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Research Article

# FACTOR PROMOTING WOUND HEALING: RADICAL SCAVENGING AND ANTI-INFLAMMATORY ACTIVITY AND GROWTH FACTOR PROMOTION OF *HELIOTROPIUM INDICUM*

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**ABSTRACT**

**Objective:** This study aims to investigate the effects of the *Heliotropium indicum* extract (HIE) on factor promoting wound healing in radical scavenging and inflammatory activity and growth factor promotion.

**Methods:** The radical scavenging capacity of HIE was evaluated by scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) radicals. Furthermore, the anti-inflammatory of HIE was determined in a cellular model. RAW264.7 macrophage cells were treated with various concentrations of HIE before activating the treated cell with lipopolysaccharide (LPS). The nitrite concentration of activated macrophage was determined by the Griess reagent kit. The cell viability of RAW264.7 was evaluated by resazurin reduction assay as well as NIH3T3 fibroblast cells. In addition, production of the growth factors (transforming growth factor-β [TGF-β] and basic fibroblast growth factor [bFGF]) of fibroblast was determined by Elisa kit.

**Results:** HIE exhibited radical scavenging activity in the DPPH and NO radicals with half maximal inhibitory concentration ($IC_{50}$) at 0.22 mg/ml and 0.52 mg/ml, respectively. In a cellular study, HIE inhibited NO production in LPS-stimulated macrophage without cytotoxic effect to the cells with $IC_{50}$ at 87 µg/ml. Furthermore, HIE promoted fibroblast cell viability at 72 h of treatment and, TGF-β and bFGF production at 24 h of treatment.

**Conclusion:** These results obtained in this study suggested that HIE promoted the factors which involved in wound healing processes, including anti-inflammatory effect with scavenged radical forming and inhibited activated-macrophage. Furthermore, HIE also stimulated growth factor production in fibroblast. These finding supported using traditional and folk medicine of *H. indicum* in wound treatment.

**Keywords:** *Heliotropium indicum*, Wound healing, Radical scavenging, Anti-inflammatory, Growth factors.

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## INTRODUCTION

Wound healing is a crucial physiological process that involves in communication of multiple cell types. The integration of cellular and molecular events occurs after the onset of tissue damage to a restoration of the injured tissues. The tissue restoration mechanism is continued with overlapping processes by hemostasis, inflammation, proliferation, and tissue remodeling [1].

Macrophages and fibroblast play critical roles in all phases of wound healing. After tissue injury, macrophages infiltrate to the lesion for phagocytosis of bacterial pathogens, foreign debris, and dead cells, and generate pro-inflammatory mediators resulting in inflammation [2]. In the inflammatory phase, macrophages are called M1 macrophages. They are activated to generate reactive oxygen species (ROS) as well as nitric oxide (NO) to contribute reactive nitrogen species (RNS) for clearance of pathogens and apoptotic cells. In addition, macrophages also generate pro-inflammatory mediators, for example, interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) as well as growth factors, for example, basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF). These pro-inflammatory mediators and growth factors recruit endothelial cells, keratinocytes, and fibroblasts to heal the injured tissues [3]. In the late inflammatory phase, the pro-inflammatory M1 macrophages transit to anti-inflammatory M2 macrophages for production of anti-inflammatory cytokines, for example, IL-4 and IL-10; meanwhile, inflammation is subsided let through the proliferative phase. In the remodeling phase, macrophages release matrix metalloproteinases to break down the temporary extracellular matrix (ECM) [4].

Fibroblasts are recruited to the wound site from the wound edge or the bone marrow after wounding. At the inflammation phase, fibroblasts produce several chemokines. At the proliferation phase, fibroblasts are activated within the wound site and differentiated to myofibroblasts to form granulation tissue. Both fibroblasts and myofibroblasts play a crucial role in generation of traction and contractile forces, respectively, to promote wound closure [5]. Fibroblasts also produce important growth factors including TGF-β and bFGF.

