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Antiangiogenic properties of *Boerhaavia diffusa* extracts in chick Chorioallantoic Membrane (CAM)

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Abstract

Boerhaavia diffusa is used in Ayurvedic medicine system to treat various health problems. Present work deals with analysis of the antiangiogenic properties of *B. diffusa* extracts by chicken chorioallantoic membrane (CAM) assay *in vivo*. The extracts of *B. diffusa* prepared in acetone, alcohol and benzene were tested administering at 48, 72 and 96 hrs of incubation to observe angiogenesis of the CAM at 144 hrs of development. These extracts of *B. diffusa* reduced neovascularization. Acetone- extract showed highest inhibitory activity in angiogenic response; followed by benzene and alcohol extracts. The quantitative and microscopic analysis indicate that these results indicated inhibition elongation and proliferation of both secondary and tertiary vessels. It seem to be consequences of interference of extracts in a) signaling of angiogenic agents from epithelial cells or b) cellular apoptosis, which in its absence results in normal CAM angiogenesis. This observations support strong claims of antiangiogenic ethnomedical properties of this plant.

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INTRODUCTION:

Angiogenesis, the formation of new blood vessels from pre-existing capillaries and circulating endothelial precursors, plays a critical role in a various physiological and pathological processes such as embryonic development, wound healing, chronic

inflammation, tumor growth, and metastasis[1-2]. Angiogenesis proceeds by a series of steps that include endothelial cell activation and breakdown of the basement membrane, followed by the migration, proliferation, and tube formation of endothelial cells [3]; which are regulated by numerous factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), tumor necrosis factor-α (TNF-α), and interleukin-8 (IL-8)[4]. In search of proangiogenic antiangiogenic drugs, numerous bioactive plant compounds and dietary products have been tested and are being tested for their antiangiogenic potentials [5]. CAM assay is more often used for proangiogenic and antiangiogenic property evaluation of different materials [6]; which is also used in present studies.

Boerrhavia diffusa the plant used in present investigation (Family; Nictaginaceae) is mainly an herbaceous creeping weed commonly known as punarnava and is widely distributed in the tropical and temperate region of the world [7]. It exhibits a wide range of medicinal properties as per Ayurvedic claims. The whole plant of *B. diffusa* has been employed for the treatment of various disorders like liver disorders, gastrointestinal disorders, and heart diseases. In earlier studies of some groups it has shown to have laxative, diuretic, antiurethritis, anticonvulsant, antifibrinolytic, antinematodal and antibacterial properties [8-12]. The plant has also been screened for anti-inflammatory, antimicrobial, immunosuppressive, hepatoprotective, antitumorogenic, antileprotic and antiasthmatic activities [13-17]. The whole plant analysis of *B. diffusa* is known to contain numerous phytochemicals constituents that include flavonoids, alkaloids, triterpenoids, steroids, lipids, lignins, tannins, phlobaphenes and ursolic acid [18-21]. Studies on its different extracts i.e. Hexane, chloroform and ethanol extracts of *B. diffusa* had shown to block the activation of DNA binding of nuclear factor-KB and AP-1, two major transcription factors centrally involved in expression of the IL-2 and IL-2R gene, that are necessary for T cell activation and proliferation [22-23]. *B. diffusa* extracts were also able to attenuate the proliferation, migration and differentiation of endothelial cells. Besides, *B. diffusa* plant showed much higher inhibition of O_2^- production [24]. Thus though plant has been screened for bioactivities its angiogenic potential remained to be studied. Our present studies as a part of our search for natural product-based antiangiogenic agents, include the antiangiogenic activity of acetone, alcohol and benzene extracts of *B. diffusa* (1mg/ml) by chicken chorioallantoic membrane (CAM) *in vivo*. The detailed evaluations of quantitative and histological analysis of the alterations in angiogenesis influenced by the extracts have been provided in following results. In earlier work from this laboratory in the mortality studies of these extracts safe doses have been already noted [25]. Which have been used for present studies.

