



Antiangiogenesis and Anticancer Activity of Leaf and Leaf Callus Extracts from *Baccharoides anthelmintica* (L.) Moench (Asteraceae)

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Authors' contributions

This work was carried out in collaboration between all authors. Author KK designed the study, wrote the protocol, and corrected the manuscript. Author VC managed the literature searches, analyses of the study performed the antiangiogenesis and anticancer studies. Author RP wrote the first draft of the manuscript and author YSJ collected the literature, helped in the manuscript preparation. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2016/28758

Editor(s):

(1) Dongdong Wang, Department of Pharmacognosy, West China College of Pharmacy, Sichuan University, China.

Reviewers:

(1) Sahdeo Prasad, Anderson Cancer Center, Houston Texas, USA.

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(3) A. Ukwubile Cletus, Federal Polytechnic Bali, Nigeria

Complete Peer review History: <http://www.sciencedomain.org/review-history/16639>

Original Research Article

Received 3rd August 2016
Accepted 3rd October 2016
Published 22nd October 2016

ABSTRACT

Baccharoides anthelmintica (L.) Moench. is an annual herb distributed throughout India, this plant has high trade value because of its many medicinal properties such as inflammatory swelling, stomachache, diuretic properties, cough, fever, diuretic, leprosy, piles, dropsy, enzyme, ringworm herpes, elephantiasis, incontinence of urine, stomach ache and rheumatism, antimicrobial, antioxidant and anti-cancer. Antiangiogenic activity of ethanol leaf and leaf callus extracts was tested through *in vivo* CAM (Chorioallantoic membrane) model. Both leaf and leaf derived callus showed higher angiogenic activity 79.33 and 66.0 percentage respectively at 40 µg/mL concentration. The average number of vessels in leaf and leaf derived callus extracts treated CAM was 2 and 3 respectively. Inhibition percentage and vessel number of both the extracts was almost

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equal in both the concentrations. The cytotoxicity of leaf and leaf derived callus was tested using the MTT assay (HeLa cell line). At a concentration of 300 mg/mL, both the tested samples produced cytotoxic effect as evidenced by the number of dead cells (24.3%, 16.34% respectively).

Keywords: CAM; MTT assay; antiangiogenesis; anticancer; HeLa cell line.

1. INTRODUCTION

Angiogenesis, or neovascularization, is the formation of new capillaries from preexisting blood vessels and is a fundamental process involved in several physiological and pathological processes [1,2]. Antiangiogenesis is a new path as alternative or complementary treatment to cancer. Without blood flow, a tumor would not be able to grow and inhibition would cause non-serious side effects on the patient [3].

Cancer is a group of diseases characterized by uncontrolled growth. It is one of the most dreadful diseases of the 20th century. Cancer may be uncontrollable and may occur at any time, any age and any part of the body. A large number of chemo preventive agents are used to cure various cancers, but they produce side effects that prevent their excess use [4]. Cancer is currently the second leading cause of death in the United States behind cardiovascular diseases. It is estimated that more than 1.6 million new cases of cancer were diagnosed in 2012 [5]. According to the World Health Organization, cancer is a leading cause of death worldwide accounting for 7.6 million deaths (around 13% of all deaths) in 2008 [6].

Medicinal importance of plants has been realized from the time of early man. The knowledge of herbal medicine has recorded and practiced well in India. India is also known as Botanical garden of the World and largest producer of medicinal plants [7]. Secondary metabolites produced by the plant during stressful environment are the main source of medicine. Some of these secondary metabolites have antimutagenic and anticancer properties. Higher quality research to evaluate the efficacy of new plant drug in the cancer field and the comparison with existing one is necessary because the plant based medicine is used by most of the people all over the World. All over the World the people have an interest to come back to plant based or natural medicine due to undesirable side effect and high cost of allopathic medicine. Medicinal plants are natural biochemical factories of the world. In developed countries the plant drugs constitute around 25% whereas in developing countries more than 80%.

The Asteraceae or Compositae (commonly referred to as the aster, daisy, composite, or sunflower family) are an exceedingly large and widespread family of flowering plants [8,9]. The group has more than 23,000 currently accepted species, spread across 1,620 genera and 12 subfamilies. *Vernonia* is one of the largest genera of flowering plants in the Asteraceae family, which includes more than 1500 species distributed widely in the tropical and sub-tropical regions of Africa, Asia and America. The majority of these plants are used as ornaments and vegetables, while others are considered as weeds in agriculture. The vegetables have a bitter taste, hence the name 'the bitter genus *Vernonia*' [10].

