RESEARCH ARTICLE

Anti-Angiogenic Effect of *Clerodendreme inerme* Leaves Extract

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Received: 18th September, 2023; Revised: 4th October, 2023; Accepted: 17th November, 2023; Available Online: 25th December, 2023

ABSTRACT

Clerodendrum inerme, a medicinal plant, exhibits diverse pharmacological properties. Various species within the *Clerodendrum* genus have been traditionally utilized in indigenous medical systems and folk remedies. This study intended to scrutinize the anti-angiogenic possibility of CI through *in-ovo*, *in-vitro*, and *in-vivo* approaches.

The screening methods employed include the chick chorioallantoic membrane test (*in-ovo*), sponge bud technique (*in-vivo*), as well as *in-vitro* assays for cell migration, cell proliferation, along with morphogenesis. Statistical analyses involved Dunnett's test for mean comparisons, utilizing one-way ANOVA.

In the chick chorioallantoic membrane assay, both angiogenic score and branching points were evaluated. Notably, CI exhibited noteworthy antiangiogenic effects.

In the sponge implantation technique, CI at specified dosages resulted in a substantial reduction in sponge weights, the formation of new vessels, and hemoglobin levels. The antiangiogenic effects were particularly pronounced with increasing doses.

Furthermore, on endothelial cells, *C. inerme* demonstrated a dose-dependent decrease in migration, proliferation, and tube length, indicating potent antiangiogenic properties.

In summary, this research underscores the considerable antiangiogenic potential of CI by various assays.

Keywords: Antiangiogenic, *Clerodendreme inerme*, Chick chorio allantoic, Sponge implantation, Cell migration.

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.4.33

How to cite this article: Kotagiri MD, Kumar GS. Anti-Angiogenic Effect of *Clerodendreme inerme* Leaves Extract. International Journal of Pharmaceutical Quality Assurance. 2023;14(4):1033-1038.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

The generation of new-fangled vessels from preexisting ones is angiogenesis (AG). Antiangiogenic (AAG) compounds are beneficial in treating a variety of diseases resulting from pathologically excess AG because they specifically target budding vessels and address the underlying cause of these diseases. Treating illness at its source yields significant therapeutic benefits with few side effects.[1-4]

Despite extensive research, the pathophysiology of Alzheimer's disease (AD) remains unknown. Pathology consists of cerebral lesions, inflammation, gene polymorphisms, and hypo-perfusion of brain cells.[5-9] Endothelial cells (ECs) have an impact on the degeneration of cortical cells.

In AD, mind endothelium discharges β-amyloid plaque and a neurotoxin harmful to cortical cells. AG activation occurs in response to cerebral hypoxia, ischemia, and inflammation, affecting a substantial number of endothelial cells. The proposition that AD is AG-dependent suggests that developing antiAG agents targeting abnormal endothelial cells in the brain could enhance both prevention and treatment.[8]

Throughout history, India has harnessed the potential of phytochemicals believed to possess antiAG properties. One such evergreen mangrove plant is wild jasmine. Revered for its anti-inflammatory, hepatoprotective, analgesic, antipyretic, antioxidant, antimicrobial, and antidiabetic properties, C.inerme is extensively utilized.[10-14]

Our objective was to investigate the impact of fractionated components from *Clerodendrum inerme* on AAG allied with AD.

MATERIALS AND METHODS

Plant Collection and Authentications

CI procured from Perrys Neutraceuticals, Chennai, and legalized by Dr. V. Sampath Kumar at Botanical Survey of India, Southern Regional Center, Coimbatore, Tamil Nadu, India.

Investigational Animals

Albino rats (150–200 g) were acquired from Teena Labs Pvt. Ltd., Hyderabad, India. All procedures adhered to CPCSEA guidelines, and study protocol approved by IAEC.

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Fractionation of *C. inerme*[11]

The leaves of *C. inerme* underwent a thorough cleaning with tap water, followed by drying at 50°C and subsequent blending. Dried leaves (1.2 kg) were macerated in ethanol (12 L) and the resulting mixture was filtered. The procedure was repeated twice. Further fractionation and sub-fractionation was carried out.