TGF-β is implicated in the control of fibroblast proliferation, and transformation of fibroblasts into myofibroblasts, production of the ECM, stimulation of collagen production, production of elastin, and synthesis of fibronectin, and inhibition of ECM degradation [6]. bFGF is also involved in the regulation of the replication and migration of epithelial, endothelial, and fibroblast cells, which participated in the production of collagen, epithelialization, and neovascularization, respectively [7].

In the case of chronic wound, it is found the excess production of ROS and RNS, which can directly interact with various biological targets. Macrophages are continuously activated to produce ROS, RNS, and pro-inflammatory cytokines including IL-1, IL-6, and TNF-α resulting in excess of inflammation where accompany with the limitation of growth factor, for example, TGF-β and bFGF, production [8]. The immoderate inflammation cause an imbalance of the healing processes indicated that the proliferative and remodeling phases do not readily occur. This event originates chronic wound which does not heal in a timely manner [9,10].

*Heliotropium indicum* is found in many paths of the world such as India, Africa, Bangladesh, Sri Lanka, Nepal, the Philippines, and Thailand. It has

been used to cure various diseases in different traditional and folklore medicine system. In folk remedies, *H. indicum* has been used for treating fever, insect bite, diarrhea, skin rash, and herpes as well as wound and ulcer [11]. Furthermore, it has been reported that the application of the leaf paste of *H. indicum* to heal rheumatism and skin infections as well as fresh cuts and wounds [12]. Previous studies in the animal model reported the healing promotion of *H. indicum* extract (HIE) in the incision and infected wound [12] as well as diabetic wound [13].

This study aims to examine the HIE on the factor promoting wound healing including (a) *in vitro* radical scavenging activity, (b) anti-inflammatory activity in macrophage, (c) production of growth factors (TGF-β1 and bFGF) in fibroblast, and (d) viability of macrophage and fibroblast cells. These results provide scientific support of using *H. indicum* in folklore medicine and a new direction for the promotion of wound healing.

## METHODS

### Plant material

*H. indicum* Linn. procured from Khaen Dong District, Buri Ram Province, Northeastern Thailand. The leaves of the plant were cleaned with water then dried at 50°C for 72 h. The dried plant was grounded and stored at room temperature in vacuum. The extract was prepared by maceration of the grounded plant in 70% ethanol and shook at 250 rpm with the shaker for 24 h. The macerated mixture was filtrated for collected supernatant to evaporate at 50°C for 24 h. The crude extract of *H. indicum* leaves was stored at −20°C until used.

### 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of HIE

The HIE was first dissolved in a 2% dimethyl sulfoxide (DMSO), approximately 75 µl of the dissolved HIE of variable concentrations (0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/ml) was mixed with 150 µl of 0.2 mM DPPH (Sigma-Aldrich USA) solution (in methanol) and allowed to stand for 30 min without direct exposure to light. The absorbance was determined at 520 nm using a microplate reader. In addition, DMSO and L-ascorbic acid were used as negative and positive controls, respectively. The DPPH scavenging capacity of the experimental HIE is presented as a percentage of DPPH radical inhibition as below, where OD is the optical density:

$$\%\text{DPPH radical inhibition} = \left[\frac{\text{OD}_{\text{without extract}} - \text{OD}_{\text{with extract}}}{\text{OD}_{\text{without extract}}}\right] \times 100$$

### NO radical scavenging of HIE

In this research, sodium nitroprusside (SNP) (Sigma-Aldrich, USA) was utilized as the NO donor. Specifically, 10 mM of SNP in a pH7.4 PBS solution was incubated with 1 ml dissolved HIE of variable concentrations (0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/ml) at 25°C for 180 min. Approximately 100 µl of the resulting solution was withdrawn to react with a Griess Reagent kit (Promega, USA) whereby the solution was reacted with 20 µl sulfanilamide for 10 min and then 20 µl N-1-naphthylethylenediamine dihydrochloride for another 10 min. The reaction mixture absorbance was measured at 560 nm, and the NO concentrations were determined as the nitrite ($NO_{2^-}$) concentrations from the standard curve of a standard nitrite solution. The reaction mixture absorbance was measured at 560 nm. DMSO and L-ascorbic acid were used as negative and positive controls, respectively. The NO scavenging capacity of the experimental pectin is expressed as a percentage of nitrite production inhibition using the following formula:

$$\%\text{NO}_2\text{\_inhibition} = \left[\frac{\text{NO}_{2-\text{without extract}} - \text{NO}_{2-\text{with extract}}}{\text{NO}_{2-\text{without extract}}}\right] \times 100$$

