Biotechnology Reports 27 (2020) e00514

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

# Rapid transient expression of functional human vascular endothelial growth factor in *Nicotiana benthamiana* and characterization of its biological activity

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ARTICLE INFO

*Article history:*
Received 19 April 2020
Received in revised form 31 July 2020
Accepted 31 July 2020

*Keywords:*
Molecular farming
*Nicotiana benthamiana*
Transient expression
Human vascular endothelial growth factor

ABSTRACT

Human vascular endothelial growth factor (VEGF) is a potent pro-angiogenic growth factor essential for wound healing. Due to its potential applications, many expression strategies have been developed to produce high levels of VEGF. Here, we have optimized the expression conditions for the production of recombinant VEGF in *Nicotiana benthamiana* by using a geminiviral vector. Four different expression constructs that differ by the location of a C- or N-terminal histidine tag and SEKDEL sequence were developed and utilized for plant transient expression. The recombinant VEGF was further purified by using affinity chromatography and confirmed by SDS-PAGE and Western blotting probed with anti-VEGF antibody. Furthermore, our results showed that the recombinant VEGF in all tested concentrations did not exhibit any cytotoxic effect on HaCaT cells and induced cell migration *in vitro*. These findings show that the plant-produced VEGF has the potential to be used in regenerative medicine and cosmetic industry.



## 1. Introduction

Wound healing is the process of tissue repair which involves a series of biologic events regulated by growth factors [1]. The process of wound healing occurs in the human body following tissue damage or cutaneous aging and proceeds with sequential events involving homeostasis, inflammation, proliferation, extracellular matrix (ECM) deposition and scar development [2]. Growth factors are known to possess tissue healing or wound healing properties by interacting with specific cell surface receptors thereby regulating the process of tissue repair. It is also essential for the process of vasculogenesis and angiogenesis, which is an important aspect during wound repair and restoration [3]. Vascular endothelial growth factor (VEGF) also known as vascular permeability factor is a highly conserved 40-46 kDa homodimeric glycoprotein, which is one of the most potent pro-angiogenic growth factors that stimulates downstream angiogenic signaling and cell migration. Alternative splicing of VEGF at proximal and distal sites generates four different isoforms *ie.,* VEGF-121, VEGF-189, VEGF-206 and VEGF-165 [4]. Among these, VEGF-165 is an abundant isoform found in the majority of cells and its mechanism of angiogenesis has been well-characterized *in vivo* [5]. VEGF is considered as a key inducer of angiogenesis such as cell proliferation, migration and survival and is the prime mediator for vascular permeability. The role of VEGF in wound healing has been extensively studied. VEGF exerts multiple effects in different stages of the angiogenic cascade through migration of endothelial cells in the extracellular matrix and wound vascularization [6]. In cases of severe and impaired wounds, reduced levels of VEGF resulted in a delayed healing process. Earlier studies tested the role of VEGF in defective healing models, where the local application of

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https://doi.org/10.1016/j.btre.2020.e00514

VEGF-165 in wounds accelerated angiogenic functions [7]. Hence, the importance of VEGF in tissue repair shows its potential application in regenerative medicine and cosmetics industry.

Most of the recombinant biopharmaceutical proteins and non-biopharmaceutical proteins were produced mainly through conventional expression platforms based on bacteria, insect and mammalian cells. The potential application of growth factors covers remarkable demands for clinical and research applications. Due to the cell survival, proliferation and tissue repair properties, the demand for recombinant VEGF is constantly increasing. Recombinant VEGF produced in *E. coli* [8], *Spodoptera frugiperda* [9] and chinese hamster ovary (CHO) [10] cells are commercially available. Despite successful manufacturing processes, extensive bio-processing timelines, contamination risks, post-translational modifications and high-capital costs place a demand for alternative expression systems that are cost-effective. The latest advancements in the development of plant expression systems – especially transient expression and viral vectors shows their potential for production of biopharmaceutical or non-pharmaceutical proteins rapidly in a short time. In addition, the effectiveness of plants for VEGF protein production has been previously explored in transgenic tomato [11] and tobacco [12].

Plants are considered as a potential cutting-edge platform for the production of commercially valuable proteins due to many advantages such as easy scale-up procedures, cheap, less initial investment, low risk of contamination of therapeutic proteins and highly capable to express complex proteins. *Nicotiana benthamiana* is one of the most commonly used host systems due to huge biomass, short life cycle, scalability and well-established transformation methods. Recent advancements on *Agrobacterium*-mediated transient transformation by using geminiviral vectors have increased the possibilities of high levels of recombinant protein accumulation in plants [13–15]. Several earlier reports reviewed the potential advantages of plant expression system over other conventional expression platforms for the rapid, cost-effective large-scale production of recombinant proteins in a short time [16–23].

In the present study, we produced recombinant VEGF protein in *Nicotiana benthamiana* by transient expression. The VEGF gene was codon optimized, cloned in a geminiviral vector, and transiently expressed in *Nicotiana benthamiana.* The transient production based on agroinfiltration using a geminiviral replicon system allowed convenient expression yields of plant-produced VEGF. Further, the plant-produced VEGF was purified and tested for its cytotoxicity and migration effect on human keratinocytes HaCat cells. Our results revealed that functional VEGF can be produced in plants suggesting that plant-produced VEGF could be used in regenerative medicine or as a biologically active ingredient in dermocosmetics.

