



Evaluation and Quantification of Angiogenesis Activity of *Terminalia Bellirica Roxb*, by Mice Sponge Implantation Method

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ABSTRACT

Angiogenesis represents an excellent therapeutic target for the treatment of cardiovascular diseases. It is a potent physiological process that underlies the natural manner in which our bodies respond to a diminution of blood supply to vital organs, namely the production of new collateral vessels to overcome the ischemic state. This present study is aimed to evaluate and quantify the Angiogenic potential of *Terminalia bellirica Roxb*, by *in vivo* mice sponge implantation assay. Here, gelatin sponge with or without Ethanolic extract of *Terminalia bellirica* leaf (EETB - 0.3 mg and 0.5 mg, respectively) were subcutaneously injected into Swiss albino mice, and 14 days later, the implanted sponges was excised and histologically examined. The stained section showed that sponge containing EETB had produced more vessels in gels than sponges alone. The new vessels were abundantly filled with intact Red blood corpuscles (RBCs), which indicate the formation of a functional vasculature inside the sponges and blood circulation in newly formed vessels by angiogenesis which is induced by EETB. It also measured that the hemoglobin content inside the sponges: Whereas, hemoglobin in control was nearly 0.3 µg, EETB cases the hemoglobin quantity was markedly enhanced to about 17 µg. Taken together, it demonstrated that Ethanolic extract of *Terminalia bellirica* leaf exhibited a profound angiogenic activity *in vivo*. The phytochemical screening and qualitative instrumental analysis of EETB reveals the presence of proteins and Phytosterols. The promising angiogenic potential may be due to the presence of the above chemical constituents. Further study is required to define more precisely the molecular mechanisms by which Ethanolic extract of *Terminalia bellirica* leaf modulates endothelial cell function and gene expression, as well as the pathological relevance of these findings.

Key words: Angiogenic growth factors, sponge implantation assay, *Terminalia bellirica*, therapeutic angiogenesis

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INTRODUCTION

Neovascularization is the growth of new capillary blood vessels in the body, and is an important natural process for healing and reproduction. The body controls neovascularization by producing a precise balance of growth and inhibitory factors in healthy tissues. When this balance is disturbed, the

result is either too much or too little angiogenesis. Abnormal blood vessel growth, either excessive or insufficient, is now recognized as a “common denominator” underlying many deadly and debilitating conditions, including cancer, skin diseases, age-related blindness, diabetic ulcers, cardiovascular disease, stroke, and many others.^[1]

Angiogenesis for cardiovascular diseases

Angiogenesis represents an excellent therapeutic target for the treatment of cardiovascular diseases. It is a potent physiological process that underlies the natural manner in which our bodies respond to a diminution of blood supply to vital organs, namely the production of new collateral vessels to overcome the ischemic state. A large number of pre-clinical studies have been performed with protein, gene and cell-based therapies in animal models of cardiac ischemia as well as models of periphery artery diseases. Administration of a particular growth factor [Table 1] to stimulate angiogenesis in the affected tissues (or) organ, and the protein therapy with single protein agent was not a viable option to treat ischemic cardiovascular diseases.^[2,3]

Therapeutic angiogenesis

Therapeutic angiogenesis is the method of stimulation of angiogenesis where it is required but lacking. This technique is used to replenish the blood supply to chronic wounds to speed healing and it prevents unnecessary amputations. New research suggests this approach can be also used to save limbs afflicted with poor circulation and even oxygen starved hearts.^[4] Therapeutic angiogenesis may even help to regenerate damaged (or) lost tissues in ways that were previously considered impossible, such as, with nerves and brain tissues. The modern clinical application of the principle of angiogenesis can be divided into two main areas: Anti-angiogenesis therapies with which angiogenesis research began, and with Pro-angiogenic therapies.^[5] Antiangiogenic therapies are being employed to fight cancer and malignancies which require an abundance of oxygen and nutrients to proliferate. Pro-angiogenesis therapy is being explored as an option to treat cardiovascular diseases. One of the first applications of pro-angiogenic methods in humans was a German trial using fibroblast growth factor-1 (FGF-1) for the treatment of coronary artery diseases. Clinical research in therapeutic angiogenesis is ongoing for a variety of diseases like coronary artery diseases, peripheral arterial diseases and wound healing disorders.^[6] The pro-angiogenesis therapies can be differentiated into three categories, namely, i) protein therapy,^[7] ii) gene therapy^[8] and, iii) cell based therapy which involved the implantation of specific cell types. Other than