MATERIALS AND METHODS

Plant material and preparation of extracts:

The properly identified whole plants of *Boerrhavia diffusa* were collected from the local areas of Kolhapur district, Maharashtra, India. It was shed dried for a week in shadow and blended in fine powder. The powder (10gm) was extracted by routine methods to get acetone, alcohol and benzene extracts. Each of the extracts was concentrated by evaporation by using high-speed vacuum evaporator (Buchi type). The yield of acetone, alcohol and benzene extract was 18.1%, 17.5% & 11.3% respectively. The dried samples of extract were dissolved in Hanks Balanced Salt Solution (HBSS-HIMEDIA, India) to prepare the stock solutions for use.

Chorioallantoic membrane (CAM) assay: (*In Vivo*)

The chick chorioallantoic membrane (CAM) assay was used to detect antiangiogenic activity of *B. diffusa* extracts. Fertilized eggs of *Gallus gallus murghi* were obtained (from Assistant commissioner of animal husbandry, central hatchery, Kolhapur, Maharashtra, India). The shells of fertilized eggs were disinfected and incubated in aseptic egg incubators. Extracts administration hours 48, 72 and 96 were selected as per the development of CAM and vitelline veins. The eggs were grouped as per initiation hrs of doses and were incubated at 37.5°C temperature with relative humidity of 70-75%. The groups were maintained independently for acetone, alcohol and benzene extracts doses **(Table 1).** The treatments of doses were initiated at hours stated above and the administration of doses was conducted by the window method [26] and development was continued for 144-hrs.i.e.on completions of CAM venation and capillary networking.

The doses of acetone, alcohol and benzene extracts of *B. diffusa* were selected on the basis of earlier work on mortality studies [27]. The dose that showed 100% survival without any abnormalities on hatching was selected in each case of the extracts used.

On completion of scheduled period of incubations the windows were prepared in embryos under aseptic conditions and *B. diffusa* extracts' doses were spread on embryonic plates in different embryos of experimental groups in final volume of 1ml HBSS. Normal group of embryos were maintained as normal group. Embryos of operative control group were sham operated for windows preparations and embryos of HBSS control group received 1ml HBSS/embryo as spread on embryonic plates. The windows made for administration were sealed with sterilized adhesive tapes and the embryos were immediately transferred to incubators to continue further incubation hrs adjusting the experimental time slot until completion of 144 hrs.

Table 1: Exposure Schedule of treatment of *B. diffusa* extracts at different development stages of chick embryo in hrs.

Groups according to developmental stages in hrs		Groups according to time of exposure to treatment			Final development in hrs
		48	*72*	*96*	
I	48	-	-	✓	144 hrs
II	*72*	-	✓	-	
III	*96*	✓	-	-	

In addition to above intervals mentioned in table; 55, 66 and 88 hrs were also used to initiate the treatment; but results were similar incase of 48 and 55hrs, 72 and 66 hrs, 88 and 96hrs. Therefore the data of only hrs given in Table 1 is presented. On 144 hrs of incubation, the shells were removed and the embryo and yolk were gently removed in the glass plate with PBS containing glycerin. The developing vasculature on CAM was imaged with a digital camera and exported to a computer for image analysis. The same was confirmed with direct stereomicroscopic observations of well spread embryonic plates on glass slides of appropriate dimensions.

Quantification of Angiogenesis:

Angiogenesis was quantified by focusing on morphometric and microscopic parameters as described in our previous work [27]. Secondary and tertiary capillaries were evaluated for antiangiogenic response. For measurement of area covered by different veins bifurcation points were used as initiation and termination markers. Area was measured on microscope and was confirmed by using graph transparencies. The observed alterations are presented in **Table 2 &3 and Figs 1&2.**

Histological preparation:

For histological evaluation, the CAM tissues removed from chicken embryos were fixed in 10% buffered neutral formaldehyde and Calcium Acetate

Formalin for 8 hrs and were processed for light microscopy as per Thompson [28].The membrane was immersed on increasing concentrations of ethanol for dehydration and was embedded in paraffin (melting point 58-60°C). Serial sections (6 µm) were cut in a plane parallel to the surface of the CAM and further processed for stained preparation of haematoxylin-eosin, as per Thompson [28].which was observed under a light photomicroscope.