Baccharoides anthelmintica (L.) Moench. (Syn. *Vernonia anthelmintica*, *Centratherum anthelmintica* Kuntzeor *Ascaradia indica* or *Conyzaascardia*or *Serratula anthelmintica*) belongs to the family Asteraceae, is an annual herb distributed throughout India [11]. The species contain 40% seed oil with 70%-80% vernolic acid which is extensively used in manufacture of adhesives, varnishes, paints and industrial coatings [12,13,14]. The seeds have a hot sharp taste; acrid, astringent to the bowels and high hiccough, applied in inflammatory swelling, good for sores and itching of the eyes. The seeds are also credited with tonic, stomachache and diuretic properties [15]. The plant has high trade value because of its many medicinal properties. Medicinally, it is effective against several diseases such as cough, fever, diuretic, leprosy, piles, dropsy, enzyme, ringworm herpes, elephantiasis, incontinence of urine, stomach ache and rheumatism [16] leucoderma, psoriasis, skin disease [17] and asthma [18]. It also has other bioactivities like antimicrobial [19], antifilarial [20], macrofilaricidal [21], anthelmintic, antioxidant [22], stimulant, anti-inflammatory [23], anti-hyperglycemic [24], antibacterial [25,26], antihyperlipidemic [27] etc.

Leaves are contain sesquiterpene lactose (centratherin) which is trypanocidal, antiinflammatory and antimicrobial [28]. In comparison with other biological activities, the anti-cancer effect is documented against breast

cancer cell lines [29]. According to Ayurveda, seeds are hot, acrid, astringent, anthelmintic; cure ulcers, veta and kapha; use in skin disease, leucoderma and fever. According to Unani system of medicine, the seeds are anthelmintic, purgative; used for asthma, kidney troubles, hiccough, and inflammatory swellings, to remove blood from the liver, sores and itching of the eyes, the seeds are anthelmintic. The powdered seeds are applied externally in paralysis of the legs at mundas of Chota Nagpur. The juice of the leaf is given to cure phlegmatic discharges from the norstils. To our knowledge, there is no report on comparative study of leaf and callus extracts of *Baccharoides anthelmintica* against anticancer and antiangiogenesis activity.

The present study reports on the comparative study of antiangiogenesis and anticancer activity of callus and leaf extracts from *B. anthelmintica*.

2. MATERIALS AND METHODS

2.1 Plant Materials

Baccharoides anthelmintica (L.) Moench. plants were collected from Sithery hills (Dharmapuri), Tamilnadu. The plant specimens were identified and authenticated by Botanical Survey of India (BSI) Southern Region, Coimbatore, India. Reference number is BSI/SRC/5/23/2013Tech/1758.

2.2 Callus Initiation and Maintenance

Leaves from plantlets of *Baccharoides anthelmintica* were washed in fresh water and surface disinfected in 70% ethanol for 30s, rinsed with sterile distilled water, than soaked in 0.12% HgCl₂ for 3 min, followed by 3 rinses in sterile distilled water. Leaf explants were inoculated in the basal medium of Murashige and Skoog (MS) supplemented with 30 g/l sucrose and 3% (w/v) agar enriched (Hi media-Bombay, India.) with cytokinin BAP (2.22 to 11.11 µM) with NAA (1.34 µM) or TDZ (1.14 µM) or GA₃ (0.72 µM) or Kinetin (1.16 µM) (Hi media-Bombay, India.). Callus culture were harvested at 30th day of cultivation and dried at room temperature.

2.3 Extract Preparation

The fresh plant leaves were carefully washed with tap water to remove soil particles and adhered debris, rinsed with distilled water, air dried for one hour. Then it was cut into small pieces and dried in room temperature for weeks.

Then they were ground into powder with the help of hand mill and stored in room temperature. The leaf derived 30 days old callus collected from our laboratory was air dried and powdered and then filtered through Whatman filter paper No.1 along with 2 gms sodium sulphate to remove the sediments and traces of water in the filtrate. Before filing, the filter paper along with sodium sulphate is wetted with ethanol. The filtrate is then concentrated by bubbling nitrogen gas into solution and reduces the volume to one mL.

