



## Effect of tagitinin C isolated from *Tithonia diversifoli* (Hemsley) A Gray on migration activity and TGF- $\beta$ 1 levels on keloid fibroblast

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### ABSTRACT

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Keloid is the formation of excessive scar tissue characterized by fibroblast hiperproliferations and collagen deposits that are similar with cancer cells. Tagitinin C is proven can inhibit proliferation and deposition of keloids collagen fibroblast. However, the mechanism of action of tagitinin C in migration activities and TGF- $\beta$ 1 levels of keloid fibroblasts has not been proved, yet. This study aimed to investigate the effects of tagitinin C isolated from *Tithonia diversifoli* (Hemsley) on migration activity and TGF- $\beta$ 1 expression of keloid fibroblast. This was quasi experimental study with post test only controlled group design using keloid fibroblasts isolated from keloid patients. The migration activity were performed by scratch assay and TGF- $\beta$ 1 levels were measured using an ELISA kits. Isolate tagitinin C was more active inhibit fibroblast keloid migration compare to the control groups ( $p < 0.05$ ) after 48 h incubation. TGF- $\beta$ 1 levels after incubation with isolate tagitinin C was lower then control group ( $p < 0.05$ ). In conclusion, isolate tagitinin C can inhibit migration and reduce TGF- $\beta$ 1 levels on keloid fibroblast

### ABSTRAK

Keloid adalah pembentukan jaringan parut berlebihan yang ditandai oleh hiperproliferasi fibroblast dan endapan kolagen yang mirip dengan sel kanker. Tagitinin C terbukti menghambat proliferasi dan deposisi kolagen fibroblast keloid. Namun demikian, mekanisme aksi tagitinin C pada aktivitas migrasi dan kadar TGF- $\beta$ 1 fibroblas keloid belum dibuktikan. Penelitian ini bertujuan mengkaji pengaruh isolat tagitinin C yang diisolasi dari *Tithonia diversifoli* (Hemsley) pada aktivitas migrasi dan ekspresi TGF- $\beta$ 1 keloid fibroblast. Penelitian ini adalah eksperimental semu dengan rancangan post test menggunakan kelompok terkontrol pada keloid fibroblast yang diisolasi dari pasien keloid. Aktivitas migrasi dilakukan dengan uji awal dan pengukuran kadar TGF- $\beta$ 1 menggunakan kit ELISA. Isolat tagitinin C lebih aktif menghambat aktivitas migrasi fibroblast keloid dibandingkan dengan kelompok kontrol setelah inkubasi selama 48 jam ( $p < 0,05$ ). Kadar TGF- $\beta$ 1 setelah inkubasi dengan isolat tagitinin C lebih rendah secara nyata dibandingkan dengan kelompok kontrol ( $p < 0,05$ ). Dapat disimpulkan, isolat tagitinin C mampu menghambat migrasi dan menurunkan kadar TGF- $\beta$ 1 pada keloid fibroblast.

### Keywords:

Keloid fibroblast  
Proliferation  
Tagitinin C  
Migration  
TGF- $\beta$ 1Levels.

## INTRODUCTION

Keloid is a benign proliferative dermis tumor or excessive formation of scar tissue formation that appears on the skin that has postoperative trauma and or lack of collagen degradation.<sup>1,2</sup> The main factors causing keloids are excessive collagen production, excessive formation of infiltration cells and the lack of elastic tissue due to increased metabolic activity of fibroblasts. Keloids also contains collagen, fibronectin, and glycosaminoglycans as constituent components.<sup>3</sup>

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 increases in keloid tissue. The increase of collagen synthesis causes the amount of destruction and excess deposition in connective tissue. The collagen connective tissue is produced by fibroblast. Disturbance of the collagen synthesis are mediated by changes in growth factor expression.<sup>4</sup> Keloid incident has a correlation with the wound healing process. TGF- $\beta$  is one of the major pathways that regulate cell behavior including keloid formation. Increased TGF- $\beta$  activity in fibroblast cell also increase the ability of tissue remodeling, so that enhancing the growth of keloid tissue. Therefore, inhibiting TGF- $\beta$  pathway is a target for therapeutic of keloid.<sup>5</sup>