Statistical analysis:

The data was expressed as Mean ± SEM and the statistical significance between groups was analyzed by using I-way ANOVA; fooled by student't' test. The values of $p<0.05$, $p<0.01$, $p<0.001$ were considered as significant.

RESULTS:

Macroscopic and histological evaluation:

The data is presented in whole mounts of embryos in microphotographs in plate 1-3. Macroscopic observation of normal and sham control CAM at 144 hrs of development showed normal angiogenesis with dendritic branching pattern of blood vessel formation at 144 hrs of development (**Plate 1A&B**). HBSS treated control CAM shows hardly any difference from natural CAM at early hrs of administration but showed numerous large and small blood vessels with virtually no disturbance of CAM structure at 72 hrs (**Plate 2A**). A dense capillary network appeared at 96th hrs of HBSS administration. It induced tortuosity to many of the smaller vessels and abundant blood vessel sprouting (**Plate 3A**). With the treatment of acetone extracts of *B. diffusa;* concentration of 1mg/ml caused a dramatic, inhibition of blood vessel numbers and branching pattern at all hrs of treatment studied but more significantly at 48 hrs of treatment where complete suppression of the angiogenesis was evidenced, which was seen as a capillary free background. It suppressed new blood vessel development not only in the treatment area but also in the surrounding area (**Plate 1D**). A similar inhibitory effect was also noted in CAM treated by alcoholic extract of *B. diffusa* at 48 hrs but at 72 and 96 hrs of treatment pre-existing and large sized vessels were not affected (**Plate 2C&3C**). Antiangiogenic effect was also observed in similar conditions with doses of benzene extract treatment where the vascular structure of the CAM was completely altered. It displayed marked hyperemia and hemorrhage significantly at 48 hrs of treatment (**Plate 1F**). It also induced the formation of avascular zones on the CAM.

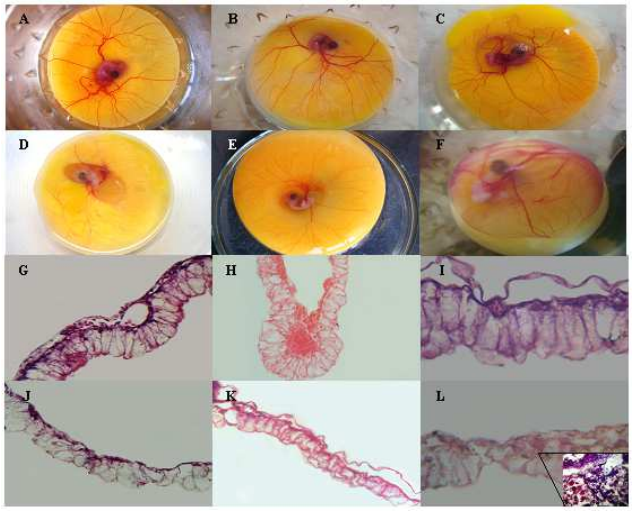

Plate 1. Antiangiogenic effect of *B. diffusa* at 48 hrs of treatment in chick CAM. A&G-Natural CAM, B&H-Sham control CAM. C&I-HBSS treated control CAM. D&J-Acetone extract treated CAM. E&K-Alcohol extract treated CAM. F&L- Benzene extract treated CAM.

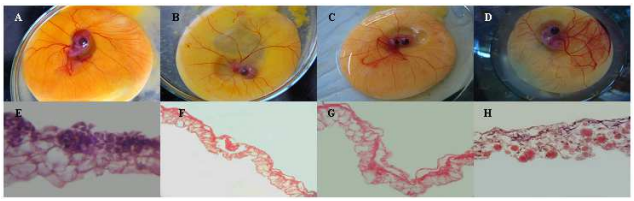

Plate 2. Antiangiogenic effect of *B. diffusa* at 72 hrs of treatment in chick CAM. A&E-HBSS treated control CAM. B&F-Acetone extract treated CAM. C&G-Alcohol extract treated CAM. D&H-Benzene extract treated CAM.