## 2. Materials and Methods

### 2.1. Construction of pBYR2e-VEGF plant expression vector

The nucleotide sequence coding for human vascular endothelial growth factor (VEGF; GenBank accession no.: NP_001165097.1) or VEGF-165 was codon optimized and synthesized by Invitrogen GeneArt® Gene Synthesis (Thermo Fischer Scientific). The optimized gene sequence along with the signal peptide (SP) and Histidine (His) tag was cloned into the plant expression vector pBYR2e [24–26]. Four different constructs were developed that vary in the location of the his-tag on the N or C-terminus and the presence of the SEKDEL retention motif on the C-terminus (Fig. 1). The list of forward and reverse primers used in the present study is provided in the Table 1. The gene sequence was further cloned into the geminiviral vector (pBYR2e) by digestion with *Xba*I and *Sac*I restriction enzymes. The ligated pBYR2e-VEGF plasmids were transformed into *E. coli* DH10B cells by heat shock. The transformed colonies were screened by PCR and the positive colonies were inoculated in Luria Bertani (LB) broth supplemented with 50 μg/mL kanamycin and grown overnight at 37 °C. The plasmid was isolated from the overnight culture by using AccuPrep® Plasmid Extraction Kit (Bioneer, Korea).

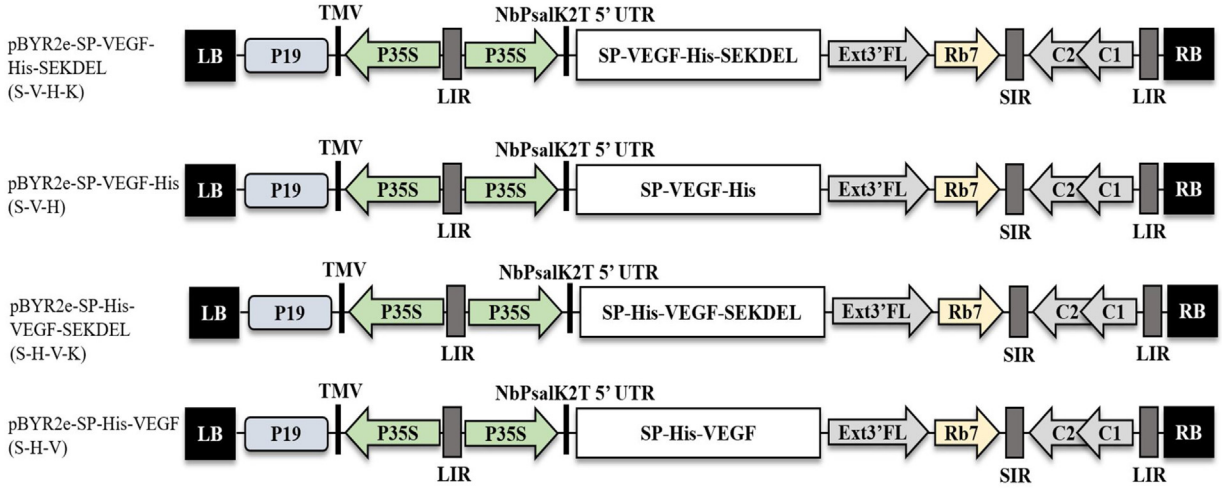


**Fig. 1.** Schematic representation of human vascular endothelial growth factor (VEGF) gene constructs in geminiviral vector (pBYR2e)that are used in the present study; P19: P19 gene from Tomato Bushy Stunt Virus (TBSV); P35S: Cauliflower Mosaic Virus 35S promoter; Ext3'FL: expressed sequence tags- 3' full length of *Nicotiana* extension gene; Rb7: tobacco Rb7 promoter; C2/C1: Bean Yellow Dwarf Virus (BeYDV) ORFs C1 and C2 which encode the replication initiation protein (Rep) and RepA; TMV: 5' untranslated region (UTR) of tobacco mosaic virus; NbP 5': 5' UTR of *Nicotiana* photosystem I reaction center subunit *psaK*; LIR: long intergenic region of the BeYDV genome; SIR: short intergenic region of the BeYDV genome; SP-VEGF-His-SEKDEL: human vascular endothelial growth factor gene with histidine residues and SEKDEL retention tag at the C-terminus and signal peptide at the N-terminus; SP-VEGF-His: human vascular endothelial growth factor gene with histidine residues at the C-terminus and signal peptide at the N-terminus; SP-His-VEGF-SEKDEL: human vascular endothelial growth factor gene with SEKDEL retention tag at the C-terminus and signal peptide and histidine residues at the N-terminus; SP-His-VEGF: human vascular endothelial growth factor gene with signal peptide and histidine residues at the N-terminus; LB and RB: the left and right borders of the *Agrobacterium* T-DNA region.