Table 1: Endogenous angiogenesis inducers

Type of angiogenesis inducer	Examples
Heparin binding peptide growth factors	VEGF PIGF FGF-1, FGF-2 Pleiotrophin PDGF
Non-heparin binding peptide growth factors	TGF-a TGF-b EGF IGF-1
Inflammatory mediators	TNF-a IL-8 IL-3 Prostaglandin E1, E2
Enzymes	PD-ECGF/TP COX-2 Angiogenin
Hormones	Oestrogens Proliferin
Oligosaccharides	Hyaluronan Gangliosides
Hematopoietic factors	Erythropoietin G-CSF GM-CSF
Cell adhesion molecules	VCAM-1 E-Selectin
Others	Nitric oxide Ang-1

Where, VEGF: Vascular endothelial growth factor; PIGF: Placental growth factor; FGF-1, FGF-2: Fibroblast growth factors; PDGF: Platelet-derived growth factor; TGF-a: Transforming growth factor alpha; TGF-b: Transforming growth factor beta; EGF: Epidermal growth factor; IGF-I: Insulin-like growth factor I; TNF-a: Tumor necrosis factor alpha; IL-8: Interleukin – 8; IL-3: Interleukin – 3; PD-ECGF/TP: Platelet-derived endothelial cell growth factor/thymidine phosphorylase; COX-2: Cyclooxygenase-2; G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte macrophage colony-stimulating factor; VCAM-1: Vascular cell adhesion molecule-1; Ang-1: Angiopoietin 1

the above three therapies there are few therapies that have been used to induce angiogenesis. Hence, this study is aimed to find potent angiogenic herbal compounds. *Terminalia bellirica* has deep roots in Indian mythology as in Ayurveda. It is part of a compound *rasayana* preparation of three *myrobalan* fruits, known as *Triphala* (or *trifla*), which are important in both Indian and Tibetan traditional medicine. The name *Vibheekaki* indicates that regular use keeps a person healthy and free from diseases. It has been previously reported that the aqueous extract of fruits of *Terminalia bellirica* inhibit the Tumor Growth and Angiogenesis, and suggests different components have different activity, hence, fresh leaves of the *Terminalia bellirica* Roxb, belonging to the family *Combretaceae*, commonly known as *Bellirica Myrobalan* have been selected to evaluate its angiogenesis activity.^[9]

MATERIALS AND METHODS

Phytochemical evaluation of plant extract

Plant material

The Fresh leaves of *Terminalia bellirica* were collected from wild area near to Mannargudi, Tiruvarur District,

Tamil Nadu, India and the same were authenticated by Prof. Dr. G.S.V. Murthy-Botanical Survey of India (BSI), Coimbatore, India and a voucher specimen No: BSI/SRC/5/23/09-10/Tech-683 was allotted for *Terminalia bellirica* Roxb. [Figure 1]. The herbarium was deposited in our University laboratory for further reference.

Preparation of ethanolic extract

Freshly collected leaves of *Terminalia bellirica* were shade dried and made into small pieces. These pieces were again shade dried and pulverized to coarse powder and passed through the sieve No: 20, and retained on sieve No: 40. About 1 kg of the dry powder of leaves of *Terminalia bellirica* were placed in a thimble of the Soxhlet extraction apparatus attached to the mouth of a round bottomed flask containing 70% ethanol as an extractive solvent. Some boiling chips were added into the flask to avoid bumping during heating. Then the dry powder of leaves of *Terminalia bellirica* was extracted for 72 hours. The extract was concentrated by rotary vacuum evaporation, and the yield was 30% w/w. The dried extract was subjected to phytochemical screening.^[10]

Phytochemical screening

Phytochemical screening of EETB was carried out using conventional protocol,^[11] for detecting the presence of different phytochemical active constituents in the plant respectively, and the result is shown in Table 2.

Qualitative instrumental analysis

Ultra violet and visible spectroscopy

The absorption spectra of plant constituents can be measured in very dilute solution against a solvent blank using an automatic recording spectrophotometer. The ultraviolet (UV)-Visible spectroscopy of plant extract shows the peak at 255 nm which reveals the presence of proteins.



