

## ETHANOLIC EXTRACT OF *TITHONIA DIVERSIFOLIA* (HEMSLEY) A. GRAY INHIBITS MIGRATION ACTIVITY AND DECREASE THE TRANSFORMING GROWTH FACTOR-BETA1, VEGF EXPRESSION ON KELOID FIBROBLASTS

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### ABSTRACT

**Objectives:** Keloid occurred due to abnormal wound healing, characterized by massive fibroblast proliferation and excessive collagen accumulation. *Tithonia diversifolia* Hemsley A. Gray has been known to show antiproliferative effect against some cancer cells *in vitro*. However, its potential as anti-keloid has not been explored. This study aims to assess the *T. diversifolia* ethanolic extract effect on fibroblast migration activity, transforming growth factor-beta1 (TGF- $\beta$ 1), and vascular endothelial growth factor (VEGF) expression of keloid fibroblasts *in vitro*.

**Methods:** Fibroblasts were isolated from keloid collected from patient keloid tissue. The migration activity of keloid fibroblasts was assessed using scratch assay. The TGF- $\beta$ 1 and VEGF expression examination was done using ELISA.

**Results:** Ethanolic extract of *T. diversifolia* treatment at a concentration of 20  $\mu$ g/ml, 10  $\mu$ g/ml, and 5  $\mu$ g/mL for 24 h on keloid fibroblasts culture showed slower migration activity compare to those on keloid fibroblast culture without treatment ( $p < 0.05$ ). The TGF- $\beta$ 1 and VEGF expression was significantly lower in ethanolic extract of *T. diversifolia* treatment group compared to those on keloid fibroblast without treatment ( $p < 0.05$ ).

**Conclusion:** Ethanolic extract of *T. diversifolia* inhibits fibroblast migration activity and decrease the expression of VEGF and TGF- $\beta$ 1 on keloid fibroblasts *in vitro*.

**Keywords:** Keloid fibroblast, *Tithonia diversifolia*, Migration, Transforming growth factor-beta1, Vascular endothelial growth factor.

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### INTRODUCTION

Keloid is a fibroproliferation dermal tumor, harmless, that grew in the scar and exceeds the wound limit. In developing countries, there are around 100 million patients who have scarring complaints. Those are giving the impact on physical condition, esthetic, psychological, and social [1,2]. Keloid is characterized by excessive accumulation of extracellular matrix components such as collagen, fibronectin, elastin, proteoglycans and growth factors [3].

Keloid therapy actually has been widely practiced with a variety of side effects that have been reported as long-term therapy with an injection of triamcinolone acetonide and mitomycin C can cause skin atrophy, telangiectasia, and pigmentation disorders [4]. Some studies about keloid have been done, but research on the natural product is still very few. One of the natural ingredients that have been studied is the *Tithonia diversifolia* (Hemsley) A. Gray. Initial studies on keloids suggested that the standardized ethanol extract of *T. diversifolia* can inhibit keloid fibroblast proliferation and collagen deposit [5]. The active compound of *T. diversifolia* shows cytotoxicity against cancer cells, is Tagitinin C which is a sesquiterpene lactones class, and was isolated from the leaves of *T. diversifolia* (Hemsley) A. Gray using a bioassay-guided isolation method [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] on cells HeLa IC<sub>50</sub>: 9.776  $\mu$ g/mL) [6]. Tagitinin C proved able to inhibit collagen accumulation on keloid fibroblasts *in vitro* [7]. Therefore, research on ethanolic extract of *T. diversifolia* (Hemsley) A. Gray against migration activity and expression of transforming growth factor-beta1 (TGF- $\beta$ 1) and vascular endothelial growth factor (VEGF) in fibroblast Keloids needs to be studied.

### MATERIALS AND METHODS

Keloid fibroblasts used were a subculture passage III-IV, obtained from the laboratory of health technology, Dermatology Venereology Division, Faculty of Medicine Gadjah Mada University. Materials used were *T. diversifolia* (Hemsley) A. Gray leaves collected from Pakem-Yogyakarta special district of Indonesia on February 2017, identified at the laboratory plant systematics, and voucher specimen no: 0579/S. Tb./IX/2017 was deposited in laboratory plant systematics, Faculty of Biology, Universitas Gadjah Mada. Amphotericin B-Fungison (Gibco™), Dulbecco's modified Eagle's medium (Gibco), dimethyl sulfoxide (DMSO) (Merck), Ceftriaxone, fetal bovine serum (Gibco), Formaldehyde 10% (Gibco), MTT (Sigma), medium Roswell park memorial Roswell park memorial Institute 1640 (Sigma), Penicillin-Streptomycin (Gibco BRL), phosphate buffer saline (Gibco), Povidone-iodine 10% (Gibco), and Trypsin EDTA 0,25% (Gibco) were used.