**Table 1**
List of primer pairs used in the present study to generate recombinant VEGFs gene constructs.

| Gene | Primer Sequence |
|---|---|
| SP-VEGF-His | **F (*XbaI*-SP)** 5'TCTAGAACAATGGGCTGG<br>**R (*SacI*-His)** 5'GAGCTCTTAATGATGGTGATGGTGGTGATGATG |
| SP-His-VEGF-SEKDEL | **F (*XhoI*-His-VEGF)**<br>5'CCTCGAGCATCATCACCACCATCACCATCATAACTTCCTGCTGTCTTGG<br>**R (*SacI*-KD-VEGF)**<br>5'CGAGCTCTCAAAGCTCATCCTTTTCAGATCTTCTAGGCTTATCGCACC |
| SP-His-VEGF | **F (*XhoI*-His-VEGF)**<br>5'CCTCGAGCATCATCACCACCATCACCATCATAACTTCCTGCTGTCTTGG<br>**R (*SacI*-His)** 5'GAGCTCTTAATGATGGTGATGGTGGTGATGATG |

### 2.2. Transient expression and purification of VEGF

The plant expression vector (pBYR2e-VEGF) was further transformed into *Agrobacterium tumefaciens* GV3101 by electroporation. Transformed colonies were screened by PCR and positive transformants were inoculated in LB broth supplemented with 50 μg/mL kanamycin, 50 μg/mL gentamicin, 50 μg/mL rifampicin and grown at 28 °C overnight. The overnight grown *A. tumefaciens* cells were centrifuged at 4000 rpm for 15 min and the pellets were resuspended in infiltration buffer (10 mM 2-N-morpholino-ethanesulfonic acid (MES) and 10 mM MgSO4, pH 5.5) to get a final $OD_{600nm}$ of 0.4. The recombinant *Agrobacterium* cells harbouring any one of the constructs *viz*., pBYR2e-SP-VEGF-His-SEKDEL (S-V-H-K), pBYR2e-SP-VEGF-His (S-V-H), pBYR2e-SP-His-VEGF-SEKDEL (S-H-V-K) and pBYR2e-SP-His-VEGF (S-H-V) were used for agroinfiltration. Briefly 5-6 week old *N. benthamiana* leaves were used for agroinfiltration. The infiltrated leaves were collected on days 2, 3, and 4 post-infiltration (dpi) and the harvested leaves were ground/extracted with extraction buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl and 5 mM Imidazole) and centrifuged at 26, 000 x g for 20 min. After centrifugation, the supernatant was collected and the total soluble protein was quantified by Bradford assay. The expression levels of VEGF expressed by the different constructs were analyzed by using SDS-PAGE and Western blots probed with HRP-conjugated rabbit polyclonal anti-His antibody (Cat. No. ab1187; Abcam, UK). Based on the results, the construct that showed the highest protein expression was used for further protein expression and purification.

For protein purification, 25 grams of infiltrated leaves was extracted with extraction buffer and the crude extract was filtered using a sterile 0.45 μm filter. The filtered extract was loaded onto a Ni-NTA affinity column and then the column was washed with washing buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl and 20 mM Imidazole). The recombinant protein was further eluted with elution buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl and 250 mM Imidazole).

### 2.3. SDS-PAGE and Western Blotting

The protein samples were mixed with 10x non-reducing loading buffer (125 mM Tris-HCl pH 6.8, 12% SDS, 10% glycerol, 0.001% bromophenol blue, pH 6.8) and denatured at 95 °C for 5 min.. The denatured proteins were separated by 12% SDS-PAGE gel and then subjected to either Coomassie staining or Western blotting. For Coomassie staining, the gel was stained with Coomassie® blue solution and the bands were visualized. For Western blotting, the separated proteins were transferred to the nitrocellulose membrane and detected either with HRP-conjugated rabbit polyclonal anti-His antibody or with mouse anti-human VEGF antibody (Cat. No: DY293B; R&D system, USA) and HRP-conjugated goat anti-mouse antibody (Cat. No. ab97245; Abcam, UK). The membrane was developed by using enhanced chemiluminescence (ECL) plus detection reagent (Abcam, UK).

### 2.4. Quantification of VEGF by Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of plant-purified VEGF was determined by Enzyme-Linked Immunosorbent Assay (ELISA) by following the manufacturer's instructions (Human VEGF ELISA kit protocol, Cat. No: DY293B, R&D system, USA). The experiments were performed in triplicate and all the data are presented as mean ± standard deviation (SD).

### 2.5. Cell Culture

The human keratinocyte line HaCat was obtained from Cell Lines Service (CLS, Heidelberg, Germany). Briefly, HaCat cells were cultured in high glucose-Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin and streptomycin (Gibco, Gaithersburg, MA, USA). Cell cultures were incubated at 37 °C under a constant humidified atmosphere of 5% $CO_2$. At 70-80% confluence, cells were trypsinized by using 0.25% trypsin and resuspended in fresh DMEM. The cell suspension was used for further experiments.

### 2.6. Cell Cytotoxicity Analysis

MTT assay was performed to assess the *in vitro* cytotoxicity of plant produced VEGF in HaCat cells. The HaCat cells were seeded at a density of 10, 000 cells per well (96-well plate) and incubated at 37 °C overnight. Then, the cells were treated with different concentrations (0.1, 0.5, 1.0, 5.0, 10.0 ng/mL) of plant-produced VEGF for 24 h. After treatment, 100 μl of MTT reagent (0.4 mg/mL) was added to each well and maintained at 37 °C for 4 h. After incubation, MTT reagent was carefully removed and 100 μl DMSO was added. The absorbance was measured in a spectrophotometer at 570 nm by a Perkin Elmer No-1 microplate reader. The cell viability percentage was calculated from the ratio of optical density (OD) between treated cells to non-treated control cells [27].