### Cell culture

RAW264.7 murine macrophage and NIH3T3 murine fibroblast cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) and incubated at 37°C in 5% $CO_2$/95% air humidified incubator. The RAW264.7 cell was subcultured using cell scraper twice a week, and 3T3 cell was subcultured using 0.25% trypsin-EDTA (Gibco, USA) trypsinization. The cell viability was determined using 0.4% trypan blue (Sigma-Aldrich, USA) with cell viability >85% and was used in all the experiments.

### NO production in the lipopolysaccharide (LPS)-stimulated macrophage

The RAW264.7 macrophage cells ($2\times10^5$ cell/ml) were pretreated with the dissolved HIE of variable concentrations (6.25, 12.5, 25, 50, and 100 µg/ml) in a 96-well plate and incubated at 37°C for 24 h. The pre-treated cells were stimulated with 1 µg/ml of LPS and incubated for another 24 h. The NO concentrations were determined from nitrite ($NO_{2^-}$) in the stimulated-cell supernatant using a Griess Reagent Kit whereby 100 µl of the supernatant was reacted with 20 µl sulfanilamide for 10 min and with 20 µl N-1-naphthylethylenediamine dihydrochloride for another 10 min. The reaction mixture absorbance was measured at 560 nm, and the NO concentrations were determined as the nitrite ($NO_{2^-}$) concentrations from the standard curve of a standard nitrite solution. About 0.2% DMSO and 100 µM of dexamethasone each with 1 µg/ml LPS were, respectively, used as the negative and positive controls.

### Determination of cell viability of RAW264.7 macrophage cells

The viability of the residual macrophage cells after the NO assay, given HIE concentrations of 6.25, 12.5, 25, 50, and 100 µg/ml, was examined by resazurin (Sigma-Aldrich, USA) reduction assay, whereby the residual cells were incubated for 2 h at 37°C in 100 µl fresh DMEM containing 50 µg/ml resazurin. The reaction mixture absorbance was determined at 560 against 600 nm. The cell viability of the RAW264.7 macrophage cells was presented as percentage cell viability using the following formula:

$$\%\text{Cell viability} = \left[\frac{(\text{OD}_{560} - \text{OD}_{600})_{\text{with extract}}}{(\text{OD}_{560} - \text{OD}_{600})_{\text{without extract}}}\right] \times 100$$

### Determination of cell viability of NIH3T3 fibroblast cells

The NIH3T3 fibroblast cells ($4\times10^5$ cell/ml) were seeded in a 96-well plate and incubated at 37°C for 24 h. The cells were treated with the variable of HIE concentrations at 6.25, 12.5, 25, 50, and 100 µg/ml for 24, 48, and 72 h. The cell viability was determined by resazurin (Sigma-Aldrich, USA) reduction assay, whereby the cells were incubated for 4 h at 37°C in 100 µl fresh DMEM containing 50 µg/ml resazurin. The reaction mixture absorbance was determined at 560 against 600 nm. The cell viability of the NIH3T3 fibroblast cells was presented as percentage cell viability using the above formula.

### TGF-β and bFGF production in fibroblast cells

The NIH3T3 fibroblast cells ($4\times10^5$ cell/ml) were seeded into 24-well plate at 37°C for 24 h. The cells were treated with the variable of HIE concentration at 12.5, 25, and 50 µg/ml for another 24 h. The supernatant was collected to determine TGF-β and bFGF levels, respectively, using TGF-β1 Mouse Elisa kit (Abcam, USA) and bFGF Mouse Elisa kit (Sigma-Aldrich, USA) according to the manufacturer's protocol.

### Statistical analysis

In this study, data from at least three independent experiments carried out in triplicate. The statistical data were expressed as mean with a standard error of the mean. The negative control group was compared against the experimental group using one-way ANOVA with Tukey's honestly significant difference (HSD) *post hoc* test, with the 5% (*$p<0.05$) and 1% (**$p<0.01$) significance level.