Figure 1: Leaves of *Terminalia bellirica* Roxb

Infrared spectroscopy

Infrared (IR) spectroscopy is the study of the reflected, absorbed or transmitted radiant energy in the region of electromagnetic spectrum ranging from wavelength of 0.8 to 500 nm. Frequency expressed in wave numbers is a more commonly used measurement. It is usually divided into 3 regions near I.R. (1250 to 400 cm^{-1}), mid I.R. (4000 to 400 cm^{-1}) and far I.R. (400 to 20 cm^{-1}). The powdered EETB was mixed with potassium bromide and a thin disc was prepared in anhydrous conditions. That disc was used for the measurement of I.R. spectra against the potassium bromide disc as reference. The Fourier transform infrared FT-IR-spectra of ethanolic extract of *Terminalia bellirica* leaf contains main absorption bands belonging to the valence vibrations corresponding to OH, C=O, C-O-C groups to aromatic rings vibrations as well shows in Figure 2. Spectra examination reveals that the valence vibrations appears in the 2916 cm^{-1} S (CH_3CH_2) band, 2849 cm^{-1} (C-H), 1713 (C=O) cm^{-1} , 1612 cm^{-1} (aromatic C=O), 1450 cm^{-1} (aromatic C=C ring), 758 and 870 cm^{-1} (benzene ring), and may be due to the presence of Phytosterols. Also, it is found that the plant contains organic compounds like carbohydrates, cardiac glycosides, chlorophyll and lignins.

Evaluation and quantification of angiogenesis activity

Animals

Seven-week-old, specific pathogen-free Male Swiss albino mice weighing between (32 – 34 gm) were purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai. They were housed in poly propylene cages (4 per cage), maintained in standard lab conditions at $25 \pm 2^\circ\text{C}$, 10% humidity under a 12 hour light/dark cycle. They were fed with standard pellet diet and autoclaved tap water *ad libitum*. All procedures were performed with the animals under general

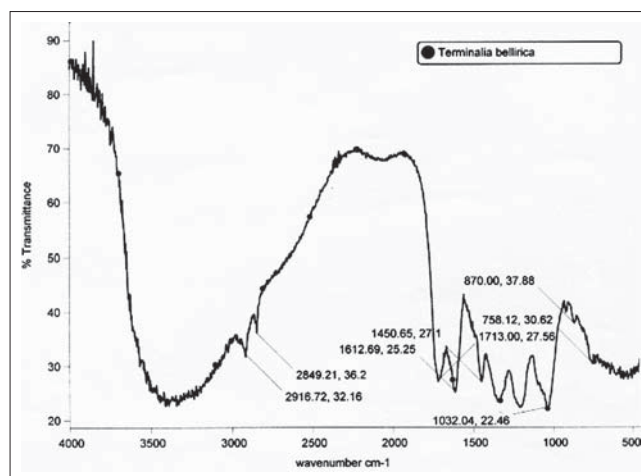


Figure 2: Fourier transform infrared (FT IR) spectra of ethanolic extract of *Terminalia bellirica* Roxb

Table 2: Preliminary phytochemical screening of *Terminalia bellirica* Roxb. during the study

Phytochemical test	Test methods/reagents	Results
Alkaloids	Mayer's	-
	Dragendroff's	-
Carbohydrates and glycosides	Molisch's	-
	Borntrager's	-
Cardiac glycosides	Legal's test	-
	Kellerkilliani test	-
Sugars	Fehling's test	-
	Benedict's test	-
Steroids	Liebermann's Burchard test	++
	Salkowski test	++
Tannins	Lead solution	-
	Aqueous gelatin solution	-
Proteins	Million's test	++
	Biuret test	++
	Ninhydrin test	++
	Xanthoprotein test	++
Terpenoids	Noller's test	+
Flavonoids	Shinoda test	-
Anthocyanins	NaOH and conc. sulphuric acid	-
Quinones	Sodium hydroxide (NaOH) test	-

+ indicates the presence of phytochemical, ++ Indicates the presence of more quantity, - indicates the absence of phytochemical

anesthesia, that is, after intraperitoneal injection of sodium phenobarbitone (30 mg/kg). This study was approved by the Institutional Animal Ethical Committee (IAEC), Approval No: Pharm/12/2009-2010, and the all the animal experiments were performed according to the strict guidelines prescribed by Committee for the purpose of control and supervision on experiments on animals (CPCSEA).

Acute toxicity studies

Acute toxicity study was carried out on plant extract using female and male Swiss albino mice. The mice were fasted overnight and the weight of each mouse was recorded just before use. The extract was subjected for acute toxicity studies by following the OECD (Organization for Economical Co-operation and Development) guidelines No. 425. A group of 5 mice was administered the plant extract in graded doses of 0.25 g/kg, 0.5 g/kg, 1.0 g/kg, 1.5 g/kg, and 2.5 g/kg mice. Mice were continuously observed for mortality and behavioral response for 48 hours. The dose selection was performed by taking 1/10th of the lethal dose (LD₅₀).

