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**Research Article**

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# Evaluation of wound healing and chemotactic activities of Embelin and Vilangin using human dermal fibroblast (*in vitro*) model

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**ABSTRACT**

*Plants or plant based products have been used in the traditional healthcare system from time immemorial for treatment of various diseases; wound healing is one among them. The aim of present study is to understand the 2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone (Embelin) and dimeric form of embelin (Vilangin) mediated attenuation of wound healing and cell migration at the cellular level. We used fibroblast cell based model for studying cytotoxicity, wound healing and Boyden's chamber migration assay. In addition to this, effect of cell migration of both embelin and vilangin was studied at the single cell level using live cell imaging technique. Results, shown that the concentration required for 50% cyotoxicity ($IC_{50}$) towards human dermal fibroblast cells was determined as 32 ± 1 µg/ml & 15 ± 2 µg/ml for embelin and vilangin respectively. Furthermore 0.1 and 1.0 µg/ml concentration were chosen for further wound healing and migration studies. Results demonstrate that both embelin and vilangin arrests wound healing (at 0.1, 1.0 µg/ml concentration level individually) and moreover human dermal fibroblast (HDF) migration was significantly reduced after treatment with both embelin and vilangin respectively. Our present findings demonstrated both embelin and dimeric form of embelin (vilangin) inhibits wound healing and cell migration. Therefore both could be used as potential drug candidates for anti-angiogenesis therapy.*

**Key words:** Embelin; vilangin; wound healing; Boyden's chamber migration assay; angiogenesis

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## INTRODUCTION

Plants have been used in the traditional healthcare system from time immemorial, particularly among tribal communities. The World Health Organization (WHO) has listed 20,000 medicinal plants globally; India's contribution is 15–20%. Since, India is sitting on a gold mine of well-recorded and well-practiced knowledge of traditional herbal medicine. Owing to increasing commercial need the government of India recently has set up a National Medicinal Plants Board (under the Ministry of Indian System of Medicine and Homeopathy) for over all development of medicinal plants and its knowledge. The Board has identified 32 prioritized medicinal plants, *Embelia ribes* is one of plant which has gained national importance owing to its therapeutically and commercial need especially of *E.ribes* berries [1]. *E.ribes* is also one among the top 20 ayurvedic drugs of India as reported by Patwardhan and co-workers [2]. *E. ribes* is commonly known as Vidanga. Ever since ancient times, the drug Vidanga has been an important ingredient in a number of ayurvedic formulations and used in all the three Indian alternative medicinal systems ( such as Ayurveda, Siddha, and Unani) as an antihelmintic and to cure skin diseases.

Wound healing is a complex biological process characterized by different overlapping phases namely, inflammation, formation of granulation tissue, re-epithelialization and tissue reorganization. These above overlapping phases,

accomplished primarily by dermal fibroblasts and keratinocytes and they are well orchestrated by bioactive molecules including growth factors, cytokines and their receptors, and matrix molecules. Key to this repair process are the proliferation, migration, and functioning of fibroblasts and keratinocytes. Many plant-based products have been shown to have therapeutic potential as promoters of wound healing [3].

Recently, Kumaraswamy & co-workers [4] reported that both ethanol extract of *E.ribes* (30 mg/kg b.w.) and embelin (4 mg/kg b.w.) showed significant wound healing and faster wound contraction in Swiss albino rats. Similarly, Alam Khan and Naidu [5] also reported significant improvement in burn wound contraction in the rats treated with embelin (0.2%) alone, as well as in the combination of embelin and silver sulphadiazine (SSD) respectively.

Recently Vilangin has been reported for antibacterial, antifungal and antioxidant activities [6]. Similarly, Vilangin has been reported to bind with collagen [7] and human neutrophil elastase [8] using molecular docking studies. This above background prompted us to investigated the effect of 2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone (Embelin) and dimeric form of embelin (Vilangin) on wound healing and migration potential of fibroblast cells.

## EXPERIMENTAL SECTION

### Materials

Dulbecco's modified Eagle's medium (DMEM), Trypsin, Antibiotics, Collagen and Fetal bovine serum (FBS) were from PAN Biotech, Germany. All other chemicals were at least of the reagent grade.