### 2.7. Nuclear Staining Assay

Cell death by apoptosis and necrosis are examined through co-staining of Hoechst33342 and propidium iodide (PI) dye. HaCat cells were seeded at the density of 10, 000 cells/ well and incubated at 37 °C overnight. Then, the cells were treated with various concentrations (0.1, 0.5, 1.0, 5.0, 10.0 ng/mL) of plant-produced VEGF for 24 h. Treated and untreated cells were stained with 10 μM of Hoechst33342 and 5 μg/mL of PI for 30 min at 37 °C. The mode of cell death was observed by using a fluorescence microscope (Olympus IX51 with DP70). Apoptotic cells were characterized by a bright blue fluorescence signal of Hoechst33342 whereas the red fluorescence signal of PI indicated necrotic cells.

### 2.8. Cell Migration Assay

Cell migration analysis was performed to evaluate the *in vitro* wound healing effect of plant-produced VEGF by a scratch assay. HaCat cells were seeded at a density of 30, 000 cells/ well and incubated at 37 °C. To inflict the wound, a scratch was created in the cell monolayer by using a small plastic pipette tip across the centre of the well. Then, the cells were treated with different concentrations (1.0, 5.0, 10.0 ng/mL) of plant-produced VEGF. Cell migration into the wound space was monitored at0, 6 and 12 h after wounding. The wound width was calculated from the average distance between the edges of the scratch. The relative migration at each time point was calculated as follows [28].

$$\text{Relative migration at } Th_{\text{treatment}} = \frac{[\text{Width of wound at 0 h–Width of wound at T h}]_{\text{treatment}}}{[\text{Width of wound at 0 h–Width of wound at 6 h}]_{\text{control}}}$$

### 2.9. Statistical Analysis

All the experiments were performed in triplicate and the data are presented as means ± SD. Multiple comparisons were investigated with the control group by using analysis of variance (ANOVA) with Tukey's post-hoc test to identify significant differences. Statistical analysis was performed using SPSS program (IBM Inc., New York, USA) and a P value less than 0.05 ($p < 0.05$) considered statistically significant.

## 3. Results

### 3.1. Optimization of expression of Vascular Endothelial Growth Factor (VEGF) *in* Nicotiana benthamiana

The human vascular endothelial growth factor (VEGF) gene flanked with the his-tag and SEKDEL sequences was codon optimized and cloned into plant expression vector pBYR2e. In order to evaluate the heterologous expression of VEGF in plants, four gene constructs were developed: pBYR2e-SP-VEGF-His-SEKDEL (S-V-H-K), pBYR2e-SP-VEGF-His (S-V-H), pBYR2e-SP-His-VEGF-SEKDEL (S-H-V-K) and pBYR2e-SP-His-VEGF (S-H-V), and protein expression of the different constructs was tested in *N. benthamiana via* agroinfiltration. The infiltrated leaves were harvested on day 2, 3 and 4 post-infiltration and analyzed by Western blots probed with anti-His antibody under non-reducing conditions (Fig. 2). Equal amounts of total soluble protein (100 μg) was used for Western blotting and the protein expression levels of each construct was assessed. All the constructs exhibited leaf necrosis in *N. benthamiana* leaves after 3 dpi (Fig. 2A). The results showed that the highest expression of VEGF protein was achieved within 4 dpi (Fig. 2B). However, the optimal level of expression was apparently observed on 3 dpi. Among the four constructs tested, the expression of VEGF was found to be higher in both S-V-H-K and S-V-H constructs when compared to S-H-V-K and S-H-V constructs. Based on these results, S-V-H-K and S-V-H constructs were used for further experiments.

### 3.2. Purification of VEGF *in* Nicotiana benthamiana

Single-step Ni-NTA affinity chromatography was performed to purify recombinant VEGF from plant crude extracts. To investigate the possible effects of the position of the his-tag and SEKDEL sequence on gene expression and purification, the two constructs that showed high protein expression *ie.,* S-V-H-K and S-V-H, were transiently expressed and the plant crude extract was purified by affinity column chromatography (Fig. 3).The western blot of the expressed VEGF protein in S-V-H-K and S-V-H crude extracts showed proteins of approximately 46 kDa as expected. However, a slight difference in the protein size was observed between both constructs tested. In addition, the results showed that VEGF protein in S-V-H-K infiltrated plant extract binds efficiently to the immobilized nickel ion column which showed the accessibility of his-tagged VEGF to the Ni-column. In contrast, the VEGF protein in S-V-H plant extract did not bind effectively to the Ni-column and

