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Research Article

Inhibition of Angiogenesis and Nitric Oxide Synthase (NOS), by Embelin & Vilangin Using *in vitro, in vivo & in Silico* Studies

Radhakrishnan Narayanaswamy[1,2], Majumder Shymatak[3], Suvro Chatterjee[3], Lam Kok Wai[4], Gnanamani Arumugam[1]*

[1] *Microbiology Division, Central Leather Research Institute (CLRI), Chennai, India.*
[2] *Laboratory of Natural Products, Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia.*
[3] *Vascular Biology Laboratory, AU-KBC Research Centre, Anna University, Chennai, India.*
[4] *Faculty of Pharmacy, Universiti Kebangsaan Malaysia (UKM), Jalan Raja Muda Abdul Aziz, Kuala Lumpur, Malaysia.*

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Abstract
Purpose: In recent year's anti-angiogenesis agents have been recognized as effective drugs for the treatment of solid tumors, this prompted us to conduct the present study.
Methods: The anti-angiogenic activity of dimeric form of embelin (vilangin) was evaluated using endothelial cell (*in vitro*) and chorioallantoic membrane (CAM) egg yolk angiogenesis model (*in vivo*) and in addition the docking behaviour of human nitric oxide synthases (NOS) with four different ligands was evaluated along with their putative binding sites using Discovery Studio Version 3.1 (*in silico*) compared with the parent compound (embelin).
Results: Vilangin exhibits 50% cytotoxic at 92 ± 1 µg/ml concentration level with reference to ECV 304 endothelial cells. Both vilangin and embelin, showed inhibitory effects on wound healing, single cell migration, nitric oxide production, and endothelial ring formation at 0.1 and 1.0 µg/ml concentration level. Similarly, CAM assay also showed inhibitory effect of vilangin and embelin with respect their reduction in length, size and junctions of blood capillaries compared to untreated egg yolk. Docking studies and binding free energy calculations revealed that vilangin has maximum interaction energy (-74.6 kcal/mol) as compared to the other investigated ligands.
Conclusion: The results suggest that both vilangin and embelin attenuates angiogenesis in similar manner.

Introduction

Recently anti-angiogenesis agents have been recognized as effective drugs for the treatment of solid tumors. Immediately after approval of Avastin by Food Drug Administration (FDA, USA), today more than 30 angiogenesis inhibitors have been either approved or are in clinical trials for cancer therapy.[1] The discovery of novel anti-angiogenic agents are likely to bring hope to the millions of sufferers related the angiogenesis associated diseases. Natural products still contributes a significant number of lead molecules for drug discovery & development program. Many of current drugs were originally derived from natural products. Recently, there has been a renewed interest in mechanistic studies and identification of active compounds from, herbal crude formulations. Such evidence-based approaches are not only important for the validation of traditional medicine, but also recognized as fundamental for the future drug discovery.[2]

2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone (Embelin) is a major active ingredient of *Embelia ribes* and well recognized for its numerous biological activities such as antitumor; anti-inflammatory; analgesic activity;[3] anticancer;[4] chemo preventive;[5] antibacterial activity;[6] cross-linking effect towards type I and III collagen[7] & inhibitory against UVB induced oxidative stress.[8] Other than embelin, dimeric form of embelin (Vilangin) has been reported from the *Embelia ribes*. Furthermore, vilangin has been reported to bind with collagen,[7] tyrosinase,[9] neutrophil elastase,[10] glutamate pyruvate transaminase,[11] & alpha amylase[12] using molecular docking studies.