Antiangiogenic Activity

In-ovo assay

- *CAM assay*

On Day '0,' all eggs were randomized into three groups for *C. inerme* treatment (each comprising six eggs), along with groups for VEGF (negative control) and bevacizumab (standard AG agent) to establish a control group. Following ethanol disinfection, the egg mixture was subjected to incubation at 37°C.

By day 3, two to three milliliters of albumin carefully introverted and reserved for subsequent incubation. A sponge containing various doses was positioned on CAM on day 7, and continued to day 14.

On Day 14, CAM was isolated, stained with 10% formalin and observed. Square area and vessel branching points were meticulously counted and AG index was determined.[13,14]

In-vivo Assay

- *Sponge implantation assay[15-20]*

The rats were anesthetized and subsequently, two sterile sponges (2 and 8 cm) were s.c. inserted along the mid-dorsal line of animals. Standard and test were administered via sponge injections to respective groups over a 13-day period.

- *Animal sacrifice and sponge analysis*

On day 14, animals killed, sponges were removed. Quantification was carried out for weight, hemoglobin content, and the number of vessels in each sponge.

- *Preparation of extract concentrations*

Process commenced by preparing utmost concentration of extract, followed by succeeding concentrations obtained via serialized dilutions from early preparation.

- *Hemoglobin content (HGC) assessment*

Here sponges were soaked in double-distilled water and homogenized for 5 minutes on an ice platform then centrifuged to gain supernatant, utilized for HGC detection (g/dL).

- *Determination of digits of blood vessels per sponge*

After an hour of saline immersion at 4°C, the sponges underwent a 30-minute soak in 75% ethanol and preserved. A total of circular spaces between fibroblast regions was conducted to conclude a number of blood vessels formed in sponges.

In-vitro assays

- *Human umbilical vein endothelial cells culture*

HUVECs were cultured on gelatin dishes using various growth complements and this medium facilitated the cultivation of HUVECs on gelatin dishes, and cells between passages 1 and 3 were employed for apiece experiment. Experiments were conducted 3 times, all instances utilizing diverse isolates and/or EC passages.[20-25]

EC proliferation assay

HUVECs were initially cultured in Dulbecco's modified Eagle's medium overnight after seeding at a density of 6000 cells/cm² in 24-well plates. Subsequent revelation to anecdotal concentrations of bevacizumab, VEGF, *C. inerme*, or vehicle, cells were allowed to propagate for entire day. After 48 hours, ECs were trypsinized, and cell counts were determined. This proliferation assay was repeated for each experimental condition.

Transwell migration assay

To assess EC migration, transwell chambers and polycarbonate membrane inserts were employed. Prior to assay, ECs were serum-starved all night. Following trypsinization, 1×10^5 ECs in 100 mL of serum-free medium containing 0.2% BSA were added to each well. Varying doses of VEGF, bevacizumab, and *C. inerme* were introduced. After a 4-hour migration era, non-migrated ECs were removed from top of well. The remaining ECs, settled at the bottom of the filter, were stained with toluidine blue and cured in Carson's solution for half an hour. Migrated ECs counted and averaged.[26]

Matrigel cord-like morphogenesis (MCLM) assay

Expansion of MCLM by HUVECs was assessed in matrigel. Each of 96 wells used for plating EC groups was precoated with 45 mL of matrigel. After 8 hour, cord-like morphology was measured.[27-34]

RESULTS

Chick CAM Assay

Table 1, and Figure 1 exemplify the effects of extracts on a number of branching points and angiogenic score, respectively. Statistical comparisons with control results were conducted for three doses of *C. inerme* (CI). All three CI test doses demonstrated noteworthy results, with highest concentration (10-4 M) showing notable anti-angiogenic bustle.

In-ovo, CI exhibited strapping anti-angiogenic bustle at utmost attention (10^{-4} M) and evidenced by diminished in sponge wt, reticence of fresh blood vessel augmentation, and lessening in HGC, surpassing typical retort (*in-vivo*).