*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 [9]. Dimeric form of embelin (Vilangin) was kindly gifted by Dr. Rao, Chennai. Human Dermal Fibroblast cells (HDF) were kindly gifted by Dr. Mary Babu, Ex-Head of Biomaterial Department, Central Leather Research Institute (CSIR, New Delhi), Chennai. Fibroblast cells (HDF) were cultured in DMEM supplemented with 10 % FBS (v/v) and 1% penicillin (w/v) and streptomycin (w/v).

### Cell viability assay

Preliminary cell viability assessment with reference to different concentrations of embelin and vilangin were made according to the method summarized by Moshmann [10]. In brief, HDF (1x $10^6$) cells were exposed to 0.1 to 100 µg concentration of embelin and vilangin dissolved in DMEM medium, for the period of 24 h incubated at 37º C in the presence of 5% $CO_2$ respectively. Cells without embelin and 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 37º 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 Scratch assay

HDF were trypsinized and (1 x $10^6$ cells/ml) seeded on 24-well plate. Twenty-four hours later, when the cells reached confluency, the fibroblast monolayer was scratched with a one mm wide sterile plastic scraper to make a linear 'wound'. As described by Staton and co-workers [11] the cells were washed with 1x PBS and incubated with embelin and vilangin (0.1 and 1.0 µg/ml individually) for 8 h. Bright field images were taken with 10x 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.

### Boyden's chamber based migration assay

Trypsinized HDF cells were used for migration assay using Boyden's chamber as described by Tamilarasan and co-workers [12] which is a two-chamber system. The upper and lower chambers are separated by a collagen coated 8-µm pore size polycarbonate membranes. HDF cells (1x$10^6$ cells/ml) were loaded in the upper well and lower well was filled with embelin and vilangin (0.1 and 1.0 µg/ml individually) and then incubated at 37°C, 5% CO2 for 3 hours. Cells were migrated across the membrane and stuck to the lower part of the membrane. After the incubation, the polycarbonate membrane was fixed with parformaldehyde and stained with 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent nuclear probe. Fibroblast cell migration activity was quantified as the number of migrated cells on the lower surface of the membrane. Cell number was counted before and after experiments to quantify the proliferation status of the loaded cells in Boyden's chamber.

**Single cell migration assay**

HDF were trypsinized and ($1x10^6$ 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(0.1 and 1.0 µg/ml individually) for 30 min. Bright field images were taken with 10x magnifications under an inverted microscope at every one-minute interval.

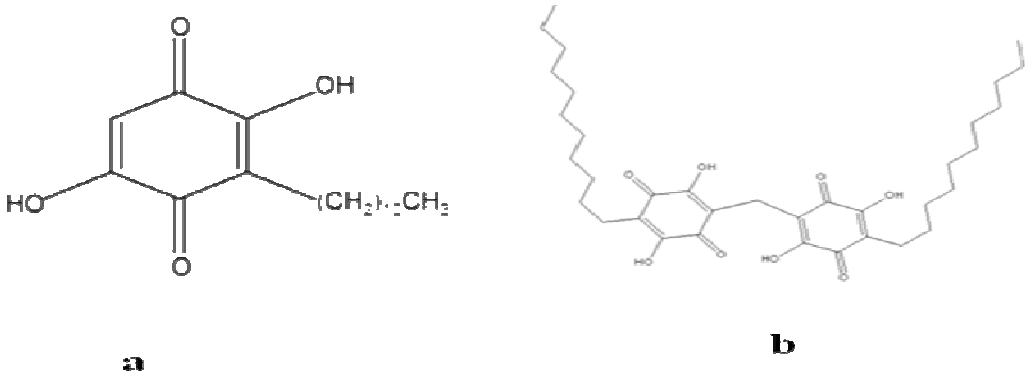
**Statistical analysis**

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 AND DISCUSSION

*Embelia ribes* is greatly admired in Ayurvedic medicine as a powerful anthelmintic and also an important ingredient in number of ayurvedic formulations. In addition decoction of *E.ribes* berries is also widely used for primarily disease treatment in India. 2, 5-Dihydroxy-3-Undecyl-1, 4-Benzoquinone (embelin; as shown in the figure 1a) is the major constituent of *E.ribes*, whereas vilangin (dimeric form of embelin; as shown in the figure 1b) is the minor constitutent of *E.ribes.* On other hand, Vilangin preparation was also reported (chemical means) by simply reacting embelin with that of formaldehyde [6].