#### Extraction of *T. diversifolia*

About 1 kg of *T. diversifolia* leaves dried powder was macerated by ethanol (70%) (2 L). The mixture was stirred periodically for 24 h. The filtrate was separated by filtration (Buchner funnel), and maceration was repeated 3 times. The filtrates obtained were combined and evaporated *in vacuo* to dryness.

#### The 96 well plate culture preparation

Cell suspension was counted based on a number of group in the study in triplicate. Fibroblast cell culture harvested, washed, and made into suspension with concentration  $2 \times 10^5$ /mL medium. Each well on a plate then filled with 200  $\mu$ L cell suspension and marked according to the research plan. Cells in 96 well plate were incubated in an incubator

CO<sub>2</sub> 5%, temperature 37°C for 24 h. Each work sample requires two plates for fibroblast proliferation and collagen accumulation, 1 plate for 72 h and 1 plate for 120 incubation time, repeated up to 3 times.

**Tested concentration preparation**

About 5 mg of the ethanolic extract of *T. diversifolia* was diluted in 100 µL DMSO to obtain a stock solution (50.000 µg/mL). Then, 3 series concentrations 5 µg/mL, 10 µg/mL, and 20 µg/mL of the extract were prepared.

**Fibroblast migration assessment with scratch assay**

Measurement of cell migration test by *in vitro* scratch assay method [8] and analyzed by the method used [9] wound creation on culture cell migration was done by scratching the well base using blue tip micro pipet. After the treatment was completed, all groups were incubated for 48 h, but every 24 h microscopic images were taken using an inverted microscope. The images obtained from each well plate sample were analyzed using Image J software to obtain the percentage of the scratch area. The percentage of cell migration is determined by 100% - percentage of scratch area.

**Measurement of TGF-β1 expression**

The protocol of TGF-β1 measurement is performed according to the procedure issued by Koma Biotech.inc as a manufacturer of the human TGF-β1 measurement kit.

**Measurement of VEGF expression**

The protocol of VEGF measurement is performed according to the procedure issued by Koma Biotech.inc as a manufacturer of the human VEGF with Catalog No: K0331132.

**Ethics approval of research**

This research has received permission from the Ethics Committee Research Faculty of Medicine, University of Gadjah Mada based eligibility letter conduct, with the number KE/FK/426/EC.

**RESULTS AND DISCUSSION**

**Fibroblast migration activity**

The mean of keloid fibroblast migration activity is optimal decreased at 24 h with 10 µg/mL of *T. diversifolia* ethanolic extract treatment. The mean of keloid fibroblast migration activity was lower significantly compare those on other groups are shown in Fig. 1. The ethanolic extract of *T. diversifolia* treatment at a concentration of 20 µg/mL, 10 µg/mL, and 5 µg/ml for 24 h on keloid fibroblast culture showed slower migration activity compare to those on keloid fibroblast culture without treatment (p<0.05). There was no difference on keloid fibroblast migration activity among *T. diversifolia* ethanolic extract treated groups on 48 hours treatment (Fig. 2). These results suggest that the effects of ethanolic extract of *T. diversifolia* on the increase in the number of fibroblast cells of keloid occur inhibition or decrease in migratory activity in keloid cell fibroblasts. This indicates that cell migration activity at 48 h may close the wound in the scratch area.

The effect of the ethanolic extract of *T. diversifolia* inhibition on migratory activity of keloid fibroblasts is greater with increased concentration of ethanolic extract of *T. diversifolia*. This suggests that the administration of the ethanolic extract of *T. diversifolia* with concentration of 5 µg/mL does not provide the inhibitory effect. However, the difference between the concentration of the ethanolic extract of *T. diversifolia* and the keloid fibroblast control group was statistically significant (p<0.05).