Table 1: Effect of CI on number of branching points in CAM assay and angiogenic score

S. No	*Treatment (μM)*	*No. of branching points*	*Angiogenic score*
1	Control	31.1 ± 1.04	4.1 ± 1.2
2	Bevacizumab	11.2 ± 1.01*	1.21 ± 1.27*
3	CI (1)	29.2 ± 0.44***	1.99 ± 0.067***
4	CI (10)	26.5 ± 1.65***	2.67 ± 0.211^{ns}
5	CI (100)	16.4 ± 1.05***	2.00 ± 0.0^{ns}
6	VEGF (500)	69.1 ± 1.21***	3.11 ± 1.1***

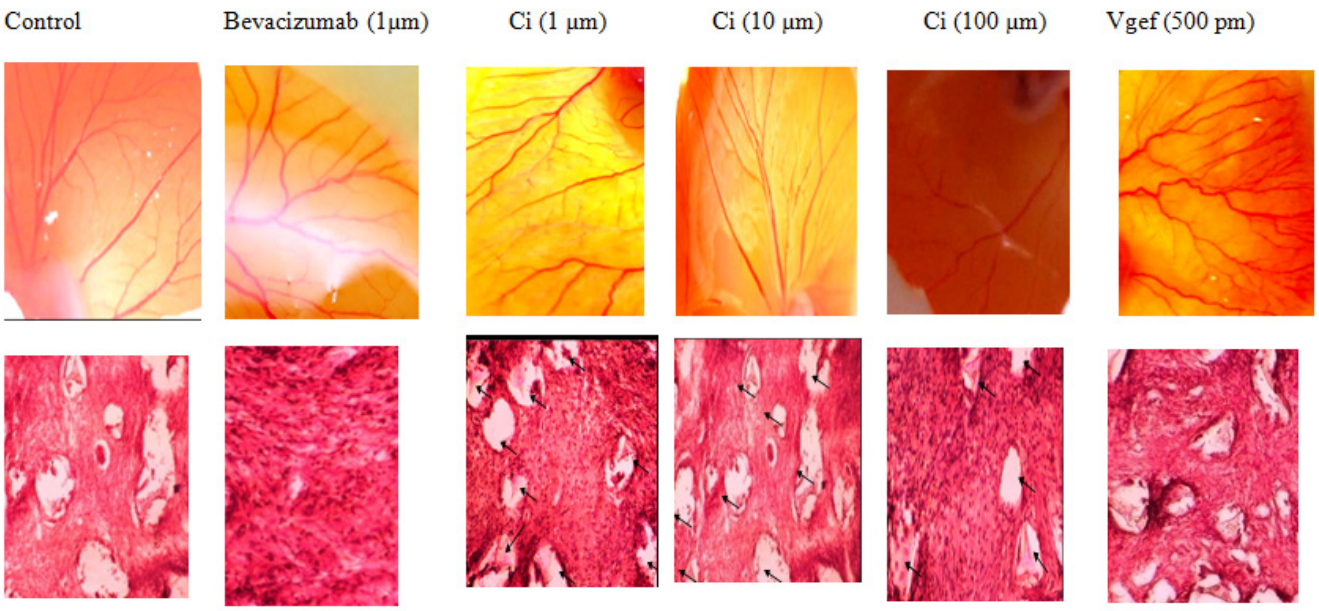

Figure 1: Photographable illustrations showing results of a) CAM assay; b) Sponge implantation assay