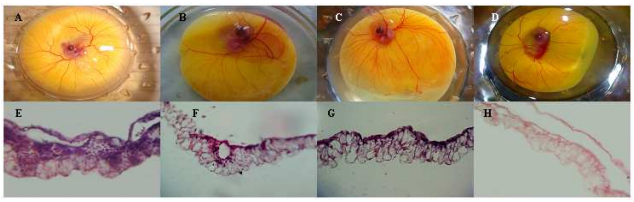

Plate 3. Antiangiogenic effect of *B. diffusa* at 96 hrs of treatment in chick CAM. A&E-HBSS treated control CAM. B&F-Acetone extract treated CAM. C&G-Alcohol extract treated CAM. D&H-Benzene extract treated CAM.

The angiogenic inhibitory activity of *B. diffusa* extracts was also confirmed by the histological observations. Normal and sham control CAM on 144 hrs of development consists of three layers (1. ectoderm, 2. mesoderm, 3. endoderm). It displays large vein in the mesoderm of the chorioallantoic membrane and thin walled capillaries just beneath the chorionic epithelium are filled with red blood cells (**Plate 1G&H**). CAM treated with HBSS, appeared well vascularized where the blood vessels were well formed and most of the ectoderm had a well-formed capillary plexus beneath the ectoderm directly touching it at all hrs of treatment **(Plate 1IA).** It also showed numerous mesodermal vessels at 96 hrs of treatment **(Plate 3A).** The pericytes surrounding the vessel appears normal, and the allantoic epithelium appears relaxed. Treatment of acetone extracts of *B. diffusa* at 48 hrs of CAM development induced a strong decrease in endothelial cell proliferation of CAM vasculature with obliteration of main blood vessels. It also caused the disproportionate thinning of all primary blood vessels (**Plate 1J**). A reduction in the total area and diameter of the primary veins was also observed after treatment with alcohol and benzene extracts **(Plate 1K&L).** It also revealed the loss of ectodermal and mesodermal integrity with both types of treatments. At 72 hrs of treatment, no blood vessels are recognizable beneath the chorion and in the mesoderm of CAMs treated with acetone extract of *B. diffusa* **(Plate 2F).** In CAM treated with alcohol extract (1mg/ml) showing no blood vessels beneath the ectoderm and in the intermediate mesenchyme, loosely arranged fibroblasts **(Plate 2G).** A similar inhibitory effect was observed when benzene extract was administered at 72 hrs of treatment **(Plate 2H).** At 96 hrs of treatment normally developed blood vessels were detectable in the alcohol extracts treated CAM. But in acetone and benzene extracts treated CAMs no blood vessels were recognizable beneath the chorion and in the mesoderm of CAMs **(Plate 3F&H).**

Effect of *B. diffusa* on number and area of secondary blood vessels in chick CAM:

The data is presented in whole mounts of embryos in microphotographs in **Plate1-3**. Quantitative data is presented in **Tables 2** and **Figs 1a&b.**Data of right hand side and left hand side differed in normal and also in experimental conditions. Left hand side remained always low or similar as in right hand side except at 96 hrs when it is high or marginally high, therefore in results and discussion only total number and areas of blood vessels/capillaries are considered.

In normal embryos secondary vessels' number with respective area covered remained nearly constant at 48, 72 and 96 hrs of treatment. In sham control embryos number of secondary vessels was nearly same as observed in normal but decreased slightly at 72 hrs of development. HBSS treatment at 48 hrs did not alter the number significantly but same treatment given at 72 hrs stimulated the number of secondary vessels significantly. These results indicate enhancement of proliferation of secondary vessels along with relative area covered by them. The same pattern showed with progressive advancement at 96 hrs of development where it was marginally high over normal. Treatment of acetone extracts of *B. diffusa* to embryos showed reduction in the number and area associated with it, at all hrs of