Mice sponge implantation method

The *in vivo* angiogenesis assay involving subcutaneous implantation of gelfoam sponges in mice was performed according to the method proposed by Vallera *et al.*, and Mc Carty *et al.*, Absorbable gel foam was cut (5 mm × 5 mm × 5 mm) and hydrated in sterile normal saline (0.9%), and strengthened with sterile 0.4% agarose.^[12] Mice were divided into four groups and each group

contains six animals. Mice were anaesthetized with sodium phenobarbitone (30 mg/kg, i.p.). The caudal region of the back was shaved and scrubbed with 95% ethanol. An incision was made and a sponge wetted with sterile normal saline was inserted into each subcutaneous pocket created under the upper flap and pushed cephalad. This helps to decrease the incident of infection, and reduces the complications resulting from scar tissue forming. Immediately, the incision was sutured. The test and Pyruvic Acid which is used as standard substances were injected slowly through the skin into the sponges by using water as a vehicle. Animals were allowed to recover for 14 days.^[13]

Hemoglobin determination in implanted sponges

On 14th day animals were sacrificed with an overdose of sodium phenobarbitone, and the sponges were harvested from the mice, and were carefully stripped of any remaining peritoneum. The sponges were weighed by placing them into preweighed 1 ml tube of double distilled H₂O and homogenized for 5–10 minutes on ice. The samples were spun at 10,000 revolutions per minute (rpm) on a micro centrifuge for 6 minutes, and the supernatants were collected for hemoglobin measurement. The supernatants were filtered through a 0.22- μ m filter, and fifty micro liters of supernatant were mixed with Drabkin's reagent, and allowed to sit at room temperature for 15–30 minutes). Hemoglobin (Hb) in the samples was quantified calorimetrically at 540 nm in a spectrophotometer. The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. The results were expressed as Hb μ g/mg wet sponges.^[14]

RESULTS

Phytochemical screening and qualitative instrumental analysis

The phytochemical screening of plant extract revealed the presence of proteins, steroids and terpenoids. The UV-Visible spectroscopy of the plant extract gives maximum absorption at 255 nm. The presence of proteins may have revealed the presence of CH₃CH₂, (C-H), C=O, aromatic C=O, aromatic C=C ring, and benzene ring in the IR-spectra of the plant extract.

Ethanollic extract of *Terminalia bellirica* leaf stimulates angiogenesis *in vivo*

The *in vivo* angiogenesis activity was then evaluated by the effect of EETB leaf on the ongoing angiogenesis process in the *in vivo* mice sponge implantation assay. Gelatin sponge with or without EETB leaf (0.3 mg

and 0.5 mg, respectively) was subcutaneously injected into Swiss albino mice, and 14 days later, the implanted sponges in mice was excised and histological examined. Gelatin sponges alone were pale in their color indicating no or less blood vessel formation. In contrast, sponges mixed with EETB leaf appeared dark-red in color. As the sponges were treated with 0.5 mg of EETB leaf, they contained too many large vessels to perform histological examination [Figure 3]. The stained section showed that sponge containing EETB leaf had produced more vessels in gels than sponges alone [Figure 4]. The new vessels were abundantly filled with intact RBCs, which indicated the formation of a functional vasculature inside the sponges, and blood circulation in newly formed vessels by angiogenesis, induced by EETB leaf. It also measured the hemoglobin content inside the sponges [Table 3] to

quantify the angiogenesis induced by EETB leaf. Whereas hemoglobin in control was nearly 0.3 µg, the hemoglobin content was markedly enhanced by EETB to about 17 µg. Taken together, It is proved that EETB leaf exhibited a profound angiogenic activity *in vivo*.

Statistical analysis

All the values are expressed as Mean ± SEM ($n=6$) and $***P < 0.001$ considered as significant when compared to control. Statistical comparisons between control and treated groups were carried out using one-way analysis of variances (ANOVA) and differences between groups assessed using Dunnet's *t* test. All statistical analysis was done by using Graph Pad Prism 5 software.