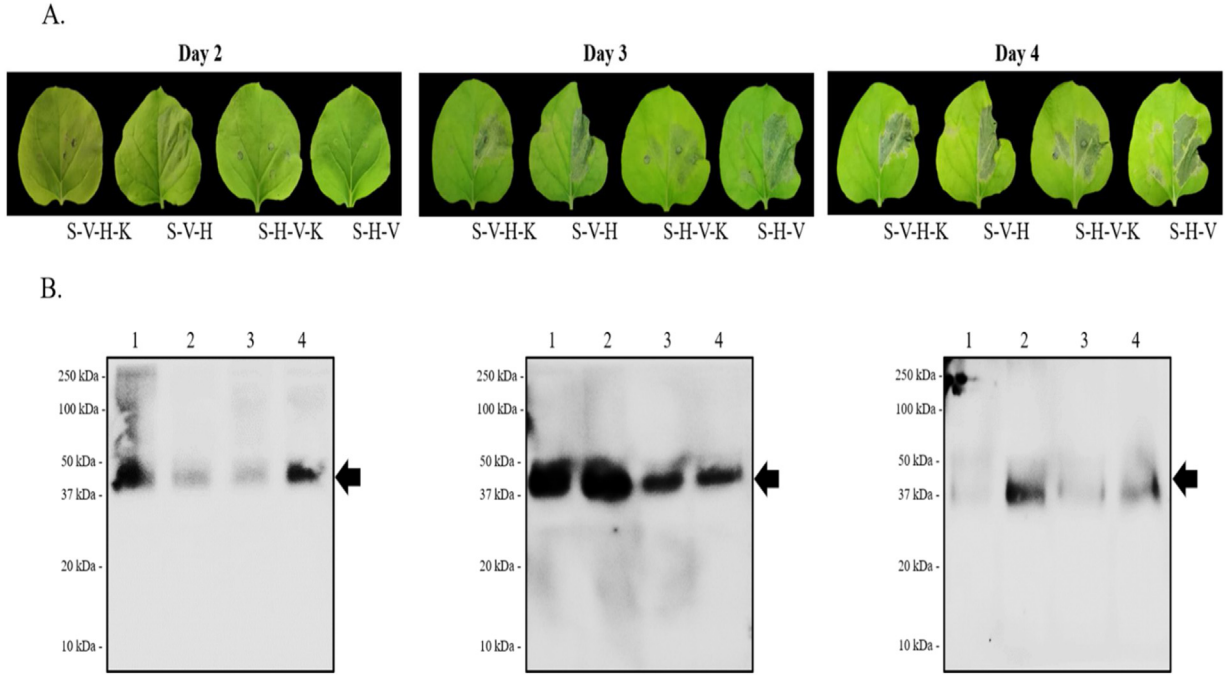


**Fig. 2.** Optimization of VEGF expression in *Nicotiana benthamiana*. Expression levels of recombinant protein in plants infiltrated with different vector constructs were determined on day 2, 3, and 4 post-infiltration. (A) The leaf necrotic symptoms on day 2, 3 and 4 post-infiltration with different constructs. (B) Expression of VEGF was shown for all the four constructs used. Equal concentrations of TSP (100 μg) was used for the experiments. Western blot probed with anti-His antibody under non-reducing conditions. Lane 1: S-V-H-K; Lane 2: S-V-H; Lane 3: S-H-V-K; Lane 4 S-H-V.

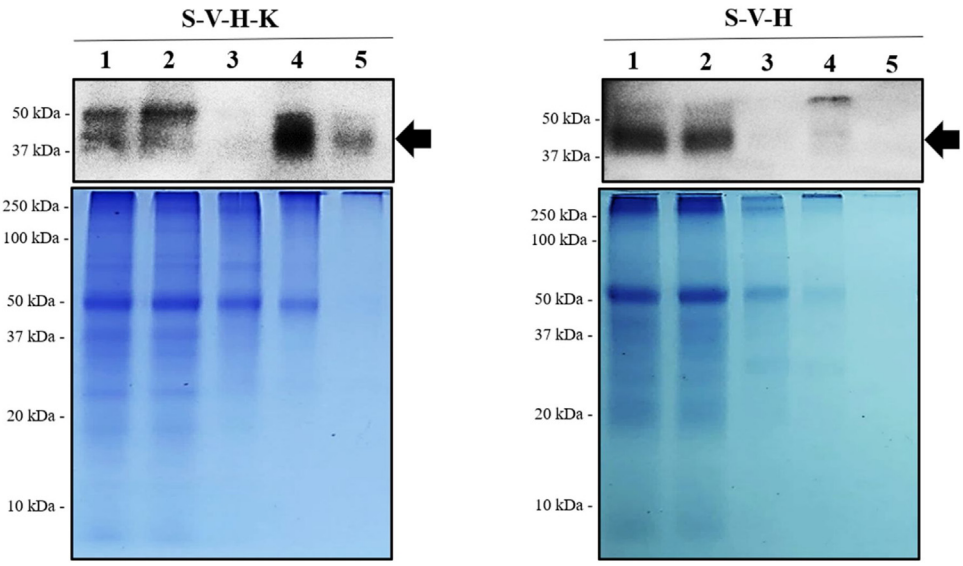


**Fig. 3.** Expression and purification of plant-produced VEGF. Purification of VEGF in S-V-H-K and S-V-H infiltrated plant leaves on day 3 post-infiltration was determined by Western blotting probed with HRP-conjugated rabbit polyclonal anti-His antibody and staining of SDS-PAGE gel with Coomassie® blue dye. Lane 1: plant crude extract; Lane 2: flow through; Lane 3: elution fraction at 20 mM Imidazole; Lanes 4 and 5: elution fractions at 250 mM Imidazole.

was observed in the flow through fraction which might be due to the inaccessibility of the his-tag to the nickel resin in the column. Based on these results, the construct, S-V-H-K was further used for large-scale protein production *via* vacuum infiltration. The crude protein was extracted from the infiltrated leaves (160 g) and the recombinant protein expression was confirmed by Western blotting probed with mouse anti-human VEGF antibody (Fig. 4). Under non-reducing condition, the recombinant VEGF protein was observed at ~46 kDa as predicted. The expression level of recombinant protein was quantified by ELISA. The highest level of VEGF accumulation up to 2.45 ng/g fresh weight was observed in S-V-H-K infiltrated leaves harvested on 3 dpi.