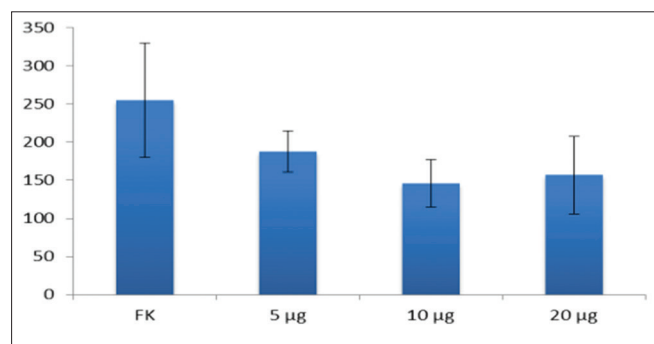
**Measurement of TGF-β1 expression**

Ethanolic extract of *T. diversifolia* on the expression of TGF-β1 in keloid fibroblasts is shown in Fig. 3. This result shows that TGF-β1 expression in keloid fibroblasts treatment with an ethanolic extract of *T. diversifolia* was lower compared to those on keloid fibroblast without treatment. The treatment by ethanolic extract of *T. diversifolia* with various concentrations of 5 µg/mL, 10 µg/mL, and 20 µg/mL in keloid fibroblasts has almost similar results shown in Fig. 3. It can be

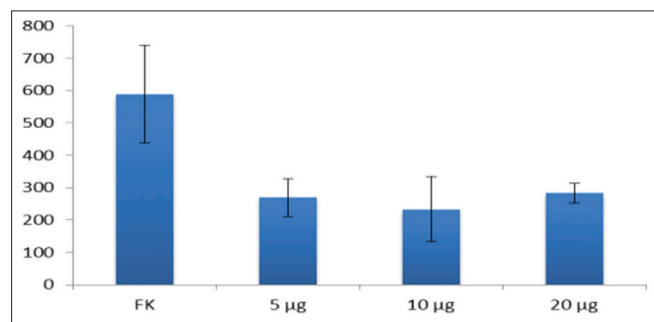
concluded that TGF-β1 expression was significantly lower in ethanolic extract of *T. diversifolia* group compared to those on keloid fibroblast without treatment (p<0.05).

**Measurement of VEGF expression**

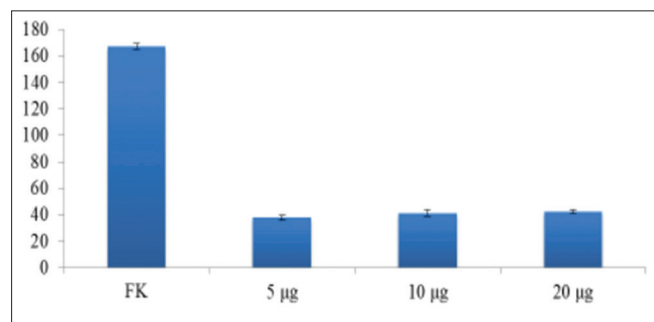
Ethanolic extract of *T. diversifolia* on the expression of VEGF in keloid fibroblasts is shown in Fig. 4. This result shows that ethanolic extract of *T. diversifolia* various concentrations (20 µg/mL, 10 µg/mL, and 5 µg/mL) in keloid fibroblasts showed different VEGF expression compare to those on keloid fibroblast control group (p<0.05). Ethanolic extract of *T. diversifolia* concentration of 20 µg/mL or which constitute the largest concentration has optimal results when compared to other concentrations of the ethanolic extract of *T. diversifolia* treatment group (p<0.05). The above data corroborate that the treatment with an ethanolic extract of *T. diversifolia* can inhibit cell proliferation



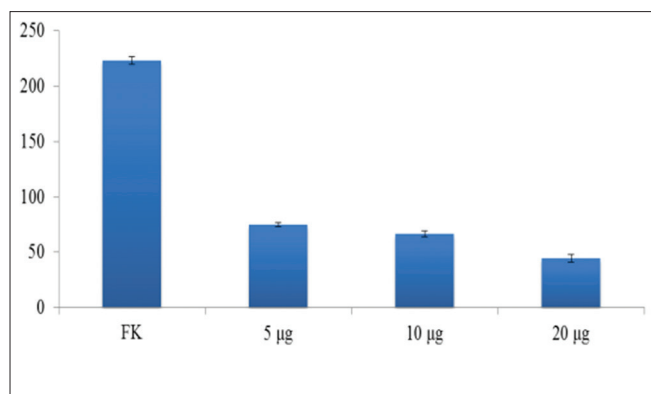
**Fig. 1: Effect of *T. diversifolia* extract on keloid fibroblasts migration activity (%) at 24 hours. Description: FK = Fibroblasts Keloid, *T. diversifolia* extract concentration 20µg/mL; 10µg/mL; and 5µg/mL**