Interestingly, Thangapazham and co-workers[13] reported anti-angiogenic property of *E.ribes*, one among the herbs of brahma rasayana. Followed by them, Zhengfang et al. (2008)[14] authenticates the anti-angiogenic profile of embelin. This prompted us to assess the anti-angiogenic property of vilangin by comparing with that of embelin (parent compound). Both *in vitro* (endothelial cell) and chorioallantoic membrane (CAM) egg yolk angiogenesis model was employed for the present study. Furthermore, embelin, 5 –O-methyl embelin, quercetin and vilangin were evaluated on the docking behaviour of nitric oxide synthase (NOS). Investigation was also done on NOS

***Corresponding author:** Gnanamani Arumugam, Tel: 91 (44) 24422024, Fax: 91 (44) 24911250, Email: gnanamani@clri.res.in

putative binding sites using Discovery Studio Version 3.1.

Materials and Methods

Dulbecco's modified Eagle's medium (DMEM), Trypsin, Antibiotics, Collagen and Fetal bovine serum (FBS) were from PAN Biotech, Aidenbach, Bavaria state, Germany. All other chemicals were at least of the reagent grade obtained from Himedia Laboratories, Mumbai, Maharashtra State, India.

Extraction and characterization of embelin

E. ribes berries was obtained from M/s Abirami Botanical Corporation, (Tuticorin, India, in April 2010) and authenticated by Dr. T. Anandan, Research Officer, Anna Hospital, Chennai. Extraction and characterization of embelin was carried out according to our previous report.[6] Dimeric form of embelin (Vilangin) kindly gifted, by Dr. Rao, Chennai.

Cell culture

Human umbilical vein endothelial cell (ECV 304) and immortalized endothelial hybrid cell line (EA.hy926) were cultured individually in DMEM supplemented with 10 % FBS (v/v) and 1% penicillin (w/v) and streptomycin (w/v). Cytotoxicity, wound healing, single cell migration and nitric oxide content assays were studied in ECV 304 cells and ring formation was assessed using EA.hy926 cells.

Cell viability studies

Preliminary cell viability assessment with reference to different concentrations of vilangin was made according to the methods summarized by Mosmann, (1983).[15] In brief, ECV 304 (1x 10^6) cells were exposed to 0.1 to 100 µg concentration of vilangin dissolved in DMEM medium, for the period of 24 h incubated at 37°C in the presence of 5% CO_2 respectively. Cells without vilangin (neat DMEM medium alone) served as control. MTT (0.5 mg/µl) was added to the incubated cells and then further incubated for another 4 h at 37°C in the presence of 5% CO_2. After incubation the cells were collected by centrifugation and then suspended in 200 µl of DMSO. Absorbance was measured in a microplate reader at 540 nm.

Wound healing assay

ECV 304 cells were trypsinized and (1x10^6 cells/ml) seeded on collagen-plated 24-well plate. Twenty-four hours later, when the cells reached confluency, the endothelial monolayer was scratched with a 1 mm wide sterile plastic scraper to make a linear 'wound'. As described by Staton et al.(2004),[16] the cells were washed with PBS and incubated with embelin and vilangin (0.1 and 1.0 µg/ml each individually) respectively for 8 h. Bright field images were taken with 10 X magnifications under an inverted microscope at every four hours interval. The rate of wound healing was quantified from the images using Scion Image, release alpha 4.0 3.2 and Adobe Photoshop version 6.0.

Single cell migration assay

ECV 304 cells were trypsinized and (1 x10^6 cells/ml) seeded on 24-well plates with 80% cell density. Twenty-four hours later, when the cells reached confluency, the cells were washed with PBS and incubated with embelin and vilangin (0.1 and 1.0 µg/ml each individually) respectively for 30 min. Bright field images were taken with 10 X magnifications under an inverted microscope at every one-minute interval.

Nitric oxide (NO) content

ECV304 cells were incubated with embelin and vilangin (0.1 and 1.0 µg/ml each individually) respectively for 8 h. NO was measured by the Griess assay protocol, as described by Nims et al (1996).[17] The concentration of nitrite (mM) was determined using Sodium nitrite as a standard. The following calibration curve equation, determined by linear regression: Absorbance at 540 nm=(0.228X [Nitrite])-0.048, R^2 = 0.998.

