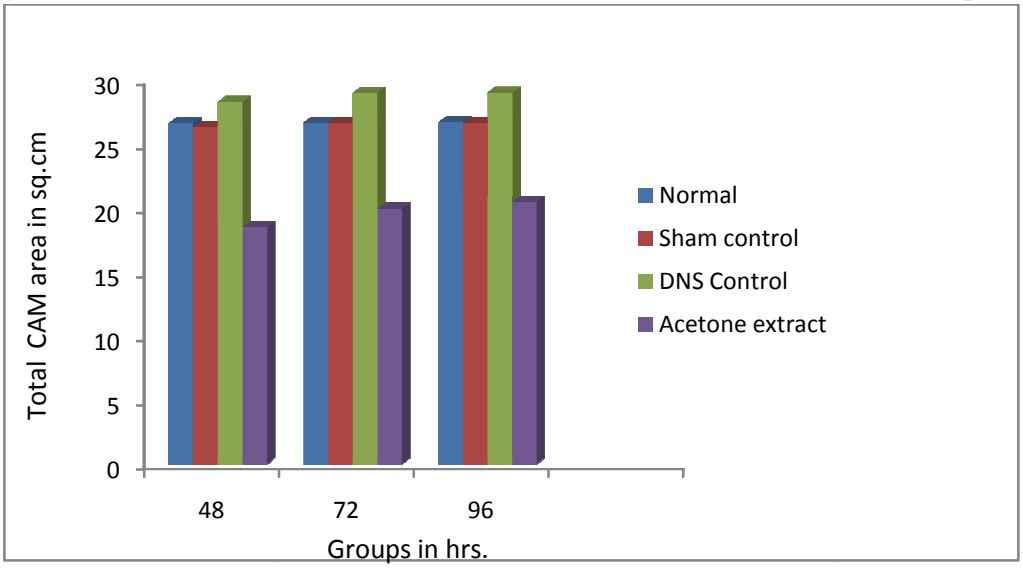


Fig. 2 : *B. sensitivum* (leaf) extract influenced alterations in total area of CAM (on 144hrs of development)



The sham controlled embryo CAM showed slight decrease in endothelial blood vessels along with mesodermal capillary plexus (Plate 2-C). Whereas in DNS control embryo number of capillary plexus along with blood vasculature were significantly increased in the mesodermal layers (Plate 2-C).

The *B. sensitivum* plant belongs to family 'Oxalidaceae' commonly known as 'Lajwanti' in Marathi. The plant leaves are sensitive, flowers dimorphic with erect stem, style nearly glabrous[29]. In eastern Nepal leaves of *B. sensitivum* plants are commonly known as "Nagbeli" against Diabetes mellitus[40,44]. While it's whole plant extract used against snake venom activity[15].

Amentoflavone is a biflavonoid isolated from plants such as *Origanum majorana, Cnestis ferruginea, Torreya nucifera Siebold* showed antioxidant, anti-inflammatory activity[2,24]. Biflavonoids like both amentoflavone and cupressuflavone compound have been reported to possess many biological activities such as antibacterial, antivenom, antioxidant, anticancer and anti-inflammatory activities[27,52]. It is also reported that amentoflavone can decrease the production of cytokines such as tumor necrosis factor (TNF-α) and arachdonate in tumor associated macrophages[31,56].

Flavonoids specifically play an important role in the process of anti-angiogenesis[41]. Many polyphenol, amentoflavone and flavonoids inhibit the process of carcinogenesis and tumorigenesis in animal experiment[8,23].

According to Melkomian and it's coworkers[37] the thickness of CAM along with number of capillary plexus increased by angiogenic substances such as suramin and cytochalastin. Phytochemically *B. sensitivum* (leaf) are rich in beneficial compounds which include two biflavonoids such as cupressuflavone and amentoflavone[33], tannins[25], saponins and some phenolic compounds[6].

Amentoflavone isolated from *B. sensitivum* inhibit tumor directed angiogenesis by disrupting endothelial cells and endogenous factors such as VEGF, TNF-α, GM-CSF, IL-1B and IL-6 required for neovascularization, which is responsible for inhibition of tumor growth and metastasis[17,21]. Amentoflavone has potential to activate the proliferation of lymphoid cells and effector cells by increasing the production of IL-2 and IFN –γ and could inhibit the proliferation of tumor cells[18]. The antiangiogenic qualities of amentoflavone is governed by distracting the integrity of endothelial cells by modulating the endogenous factor required for neovascularization and tumor metastasis[20,21].

It is clearly elucidated that *B. sensitivum* (leaf) extract significantly inhibits the development of capillary networks in CAM. In this direction, *B. sensitivum* extract exhibit a strong anti-angiogenic activity. It may have the potential to be useful deactivator of numerous serious diseases characterized by regulated angiogenesis.

Medicinal plants still remains as thriving source of life-saving drugs due to non-toxicity and non-side effects for the large majority of people treating with severe health problems. The anti-angiogenic activity

of *B. sensitivum* attributed due to phytoconstituents present in it. It could be either due to individual or the additive effects of the constituents.

CONCLUSION AND RECOMMENDATIONS

From quantitative, macroscopic and microscopic analysis it was observed that, the use of *B. sensitivum* (leaf) extract has potential anti-angiogenic property. The average branch point in the chick CAM was decreased in a dose dependent manner. The *B. sensitivum* (leaf) extract preventing signaling of angiogenesis from epithelial cells and thus significantly inhibits development of capillary networks in CAM. It has been concluded that *B. sensitivum* (leaf) extract has a promising source of chemo-therapeutic agents against tumors. The phytoconstituents of these plants actively explored as a source of new chemical drugs that can inhibit the process of angiogenesis.

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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