## RESULTS

### DPPH scavenging capacity of HIE

In this study, the DPPH scavenging capacity of HIE is evaluated by the percentage of DPPH free radical inhibition and half maximal inhibitory concentration ($IC_{50}$). $IC_{50}$ is the IC at which the DPPH radicals are scavenged by 50%. The results revealed that with 0.0156–2 mg/ml (0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/ml) of HIE concentrations, HIE significantly reduces DPPH radicals, achieving the inhibition performance in the range of 29.17±5.62–90.78±1.17% (Fig. 1); and an $IC_{50}$ of 0.22 mg/ml. Meanwhile, L-ascorbic acid at the same concentration which used as the positive control exhibits the inhibition efficiency of 31.53±3.90–84.88±0.29% (Fig. 1); and an $IC_{50}$ of 0.030 mg/ml (Table 1).

**Table 1: DPPH and NO half maximal inhibitory concentrations of HIE and L-ascorbic acid**

| Testing compound | $IC_{50}$ of free radical scavenging (mg/ml) | |
|---|---|---|
| | DPPH | NO |
| HIE | 0.22 | 0.52 |
| L-ascorbic acid | 0.030 | 0.034 |

DPPH: 2,2-diphenyl-1-picrylhydrazyl, NO: Nitric oxide, HIE: *Heliotropium indicum* extract, $IC_{50}$: Half maximal inhibitory concentration

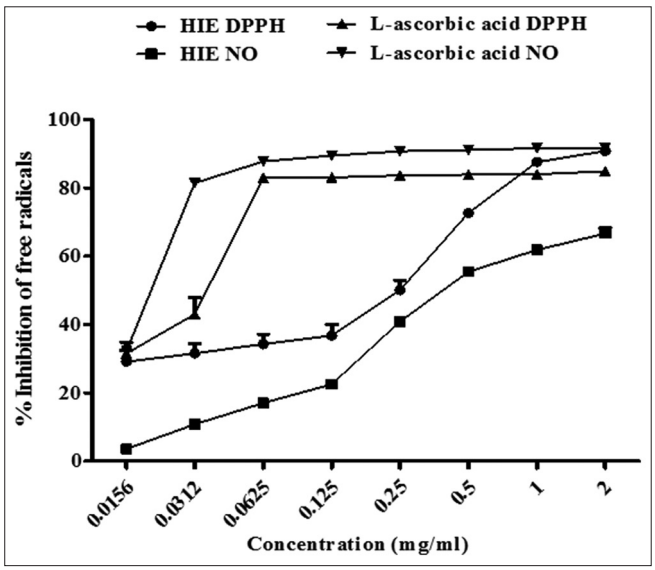


**Fig. 1: The nitric oxide (nitrite) inhibition efficiency (%) under variable *Heliotropium indicum* extract (HIE) and L-ascorbic acid concentrations, compared with HIE-free dimethyl sulfoxide (the negative control). The values are means ± standard error of the mean. L-ascorbic acid is used as the positive control**

### NO scavenging capacity of HIE

The experimental results reveal that HIE could scavenge the NO radicals. In the negative control with the absence of HIE (DMSO) presented a high nitrite (a stable product of NO) concentration while the positive control (L-ascorbic acid) inhibited the nitrite ($NO_2^-$) formation. The resulted revealed that with 0.0156–2 mg/ml (0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/ml) of HIE concentrations, HIE significantly reduces $NO_2^-$ formation, achieving the scavenging performance in the range of 3.59±0.46–66.61±2.87% (Fig. 1); and an $IC_{50}$ of 0.52 mg/ml (Table 1), where $IC_{50}$ is the IC at which the NO radicals are scavenged by 50%. By comparison, L-ascorbic acid (the positive control) at the same concentrations achieved the inhibition performance of 32.26±0.86–91.72±0.19% (Fig. 1); and an $IC_{50}$ of 0.034 mg/ml (Table 1).

