

## Evaluation of Antiangiogenic Effect of the Leaves of *Justicia gendarussa* (Burm. f) (Acanthaceae) by Chrio Allontoic Membrane Method

K. Periyanyagam, B. Umamaheswari, L. Suseela,  
M. Padmini and M. Ismail

<sup>1</sup>Department of Pharmacognosy,

College of Pharmacy, Madurai Medical College, Madurai, India

<sup>2</sup>Institute of Microbiology, Madurai Medical College, Madurai, India

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**Abstract: Problem statement:** Angiogenesis involves the formation of new blood vessels from pre-existing blood vessels plays a crucial role in pathological processes such as diabetic retinopathy, arthritis and in the growth of solid tumors. Numerous reports had pointed out to the crucial role of neovascularization in the malignancy of tumors and other angiogenesis dependant diseases. Antiangiogenic therapy which targets activated endothelial cells having several advantages over therapy directed against tumor cells. Determine the antiangiogenic potential of the leaves of *Justicia gendarussa* by CAM (Chrio Allontoic Membrane assay) method. **Approach:** A chick chrio allontoic membrane assay was carried out. Six eggs were used per experiment to test one extract at a given dose. The upper part of the shell of the eggs were removed like a window, covered with a plastic film and incubated for 72 h. When the CAM is about 1.8-2.6 cm development the pellets containing test solution both aqueous and ethanolic placed on the CAM by means of micropipette. After 24 h antiangiogenic effect was measured by means of a stereomicroscope to observe the avascular zone surrounding the pellet. **Results:** Below 10  $\mu\text{g}$  both extracts showed no effect. In the dose range of 50  $\mu\text{g mL}^{-1}$  of ethanolic extract and 100  $\mu\text{g mL}^{-1}$  of aqueous extract showed inhibition of neovascularization. The effect was in dose dependant manner. **Conclusion:** These results indicated that both aqueous and ethanolic extracts of the leaves of *Justicia gendarussa* inhibits the angiogenesis in dose dependant fashion and it provides a scientific basis for its traditional use in the treatment of arthritis which is an angiogenesis dependant disease.

**Keywords:** Angiogenesis, CAM, *Justicia gendarussa*,

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### INTRODUCTION

Angiogenesis or neovascularisation is a complex process involving the activation, adhesion, proliferation and transmigration of endothelial cells from preexisting blood vessels. It plays a critical role in normal physiological process such as wound healing but also in a number of pathological processes for instance diabetic retinopathy, arthritis and the growth of solid tumors<sup>[1]</sup>.

Any imbalance in this process may promote numerous angiogenesis. Natural products represent an important source of interesting leads for new drug development.<sup>[2]</sup> Vascular Endothelial Growth Factor (VEGF) Fibroblast Growth Factor (FGF-2) and Epidermal Growth Factor (EGF) are the major angiogenic regulators Isoliquirtin, a magnoshinin isolated from liquorice root, Magnolia salicifolia and

resveratrol from grape seed have been shown as potential inhibitors of these “deadly” processes<sup>[2,3]</sup>.

***Justicia gendarussa* Family:** Acanthaceae is a shade loving, quick growing, evergreen scented shrub found through out India and also in all Asian countries like Malaysia, Indonesia, srilanka<sup>[4,5]</sup>. The plant is used in traditional medicinal practice for chronic rheumatism, inflammations, bronchitis, vaginal discharges, dyspepsia, eye diseases and fever<sup>[4,6]</sup>. *Justicia* found to contain lignans, naturally occurring phenolic dimers<sup>[6]</sup> and triterpenoids<sup>[7]</sup> Lignans have been used as lead compounds for the development of antirheumatic agents<sup>[8]</sup>. Among the known angiogenic inhibitors triterpenoids play a prominent role<sup>[1]</sup>. Its medicinal properties for the treatment of arthritis and the presence of lignans, triterpenoids inspired us to take up this

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**Corresponding Author:** K. Periyanyagam, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai, India Tel: ++919443772991

study. We have performed preliminary CAM assay to study its effect on angiogenesis.

## MATERIALS AND METHODS

**Collection of plant materials:** The leaves of *J. gendarussa*, *Acanthaceae* were identified and collected in the Covenant Centre for Development, (CCD) Kariapatti, Virudhunagar district, Tamilnadu, India during Aug 2008 and was authenticated by the Taxonomist Dr. D. Stephen, Department of Botany, The American college Madurai. A voucher specimen (PCG 267) was preserved in the Department of Pharmacognosy, Madurai Medical college, Madurai.

Fertilized white leghorn chicken eggs were obtained from Department of Poultry Science, Veterinary Research Institute (Nammakkal, India). Buchi Rota vapor R-210,  $\beta$ 1, 4 galactan sulphate (Sigma laboratories), stereozoom microscope (Hertel and Reuss), Egg incubator.

**Preparation of ethanolic extract (EJG):** 500 g of shade dried powdered leaves were sieved (60 mesh) and extracted by cold maceration using absolute alcohol for 72 h. The green organic phase was filtered through Whatmann paper No: 1 and concentrated in vacuo and a dark green color viscous residue was obtained (2.1%).