2.4 Antiangiogenesis Activity

Antiangiogenic activity of crude extracts of *B. anthelmintica* leaf and *in vitro* callus was conducted on fertilized eggs by modified CAM assay method [30]. Fertile white Leghorn chicken eggs (*Gallus domesticus*) were obtained from a local hatchery (SV farms, Idekari, Coimbatore, Tamil Nadu, India.) with 3 days incubation. The eggs were incubated at 37°C in humidified 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 was 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 ethanol and methanol 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 three eggs were used for each extract dose. NaCl and NaOH was used as negative and positive control [31]. Percentage inhibition was calculated using the following equation. % inhibition = [(vessel number of untreated CAM-vessel number of CAM treated with herbal extract)/vessel number of untreated CAM] x 100.

2.5 *In vitro* Cytotoxicity Assay

Human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune, India and grown in Eagles

Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) (Hi media-Bombay, India.). All cells were maintained at 37°C, 5% CO₂, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

2.6 Cell Treatment

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted by trypan blue exclusion assay using a hemocytometer. The cell suspension was diluted with medium containing 5% FBS to give final density of 1x10⁵ cells/mL. One hundred microliters per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO₂, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. The samples were solubilized in dimethylsulfoxide and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µL of these different sample dilutions were added to the appropriate wells already containing 100 µL of medium, resulted the required final sample concentrations. Following drug addition the plates were incubated for an additional 48 h at 37°C, 5% CO₂, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

2.7 MTT Assay

3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48 h of incubation, 15 µL of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The

percentage cell viability was then calculated with respect to control as follows:

$$\% \text{ Cell viability} = \text{OD of treated cells} / \text{OD of control cells} \times 100.$$

3. RESULTS

3.1 Antiangiogenic Activity

As a measure of testing the medicinal properties of *B. anthelmintica* against angiogenesis the extracts obtained from *in vivo* leaf and leaf derived callus were subjected to anti-angiogenesis studies. Two different concentrations (40 µg/mL and 80 µg/mL) of the extracts were prepared in ethanol and tested antiangiogenic activity. Antiangiogenic activity of ethanol extracts of *in vivo* leaf and leaf derived callus samples was 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 vessels was based on evaluation of angiogenesis by measuring the area of inhibition surrounding the applied disc. The inhibition percentage is shown in the Table 1. Ethanol extract of both *in vivo* leaf and leaf derived callus showed the higher angiogenic activities 79.33 and 66.0 percentage respectively at 40 µg/mL concentration. The Fig. 1E respects normal vascularization in the untreated CAM which consists of primary, secondary and tertiary microvessels. In comparison, the CAM treated with ethanol extracts of both *in vivo* leaf and leaf derived callus displayed distorted vascularization as well as perturbation on existing vasculature (Fig. 1A, B, C, D). The percentage of inhibition in *in vivo* leaf and leaf derived callus ethanol extract treated CAMs was 79.33 and 66.0 percentage in 40 µg/mL concentration while that in 80 µg/mL treatment was 78.0±4.04 and 60.6±2.90 percentage (Table 1). The average number of vessels in *in vivo* leaf and leaf derived callus ethanol extracts treated CAM was 2 and 3 at 40 µg/mL concentration and in 80 µg/mL treatment was 3 and 4. From the above result the inhibition percentage and vessel number of *in vivo* leaf and leaf derived callus extracts of ethanol was almost equal in both concentrations.

3.2 Anticancerous Activity

The results of antiproliferative assay reveal that the ethanol extract used *in vivo* leaf and *in vitro* callus exert antiproliferative action on HeLa cell lines (Tables 2 and 3). The dying cells exhibited ultra-structural and biochemical features that characterize loss of viability. Some cells were

beginning to detach from the plate and become rounded after 5-6 h of treatment. All the concentrations used exhibited lower levels of cytotoxicity like cell rounding, shrinkage, aggregation and cell death, depending on the concentration of the extract. At a concentration of

300 µg/mL, both the tested samples produced cytotoxic effect as evidenced by the number of dead cells (24.3%, 16.34% respectively). This is followed by 150 µg/mL concentration with 20.7, 10.7% dead cells. Cytotoxicity of both the extracts was very less (Tables 2 and 3).