One of the medicinal plants that has been studied for its antikeloid activity is the moon flower [*T. diversifolia* (Hemsley) A. Gray]. Preliminary studies of keloids suggested that the standardized ethanol extract of *T. diversifolia* using tagitinin C can inhibit keloid fibroblast proliferation and collagen deposit.<sup>6</sup> Tagitinin C is a sesquiterpene lactones (SLs) class isolated from the leaves of *T. diversifolia* (Hemsley) A. Gray using a bioassay guided isolation (MTT HeLa cells IC<sub>50</sub>: 9.776  $\mu$ g/mL) method.<sup>7</sup>

Tagitinin C can inhibit keloid fibroblast viability. It also can decreasing

keloid collagen deposition concentration-dependent and time-dependent manner.<sup>8</sup> However, the mechanism of action of tagitinin C in migration activities and TGF- $\beta$ 1 levels of keloid fibroblasts has not been proved, yet. In this study we reported the effects of tagitinin C isolated from *T. diversifoli* (Hemsley) A. Gray on migration activity and TGF- $\beta$ 1 expression of keloid fibroblast.

## MATERIALS AND METHODS

### Tested compound

Tagitinin C was isolated from the leaves of *T. diversifolia* (Hemsley) A. Gray as conducted based on the previous studies.<sup>6</sup> Protocol of the study has been approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta (ref. KE/FK/426/EC).

### Isolation of keloid fibroblast culture

Keloid fibroblast was isolated from keloid fibroblast sample of adult patients and then and cultured. The *in vitro* keloid fibroblast culture was maintained in Dulbecco's modified essential medium (DMEM)-Gibco which was supplemented with 10% fetal bovine serum (FBS), 50  $\mu$ g/mL of gentamicin sulfate, and 2.5  $\mu$ g/mL fungizone. The cells were incubated at 37°C incubator with 5% CO<sub>2</sub> for 24 h.

The primary culture was harvested after fulfilling all petri fibroblasts. The next step was subculture processed. The whole process of subculture can be repeated with PBS sterile for washing, DMEM for medium, and 2 mL 0.25% trypsin for trypsinization until mature subcultured on passage 3.<sup>11</sup>

### Culture on 96 well plates

Cell suspension was calculated based on the number of groups used in the study along with its triplication. The fibroblast cells culture were harvested and then

washed, then made into cells suspension with a concentration of  $2 \times 10^5$ /mL of medium. Afterwards, the distribution of the wells were designed based on the planned mapping. Subsequently each well was filled with 200  $\mu$ L of cell suspension and marked according to the study design. Cells in 96 well plates were incubated in a 5% CO<sub>2</sub> incubator at temperature of 37°C for 24 h.<sup>10</sup>

### Preparation of tested compound

Tagitinin C solution was prepared according to the previous study.<sup>8</sup> It was weighed as much as 1.6 mg and dissolved in 160  $\mu$ L of dimethyl sulphoxide to obtain the stock solution in concentration of 10 mg/mL. Then it was made into 8 concentrations with serial dilution.

After 24 h incubation, each treatment group at 96 well plates was added isolate tagitinin C with serial concentration of 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125  $\mu$ g/mL with each series of concentration underwent in triplicate. The negative control and media only contain keloid fibroblasts. Afterwards the cells at 96 well plate were incubated for 3 days then calculate the IC<sub>50</sub> as well as the experimental results elisat storage for later use in the measurement of TGF- $\beta$ 1 levels using the protocol kit attached from ELISA kit manufacturers.