### 3.3. Effect of Plant-Produced VEGF on HaCat Cell Cytotoxicity

The cytotoxicity of the plant-purified VEGF was tested on HaCat cells by MTT assay (Fig. 5A). The HaCat cells were grown in a 96-well plate and were treated with 0.1, 0.5, 1.0, 5.0 and 10.0 ng/mL of plant-produced VEGF. The untreated cells were considered as negative control. The concentration-dependent effect of VEGF on the viability of HaCat cells were analyzed after 24 h incubation. The results showed that the plant-produced VEGF did not exhibit any cell toxicity, irrespective of concentration tested. The cells treated with high doses of VEGF showed 95-100% cell viability after 24 h treatment. These results demonstrated that plant-produced VEGF did not induce any cytotoxicity *in vitro*.

Further, the cell death was assessed through Hoechst and PI co-staining on HaCat cells (Fig. 5B). Neither apoptosis nor necrosis was observed in VEGF treated cells irrespective of the dose range. This result showed that the plant-produced VEGF did not induce cell death *in vitro*.

### 3.4. Effect of Plant-Produced VEGF on HaCat Cell Migration

The plant-purified VEGF was tested for cell migration activity on HaCat cells by *in vitro* scratch assay (Fig. 6). The cells were cultured with 1.0, 5.0 and 10.0 ng/mL of plant-produced VEGF and were monitored for 0, 6 and 12 h. The non-treated HaCat cells were used as negative control. Cell images were calculated for the change in gap area of treated cells over the control. The results showed that recombinant VEGF induced cell migration irrespective of the concentration used. Notably, the relative migration of HaCat cells treated with VEGF was moderately induced after 6 h of treatment compared to non-treated control cells (Refer supplementary table). Further findings showed significant difference in cell migration induced by the plant-produced VEGF after 12 h of treatment compared to the control, with a two-fold increase observed at a high concentration *ie.*, 10 ng/mL. This data suggests that the plant-based VEGF is biologically active and promotes cell migration in human keratinocyte cells.

## 4. Discussion

Plant expression systems have many advantages such as rapid scalability, flexibility, improved safety and high-yield of recombinant proteins at short time [29,30]. Growth factors are extensively studied for their skin renewal activities, wound healing properties, cell growth and collagen biosynthesis. Specifically, human vascular endothelial growth factor (VEGF) plays a major role in cell survival and regenerative medicine. Hence, in this study, we produced recombinant VEGF by transient expression in *N. benthamiana* plants. Different constructs were tested and the expression of recombinant VEGF in plants was optimized. The geminiviral replicon system based on the bean yellow dwarf virus was utilized for rapid production of recombinant VEGF. In order to determine the optimal time to harvest the biomass for high protein accumulation, a time course experiment was performed. The ER retrieval peptide, SEKDEL, in the C-terminal region has been shown to enhance production of recombinant proteins in plants [31]. Previous studies demonstrated that SEKDEL directs protein to the ER *via* – retrograde transport, ensuring proper folding and increasing protein stability [32,33]. However, improved protein accumulation was not consistently observed in all cases [34].The expression of recombinant VEGF expressed in plant leaves infiltrated with different constructs was assessed by Western blotting. The results showed that the highest level of expression was attained on day 3post-infiltration with no significant difference observed when using the constructs with or without the C-terminal SEKDEL motif – in contrast to other studies which reported that the inclusion of SEKDEL improves protein accumulation and stability [35–37]. Furthermore, the localization of histidine residues was analyzed to determine its effect on protein