**Preparation of Aqueous of extract (AJG):** Five hundred gram of the shade dried powdered leaves were sieved (60 mesh) and extracted by hot reflux using distilled water for 4 h. The dark brown aqueous phase was filtered through whatmann paper No: 1 concentrated in vacuo and a dark brown color viscous residue was obtained (4.18%).

Both of the extracts were kept in the refrigerator till the study.

**Acute toxicity assessment (brine shrimp lethality assay)<sup>(9,10)</sup>:** In order to study the acute toxicity of the EJG and AJG we performed brine shrimp lethality bio assay which based on the ability to kill laboratory cultured brine shrimp (*Artemia nauplii*) for the preliminary assessment of toxicity. It was found that LC<sub>50</sub> values both the extract were >1000 ppm.

### Method:

**CAM assay<sup>(2,3)</sup>:** This assay is based upon the formation of a chorioallantoic membrane, in which angiogenesis takes place, in fertilized chicken eggs. Test extracts in agarose pellets were placed on the vascular membrane of the opened eggs and the influence of neovascularisation was studied. Six eggs were used per experiment to test one extract at a given dose. The eggs

were incubated at 37°C and 80% relative humidity condition in an egg incubator. The shells of the eggs were cleaned with 70% ethanol to avoid infections (e.g., Salmonella). After 72 h, 2 mL of albumin was removed with a syringe at the lower side of the egg and the hole was sealed with a sterilized tape. The upper part of the shell has removed like a window and the eggs were covered with a plastic film and incubated for another 72 h. In this period, when the CAM is about 1.8 and between 2.6 cm, the pellets containing test solution was placed on the CAM by means of a micropipette. Test substances were dissolved in a 2.5% agarose solution. After 24 h the antiangiogenic effect was measured by means of a stereomicroscope to observe the a vascular zone surrounding the pellet. Antiangiogenic effect is expressed as a score.  $\beta$ , 1,4 galactan sulphate and agarose pellets were used as positive control and vehicle control respectively. Antiangiogenic activity is expressed as a score where 0 = no or weak effect, 1 = medium effect and 2 = strong effect (capillary free zone is at least twice as large as the pellet).

## RESULTS

The aqueous and ethanolic extracts of the leaves of *J. gendarussa* were studied for their antiangiogenic activity by means of CAM assay. Below 10  $\mu$ g the AJG and EJG showed no effect on vascular network. In doses ranges of 10-100  $\mu$ g mL<sup>-1</sup> the test extracts were able to decrease capillary development on the CAMs and the inhibition was increasing with the dose. At 50  $\mu$ g mL<sup>-1</sup> concentration of ethanolic extract and 100  $\mu$ g mL<sup>-1</sup> concentration of aqueous extract treated CAMs presented avascular zones, indicating that new vessels were not formed (Table 1 and 2).

The data demonstrates that ethanolic and aqueous extracts of *J. gendarussa* inhibits angiogenesis *in vitro*.

Table 1: Antiangiogenic activity of the AJG in the CAM Assay n = no of experiments (n = 2)

Crude diluted extract	Dose ( $\mu$ g pellet <sup>-1</sup> )	Inhibition $\pm$ SD
Aqueous extract	25	0.70 $\pm$ 0.20
Aqueous extract	50	0.90 $\pm$ 0.10
Aqueous extract	100	1.00 $\pm$ 0.20

AJG (Aqueous extract of *J. Gendarussa*) n = no of experiments

Table 2: Antiangiogenic activity of the EJG in the CAM assay n = 2

Crude diluted extract	Dose ( $\mu$ g pellet <sup>-1</sup> )	Inhibition $\pm$ SD
Ethanolic extract	10	0.40 $\pm$ 0.10
Ethanolic extract	25	1.00 $\pm$ 0.20
Ethanolic extract	50	1.30 $\pm$ 0.10
Agarose pellet (blank)		0.20 $\pm$ 0.10
$\beta$ ,1,4 galactan sulphate	50	1.20 $\pm$ 0.20

EJG (Ethanolic extract of *J. Gendarussa*) n = no of experiments

## DISCUSSION

The presents study reveals that ethanolic and aqueous extracts of *J. gendarussa* inhibits the angiogenesis *in vitro* in dose dependant manner. It was already reported in the previous study that it contains triterpenoids and lignans<sup>[6,7]</sup> but we cannot presumed that these two constituents alone were not responsible for this activity as aqueous extract also possess significant inhibition. *In vitro* preliminary acute toxicity assessment reveals the safety of this crude drug. It needs further evaluation in animal model.

Further investigation is in progress in our laboratory to isolate the bio active fraction which is responsible for the activity. It was already reported that *J. gendarussa* contains friedelin,  $\beta$  sitosterol, lupeol and four simple O-substituted aromatic amines which may be the reason for the inhibition by single (or) in combined manner.

## CONCLUSION

We conclude that this preliminary study provide scientific basis for its traditional use in the treatment of arthritis which is an angiogenesis dependant disease. Further research may provide a safe lead drug molecule for the treatment of angiogenesis dependant diseases especially for the arthritis.

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