### Observation on fibroblast migration activity

Measurement of cell migration was tested by *in vitro* scratch assay method<sup>11</sup> and analyzed by these method.<sup>12</sup> Wound creation on cell migration was done by scratching the well base using the tip of micropipette. After the treatment was completed, all groups were incubated for 48 hours. Medium was sucked and rinsed in PBS to the extent that cells were lifted off. During the final rinse, the culture was recorded by a moticam, and then a sample medium was added to each well and incubated at 37°C in

5% CO<sub>2</sub> for 3x72 h. The medium was replaced every 72 h. After incubation, the culture was rinsed twice in PBS and 500- $\mu$ L Mayer's hematoxylin was added to each well and incubated for 1 min at ambient temperature. The culture was then rinsed and filled with 1 mL PBS. The cell migration was counted under a microscope. The cell migration rate was calculated using the migration formula=  $100\% - (\text{empty pixels}/\text{empty pixels} + \text{blue pixels}) \times 100\%$ .

### Measurement of TGF- $\beta$ 1 Level

The protocol of TGF- $\beta$ 1 measurement was performed according to the procedure issued by Koma Biotech Inc. as the manufacturer of the Human TGF- $\beta$ 1 measurement kit.

### Statistical analysis

Cell migration activity was analyzed using ANOVA and Post-hoc analysis. A p value < 0.05 was considered as significant.

## RESULTS

### Cell migration activity

The keloid fibroblasts cell migration after incubation with tagitinin C at concentration of 0.25, 0.5 and 1  $\mu$ g/mL for 24 h is presented in FIGURE 1. The cell migration after incubation with tagitinin C at concentration of 1  $\mu$ g/mL (2 IC<sub>50</sub>) was lower than that negative control (FK + medium), at concentration of 0.25 and 0.5  $\mu$ g/mL. In contrast, at the concentration of 0.25 (0.5 IC<sub>50</sub>) and 0.5 (IC<sub>50</sub>) was higher than that negative control (FK + medium). However, it was not significantly different (p>0.05). The increase of cell migration after incubation with tagitinin C at concentration 0.25 and 0.5  $\mu$ g/mL indicated that at low concentration tagitinine stimulates cell migration.

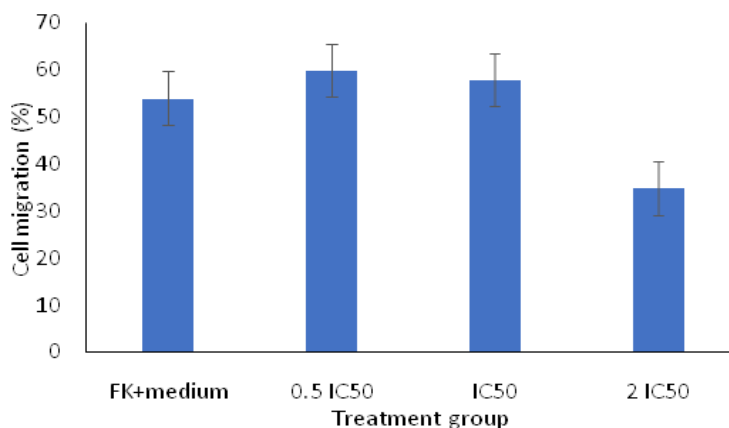


FIGURE 1. The migration of keloid fibroblasts cell after incubation with tagitinine C at concentration of 0.25 (0.5 IC<sub>50</sub>), 0.5 (IC<sub>50</sub>) and 1 (2 IC<sub>50</sub>) µg/mL for 24 h.

The keloid fibroblasts cell migration after incubation with tagitinin C at concentration of 0.25, 0.5 and 1 µg/mL for 48 h is presented in FIGURE 2. The cell migration after incubation with tagitinin C at concentration of 1 µg/mL (2 IC<sub>50</sub>) was significantly lowest compared to that negative control (FK + medium), at concentration of 0.25 (0.5 IC<sub>50</sub>) and 0.5 (IC<sub>50</sub>) (p<0.05). However, there was no significantly difference in the cell migration between the negative control (FK + medium) with at concentration 0.25 and 0.5 µg/mL as was as between 0.25 µg/mL and 0.5 µg/mL (p>0.05). This result indicated that the optimum cell migration inhibition of tagitinin C was

observed at the concentration of 1 µg/mL after incubation time of 48 h.