Endothelial ring formation assay

An EAhy926 cell line was used for endothelial ring (ER) formation assay (Sinha et al., 2011).[18] The cells were seeded in 12 well plates in such a way that they reach 20% confluence on the day of experimentation. The cells were treated with respective compounds mentioned for 30 min unless otherwise mentioned, then washed with 1× PBS and fresh media was added and then cells were incubated at 37°C for 330 min. After a total time of 360 min the number of ring like structures were counted under bright field microscope (20 × objectives).

Egg yolk angiogenesis (CAM) assay

Four day incubated eggs were collected from the Poultry Research Station, Nandanam, Chennai. Eggs were broken and gently plated on a cellophane bed in Petri dishes under sterile conditions. Embelin (1.0 µg/ml) and vilangin discs (0.1 & 1.0 µg/ml individually) were then placed on the egg yolks and were incubated for another 6 hours. Images were taken using a Kodak digital camera at 0, 6 and 12 hours of incubation. Quantification of angiogenesis was performed by using Scion Image, Release Alpha 4.0 3.2 and Adobe Photoshop version 6.0 (Tamilarasan et al., 2006).[19]

Docking studies

Docking studies were carried out on the crystal structure of Nitric oxide synthases (NOS) retrieved from Protein Data Bank (pdb id: 4NOS with resolution 2.3° A) using the CDOCKER protocol under the protein-ligand interaction section in Discovery Studio® 3.1 (Accelrys, San Diego, USA). The docking protocol was followed as described by Singh and Konwar.[20] In every docking experiment, 10 ligand conformations were generated for each ligand respectively. Highest

CDOCKER interaction energy pose was chosen, *in situ* ligand minimization were using standard protocol.

Statistical analysis

Statistical analysis was performed using one-way ANOVA on sigma stat (Version.2) and the pair comparison is carried by Turkey test. Standard derivation and standard error were calculated using the same. A value of $P<0.05$ was considered as statistically significant.

Results

About 1.9± 0.1 g of embelin was obtained from 100 g of powdered berries (*E. ribes)*, which accounts approximately 2% of total weight of raw material taken for study. Spectral characterizations of embelin are on par (results not shown) with our previous report.[8] Concentration required for 50 % cell viability (IC_{50}) was assessed for both embelin and vilangin using ECV 304 endothelial cells, before assessing anti-angiogenic activity. Cell viability results reveal, that decreasing the cell viability was observed with increasing concentration of vilangin in dose dependent manner. The IC_{50} concentration of vilangin was determined as 92 ± 1 µg/ml. Further, no cell toxicity was observed up to 10 µg/ml concentration of embelin (results not shown) and vilangin. Hence 0.1 and 1.0 µg/ml concentration was chosen for the further assessment of anti- angiogenesis activity, irrespective of sample. Wound healing assay results reveals that both vilangin & embelin treated ECV 304 cells showed significant inhibition in wound healing, irrespective of the concentrations studied (as shown in the Figure 1). Lamellipodia and filopodia are the two key migratory bodies responsible for cellular migration. In order to determine whether embelin and vilangin promotes the formation of these structures in ECV 304 cells or not, cells were exposed to 30 min in the presence of embelin and vilangin (0.1 and 1.0 µg/ml each individually) respectively and with respective control. Microscopy image observations revealed that no significant difference in the cells with and without the chosen compounds (results not shown). In order to understand further the anti-angiogenic property of dimeric form of embelin (vilangin), Nitric oxide (NO) content was measured after the treatment with embelin and vilangin. Owing to the extremely short half-life of NO, we measured nitrite as stable bio-marker of NO production. NO production was determined by measuring the accumulation of nitrite in the culture medium (using the Griess reaction). In the present study, basal level of NO production from ECV 304 cells was less than 1.5 mM of nitrite concentration. It seems that no statistically significant reduction in NO content was observed for both the tested compounds (irrespective of the concentrations) studied. NO content also assayed using diamino fluorescein-2-diacetate (DAF-2DA), similarly no significant reduction was observed for both the tested compounds studied with that control (results not shown). With regard to ring formation assay, we observed upon treatment with vilangin and embelin, no ring formation was observed irrespective of the concentrations studied (as shown in the Figure 2). Results obtained from CAM assay revealed suppression on formation of new microvessles irrespective of the concentrations studied as shown in the Figure 3 a & b. Docking studies was performed, in order to substantiate our *in vivo* results, where were docked with four ligands namely embelin (ID: 3218), 5 –O-methyl embelin (ID: 171489), quercetin (ID: 5280343) & vilangin (ID: 417182) with that human nitric oxide synthase (NOS) A-chain (PDB ID: 4NOS). Table 1 shows the docking studies and binding free energy calculations in which vilangin exhibited the maximum interaction energy (-74.6 kcal/mol), where as quercetin (reference compound) showed least interaction energy (-39.1 kcal/mol) compared to three other ligands. In the present study, embelin showed interaction with Asn370 and Gly371amino acid residues, whereas 5-O- methyl embelin showed interaction with Glu377 amino acid residue as shown in Table 1. Furthermore, we interesting observed that both quercetin and vilangin shares interaction with same amino acid residues (Arg199 & Glu377) of NOS enzyme.