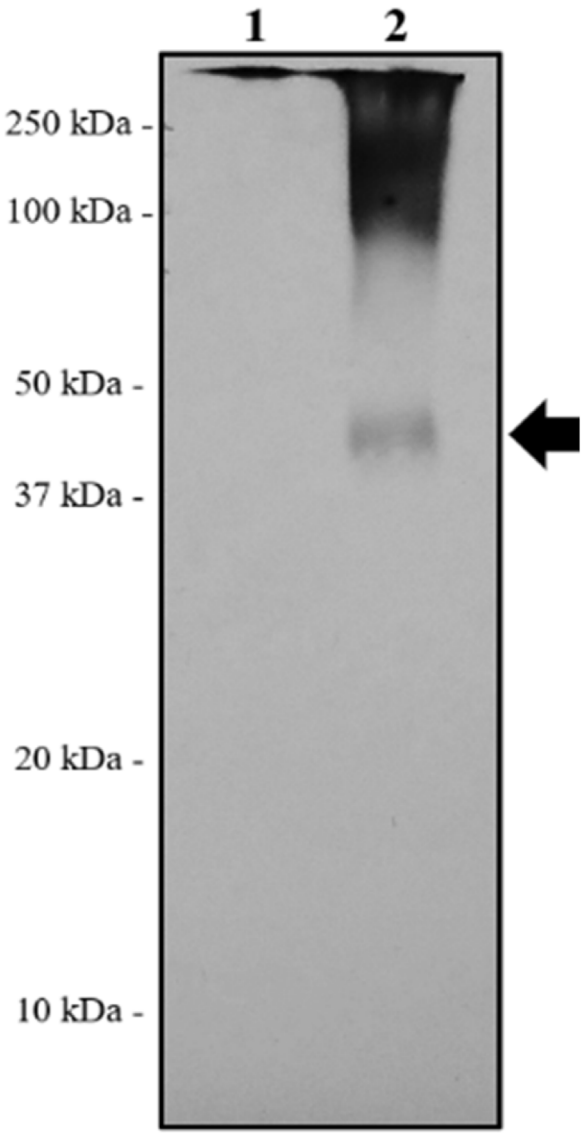


**Fig. 4.** Western blot analysis of purified plant-produced VEGF with S-V-H-K construct analyzed with mouse anti-human VEGF antibody as the primary antibody and detected with HRP-conjugated goat anti-mouse antibody. Lane 1: non-infiltrated plant purified extract; Lane 2: purified plant-produced VEGF.

expression and purification efficiency. The his-tag was added with VEGF either on the N-terminus (N-His) or on the C-terminus (C-His) in order to facilitate simple purification of proteins by immobilized metal affinity chromatography. Although several reports have shown that there is negligible or no effect on the location and attachment of the his-tag with the protein stability and structure [38], few reports showed the differential effect of location of the his-tag at the N- and C-termini [39]. The Western blotting results showed that C terminal-his-VEGF constructs had higher protein expression levels compared to the N terminal his-VEGF constructs.

The recombinant VEGF was expressed with the C-terminal his-tag (S-V-H-K and S-V-H) within 3 days of post-infiltration and the expressed protein was detected by Western blotting probed with anti-his antibody. The VEGF protein was detected at a size of ~46 kDa with slight difference in the protein size observed between two different constructs tested which might be due to plant-specific glycosylation of recombinant proteins [40].The native VEGF contains one glycosylation site which is critical for the efficient secretion of the protein [41]. The recombinant VEGF protein was purified and used for further analysis. The VEGF protein in S-V-H extract was observed in the flow through suggesting insufficient exposure of the his-tag for binding that could be due to differential protein folding. In some cases, his-tagged proteins are not able to bind to the immobilized resin due to inaccessibility of the his-tag with the divalent metal ion on the column [42], leading to the loss of target protein in the flow through fraction during purification process. Accordingly, a prominent band was observed in the elution fraction of S-V-H-K showing the efficient binding and accessibility of the his-tagged VEGF to the nickel column. The molecular weight of VEGF protein is approximately 23 kDa and 46 kDa in monomeric form [8] and dimeric form [4], respectively. The purified plant-produced VEGF was confirmed by using both anti-His and anti-VEGF antibodies under non-reducing conditions and the band size of ~46 kDa was observed as expected.

The cytotoxic effect of purified VEGF on human keratinocytes HaCat cells was examined by MTT assay. In accordance with previous reports [43,44], various concentrations ranging from 0.1 to 10 ng/mL of recombinant VEGF was tested in the study. The results showed that the plant-produced VEGF did not show any cytotoxic effect on HaCat cells in all the concentrations tested. This is consistent with the earlier reports which showed that recombinant VEGF [43,45,46] effectively promotes the growth of epithelial cells, oral primary cells and endothelial cells at low concentration.

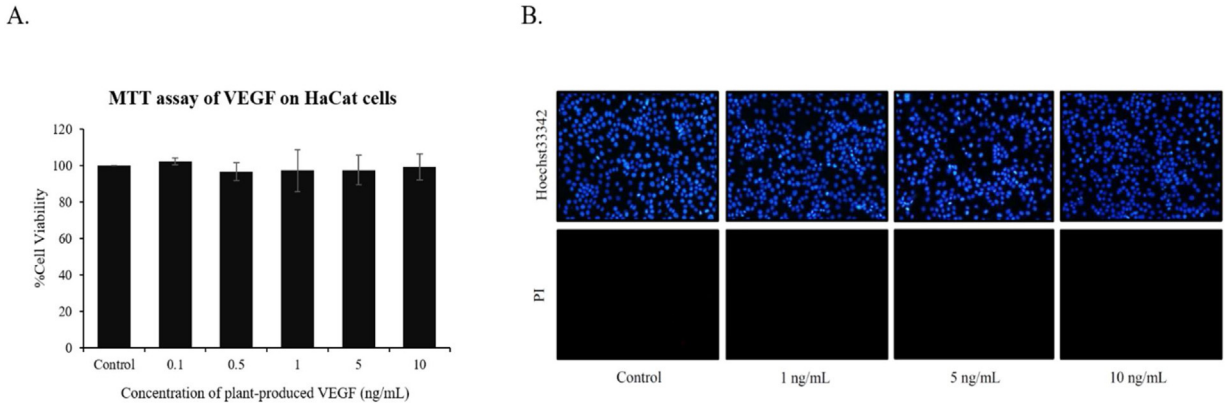


**Fig. 5.** Effect of plant-produced VEGF on cell cytotoxicity. (A) HaCat cells (10,000 cells) were seeded in 96-well cell culture plate and incubated with 0.1 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 5.0 ng/mL and 10.0 ng/mL of plant-produced VEGF for 24 h. After incubation, MTT assay was performed. Data corresponds to the percentage (%) of cell viability. (B) Representative images of cells after treatment with increasing concentration of plant-produced VEGF. HaCat cells (10,000 cells) were cultured with 1.0 ng/mL, 5.0 ng/mL and 10.0 ng/mL plant-produced VEGF for 24 h. Cells were co-stained with Hoechst 33342 and propidium iodide (PI) dyes to assess mode of cell death.