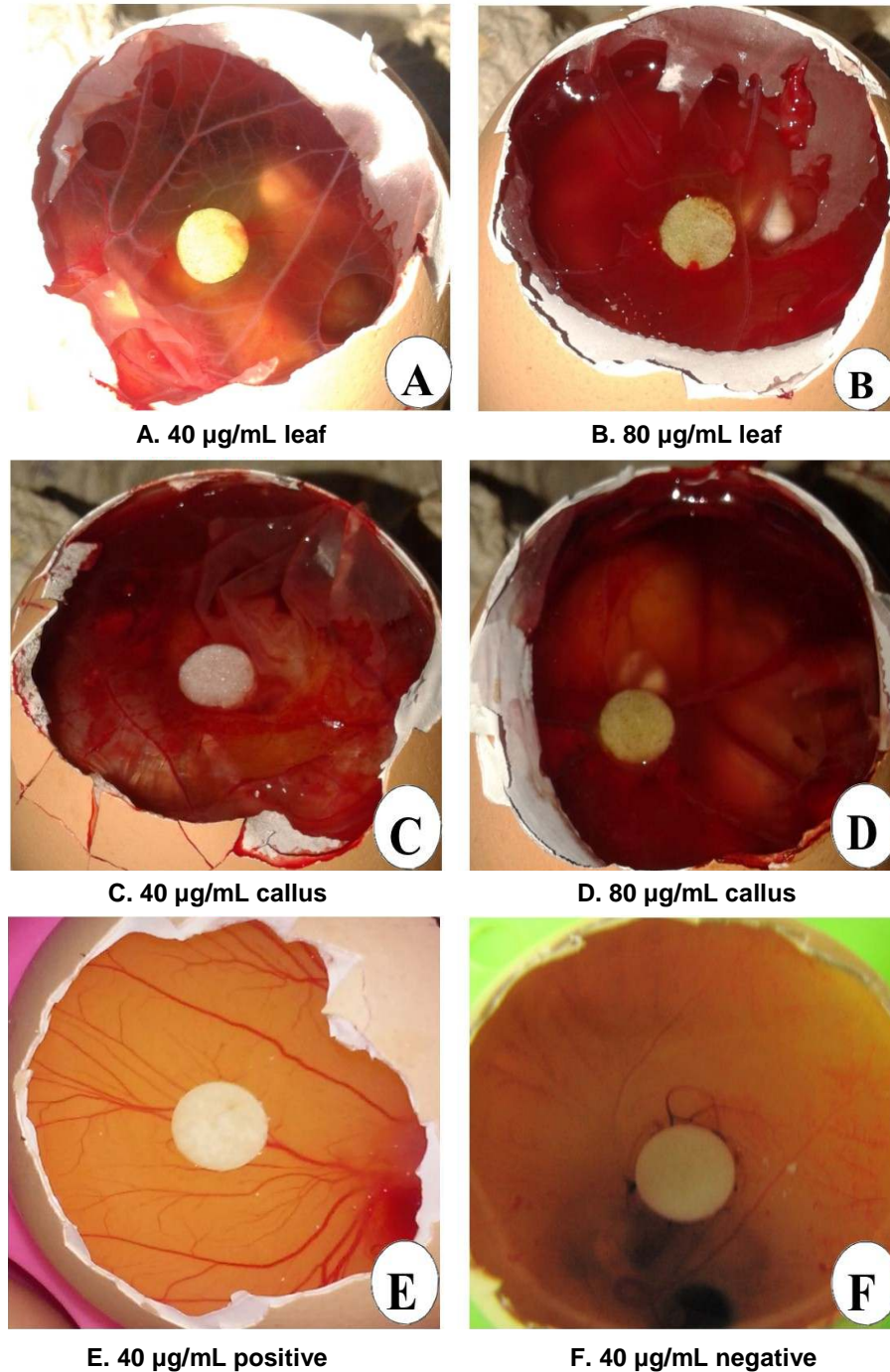


Fig. 1. Anti-angiogenic activity of *Baccharoides anthelmintica* in chick CAM
A & B- Ethanol *in vivo* leaf extract. C & D- Callus ethanol extract. E- Positive, F- Negative

Table 1. Anti-antigenic activity of ethanol extracts of *in vivo* leaf and leaf callus *B. anthelmintica*