#### TGF-β<sub>1</sub> level

The TGF-β<sub>1</sub> level after incubation with tagitinin C at all concentration was significantly lower than that the negative control (FK + medium) (p<0.05). However, there was no significantly different in TGF-β<sub>1</sub> level after incubation of tagitinine C at all concentration and the normal fibroblast (FN + M) (p>0.05) as presented in FIGURE 3. This result indicated that the tagitinin C can decrease the TGF-β<sub>1</sub> level until normal range.

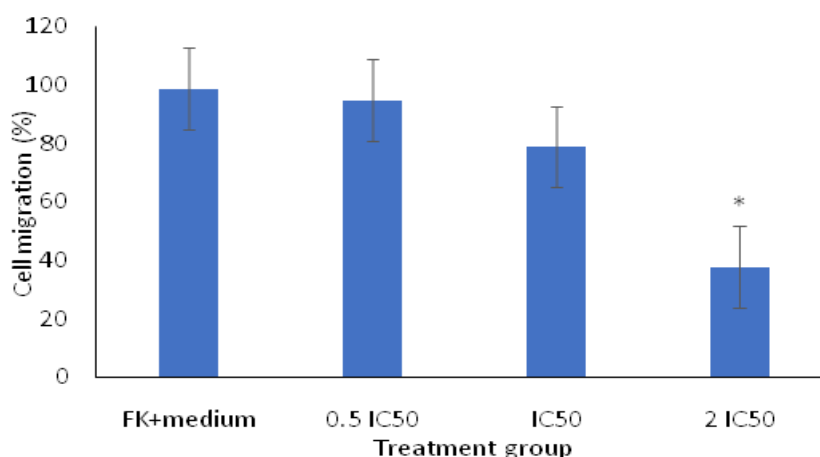


FIGURE 2. The migration of keloid fibroblasts cell after incubation with tagitinine C at concentration of 0.25 (0.5 IC<sub>50</sub>), 0.5 (IC<sub>50</sub>) and 1 (2 IC<sub>50</sub>) µg/mL for 48 h.

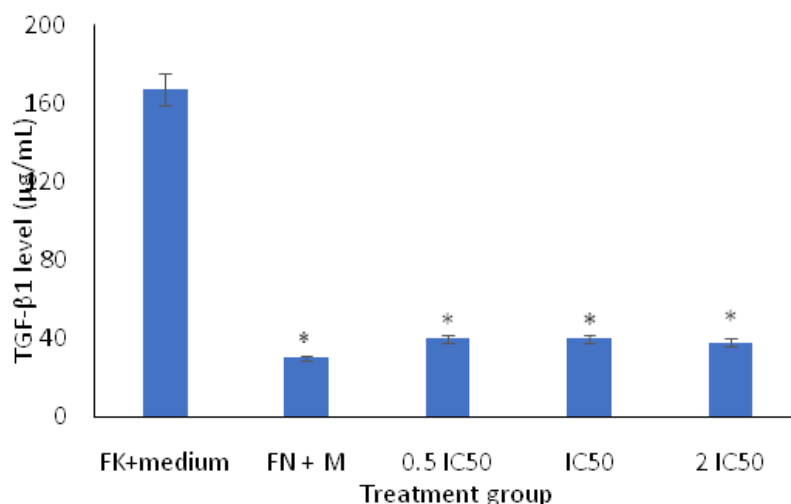


FIGURE 3. TGF-β1 level after incubation with tagitinine C at concentration of 0.25 (0.5 IC<sub>50</sub>), 0.5 (IC<sub>50</sub>) and 1 (2 IC<sub>50</sub>) µg/mL for 48 h.

