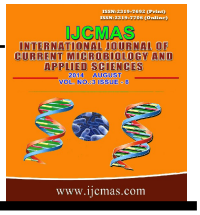
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 8 (2014) pp. 107-114**
http://www.ijcmas.com



Original Research Article

Antiangiogenic activity of *Boucerosia diffusa* and *Boucerosia truncato-coronata* extracts in chick Chorioallantoic Membrane (CAM)

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A B S T R A C T

Keywords

Antiangiogenic activity Boucerosia diffusa,

Antiangiogenic activity of crude ethanolic, methanolic, ethyl acetate and chloroform extracts of *Boucerosia diffusa* and *B. truncato-coronata* were assessed through *in vivo* CAM assay. Chloroform and ethanolic extract of *B.diffusa* showed higher angiogenic activities (45.7 and 44.4 respectively) and inhibition of vessel number. In *B. truncato-coronata,* higher angiogenic activity 65.90 and less vessel number was observed in methanolic extract. *B. diffusa* and *B. truncato-coronata* extracts inhibited angiogenesis by blocking normal vascularization

Introduction

Angiogenesis is the formation of new blood vessels involves the migration, growth, and differentiation of endothelial cells, which line the inside wall of blood vessels. The process of angiogenesis is controlled by chemical signals in the body. These signals can stimulate both the repair of damaged blood vessels and the formation of new blood vessels. Other chemical signals, called angiogenesis inhibitors, interfere with blood vessel formation. Normally, the stimulating and inhibiting effects of these chemical signals are balanced so that blood vessels form only when and where they are needed (Auerbach et al., 2003).

The family Asclepiadaceae is commonly known as Milky weed family and found in tropical regions of the world. About 2900 species belonging to 345 genera are present in the world. A total of 332 species (35 genera) and 48 species (28 genera) reported from India and Andhra Pradesh respectively. The genus *Caralluma* comprises about 200 genera and 2,500 species. The generic name is derived from the Arabic word-gahral-luhum meaning wound in the flesh or abscess referring to the floral odours. The member of this genus is a small erect fleshy plant which has four grooved round shaped stems devoid of leaves with small flowers of

several dark colours. Most of the species occur in Africa including several taxa valued by people for their medicinal properties, one species *Caralluma edulis* eaten as vegetable. The species of *Caralluma* found in India are edible and forms a part of the Traditional Medicine System of the country (Deepaket al.,1997). A total of 13 species and 7 varieties of *Caralluma* occur in India out of which 11 species are solely endemic to South India (Jagtap and Singh, 1999).

Caralluma diffusa (Wight) N.E.Br is rare and an endemic medicinally important plant belonging to the family Asclepiadaceae (Henry et al.,1979). *C.diffusa* was first collected by Robert Wight, and named by him as *Boucerosia diffusa*, from Coimbatore in 1850. After 160 years it was rediscovered from Southern Western Ghats of Coimbatore district, Tamil Nadu, India (Ramachandran et al.,2011). The Flora of Tamil Nadu states that *C.diffusa* distribution is only from Coimbatore district in Tamil Nadu and ecological status is mentioned as rare and threatened species (Srinivasan, 1987). Distribution of this species as Deccan, arid rocky hills near Coimbatore at an elevation of about 600m (Gamble and Fischer, 1923).This plant is usually seen in rock crevices. The local people use the sap of young stems to treat obesity. This wild plant is well suited for rock gardens and it can be conserved through *ex situ* cultivation. This species is unique for its diffuse branches, very stout and attractive flowers in dense umbels (Hooker, 1885).

The *Boucerosia truncato-coronata* belongs to the family Asclepiadaceae is an important indigenous endemic medicinal herb. They are succulent, perennial herb, erect and fleshy grow to a height of up to 9.5cm seen in rock crevices and grow on different regions of India. They have quadrangular stem devoid of leaves and small flowers. Around twenty chemical constituents have been identified from ethanolic extract of the whole plant of *B. truncato-coronata*. The presence of these bioactive compounds may be responsible for medicinal properties(Kalimuthu et al.,2013).

As there is no report on antiangiogenic activity, different extracts of these plants were tested for their antiangiogenic activity by CAM assay.