### Effect of HIE on NO production and cell viability in LPS-stimulated macrophage

In this study, the RAW264.7 macrophage cells were treated with variable concentrations of HIE (6.25, 12.5, 25, 50, and 100 µg/ml) for 24 h before activating with 1 µg/ml LPS. Furthermore, DMSO and 100 µM of dexamethasone each with LPS were, respectively, used as the negative and positive controls. In Fig. 2a, the experimental results showed that the nitrite ($NO_2^-$) concentration in the LPS-stimulated macrophage was decreased in HIE-treated cells, achieving the inhibition performance in the range of 16.64±1.70–54.87±1.86% (Fig. 2a); and an $IC_{50}$ of 87 µg/ml. The NO inhibition is more obvious in the treatment with 12.5–100 µg/ml HIE concentrations. The dexamethasone-treated cells (positive control) achieved NO inhibition performance by 64.51±6.63% (Fig. 2a).

In Fig. 2b, the viability of the RAW264.7 macrophage cells was assessed by a resazurin reduction assay and the effective HIE concentrations determined. DMSO and dexamethasone each with 1 µg/ml LPS were, respectively, used as the negative and positive controls. In general, the cell viability and NO production are positively correlated. Given the non-cytotoxicity of HIE, the HIE-treated cells could achieve high NO inhibition performance with no effect to cell viability.

### Effect of HIE on fibroblast cell viability

The cell viability of the NIH3T3 fibroblast cells was examined by resazurin reduction assay. DMSO was used as negative control. In Fig. 3, the fibroblast cells were treated with variable concentration of HIE 6.25–100 µg/ml. The results indicate that the cell viability of HIE-treated cell for 24, 48, and 72 h trend to increase. However, the cell viability of 24 and 48 h of treatment was not significantly different from the control (DMSO treated-

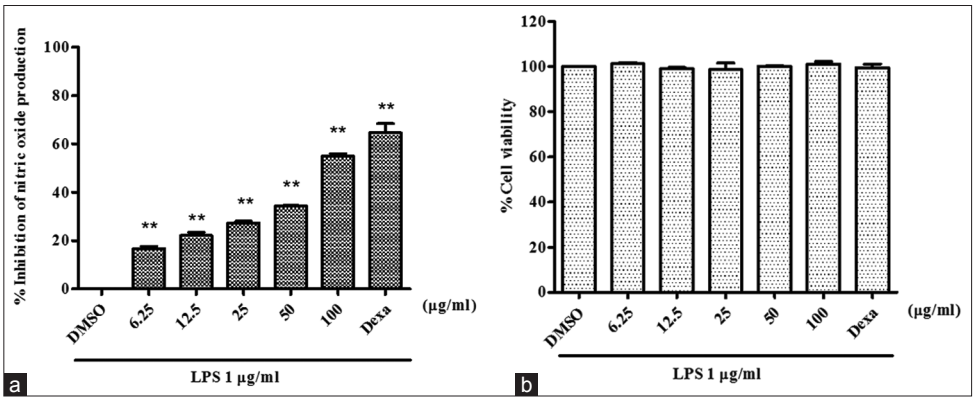


**Fig. 2: Effect of variable *Heliotropium indicum* extract concentration in 1 µg/ml lipopolysaccharide-stimulated RAW264.7 macrophage: (a) Percentage of nitric oxide inhibition, (b) cell viability. The values are means ± standard error of the mean. **denote p<0.01. Dimethyl sulfoxide and dexamethasone are, respectively, used as negative and positive controls**

cells). For 72 h of HIE-treated cells, the cell viability is more pronounced in treatment with 50 µg/ml HIE concentration-treated cell (Fig. 3).

**Effect of HIE on TGF-β1 and bFGF production in fibroblast cells**

The growth factors, TGF-β1, and bFGF are determined by ELISA kit. The results exhibited that HIE with a concentration of 12.5, 25, and 50 µg/ml significantly increase the production of TGF-β1 (Fig. 4a) and bFGF (Fig. 4b).