## DISCUSSION

Cell migration is an essential process to stimulate the synthesis of new extracellular matrix, thereby contributing to a wound-healing process. A previous study reported that fibroblasts taken from keloid tissue by cell culture will show an increase in migration activity.<sup>13</sup> Incubation for 48 h with tagitinin C at concentration of 1 µg/mL (2 IC<sub>50</sub>) significantly inhibit cell migration ( $p < 0.05$ ). The cell migration inhibition is probably caused by decreased activity of mitochondria in fibroblast cells due to increased reactive oxygen species (ROS). Increased ROS may cause migration disorders because of changes in the function of tubulin proteins and actin. There is also a change in the growth factor activity of connective tissue growth factor (CTGF) in improving cell adhesion, Rac1 activity and also phosphorilation disturbance c-Jun N-terminal kinase (JNK) resulting in migration disorders in fibroblast.<sup>14,15</sup>

Activity of cell migration inhibition by active compounds isolated from plants has been reported in the previous studies. 5α-Oleandrin isolated from *Nerium indicum* could significantly decrease cell

migration activity of keloid fibroblasts after 48 h incubation compared to keloid fibroblasts control.<sup>16</sup> Syed *et al.*<sup>17</sup> reported that palomid 529 (P529) at a low concentration (5 ng/mL) inhibits cell spreading, attachment, proliferation, migration, and invasive properties and induced keloid fibroblast apoptosis. P529 also caused tissue shrinkage, growth arrest, and apoptosis in keloid organ cultures and substantially inhibited angiogenesis as well as suppressed *in situ* of pS6, pAkt-Ser473, and mTOR phosphorylation.

This study showed that incubation of tagitinin C for 24 h at concentration of 2 µg/mL did not affect cell migration activity. The cell migration examination is affected some factors included phases of cell growth, time of incubation and quality staining reagents.<sup>18</sup>

TGF-β is the main pathway that regulates cell behavior both in homeostatic state and in a pathological state including keloid formation. Activation of TGF-β signaling in fibroblast cells causing phenotypic changes that may enhance the ability to proliferate and migrate or invasion. Increased TGF-β activity in fibroblast cell may also increase the ability to stimulate tissues



re-modeling so as to further enhance the growth of keloid tissue. Thus the inhibition of the TGF- $\beta$  pathway is a pathway or indicator of successful keloid therapy.<sup>19</sup>

TGF- $\beta$  ca stimulates of protein synthesis of normal fibroblasts but not keloids fibroblasts. This indicates that the TGF- $\beta$  regulatory signals in the keloid fibroblast tissue are altered. Increased fibronectin biosynthesis in keloid fibroblasts is faster than normal fibroblasts by involving transcriptional mechanisms and increased production of extracellular matrix in keloid fibroblast with the development of modification on the TGF- $\beta$  regulatory program.<sup>20</sup>

Incubation with tagitinin C significantly decreased TGF- $\beta$ 1 level of keloid fibroblasts in this study. This result is consistence with the previous study that reported extract of *Nigella sativa* can decrease TGF- $\beta$ 1 synthesis.<sup>21</sup> Another previous study reported that 5 $\alpha$ -oleandrin isolated from *N. indicum* significantly decrease TGF- $\beta$ 1 level of keloid fibroblast.<sup>16</sup> Furthermore, administration of *Xanthium strumarium* and *Psoralea corylifolia* combined with UVA1 radiation can inhibit TGF- $\beta$ 1 expression and collagen synthesis in keloid fibroblast.<sup>22</sup>

## CONCLUSION

In conclusion, incubation with tagitinin C isolated from *T. diversifolia* (Hemsley) A Gray at concentration of 2  $\mu$ g/mL for 48 h inhibits migration activity and decreases TGF- $\beta$ 1 level on keloid fibroblast.

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