**Fig. 2: Effect of *T. diversifolia* extract on keloid fibroblasts migration activity (%) at 48 hours. Description: FK = Fibroblasts Keloid, *T. diversifolia* extract concentration 20µg/mL; 10µg/mL; and 5µg/mL**



**Fig. 3: Effect of *T. diversifolia* extract on keloid fibroblasts TGF beta 1 expression. Description: FK = Fibroblasts Keloid, *T. diversifolia* extract concentration 20µg/mL; 10µg/mL; and 5µg/mL**



**Fig. 4: Effect of *T. diversifolia* extract on keloid fibroblasts VEGF A expression. Description: FK = Fibroblasts Keloid, *T. diversifolia* extract concentration 20µg/mL; 10µg/mL; and 5µg/mL**

through inhibition of keloid fibroblasts and decrease the synthesis of VEGF supernatant concentration groups with optimal concentrations decrease at 20 µg/mL compared to the control group keloid fibroblasts.

## DISCUSSION

Cell migration is an important process to trigger the synthesis of new extracellular matrices and contributes to the wound healing process. On previous research [10], it is stated that fibroblasts taken from keloid tissue by cell culture will show an increase in migration activity. Treatment with ethanolic extract of *T. diversifolia* at a concentration of 20 µg/mL, 10 µg/mL, and 5 µg/mL for 24 h in this study showed a higher percentage of migration compared to keloid fibroblast untreated, but there was a significant difference at 48 h after treatment ( $p < 0.05$ ). This insignificant result is probably caused by decreased activity of mitochondria in fibroblast cells due to reactive oxygen species (ROS) increase. The rise of ROS may cause migration disorders due to changes in the function of tubulin proteins and actin, there is also a change in the growth factor activity of connective tissue growth factor in improving cell adhesion, Rac1 activity and also phosphorylation disturbance c-Jun N-terminal Kinase resulting in migration disorders in fibroblast cells [11,12]. Another study also showed similar keloid fibroblast migration between treated group and control group which might happen because the fibroblast cells are cultured many times which somehow can affect the cells ability to migrate. In addition, the method of scratching and washing of cells after a determined intervention time of 24 h and 48 h is done with caution because at the final washing before staining with hematoxylin Mayer many cells is detached. This may affect the migration measurement results using the Image J software.

Keloid formation may involve TGF- $\beta$  expression by some neovascular endothelial cells by producing TGF- $\beta$  autocrine. Gene expression of collagen Type I and IV also increased in keloid tissue. Increased collagen synthesis exceeds the amount of destruction and excess deposition in connective tissue. The collagen connective tissue is produced by fibroblast cells. Disturbed synthesis activities are mediated by changes in growth factor expression [13]. TGF- $\beta$  especially TGF- $\beta_1$ , is the main cytokines involves in the development of organ fibrosis including keloid. Activation of TGF- $\beta$  signaling in fibroblast cells causing phenotypic changes that may enhance the ability to proliferate and migrate or invasion. Increased TGF- $\beta$  activity in fibroblast cells may also increase the ability to stimulate tissues re-modeling so as to further enhance the growth of keloid tissue. Thus, the inhibition of TGF- $\beta$  pathway is a pathway or indicator of successful keloid therapy [14]. Treatment with ethanolic extract of *T. diversifolia* on keloid fibroblasts can lower TGF- $\beta_1$  expression significantly ( $p < 0.05$ ) compared with keloid fibroblasts control. This result which is consistency with the previous study [15] suggest that giving isolates from the butter flower leaf (*Nerium indicum* Mill.) can significantly lower the level of TGF- $\beta_1$  synthesis compared

to the keloid fibroblast control group ( $p < 0.01$ ) because TGF- $\beta_1$  is the major pathway in keloid formation. In a study [16], it was found that the treatment of *Xanthium stramarium* and *Psoralea corylifolia* combined with Ultraviolet A1 radiation can inhibit TGF- $\beta_1$  expression and collagen synthesis in keloid fibroblasts ( $p < 0.05$ ).