Materials and Methods

Plant Collection

The fresh plants of *Boucerosia diffusa* (Wight) N.E.Br(synonym: *Caralluma diffusa*) and *Boucerosia truncato-coronata* (Sedgw.) Gravely Mayur(synonym: *Caralluma truncato-coronata*) were collected from Madukkarai hills, Coimbatore District, Tamilnadu (India) and carefully washed with tap water, rinsed with distilled water, and air dried for one hour. Then it was cut into small pieces, dried in room temperature for two weeks, grounded into powder with the help of hand mill and stored in room temperature. The whole plant powder was macerated in different solvents including methanol 95% (v/v), ethanol, ethyl acetate and chloroform in 1:3 proportion at room temperature, undergoing mechanical shaking for 4 hours followed by filtration. The extracts obtained were concentrated in a rotary evaporator at 40°C and the residue was extracted twice again analogously, there by obtaining the crude solvent extracts.

Chicken egg Chorioallantoic Membrane Assay (CAM)

Antiangiogenic activity of crude plant extracts of *B. diffusa* and*B. truncato-coronata*was conducted on fertilized eggs by modified CAM assay method (Parivash Seyfi, 2010). Fertile

white Leghorn chicken eggs (*Gallus domesticus*) were obtained from a local hatchery with 3 days incubation. The eggs were incubated at 37ºC in humified incubator for 48 h, placed in horizontal position and rotated several times. The eggs were grouped as per type of extracts and sprayed with 70% ethanol and air-dried to reduce contamination from the egg surface. On day 6, 26- gauze needle was used to puncture a small hole in the air sac of the egg, and 2-3 mL of albumen was sucked and sealed. This allows separation of vascularized CAM from the vitelline membrane and the shell. A window was then cut in the shell using a sterile blade and shell was removed with sterile forceps, under Laminar air flow. The window is closed with a cellophane tape after capturing the photographs of the embryo. The eggs were returned to the incubator after the filter paper discs (100 micrograms of extract) of ethanolic, methanolic, ethyl acetate and chloroform extracts are placed on blood vessels of embryo using sterile forceps. After 48 h of incubation on 8th day photographs of embryos were taken to obtain the image of CAM after treatment with various extracts. At least six eggs were used for each extract dose. The percentage inhibition was calculated using the following equation(Shanshan Wanga et al., 2004).

% inhibition = [(vessel number of untreated CAM-vessel number of CAM treated with herbal extract)/vessel number of untreated CAM] x 100.

Results and Discussion

Antiangiogenic activity of crude ethanolic, methanolic, ethyl acetate and chloroform extracts of *B.diffusa* and *B. truncato-coronata* samples were tested through *in vivo* CAM model. The 8th day old embryo after treatment for number of blood vessels and their reduction was examined. The analysis of blood vessel was based on the evaluation of angiogenesis by measuring the area of inhibition surrounding the applied disc. The *B.diffusa* inhibition percentage is shown in the table 1. Chloroform and ethanolic extracts of *B.diffusa* showedthe higher angiogenic activities 45.7±9.99 and 44.4±9.64 (Plate 1 A &G) respectively than the methanolic and ethyl acetate extracts. In *B.truncato-coronata,* higher angiogenic activity 65.90±13.82 was observed in methanolic extract (Plate 1 D), then the other extracts. In untreated CAM the normal vascularisation with primary, secondary and tertiary micro blood vessels was observed (Plate 1, I and J). All the four extracts showed distorted vascularisation as well as perturbation of existing vasculature, percentage of inhibition and average number of vessels are represented in the table 1 and 2; Plate 1.

The inhibition percentage in *B. diffusa* and *B. truncato-coronata* ethanolic extract treated CAM was 44.4±9.64 and 32.480±11.61, methanolic extract treated CAM was 27.42±2.50 and 65.90±13.82 ethyl acetate extract treated CAM was 20.13±2.02 and 36.37±16.7 and chloroform extract treated CAM was 45.7±9.99 and 19.783±8.80 respectively (Table 1 and 2).The average number of vessels in *B. diffusa* and *B. truncato-coronata* ethanolic extract was 5 and 8 where as in untreated CAM was 9 and 12, in methanolic extract was 8 and 3 where as in untreated CAM was 11 and 9, in ethyl acetate was 8 and 5 whereas untreated CAM was 10 and 8 and in chloroform extract treatment was 7 and 8 where as in untreated CAM was 13 and 10 respectively.

From the above results the inhibition percentage and vessels number of

chloroform and ethanolic extract of *B. diffusa* was almost equal. But in *B.truncato-coronata* the ethanolic extract showed the more inhibition percentage (65.90±13.82) and less vessel number (3) when compare to all other extracts. This is followed by the ethyl acetate (36.37±16.7 and 5) and ethanol (32.480±11.61and 8) respectively.