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

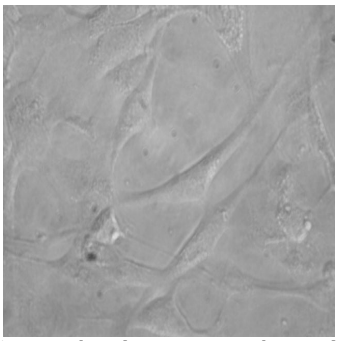



Embelin is well recognized for its various biological activities which includes anti-angiogenic activity. Accordingly, is worth to be investigated whether dimeric form of embelin (vilangin) also inhibits angiogenesis. Then through comprehensively analyzing the research references about embelin, we theoretical assumed that the dimeric form of embelin (vilangin) should be more superior to parent compound embelin (2, 5-Dihydroxy-3-Undecyl-1, 4-Benzoquinone).

Therefore, in the present study is the first step to determine whether vilangin could exhibit better wound healing & cell migration activity and compare with that of embelin (parent compound). 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. Moreover, spectral characterizations of embelin were on par with previous report [9], however results were shown presently.

With regard to cell viability, studies carried out with varied concentrations of embelin and vilangin reveals, increase in concentration of embelin and vilangin respectively results in decrease in cell viability which was observed in dose dependent manner.

**Figure 2 Represents the morphology of human dermal fibroblasts (HDF) in cell culture[*]**

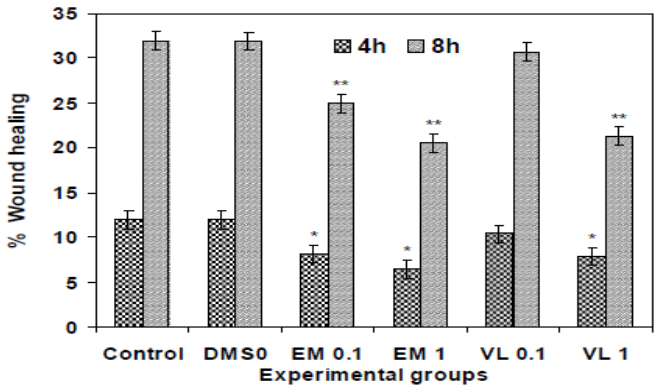


*[*- Where, Human dermal Fibroblast cells (HDF) were cultured in DMEM supplemented with 10 % FBS (v/v) and 1% penicillin (w/v) and streptomycin (w/v)].*

The inhibitory concentration required for 50% cyotoxicity of embelin and vilangin ($IC_{50}$) to HDF cells were determined as 32 ± 1 & 15 ± 2 µg/ml respectively. Similarly, Feresin et al. [13] reported embelin at 217 µg /ml cause only 50% cell death in human lung fibroblasts and Podolak et al. [14] observed >20 µg /ml embelin as effective dosage for human fibroblasts. Joy and Lakshmi [15] reported the active fraction containing embelin ($IC_{50}$ value) display cytotoxicity at 370 and 114 µg/ml respectively to DLA cells and K-562 cells. Both embelin and vilangin does not show any cytotoxicity up to 1.0 µg/ml level in the HDF cells. Hence 0.1 and 1.0 µg/ml concentration were chosen for further angiogenesis inhibitor studies.

In present study both embelin (0.1 and 1.0 µg/ml individually) and vilangin (1.0 µg/ml) treated cells inhibits /impairs the wound healing as shown in the fig. 2.

**Figure 2. Embelin and Dimeric form of embelin (Vilangin) impairs wound healing in human dermal fibroblast cell (HDF) monolayer[♦]**