## DISCUSSION

Wound healing is the coordination and dynamic process that involves in many cell types, including macrophages and fibroblasts as well as their cytokines and growth factors. Macrophages promote and resolute inflammation, remove apoptotic or debris cells, and support cell proliferation, which makes equable of wound healing process [14]. However, macrophages have the potential to disturb different phases of repair, and persistent macrophage activation can lead to maladaptive, chronic inflammation, and dysfunction wound healing [15]. Macrophages continue to produce NO and ROS in prolonging inflammation that can cause to wound surrounding cell injury and chronic wound [16]. The previous study demonstrated that the chronic wound both infected and noninfected wounds improved by antioxidant, where it scavenges the excess free radicals to reduce the damage caused. In addition, the previous study revealed the correlation of radical scavenging properties of *Sphaeranthus amaranthoides* and wound healing improvement in mice [17]. This current study showed that HIE effectively scavenged free radical, DPPH, and NO. Based on these results, radical scavenging activity of HIE belongs to the enhancement of wound healing.

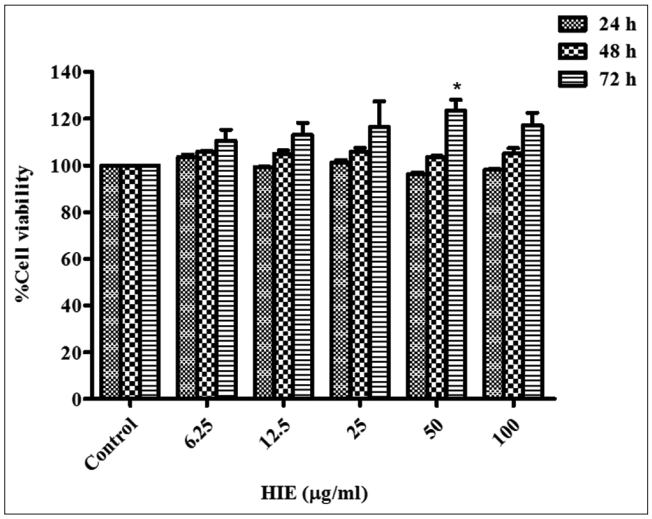


**Fig. 3: The effects of variable *Heliotropium indicum* extract concentrations treated cell for 24, 48, and 72 h on cell viability of NIH3T3 fibroblast cells. The values are mean ± standard error of the mean. *denote p<0.05. 0.2% dimethyl sulfoxide is used as negative control**

Moreover, the results also revealed the anti-inflammatory property of HIE through inhibition of NO production in activated macrophages. In general, although NO is a physiological signaling molecule, inordinate inducible NO becomes oxidative molecule which can interact with $O_2^-$ to produce cytotoxic oxidant peroxynitrite ($ONOO^-$). The peroxynitrite is a powerful oxidant initiate cleavage DNA resulting in cell damage [18]. Prolong activation of macrophages cause to continue production of NO associated with chronic inflammation that impedes proliferation stage in the wound healing process [19]. The previous study revealed that failure in the change of M1 inflammatory macrophage to M2 anti-inflammatory macrophage phenotype caused to prolong inflammatory stage of wound healing where delay in transition to the proliferative stage [20]. Furthermore, suppression of M1 inflammatory macrophage and enhancement of M2 anti-inflammatory macrophage polarization together with degradation of receptor interacting protein 140 became anti-inflammation to promote diabetic chronic wound healing in mice [21]. In this study, HIE inhibited the production of NO in activated macrophage and given non-cytotoxic to the cells, suggested that the limitation of inflammation, where to support the initiation of proliferation stage of wound healing.

Proliferative stage of wound healing occurs after the subsidence of inflammation where the major focus on re-epithelialization, vascular network restoration, and granulation tissue formation. Fibroblasts play important roles to form the granulation tissue. They migrate from the nearby dermis to the wound in response to cytokines and growth factors produced by platelets and macrophages in the wounds, for example, PDGF, TGF-β, and bFGF. In addition, fibroblasts also produce cytokines, chemokines, and growth factors to enhance angiogenesis [22]. bFGF and TGF-β1 are key growth factors implicating in the process of wound healing.