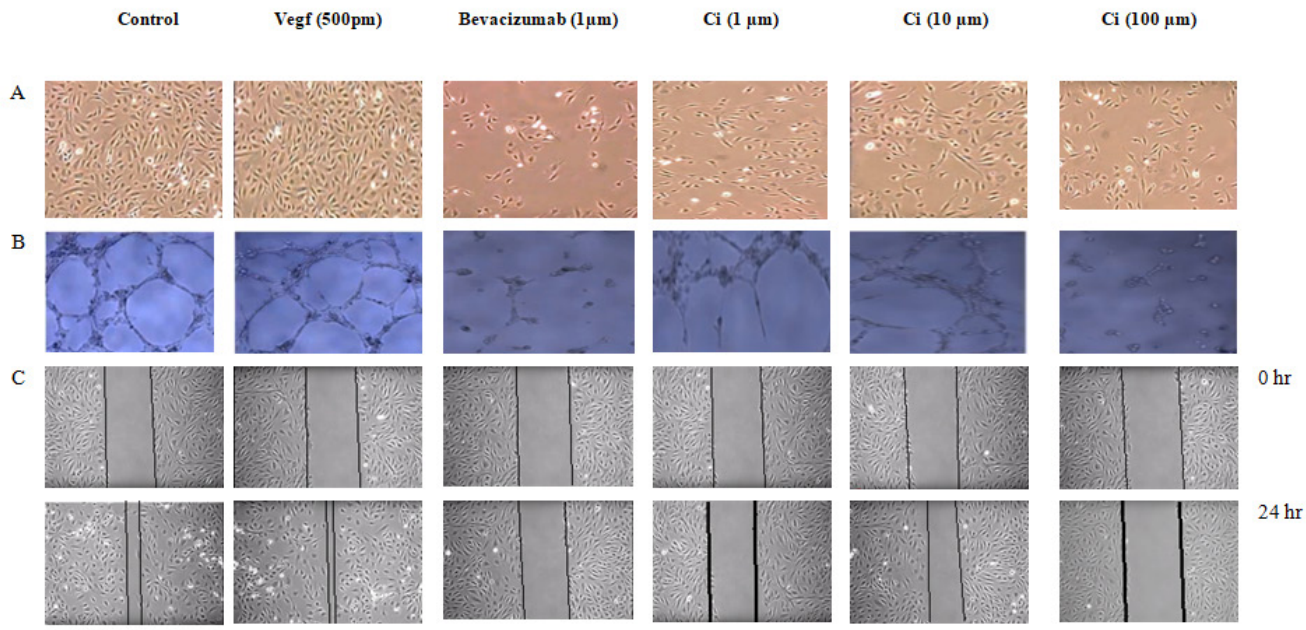

Figure 2: Intonation of endothelial cell responses to CI, bevacizumab and VEGF

Table 2: Effect of CI on weight of sponge, number of vessels/sponge and HGC/ sponge

S.No	*Treatment (µM)*	*Weight*	*No. of vessels/sponge*	*HGC/sponge (g/dL)*
1	Control	2.10 ± 1.02	29.3 ± 1.20	1.12 ± 1.11
2	Bevacizumab (1)	0.523 ± 0.0557**	16.4 ± 1.12***	0.311 ± 0.023***
3	CI (1)	1.20 ± 0.147^{ns}	31.3 ± 1.28^{ns}	0.933 ± 0.115*
4	CI (10)	0.821 ± 0.112*	28.3 ± 1.61***	0.900 ± 0.0683**
5	CI (100)	0.898 ± 0.144 ^{ns}	20.8 ± 1.92***	0.550 ± 0.0957***
6	VEGF (500)	2.11 ± 0.06***	69.4 ± 2.11***	2.19 ± 0.055***