VEGF is a proangiogenesis cytokine that has an important role in normal as well as pathologic conditions of wound healing [17]. VEGF belongs to the group of growth factors have a major role in keloid incident, VEGF-induced angiogenesis directly through mitogenesis endothelial cells and indirectly by increasing the permeability of blood vessels and promotes deposition of extravascular fibrin matrix [18]. VEGF plays an important role in the formation of keloids by changing the extracellular matrix and increased endothelial cell proliferation [19]. VEGF is also important for vascular permeability that promotes neovascularization and cell growth [20,21]. Increased expression of VEGF in keloid is showed by the higher amount of VEGF expression in keloid fibroblast group compared with those on treated group. Increased expression of VEGF in keloid fibroblasts is caused by local hypoxia conditions that contributed to hypoxia-inducible factor (HIF-1), a VEGF expression stimulator [22]. Hypoxic conditions generally occur in the inflammatory phase of wound tissue due to disruption of the vascularization process around the wound so that oxygen delivery to other tissues is inhibited. This is exacerbated by the rapid influx of inflammatory cells in the wound tissue. The inflammatory cells play a role in the increase of granulation, re-epitelisasi, and proliferasi [23], cell response to hypoxic conditions is also called the HIF protein expression which is a transcription factor that plays an important role in maintaining the balance of oxygen. Increased of HIF-1 $\alpha$  protein in keloid tissue result in increased expression of TGF- $\beta$ , thrombospondin-1, plasminogen activator inhibitor-1, and VEGF which play a major role in driving the fibroblast proliferation [22]. Fibrosis in keloid tissue is caused by increased of exogenous and endogenous VEGF in keloid fibroblasts. Exogenous VEGF will increase the VEGF receptors, whereas endogenous VEGF will increase with increased transcription factor VEGF and insulin-like growth factor 1 in tissues fibroblasts keloid [19]. VEGF as a pro-angiogenic growth factor increase the keloid tissue angiogenesis that chronic inflammation occurs and persistent increase fibroblast proliferation. Ethanolic extract of *T. diversifolia* treatment of various concentrations (20; 10; and 5) µg/mL showed lower VEGF expression compared with those in keloid fibroblast control, with optimal decrease occurring at the concentration 20 µg/mL with VEGF expression value of 44.82 pg/mL when compared with keloid fibroblast group ( $p < 0.05$ ). The lowest VEGF expression is showed in the concentration of the ethanolic extract of *T. diversifolia* 20 µg/mL which is the highest treatment concentration. VEGF expression is optimal at that concentration. Another research results that VEGF is released in large amounts from degranulating platelets, and it is present in wound fluid, particularly early after injury. The expression of VEGF related antigen, a marker of vascular endothelial cell, a marker of proliferation, in the wounds showed that there was an increase in the number of new capillary sprouts in the three evidence-based practice (EBP) gel groups at varied extent, especially in 75% EBP gel groups, compared with that in control group on day 12 and day 16 after wounding [24]. Other research also concluded that ethyl acetate fraction of *Andrographis paniculata* and doxorubicin combination increase apoptosis and decrease VEGF protein expression of mice fibrosarcoma cells [25].

Based on the results of VEGF expression, ethanolic extract of *T. diversifolia* might inhibit the proliferation of keloid fibroblasts cells through the inhibition of the expression of VEGF and specifically inhibit the formation of a transcription factor of endogenous VEGF in keloid fibroblasts. VEGF is abundant in the dermis underlying keloids. *In vitro* studies have shown that VEGF is expressed at high levels in keloid fibroblasts derived from normal skin fibroblast [26].

## CONCLUSION

Ethanolic extract of *T. diversifolia* can decrease migration activity on keloid fibroblast at 24 h in concentration 10 µg/mL. The TGF- $\beta_1$

expression of Keloid fibroblast in 5 µg/mL ethanolic extract of *T. diversifolia* was lower compared to those on the control group. The expression of VEGF keloid fibroblast in all concentration ethanolic extract of *T. diversifolia* was lower compared to those on the control group.

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#### AUTHORS' CONTRIBUTION

MSH contributed to drafting the manuscript, research design, data collection, analysis, and interpretation. DAAN and AB contributed to the revision of the manuscript. All the authors read and approved the final manuscript.

#### CONFLICTS OF INTEREST

Authors declare that there are no conflicts of interest.

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