S. no	Sample	Concentration µg/mL	No. of vessels in untreated CAM				Average no. of vessels	No. of vessels in treated CAM				Average no. of vessels	% inhibition			% Inhibition (Mean ± SED)
			Egg 1	Egg 2	Egg 3			Egg 1	Egg 2	Egg 3			Egg 1	Egg 2	Egg 3	
1	<i>In vivo</i>	40	10	09	11	10	02	02	03	02	80	77	81	79.3±1.20		
2	Leaf ethanol	80	12	11	10	11	02	03	03	03	83	81	70	78.0±4.04		
3	<i>In vitro</i>	40	09	08	10	09	03	03	03	03	66	62	70	66.0±2.30		
4	Callus ethanol	80	09	11	10	10	03	05	04	04	66	56	60	60.6±2.90		
5	Negative control (0.9% NaCL)	40	08	08	08	08	08	08	08	08	0	0	0	0		
6	Positive control (1NNaOH)	40	09	10	08	09	02	02	03	02	77	80	62	73±5.56		

Three eggs used for each samples
Mean ± SD was calculated for % inhibition of each sample

Table 2. Anti-cancer activity of ethanol extracts of *in vivo* leaf of *B. anthelmintica*

S. no	Concentration					
	18.75 µg/mL	37.5 µg/mL	75 µg/mL	150 µg/mL	300 µg/mL	Control
1	98	99	90	79	75	100
2	99	100	90	80	76	100
3	100	98	89	79	76	100
Cell viability (%)	99.0	99.0	89.66	79.33	75.66	100

Table 3. Anti-cancer activity of ethanol extracts of leaf callus of *B. anthelmintica*

S. no	Concentration					
	18.75 µg/mL	37.5 µg/mL	75 µg/mL	150 µg/mL	300 µg/mL	Control
1	100	97	90	89	84	100
2	99	98	91	90	83	100
3	100	97	91	89	84	100
Cell viability (%)	99.66	97.33	90.66	89.33	83.66	100

4. DISCUSSION

Angiogenesis is essential in tumor growth and metastasis as the process provides necessary oxygen and nutrition for the growing tumour [32]. In the past sixteen years investigators have been looking for angiogenesis inhibitors and promoters from plants. Our results showed that both ethanol leaf and lead derived callus extracts potently inhibited the outgrowth of new blood vessels in dose dependent manner. Our result was confirmed by other earlier report on medicinal plants like *Ceropegia pusila* [33], *Bouerhaavia diffusa* [34]. The medicinal and pharmacological actions of medicinal herbs are often dependent on the presence of bioactive compounds, the secondary metabolites [35]. The use and search for, drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists, biochemists, botanists and natural-products chemists all over the world are currently investigating medicinal herbs for phytochemicals and lead compounds that could be developed for treatment of various diseases [36]. Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. Medicinal plants represent a vast potential resource for anticancer compounds.

The anticancer activity of medicinal plant derived compounds may result from a number of mechanisms, including effects on cytoskeletal proteins that play a key role in cell division, inhibition of DNA topoisomerase enzymes, antiprotease or antioxidant activity, stimulation of the immune system etc.

The value of medicinal plants lies in the potential access to extremely complex molecular structures that would be difficult to synthesize in the laboratory [37]. The limitations of the available cancer management modalities create an urgent need to screen and generate novel molecules. Despite, well-documented illustrations of phytochemicals being used for prevention and treatment of cancer, their importance in modern medicine remains underestimated. Plants are the storehouse of "pre-synthesized" molecules that act as lead structure, which can be optimized for new drug development. In practice, a large number of cancer chemotherapeutic agents that are currently available in the market can be traced back to their plant resource [38]. *In vivo* leaf and leaf derived callus ethanol extracts of *B. anthelmintica* were tested for their antiproliferative activity. *In vitro* antiproliferative property of the extracts of *B. anthelmintica* was minimum in the present results. Morphological analysis of the cells exhibited that the extract treatment had initiated apoptotic mechanism to trigger cell death. At 300 µg/mL concentration over 17%, 25% of the cell population had been rendered apoptotic by both the extracts respectively through slow cytotoxic observation. In general both the extracts showed very less inhibition percentage. So it is confirmed that the *in vivo* leaf and leaf derived callus was having very less anticancerous activity. Even though there are some reports on anticancerous activity of *B. anthelmintica* seed extracts [39], the presence of oil in seed might be the reason for the anti-cancerous activity. There is less or no oil content present in the leaf and callus may be the reason for less anticancerous activity.

5. CONCLUSION

The present study confirm the presence of biologically activity constituents in leaf derived callus, which is comparable to that of leaf. It also shows similarities and differences in the production of such compounds *in vitro* than these produced *in vivo*. So, further studies are needed, for the isolation identification and purification of active antiangiogenesis and anticancer principles from *B. anthelmintica* extracts for pharmaceutical uses.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
 The peer review history for this paper can be accessed here:
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