The medicinal and pharmacological actions of medicinal herbs are often depended to the presence of bioactive compounds called secondary herbal metabolites (Bruneton, 1999; Heinrich et al.,2004). Secondary herbal metabolites with reported medicinal properties consist of waxes, fatty acids, alkaloids, terpenoids, phenolics (simple phenolics and flavonoids), glycosides and their derivatives (Satyajit et al.,2006, Eloff, 2001; Cowan, 1995).Angiogenesis is essential in tumor growth and metastasis as the process provides necessary oxygen and nutrients for the growing tumor (Folkman, 1971). The present results showed that both *B.diffusa* and *B. truncato-coronata*extracts changed the vascularization pattern; all the extracts inhibited the new blood vessels formation in the treated CAMs as well as distortion of existing vasculature.

Angiogenesis is a strictly controlled process in normal human body and regulated by a variety of endogenous angiogenic and angiostatic factors (Folkman and Klagesburn 1987). The new pharmaceutical effects of *B.diffusa* and *B. truncato-coronata* have been confirmed by the inhibition of angiogenesis through Chick CAM model. In the present study both plant extract had significant antiangiogenic activity by reducing neovascularisation of the CAM. Among the four solvent used for extraction chloroform extract of *B. diffusa* and methanol extract of *B.truncato-coronata* showed the higher percentage of inhibition in angiogenic response followed by ethanol and ethyl acetate (Muslim et al., 2012).

In contrary to this study, in Asclepiadaceae member *Ceropegia pusilla*, the ethanolic extract showed the higher percentage of inhibition (Prabakaran et al., 2014). Chloroform extract of *B. diffusa* and methanol extract of *B. truncato-coronata* suppressed normal branching of blood vessels in the developing CAM. This may be due to the induction of apoptosis by phytochemical present in these plants.The presence of phenolic compounds especially pregnane glycosides and megastimane glycosides in this *Caralluma* genus may contribute to their cytotoxic activity (Lawrence and Chaudhary, 2004). The antiproliferative activity of methanolic extract of *B. umbellata* and *B. lasiantha*was reported (Madhuri Vajha et al.,2010) which induced to evaluate antiangiogenic potential of *B. diffusa* and *B. truncato-coronata.* In the present study the methanolic extract of *B. truncato-coronata* showed the highest percentage of inhibition and suppression of blood vessels. In contrary to this in *B. diffusa* chloroform extract showed the highest percentage inhibition of blood vessels.

The angiogenic properties of *B. diffusa* and *B. truncato-coronata* may be attributed due to the phytochemical present in these plants. It might be either the individual or the collective effects of phytoconstituents. In *Caralluma* and other genera of the Asclepiadaceae family are rich in esterified polyhydroxy pregnane glycosides, exhibited anticancerous and antitumor effect (Deepak et al.,1997). This properties of *B. diffusa* and *B. truncato-coronata* might have played an important role in the inhibition of CAM angiogenesis as observed in the present study.In *B. diffusa* and *B. truncato-coronata* plant extracts from all these observations it is conformed that both the plant extracts revealed high level of antiangiogenic activity.

Table.1 Antiangiogenic activity of *in vivo* ethanolic, methanolic, ethyl acetate and chloroform extracts of *B.diffusa*

Extracts	Egg-1#	Egg-2#	Egg-3#	Average No. of vessels	Egg-1#	Egg-2#	Egg-3#	Average No. of vessels	Egg-1#	Egg-2#	Egg-3#	% inhibition (Mean ± SD)*
	No. of Vessel in untreated CAM				No. of Vessel in treated CAM				% inhibition			
Ethanol	9	8	10	**9**	6	4	5	5	33.3	50.0	50.0	44.4 ± 9.64[a]
Methanol	11	10	12	11	8	7	9	8	27.27	30.0	25.0	27.42 ± 2.50[b]
Ethyl acetate	10	11	9	10	9	8	7	8	20.0	18.18	22.22	20.13 ± 2.02[b]
Chloroform	13	12	14	13	8	7	6	7	38.46	41.66	57.14	45.7 ± 9.99[a]

Values are mean ± SD of three samples in each group
A means followed by a different superscript are significant at 5% level by DMRT