DISCUSSION

Therapeutic angiogenesis is the method of stimulation of angiogenesis where it is required but lacking. This technique is used to replenish the blood supply to chronic wounds to speed healing and it prevents unnecessary amputations. New research also suggests that this approach can be also used to save limbs afflicted with poor circulation and even oxygen starved hearts. Therapeutic angiogenesis may even help to regenerate damaged (or) lost tissues in ways that were previous considered impossible such as with nerves and brain tissues. Developing novel angiogenic agents that have a well-developing mechanism of action is most important in angiogenesis field. Three types of therapies were developed up to now: Namely, protein therapy; gene transfer technology; and, stem cell transplantation which were most costly and have side effects. In this direction, the plants are being actively explored as a source of new molecules that can induces angiogenesis. In the present study, we have clearly elucidated the angiogenic activity of EETB leaf by performing *in vivo* angiogenesis assays. In mice sponge implantation assay, EETB highly elevated the formation of neovessels in implanted sponges and these newly synthesized vessels participated actively in the circulating of blood cells in mice [Figure 3]. Taken together, the observations in the present study suggest that EETB leaf exhibits strong angiogenic actions, and also may have the potential to be a useful modulator of the large number of serious diseases characterized by deregulated angiogenesis.

Table 3: Angiogenesis activity induced by ethanolic extract of *Terminalia bellirica* leaf in mice sponge implantation method

Group	Treatment	Hemoglobin present hemoglobin µg/mg wet sponge (mean±standard error of Mean)
Control	Gel foam	0.317 ± 0.031
Standard	Gel foam+P.A. (500 µg)	22.667 ± 0.667***
Test A	Gel foam+T.B. (300 µg)	10.667 ± 0.882***
Test B	Gel foam+T.B. (500 µg)	17.667 ± 0.882***

All the results were expressed as mean±SEM and $***P < 0.001$, $**P < 0.005$ and $*P < 0.05$ considered as a significant where (n.s) as not significant

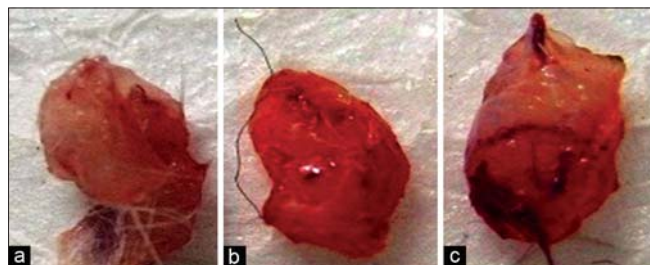


Figure 3: Angiogenesis induced by *Terminalia bellirica* in *in-vivo* mice sponge implantation assay. a) The sponge alone. b) Sponge with Pyruvic acid (500 µg). c) Sponge with *Terminalia bellirica* extract (500 µg)

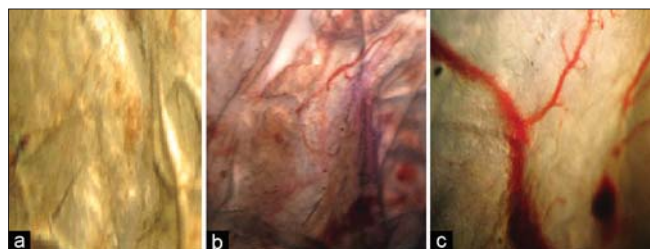


Figure 4: (a) The sponge without pro-angiogenic agent does not show blood vessel supply. However, the sponge with Pyruvic acid 500 µg (b) shows angiogenesis and the sponge with *Terminalia bellirica* leaf extract (500 µg) shows (c) many blood vessels appeared

CONCLUSION

The preliminary phytochemical screening of the EETB has showed the presence of proteins, sugars,

phytosterols, Terpinoids and tannins. The acute toxicity study was carried out and a dose level of 2000 mg/kg body weight orally in mice was considered to be safe for pharmacological treatment. The results of this study support the development and use of *Terminalia bellirica* as a well-tolerated, safe and less cost angiogenic agent with potential angiogenic therapy. The hemoglobin present in the extract treated group was increased when compare to the control group implanted sponges. The histopathology of the implanted sponges shows the presence of the blood vessels in the extract treated groups. Thus, from the foregoing findings it was observed that leaf of *Terminalia bellirica* is a promising angiogenic agent, and this angiogenic activity may be due to the presence of proteins. This is the first report which is not previously said, as well as not yet it is reported that plant proteins inhibit angiogenesis. Further study is required to define more precisely the molecular mechanisms by which proteins of EETB leaf modulate endothelial cell function and gene expression, as well as the pathological relevance of these findings.

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