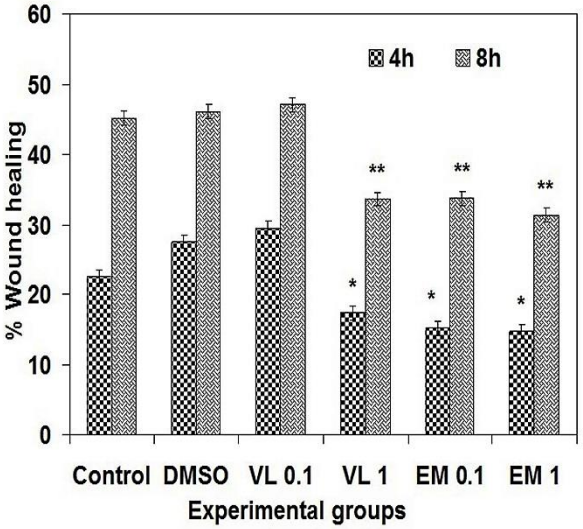

Figure 1. Embelin and Dimeric form of embelin (Vilangin) impairs wound healing in Endothelial cell (EC) monolayer (*, **- Significance between control and treated groups at 4 & 8th h respectively).

Table 1. The interaction energy analysis of four ligands (embelin, 5 –O-methyl embelin, quercetin & Vilangin) with that of human nitric oxide synthase (NOS).

Ligand name	cDocker interaction energy (kcal/mol)	Interaction amino acid residue	Bond distance (Å)
Embelin	-42.1	Asn370	1.7
		Gly371	3.0
5-O-methyl embelin	-43.5	Glu377	1.4
Quercetin	-39.1	Arg199	1.9
		Glu377	1.7
Vilangin	-74.6	Arg199	2.2
		Glu377	1.1

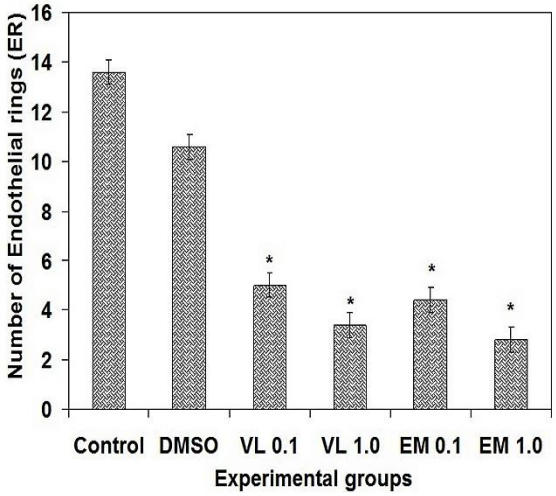

Figure 2. Effect of embelin and dimeric form of embelin (Vilangin) on Endothelial ring (ER) formation assay which was performed in EAhy926 cultures (*- Significance between control and treated groups).