treatment but more significant reduction in both the categories was noted in the embryos treated at 48 hrs of treatment (38.8% and 33.3% respectively). This indicates that at early hrs of administration of acetone extract of *B. diffusa* influences both proliferation and extension. Secondary vessels were significantly suppressed. When alcohol extract of *B. diffusa* (1mg/ml) was given to normal embryos at 48 hrs reduced the number of secondary vessels significantly (30%) and also the area covered by them (8.57%). This trend remained same at 72 hrs in number and area (moderately significant). At 96 hrs number of the secondary vessels was normalized but associated area covered by them was decreased by 8.25%. By the treatment of benzene extract to normal embryos, similar inhibitory observations were observed but the influence appeared strong at early hrs of treatment (48hrs) where it showed significant suppression of secondary blood vessels (16.6%) with continued decrease in area occupied by them (26.8%). At 72 and 96 hrs of treatment also, moderate reduction in the number and area associated of secondary vessels was noted i.e. benzene extracts seems to inhibit proliferation of secondary vessels at this hrs of administration or stages initiated and or modified that are set in at this hr of development.

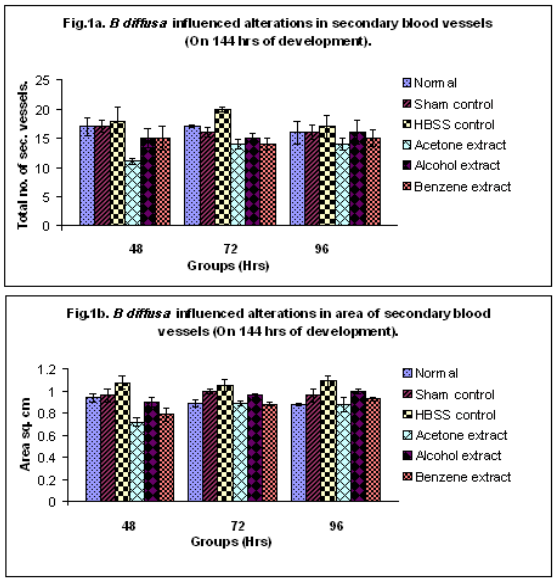

Fig.1a&b. *B. diffusa* influenced alterations in secondary blood vessels and area occupied by them on 144 hrs of development at 48, 72 and 96 hrs of initiation treatment.

Table 2: Effect of *B. diffusa* extracts on secondary blood vessels on chick CAM.

Initiation of treatment (hrs)	Groups	Right		Left		Total	
		No.	Sq. cm.	No.	Sq. cm.	No.	Sq. cm.
48 hrs (1mg/ml)	**Normal**	9±1.09	0.45±0.033	8±0.67	0.49±0.04	17±1.6	0.94±0.041
	Sham control	8±0.89	0.50±0.041	9±0.78	0.46±0.043	17±1.08	0.96±0.062
	HBSS control	9±1.24	0.58 ± 0.026^{a}	9±1.02	0.50±0.03	18±2.2	1.08±0.061
	Acetone extract	6 ± 0.37^{ax}	0.38 ± 0.028^{px}	5 ± 0.31^{bqy}	0.34 ± 0.036^{ay}	11 ± 0.5^{brx}	0.72 ± 0.036^{bqz}
	Alcohol extract	9±1.36	0.44 ± 0.020^{y}	6 ± 1.14^{p}	0.46±0.021	15±1.7	0.90 ± 0.043^{x}
	Benzene extract	7±1.01	0.38 ± 0.023^{pz}	5 ± 1.05^{apx}	0.41 ± 0.026^{x}	15±2.03	0.79 ± 0.062^{y}
72 hrs (1mg/ml)	***Normal***	9±0.43	0.47±0.023	8±0.45	0.42±0.033	17±0.18	0.89±0.031
	Sham control	9±0.20	0.50±0.032	7±0.36	0.50±0.03	16±0.82	1.00 ± 0.021^{a}
	HBSS control	10±0.65	0.53±0.026	10±1.62	0.52±0.047	20 ± 0.45^{c}	1.05 ± 0.052^{a}
	Acetone extract	7 ± 0.31^{brx}	0.42±0.043	7±0.75	0.47±0.055	14 ± 0.81^{az}	0.89 ± 0.023^{ax}
	Alcohol extract	7±1.03	0.48±0.031	8±1.06	0.48±0.024	15 ± 0.73^{az}	0.96±0.019
	Benzene extract	8±0.58	0.46±0.071	6±1.03	0.42±0.038	14 ± 1.01^{az}	0.88 ± 0.018^{qx}
96 hrs (1mg/ml)	***Normal***	7±0.97	0.46±0.069	9±1.65	0.42±0.027	16±1.99	0.88±0.013
	Sham control	8± 0.62	0.52±0.028	8±1.0	0.44±0.055	16±1.20	0.96±0.062
	HBSS control	9±1.24	0.50±0.039	8±1.64	0.59 ± 0.029^{bp}	17±1.93	1.09 ± 0.048^{b}
	Acetone extract	7±1.39	0.42±0.076	7±1.2	0.46±0.089	14±1.02	0.88 ± 0.068^{x}
	Alcohol extract	7±1.06	0.48±0.012	9±1.24	0.52±0.072	16±2.09	1.0 ± 0.024^{b}
	Benzene extract	6±1.53	0.49±0.019	9±0.50	0.44 ± 0.052^{x}	15±1.46	0.93 ± 0.019^{x}