In this study, TGF-β1 and bFGF were upregulated in HIE-treated fibroblast cells. The previous study demonstrated that aloe vera can stimulate TGF-β1 and bFGF production in mouse embryonic fibroblast cell [23] as well as *Panax ginseng* encouraged TGF-β1, collagen type I, and VEGF production in NIH3T3 fibroblast cells [24]. These growth factors are important in the improvement of chronic wound healing. TGF-β1 regulates several cell functions, for example, proliferation, differentiation, apoptosis, cell adhesion, cell motility, and ECM production [25]. bFGF regulates replication and migration of epithelial, endothelial, and fibroblast cells which implicated in collagen production, epithelialization, and neovascularization of the wound healing process [26]. The upregulation of these growth factors in

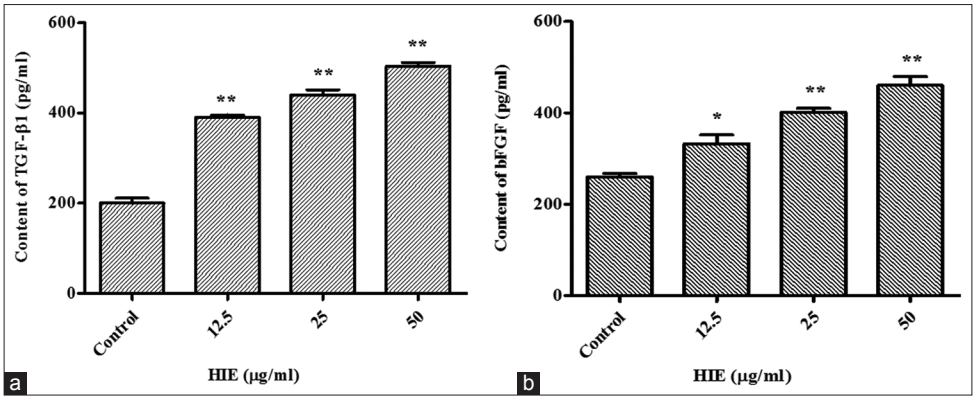


**Fig. 4: The effects of variable *Heliotropium indicum* extract concentrations treated cell for 24 h on growth factor production in NIH3T3 fibroblast cells: (a) Transforming growth factor-β1, (b) basic fibroblast growth factor. The values are mean ± standard error of the mean. *and **, respectively, denote p<0.05 and p<0.01. 0.2% dimethyl sulfoxide is used as negative control**

function and production can help wound repair, especially the chronic wounds which presented growth factor deficiency.

In an animal model study, HIE promoted the healing of excision (normal and infected), incision, and dead space wound models [12], and excision wound in streptozotocin-induced diabetic rats [13]. The findings from our research in the cellular study provide a better understanding of the function of HIE in wound healing improvement. Given antioxidant, anti-inflammatory properties, and growth factors promotion of HIE, the extract could thus be applied to encourage chronic wound healing and increase the possibility of therapeutic application of HIE in wound healing. However, further phytochemical studies of HIE are required to separate the active compounds responsible for these pharmacological activities.

## CONCLUSION

This experimental research has investigated the scavenging ability of HIE of variable concentration (0.0156–2 mg/ml) on DPPH and NO free radicals; anti-inflammatory property through inhibition of NO production in LPS-stimulated macrophage cells; and cell viability of LPS-stimulated macrophage and fibroblast cells, given the HIE concentration 6.25–100 µg/ml; and promotion of TGF-β1and bFGF growth factors in fibroblast cells, given the HIE concentrations of 12.5–50 µg/ml. The experimental results revealed that the DPPH and NO scavenging performance and the HIE concentration are positively correlated, with the corresponding $IC_{50}$ of 0.22 and 0.52 mg/ml. In addition, inhibition of NO production in LPS-stimulated RAW264.7 macrophage cells is also positively correlated to the HIE concentrations with no effect to cell viability. The experimental results also revealed that the cell viability of fibroblast trend to increase in 72 h of HIE treatment. Moreover, the results also presented the promotion of TGF-β1and bFGF growth factors production in HIE-treated fibroblast cells. The findings demonstrate the benefit of HIE in wound healing promotion.

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