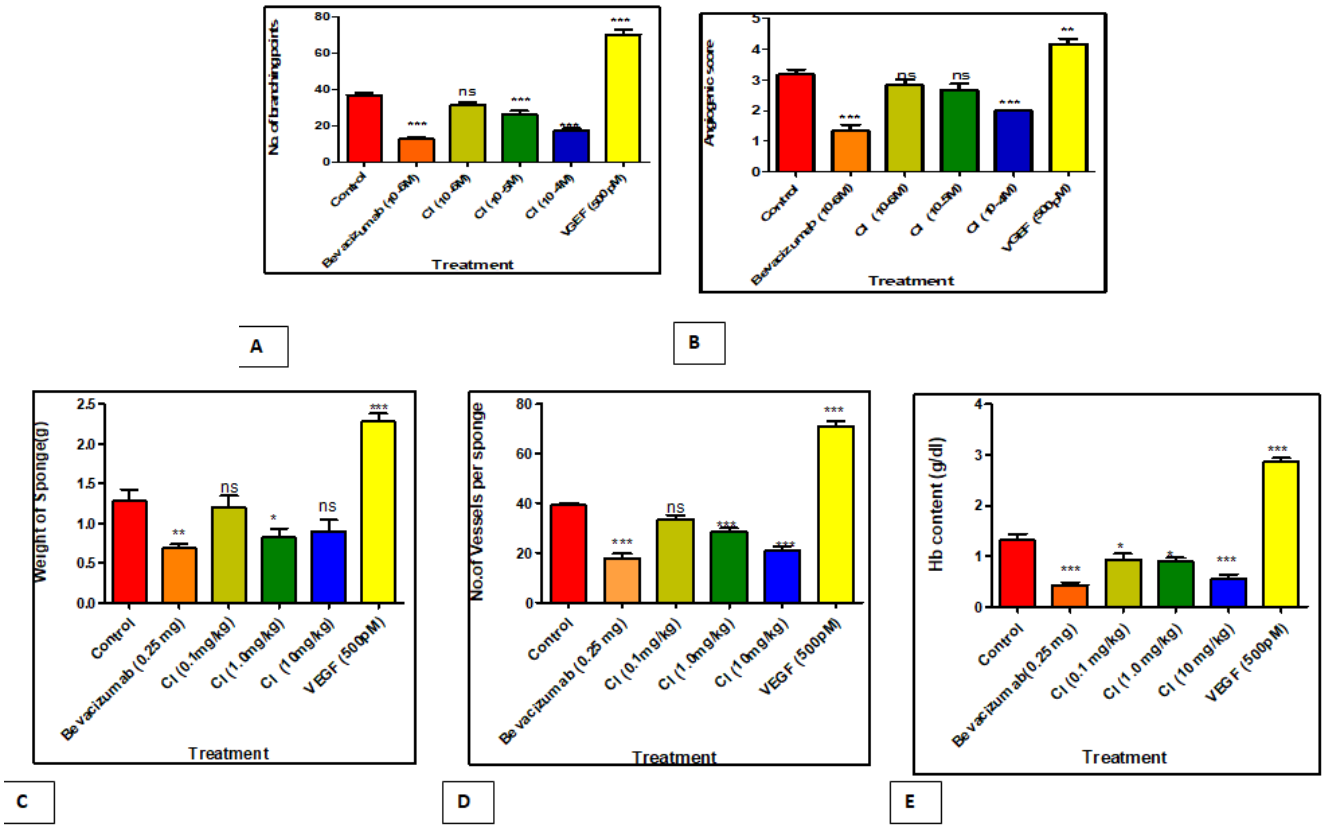

Figure 3: Graphs representing effect of CI on various assays

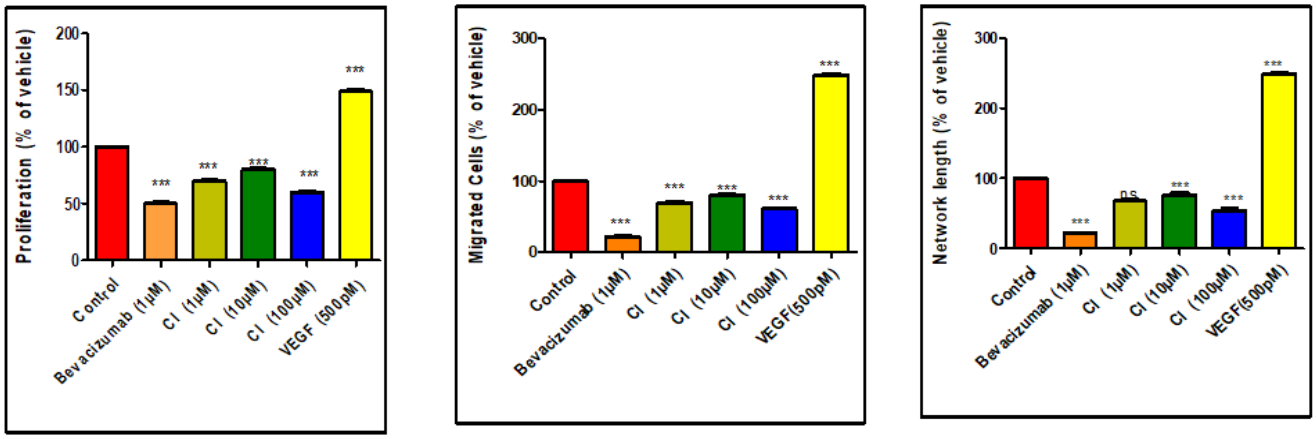

Figure 4: Graphs depicting effect of HCL on the modulation of endothelial cell responses to CI