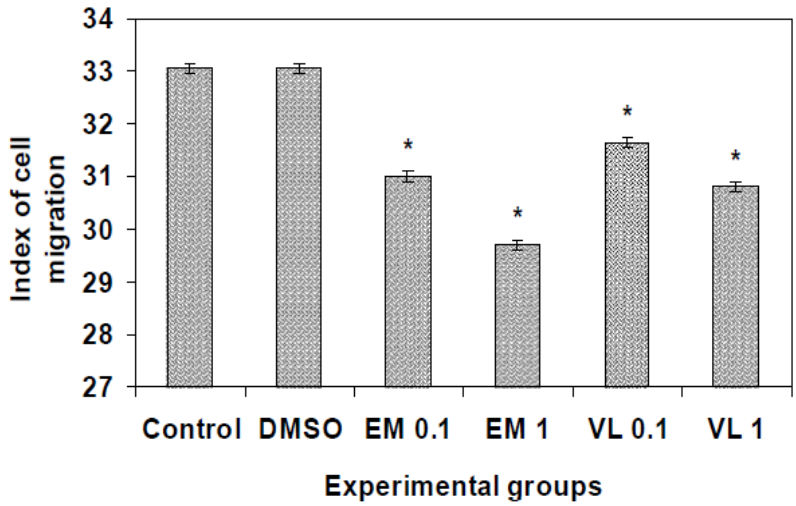



*[♦- Where, Wound was created with a sterile plastic scraper and images were taken at 0, 4 and 8 hours. Analysis of the data depicts that the rate of wound healing is slower in embelin (0.1 & 1.0 µg/ml concentration level) and vilangin (1.0 µg/ml). The asterisks (*) represent the level of significance. * P < 0.05 compared to control (4 h). The asterisks (**) represent the level of significance. ** P < 0.05 compared to control (8 h) Similarly, where DMSO; EM & VM were DMSO solvent, embelin and vilangin treated groups at concentration of 0.1 & 1.0 µg/ml respectively].*

Wound healing assay carried out at 4 and 8 hr displays, significant reduction in percentage of wound healing for the cells treated with embelin and vilangin compared to control irrespective concentration studied. However, no significant difference was observed between 4 and 8hr period for both the compounds. Similarly, Tingfang et al. [16] reported thymoquinone from *Nigella sativa*, inhibited wound healing process in human umbilical vein endothelial cell (HUVEC). Dexin et al. [17] reported sesquiterpene aminoquinone from *Dactylospongia elegans*, inhibited wound healing process in HUVEC. Our results were converse to *in vivo* findings of Kumaraswamy et al. [4] where he reported both ethanol extract of *Embelia ribes* (30 mg/kg b.w.) and embelin(4 mg/kg b.w.) showed significant wound healing and faster wound contraction in Swiss albino rats. Similarly, Alam Khan and Naidu [5] also reported significant improvement in burn wound contraction in the rats treated in the combination of embelin with silver sulphadiazine and they also compared with that of embelin (0.2%) alone.

Chemotactic migration is a key event in angiogenesis, and we investigated the effect of embelin and vilangin on HDF migration using a Boyden's chamber migration assay. HDF migration was reduced after both embelin and vilangin treatment (0.1 and 1.0 µg/ml individually) as shown in figure 3. Even with prolonged the incubation period (by another 3 hours), which also clearly shows no much reduction in migration of fibroblast cells (results not shown).

**Figure 3. Migration assay by using modified Boyden's chamber▲.**




*[▲- Where, Human dermal fibroblast cells were trypsinized and seeded on the upper chamber of Boyden's chamber, which is a two chamber system separated by a collagen coated 8 µm pore size polycarbonate membranes. Lower well was filled with DMEM. The chambers were then incubated at 37°C, 5% $CO_2$ for 3 h. Cells were migrated across the membrane and stuck to the lower part of the membrane. After the incubation, the polycarbonate membrane was fixed and stained with stained 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent nuclear probe. Cell number was counted before and after experiments to quantify the proliferation status of the loaded cells in Boyden's chamber. The asterisks (*) represent the level of significance. * P < 0.05 compared to control. Similarly, where DMSO; EM & VM were DMSO solvent, embelin and vilangin treated groups at concentration of 0.1 & 1.0 µg/ml respectively].*

Lamellipodia and filopodia are two key migratory bodies for cellular migration. In order to determine if embelin and vilangin promotes the formation of filopodia and lamellipodia-like structures, HDF were exposed to 30 min in the presence of both embelin and vilangin (0.1 and 1.0 µg/ml individually). The structures of (both embelin and vilangin treated) cell lamellipodia and filopodia were in par with that of control cells, which also again clearly indicates that no increase in cell migration. Similarly, Xu et al. [15] reported that both 5- O-ethylembelin and 5- O-methylembelin as antimitotic and anticancer molecules targeting microtubular proteins. Kantham Srinivas. et al. [16] reported embelin derivatives exhibits antimitotic activity in the order of IIf, IIa, IIb, IIe, IId, IIc. Among all the derivatives of embelin, benzyl derivative (IIf) has reported as significant antimitotic activity against germinating Bengal gram seeds and germinating Onions, when compared to rest of the compound.

## CONCLUSION

Both 2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone (embelin) and dimeric form of embelin (vilangin) inhibits wound healing and cell migration. To our knowledge, this is the first report about the wound healing and cell migration property of dimeric form of embelin (vilangin).

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