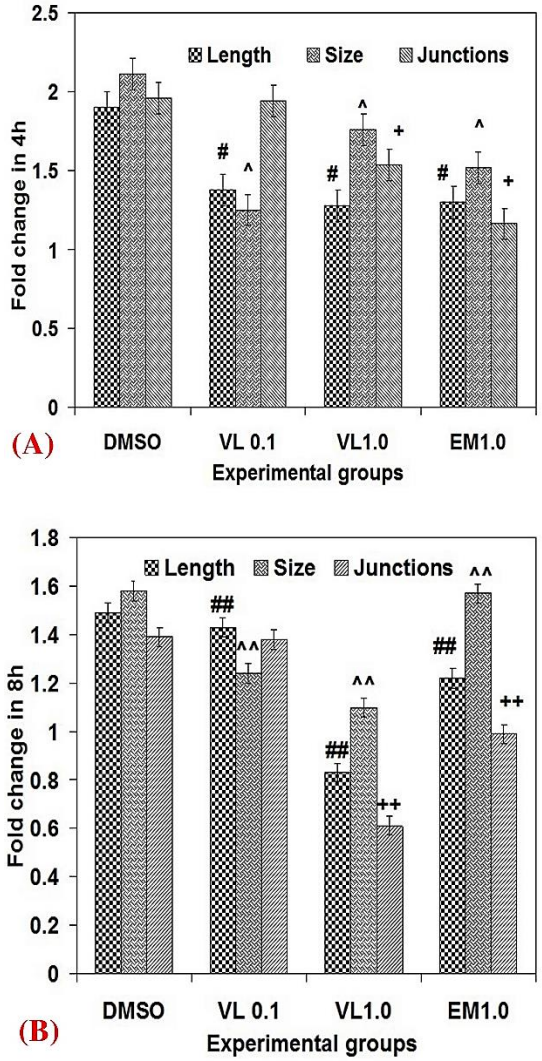

Figure 3. a & b: Embelin and dimeric form of embelin (Vilangin) inhibits angiogenesis which was performed in egg yolk at (a) 4 & (b) 8th h respectively (#, ##; ^, ^^; +, ++ Significance between control and treated groups at 4 & 8th h respectively).

Discussion

Embelia ribes is greatly admired in ayurvedic medicine as a powerful anthelmintic agent and also serve as important ingredient in the number of ayurvedic formulations. 2, 5-Dihydroxy-3-Undecyl-1, 4-Benzoquinone (embelin) is the major constituent of *E.ribes*, on other hand vilangin (dimeric form of embelin) is the minor constituent as shown in the Figure 4 a & b. Hypothetical, we assumed that anti-angiogenic activity of dimeric form (vilangin) might be having more promising effect compare to that monomeric form (embelin). Hence, we validate this hypothesis by undertaking present study. Cell viability results are in excellent agreement with those of Joy et al (2010)[21] and Schmidt et al (2010)[22] report on cytotoxicity activity of embelin towards human umbilical cord endothelial cell and porcine aortic endothelial cells (PAECs) respectively. Similarly, wound healing inhibition in HUVEC by thymoquinone of *Nigella sativa*[23] and sesquiterpene aminoquinone of *Dactylospongia elegans*[24] are in reports and these results corroborate well with present findings. However, in contradict to the observation on *in vitro* studies, Kumara swamy et al (2007)[25] and Alam Khan & Naidu (2009)[26] reported significant wound healing and faster wound contraction in albino rats followed by the topical application of alcohol extract of *E.ribes* and embelin alone respectively. Since, Kumara swamy et al (2007)[25] uses a blend of alcohol extract of *E.ribes* and sodium alginate, the faster healing may be due to the presence of the blend, sodium alginate. Single cell migration assay results suggested that further exploration is necessary to understand the principle behind the study. Furthermore, at this time we do not have any plausible explanation for this difference. However, Xu et al (2005)[4] reported, 5- O-ethylembelin and 5- O-methylembelin acts as anti-mitotic and anti-cancer molecules targeting microtubular proteins. Kantham Srinivas et al (2010)[27] reported embelin derivatives exhibits anti-mitotic activity of derivatives of embelin and among all the derivatives reported, benzyl derivative displayed a significant anti-mitotic activity against germinating seeds of Bengal gram and Onions respectively. Nitric oxide production results corroborate well those of Niwa et al (1997)[28] where, they reported quinone derivatives inhibit NO production in dose dependent manner. Similarly, Sun et al (2006)[29] also reported that 1, 2-naphthoquinone from diesel exhaust particles, inhibits endothelial nitric oxide synthase (eNOS). However, contradict Schmidt et al (2010) reported that embelin activates endothelial nitric oxide synthase (eNOS) and they added that some discrepancy was observed between nitric oxide-mediated cyclic GMP accumulation and L-citrulline formation, which might be reason for activation of endothelial nitric oxide synthase (eNOS). Similarly, results on endothelial ring formation assay also correlates well with above said experiments and suggests as test compounds posses anti-angiogenic activity. Furthermore, CAM assay also showed inhibitory effect of vilangin and embelin with respect