Table 3: Effect of CI on modulation of endothelial cell responses to CI, bevacizumab and VEGF

S. No	*Treatment*	*Proliferation*	*Migrated cells*	*Network length*
1	Control	99.01 ± 0.21	98.1 ± 1.01	100.0 ± 0.10
2	Bevacizumab (1)	49.2 ± 1.11***	20.2 ± 1.97***	21.31 ± 0.15***
3	CI (1)	69.5 ± 1.23***	69.7 ± 1.29***	64.5 ± 1.00^{ns}
4	CI (10)	80.0 ± 1.18***	80.3 ± 0.30***	76.99 ± 1.30***
5	CI (100)	60.0 ± 1.29***	60.8 ± 1.01***	49.3 ± 2.01***
6	VEGF (500)	165.0 ± 1.99***	240.0 ± 2.01***	222.03 ± 2.14***

Sponge Implantation Method

Here modest lessening in sponge mass and swift preserve of novel vessel growth, HGC were observed at two doses of CI extract. Microscopic examination revealed reducing digit per sponge in CI test extract group, as opposed to large numbers in the VEGF group and very little in standard (Figures 1 and 3; Table 2).

ECP Assay, Transwell Matrigel, and CLM Assay

VEGF exhibited augmented proliferation by 50%, while bevacizumab and three CI doses demonstrated noteworthy reserve. CI inhibited cell motility as compared to vehicle. Additionally, a CLT configuration attempt revealed significant inhibition with CI (Figures 2 and 4; Table 3).

DISCUSSION

The normal process of angiogenesis, or the creation of fresh blood vessels from preexisting ones, aids bodily growth, embryogenesis, the development of the female reproductive system, and the healing of damaged tissue. When it happens under unusual circumstances, growth factors aid in the tumor's progression. Angiogenesis is a critical stage in the development of a tumor into a potentially fatal malignancy. When tumor cells go through this stage, they start to produce their own proteins with this ability and call on proteins that promote capillary growth. Vascular endothelial growth factor is one of these proteins (VEGF). Endothelial cells are stimulated by VEGF to enter a tumor nodule and initiate the formation of new capillaries. Growth factors that stimulate the growth or motility of tumor cells are secreted by the dividing endothelial cells. As a result, tumor cells and endothelial cells stimulate one another reciprocally.

Research indicates that angiogenesis happens when there is a lower ratio of angiogenesis inhibitors to angiogenesis activators. Angiogenesis inhibitors are thought to be promising medicinal treatments for a range of cancerous and other diseases. The chemicals that prevent angiogenesis are called angiogenesis inhibitors, or anti-angiogenic agents. They obstruct the different stages of the angiogenesis mechanism, ultimately preventing the development of fresh blood vessels. Disrupting this vital process slows the growth of the tumor and is therefore a useful treatment for a variety of cancers. The endothelial cells have a variety of ion channel types. These ion channels are essential to the several stages of the angiogenesis process. Numerous pathological conditions can have their origins in excessive angiogenesis.

Utilizing CCM assay, angiogenic score and number of branching points were evaluated. Ethanolic leaf extract of CI, administered at doses of 10^{-5} M and 10^{-4} M, demonstrated noteworthy antiangiogenic effects, as evidenced by a decline in both angiogenic score and figure of branching points.

In the sponge implantation technique, CI doses led to a substantial reduction in sponge weights, the formation of new vessels, and HGC. These observations were made at selected CI doses, indicating a notable impact on angiogenesis *in-vivo*.

On endothelial cells, CI demonstrated a potent dose-dependent reduction in proliferation, migration, and tube length. Across doses ranging from 1 to 100 nM, CI consistently exhibited significant antiangiogenic effects *in-vitro*.

In summary, this study provides comprehensive evidence of effective antiangiogenic properties of CI, as demonstrated through a range of assays.

CONCLUSION

C. inerme consistently demonstrates robust antiangiogenic properties. In the context of anti-angiogenic therapy, their phytochemicals serve as a promising template that can be subjected to structural modifications to achieve a site-specific effect.

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