(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.*HBSS control embryo).

Effect of *B. diffusa* on number and area of tertiary blood vessels in chick CAM:

The data is presented in whole mounts of embryos in microphotographs in **Plate 1-3.** Quantitative data is presented in **Table 3** and Figs **2a&b.**

In normal embryos tertiary blood vessels' number was 172 and associated area was 1.16 sq. cm. At early hrs (48 hrs) of development of embryo, HBSS treatment did not alter the total number of tertiary vessels with marginal increase in the associated area. These observations indicted that the HBSS administration stimulated the elongation of these vessels but proliferation of the vessels remained unaffected at early hrs of treatment. Same treatment given at 72 and 96 hrs indicated significant increase in the number of veins with its associated area. At this hrs of intervals CAM tissues of embryos seem to respond to the factors in extracts.

Initiation treatment of acetone extract of *B. diffusa* had reduced the total number and total area covered by tertiary vessels by 27.9% and 21.5% respectively. As compare to the HBSS treated control embryos reduction in the number of tertiary vessels and associated area was 30.3% and 24.1% respectively. This indicates that at early hrs of treatment of acetone extracts of *B. diffusa* both proliferation and extension of vessels was significantly suppressed. Treatment of 1mg alcohol and benzene extract of *B. diffusa* had also decreased the total number of vessels (13.9% and 23.8% respectively) and area associated with them (18.9% and 14.6% respectively). As compared to the HBSS control embryos' results showed significant reduction in vessel number (16.8% and 26.4%), while reduction in area observed was normal. Similar type of inhibitory response was observed when the experimental schedule was initiated at 72 hrs but more significant angiogenic inhibitory response was noted with acetone extract of *B. diffusa* at all hrs of initiation variation studies. At 72 hrs of acetone extracts treatment initiation significant reduction in total number of tertiary vessels and area associated with them was noted (by 28.2% and 29.05% respectively). While initiation of alcohol extract at this hrs showed reduction in number of vessels and area covered by them was 17.6% and 22.9% respectively. Administration of benzene extract also showed depletion in number of tertiary vessel (24.2%) and area covered by them (27%). At 96 hrs of treatment HBSS stimulated the area covered very significantly (26%) by promoting vessel growth and cell proliferation more significantly at late hrs of treatment. Thus it seems that at 96 hrs HBSS is stimulatory for length increase of tertiary vessels (1.14 fold increase). Treatments of acetone extract of *B. diffusa* initiated at 96 hrs reduce the total number of tertiary vessels (23.15%) and the area covered (14.28%) significantly. Treatment of alcohol and benzene extract also inhibited the total number (5.7% and 19.4% respectively) and area (12.6% and 14.2%) covered but less significant as compared to the acetone extract of *B. diffusa* plant.