their reduction in length, size and junctions of blood capillaries compared to untreated egg yolk. In human beings three isoforms of nitric oxide synthases (NOS) are exists, which produce nitric oxide (NO) and citrulline by catalyzing NADPH and O_2 dependent oxidation of L-arginine.[30] More over the inducible nitric oxide synthase (iNOS) isoform is a homodimer and it is located on chromosome 17.[31] Docking studies and binding free energy calculations revealed that vilangin has maximum interaction energy (-74.6 kcal/mol) as compared to the other investigated ligands.

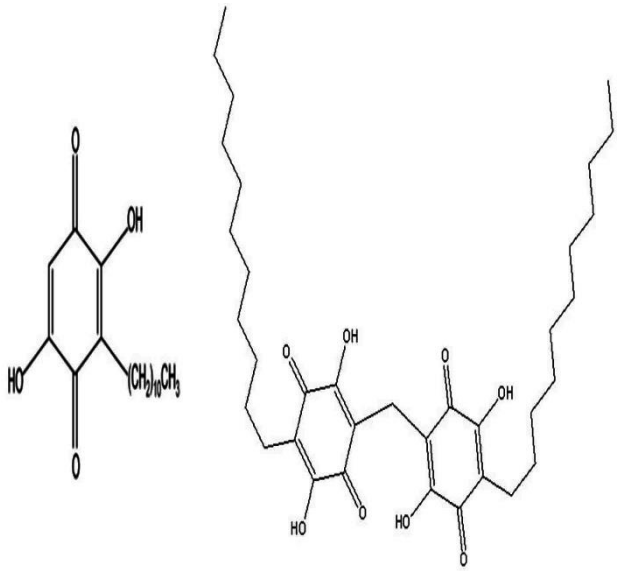

Figure 4. a) Structure of embelin (2, 5-Dihydroxy-3-Undecyl-1, 4-Benzoquinone) & **b)** Structure of dimeric form of embelin (Vilangin)

Conclusion

The present study emphasizes, both 2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone (embelin) and dimeric form of embelin (vilangin) inhibits wound healing, single cell migration, nitric oxide production, endothelial ring formation (*in vitro*) and also inhibits *in vivo* angiogenesis in the CAM assay. Furthermore docking studies and binding free energy calculations revealed that vilangin has maximum interaction energy (-50.1 kcal/mol) and interaction with amino acid residues (Arg199 & Glu377) of Nitric oxide synthase (*in silico*). However, compared to the parent compound (embelin) the dimeric one is not much effective as for *in vitro* & *in vivo* study observations. Further, to our knowledge this is the first report about the antiangiogenic activity of dimeric form of embelin (vilangin).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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