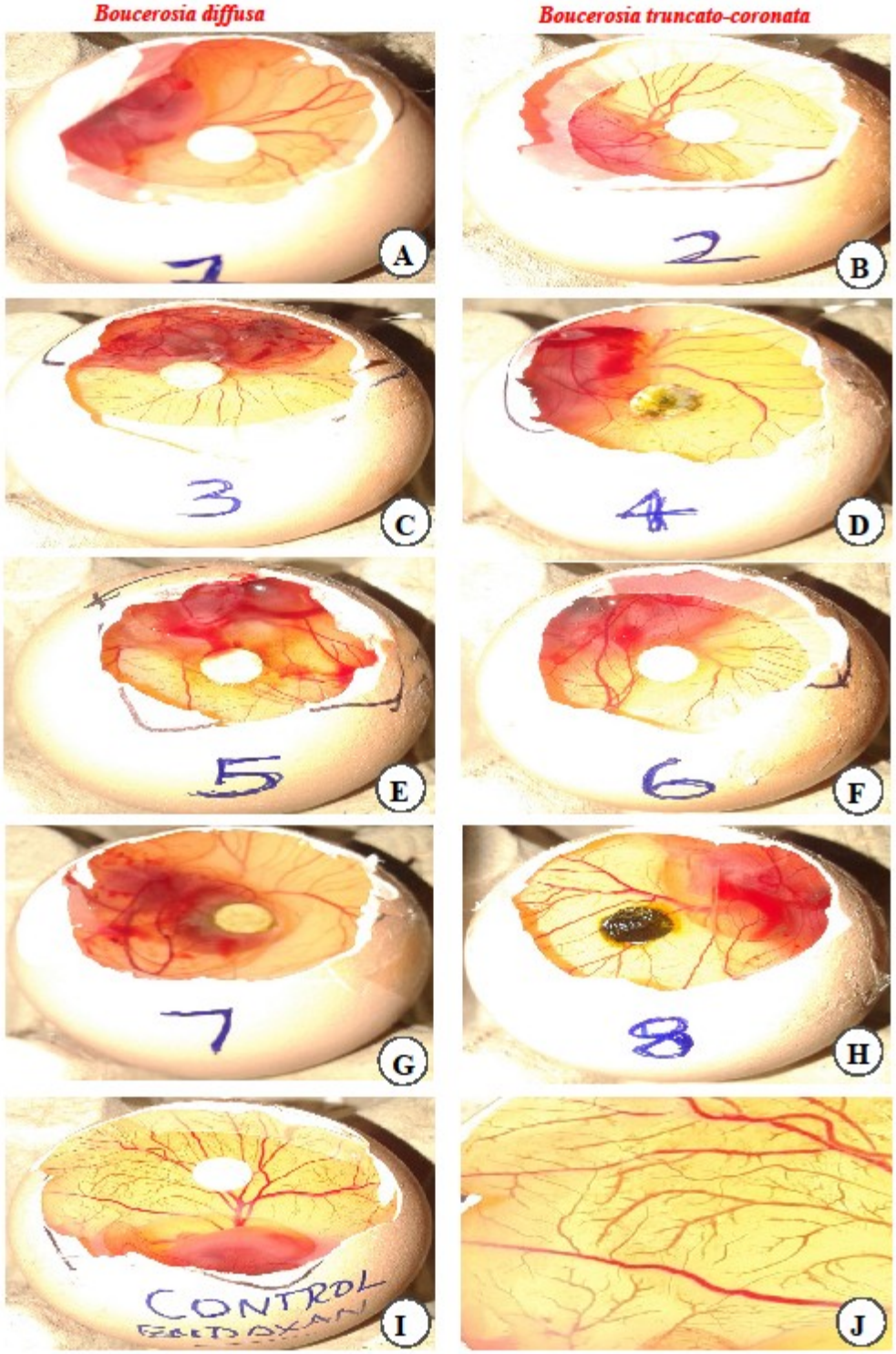
Table.2 Antiangiogenic activity of *in vivo* ethanolic, methanolic, ethyl acetate and chloroform extracts of *B. truncato-coronata*

Extracts	Egg-1#	Egg-2#	Egg-3#	Average No. of vessels	Egg-1#	Egg-2#	Egg-3#	Average No. of vessels	Egg-1#	Egg-2#	Egg-3#	% inhibition (Mean ± SD)*
	No. of Vessel in untreated CAM				No. of Vessel in treated CAM				% inhibition			
Ethanol	11	13	12	**12**	8	7	9	**8**	27.27	46.15	25.0	32.80 ± 11.61[b]
Methanol	8	8	11	9	2	4	3	3	75.0	50.0	72.72	65.90 ± 13.82[a]
Ethyl acetate	7	9	8	8	5	4	6	5	28.57	55.55	25.00	36.37 ± 16.70[b]
Chloroform	11	10	9	10	8	9	7	8	27.27	10.0	22.22	19.83 ± 8.80[b]

Values are mean ± SD of three samples in each group
A means followed by a different superscript are significant at 5% level by DMRT

PLATE – 1. Antiangiogenic activity of *Boucerosia diffusa* and *B.truncato-coronata* in chick CAM

A& B . Ethanolic extract. C& D . Methanolic extract.
E& F. Ethyl acetate extract. G& H. Chloroform extract. I & J. Control



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