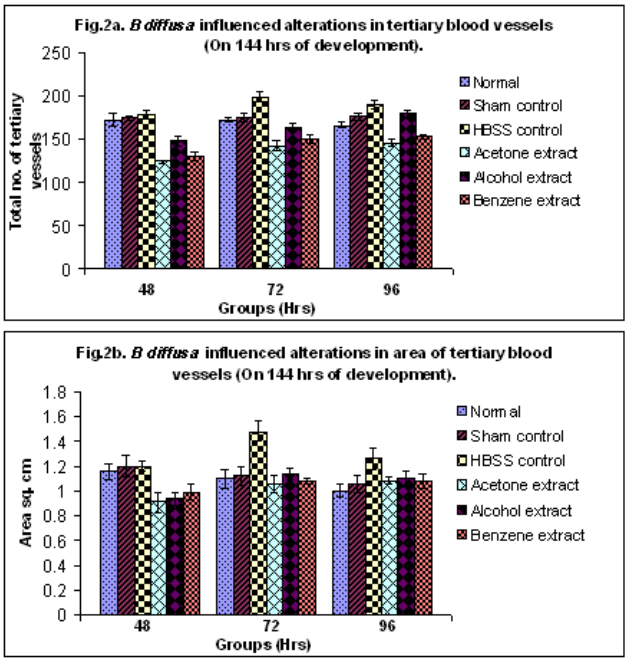

Fig.2a&b. *B. diffusa* influenced alterations in tertiary blood vessels and area occupied by them on 144 hrs of development at 48, 72 and 96 hrs of initiation treatment.

Table 3. Effect of *B. diffusa* extracts on tertiary blood vessels on chick CAM.

Initiation of treatment (hrs)	Groups	Right		Left		Total	
		No.	Sq. cm.	No.	Sq. cm.	No.	Sq. cm.
48 hrs	**Normal**	94±4.22	0.62±0.033	78±3.21	0.54±0.021	172±7.00	1.16±0.06
(1mg/ml)	***Sham control***	88±2.41	0.64±0.022	86±1.92	0.56±0.019	174±2.10	1.20±0.084
	HBSS control	98±3.35^{p}	0.64±0.034	80±3.76	0.56±0.084	178±4.71	1.20±0.041
	Acetone extract	63±2.55^{crz}	0.39±0.021^{crz}	61±1.60^{bry}	0.52±0.041	124±1.59^{crz}	0.91±0.08^{apx}
	Alcohol extract	82±5.08^{x}	0.48±0.022^{bry}	66±1.93^{arx}	0.46±0.04	148±5.6^{aqy}	0.94±0.054^{apy}
	Benzene extract	72±3.62^{bqz}	0.52±0.026^{aqx}	59±1.58^{crz}	0.47±0.021^{p}	131±4.87^{brz}	0.99±0.062^{x}
72 hrs	***Normal***	94±3.57	0.58±0.029	78±3.76	0.52±0.045	172±3.16	1.10±0.072
(1mg/ml)	***Sham control***	88±1.22	0.60±0.034	86±2.14	0.52±0.028	174±4.6	1.12±0.078
	HBSS control	97±1.86	0.76±0.024^{bq}	101±4.6^{bp}	0.72±0.012^{br}	198±5.73^{bp}	1.48±0.081^{bp}
	Acetone extract	72±1.95^{crz}	0.56±0.014^{z}	70±3.71^{qz}	0.49±0.033^{z}	142±5.47^{bqz}	1.05±0.071^{y}
	Alcohol extract	84±4.12^{x}	0.60±0.064^{x}	79±1.82^{y}	0.54±0.036^{y}	163±5.43^{y}	1.14±0.041^{y}
	Benzene extract	78±3.32^{apy}	0.58±0.051^{x}	72±1.01^{rz}	0.50±0.052^{y}	150±3.96^{bqz}	1.08±0.026^{y}
96 hrs	***Normal***	94±4.27	0.46±0.025	72±1.98	0.54±0.033	166±3.63	1.0±0.054
(1mg/ml)	***Sham control***	98±2.98	0.52±0.032	78±2.65	0.54±0.019	176±4.52	1.06±0.074
	HBSS control	104±2.45	0.62±0.041^{a}	86±2.27^{b}	0.64±0.036^{p}	190±4.30^{b}	1.26±0.084^{a}
	Acetone extract	81±3.66^{aqz}	0.52±0.014^{x}	65±3.45^{pz}	0.56±0.021	146±3.82^{brz}	1.08±0.032
	Alcohol extract	93±2.01^{y}	0.58±0.012^{b}	86±2.0^{bp}	0.52±0.017^{x}	179±3.33^{a}	1.10±0.062
	Benzene extract	82±1.80^{aqz}	0.52±0.026	71±1.88^{z}	0.56±0.042	153±2.46^{aqz}	1.08±0.06

(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*. HBSS control embryo).