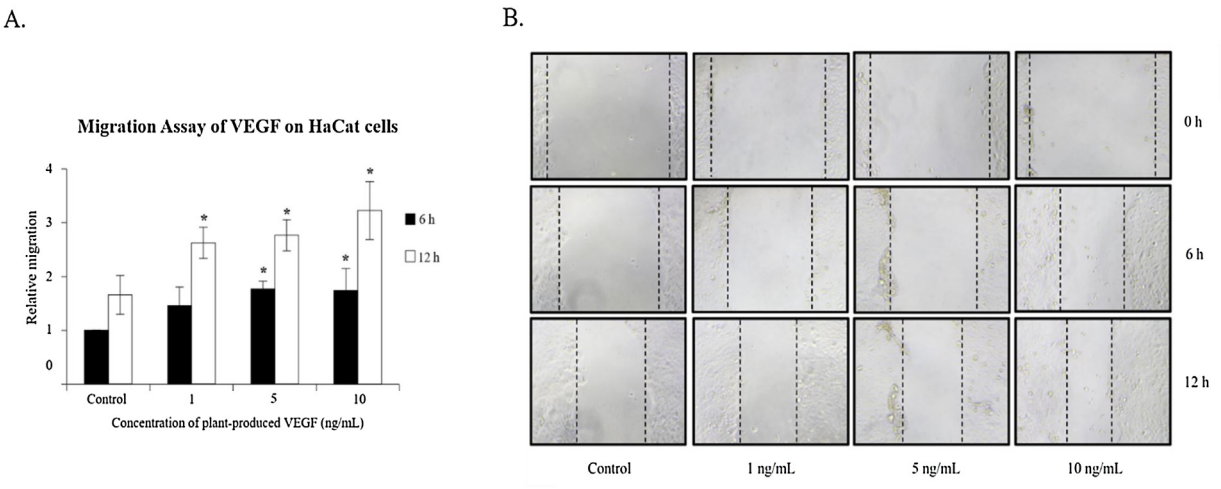


**Fig. 6.** Effect of plant-produced VEGF on cell migration. (A) The HaCat cells at a density of 30, 000 cells/ well in a 96-well cell culture plate were treated with 1.0 ng/mL, 5.0 ng/mL and 10.0 ng/mL of plant-produced VEGF for 6 and 12 h. Relative migration was measured by using the scratch assay. The result shown are means ± SD ($p < 0.05$ compared with the untreated control cells at the same time point). (B) The cells were observed t0, 6 and 12 h after treatment under an inverted microscope.

VEGF plays a major role in the migration of endothelial cells, epithelial cells, fibroblasts, and epidermal keratinocytes cells [47–49]. In this study, the activity of plant-produced VEGF was tested by *in vitro* scratch assay. The migration effect on HaCat cells induced by plant produced VEGF was tested at different time points and the results showed that the recombinant VEGF induced migration in a dose-dependent manner. Similar to other studies, relative migration of endothelial cells was induced within the gradient concentration of VEGF [49–51]. The results showed the preliminary findings of plant produced VEGF in inducing the migration and proliferation of HaCat cells. As the doubling time of human keratinocyte HaCat cells is about 21-24 h [52], the experiment on migration effect was performed for 6-12 h after treatment with plant-produced VEGF in order to minimize the effect of cell proliferation.

In conclusion, the present study demonstrates the feasibility of using a transient plant expression system to produce the functional VEGF protein in *N. benthamiana.* The plant-produced VEGF was purified by single-step Ni-affinity chromatography and tested *in vitro*. The plant-produced VEGF did not show any cytotoxic effect and activates the migration of human keratinocyte cell HaCaT *in vitro*. However, the underlying cellular mechanisms for plant-produced VEGF on wound healing effects on HaCat cells were not elucidated. Future studies will focus on elucidating the wound healing pathways and angiogenic effect of plant-produced VEGF on HaCat cells. Altogether, these results showed that the functional VEGF protein can be produced in plants that can potentially be exploited for their application in tissue engineering and dermo-cosmetics.

## CRediT authorship contribution statement

**Christine Joy I. Bulaon:** Investigation, Methodology, Writing - original draft, Writing - review & editing. **Balamurugan Shanmugaraj:** Methodology, Writing - original draft, Writing - review & editing. **Yamin Oo:** Methodology, Writing - original draft, Writing - review & editing. **Kaewta Rattanapisit:** Investigation, Methodology. **Taksina Chuanasa:** Investigation. **Chatchai Chaotham:** Writing - original draft, Writing - review & editing. **Waranyoo Phoolcharoen:** Investigation, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Acknowledgement

This study was supported by Thailand Research Fund grant No. RSA6280006 and the Chulalongkorn Academic Advancement its 2nd Century Project. C.J.I.B and Y.O was supported by the Scholarship Program for ASEAN Countries; B.S. was supported by the Second Century Fund (C2F), Chulalongkorn University; K.R. was supported by the Ratchadaphiseksomphot Fund, Chulalongkorn University.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00514.

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