DISCUSSION:

Using chick CAM model, the new pharmacological effects of *B. diffusa* have been confirmed by the proven inhibition of angiogenesis. We showed that *B. diffusa* plant extracts had significant antiangiogenic activity at all hrs of treatment studied; where these extracts of *B. diffusa* reduce neovascularization of the CAM. Out of three solvents used for extraction, the acetone extracts showed the highest inhibitory activity in angiogenic response, followed by benzene and alcohol extracts. Treatment of HBSS did not affect the formation of vascular network of the CAM. Therefore, *B. diffusa* extracts seems to specifically inhibit the microvascular formation that occurs normally during embryogenesis.

Treatment of acetone and benzene extracts of *B. diffusa* suppressed normal branching of blood vessels in the developing CAMs at all hrs of treatment but more significantly at early hrs of treatment resulted in frequent avascular zones. This may be due to the induction of apoptosis by phytocompounds in *B. diffusa* as observed in histological screening. It also inhibited the migration of mesodermal blood vessels to the ectoderm and the subsequent formation of the capillary plexus at early hrs of (48,55& 66 hrs) doses. The absence of neovascularization in these extracts treated CAM suggests that *B. diffusa* exert inhibition of normal CAM angiogenesis. No thrombosis, hemorrhage or marked distortion of large primitive blood vessels were observed at late hrs (72, 88&96 hrs) of

treatment. Alcohol extract of the same plant showed inhibitory activity at early hrs of treatment only.

Several growth factors like VEGF and FGF-2 and signaling pathways play a role in angiogenesis [29-31]. It appears that the anti-angiogenic action of this plant might be due to the inhibition of VEGF and bFGF signaling. Thus through this extract seem to influence the suppression of secondary and tertiary blood vessel proliferation and extension and inhibited the natural process of CAM angiogenesis.

The antiangiogenic property of *B. diffusa* may be attributed to the phytoconstituents present in the plant. It could be function of either the individual or the additive effects of the phytoconstituents. Many polyphenols, terpenoids and flavonoids have shown to inhibit carcinogenesis and tumorigenesis in animal experiments [32-33] and inhibit proliferation and angiogenesis of tumor cells in vitro [34-36]. The flavonoids specifically have been reported to play such roles [37]. Flavonoids and triterpenoids isolated from *Ginkgo biloba* inhibited angiogenic activity by down regulating VEGF [38-39]. It is also reported that two triterpenoids isolated from *Rabdosia rubescens* possesses antiangiogenic activity[40]. Moreover, several alkaloids are considered to be antiangiogenic natural products [41-45]. Furthermore, *B. diffusa* plant showed much higher inhibition of O_2^- production [24]. It has recently reported that reactive oxygen species induce migration and proliferation of endothelial cells [46]. Antioxidants serve as a potent inhibitor of angiogenesis [47]. This property of *B. diffusa* might have played a synergic role in the inhibition of CAM angiogenesis as observed in the present investigation. It is also clear that the *B. diffusa* extracts were also able to attenuate the proliferation, migration and differentiation of endothelial cells.

CONCLUSION:

From quantitative, macroscopic and microscopic analysis it was observed that *B. diffusa* has antiangiogenic potential. This property of *B. diffusa* extracts might have been executed either by preventing signaling of angiogenic agents from epithelial cells or by induction of apoptosis, preventing promotional events in the CAM tissue through free radical scavenging mechanism. However, further studies are required to elucidate the exact mechanism underlying the antiangiogenic property of *B. diffusa*. The strong antiangiogenic properties of this plant support the ethnomedical claims for the plant. It is tempting to speculate that *B. diffusa* extract might contribute to the long known preventive effect of a plant based direct on chronic neovascular diseases including solid tumor growth.

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