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Research Article

Antifibrotic Effect of Standardized Ethanol Extract of *Tithonia diversifolia* (Hemsley) A. Gray on Keloid Fibroblasts

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ABSTRACT
Keloid occurred by abnormal wound healing, characterized by massive fibroblast proliferation and excessive collagen accumulation. Therapy for keloid is relatively limited and mostly has side effects. *Tithonia diversifolia* has widely been studied for having anti-proliferative effect against some cancer cells *in vitro*. This study was conducted to assess *T. diversifolia* potential as an antifibrotic agent. Antifibrotic activity of standardized ethanolic extract of *T. diversifolia* leaf on cell proliferation of keloid fibroblasts and its collagen accumulation were expressed by IC_{50} values using probit regression analysis. Keloid fibroblast proliferation inhibition percentage was elevated gradually along with the given doses of *T. diversifolia* extract with very strong correlation (r=0.838;p=0.000 in 72 hours; r=0.924;p=0.000 in 120 hours of incubation) with the lowest IC_{50} value 3.624 µg/mL in 120 hours incubation time. There was also significant time-dependent effect (p=0.005). As well for collagen accumulation with moderate correlation (r=-0.797;p=0.000 for 72 hours incubation time; r=-0.583;p=0.000 for 120 incubation time) with lowest IC_{50} value 2.280 µg/mL in 120 hours incubation time. This study concluded that the ethanol extract of *T. diversifolia* is potential to be developed as antikeloid in the future although some additional data such as toxicity and clinical study.

Keywords: Keloid fibroblast, *Tithonia diversifolia*, proliferation, collagen, IC_{50}

INTRODUCTION

Keloid is a benign fibroproliferative tumor on dermis layer results from excessive wound healing response. It is only found in humans. Keloid can occur to all races with incidence from highest to lowest are in Africans, Asians, Hispanics, and Mediterranean. There is tendency that keloid occurs 15% higher in black than in white people. Moreover, its occurrence is higher in women and in second decade of life[1]. There are many methods available for treating keloid. Some of them which usually used in clinical practice are surgery, steroid injection, pressure therapy, radiation, laser therapy, and cryotherapy. In clinical practice combination therapy also often performed. Research showed that combination 5-fluorouracil and triamcinolone showed the best potential. Combination of these two drugs was proved to give fast response with minimal side effects[2]. But until now there has not been discovered a really effective method for treating keloid. Thus researches are still being conducted especially in herbal field that believed to yield potent drugs with minimal to no side effects at all[3].

Recently there are many researches conducted to find alternative solution for treating keloid using natural products that is believed to be able to cure keloid and repress the side effects. One of those products is *Tithonia diversifolia* (Hemsley) A. Gray. The study on *T. diversifolia* as anti-cancer has been extensively conducted. Etheric extract of *T. diversifolia* showed cytotoxic activity against HTC-116 cells, and it's ethyl acetate extract inhibited proliferation of colon cancer cells (Col-2), moreover the alcohol extract showed anti-leukemia activity[4,5,6]. Tagitinin C is one of sesquiterpen lactones (SLs), isolated from *T. diversifolia* using Bioassay Guided Isolation method (MTT in HeLa cell IC_{50}: 9.776 µg/mL)[7]. Cytotoxicy test of tagitinin C was performed on against cancer cell cultures and normal cells *in vitro*. Tagitinin C actively and selectively inhibited melanoma cells and skin cancer with selectivity index 40.536 (IC_{50} = 0.996 µg/mL)[8].

Nowadays there are many researches conducted to assess anti-cancer drug potential as antifibrotic agent which inhibits keloid fibroblast proliferation and collagen accumulation. The ethanol extract of *T. diversifolia* is developed to be standardized herbal which needs specific marker. In this case, tagitinin C is perfectly utilized as a marker for this extract. Therefore, this research is aimed to assess whether the standardized ethanolic extract of *T. diversifolia* displayes antifibrotic activity against keloid.

MATERIALS AND METHODS

Keloid fibroblasts used was subculture passage III, obtained from Laboratory of Health Technology, Dermato-Venereology Division, Faculty of Medicine

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Gadjah Mada University. Materials used were *Tithonia diversifolia* (Hemsley) A. Gray leaves collected from Pakem-Yogyakarta special district of Indonesia on February 2014, identified at the laboratory plant systematics, and voucher specimen no: 0579/S.Tb./IX/2014 was deposited in Laboratory plant systematics, Faculty of Biology, Universitas Gadjah Mada; Povidone Iodine 10%, DMEM (Gibco), Fetal Bovine serum (FBS) (Gibco), Amphotericine B (Fungison-GibcoTM), Penicillin – Streptomycine (Gibco BRL), Ceftriaxon, Trypsin EDTA 0,25% (Gibco), MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} (Sigma), Dimethyl Sulfoxide (DMSO) 99%, Formaldehyde 10%, Phosphate Buffered Saline (PBS). KCl, KH_2PO_4, NaCl, Na_2HPO_4, Aquadest (H_2O), Bouin solution, picrosirius red staining solution, HCl 0,1N, NaOH 0,5N.

Equipments used Laminary air flow sterile hood (Ebsco), sterile flask sized 25cm^2 and 75cm^2, 96 *well plate* (Iwaki), pasteur pipette, 10 ml sized pipette (Pyrex), aid pipette (Falcon), micropipette (Biohit), *yellow tip* (20-200µl), *blue tip* (200-1000µl), *white tip* (0,5-10µl), Centrifuge tube sized 10ml and 15ml (Biologix), Tube effendrorf 1,5ml (Stardec), reaction tube shelf, incubator CO_2 (Galaxy S), sterile petri dish, gloves, mask, aluminium foil, pinset, scissor, scalpel, spiritus lamp, centrifuge, inverted microscope (Euromed), *Handheld Automatic Cell Counter* (Milipore Scapter), *plate shaker*, micro filter 0,22ηm (Millex^{TM}), *multiplate reader* (Imark biorad).

Procedure

Extraction of T. diversifolia

One kg of *T. diversifolia* leaves dried powder was macerated by ethanol (70%) (2 liters). The mixture was stirred periodicaly for 24 hours. 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.

Extract standardization

Thin layer chromatography [TLC; SiO_2, washbenzene:ethyl acetate (2:1 v/v)] method was used to observe the present of Tagitinin C (marker) in the ethanol extract of *T. diversifolia*. Identical Rf value of the spot and their performance in the TLC of tagitinin C and the ethanol extract was indicative the present of marker in the ethanol extract. TLC Scanner was used to quantate relative concentration of tagitinin C in the ethanol extract of *T.diversifolia.*

Tested concentration preparation

Five mgs of ethanolic extract of *T. diversifolia* was diluted in 100 µL DMSO to obtain stock solution (50.000 µg/mL). Then 8 series concentrations of the extract were prepared according to equation below,

$V_1.N_1 = V_2.N_2$

Where V_1 = Volume of solution to be diluted; N_1 = Concentration of solution to be diluted; V_2 = Volume of diluted solution; N_2 = Concentration of diluted solution

The 96 well plate culture preparation

Cell suspension was counted based on number of group in the study in triplicate. Fibroblast cell culture harvested, washed, and made into suspension with concentration 2 x 10^5/mL medium. Each well on plate then filled with 200 µL cell suspension and marked according to research plan. Cells in 96 well plate was incubated in an incubator CO_2 5%, temperature 37^{o}C for 24 hours. Each work sample requires two plates for fibroblast proliferation and collagen accumulation, 1 plate for 72 hours, and 1 plate for 120 incubation time, repeated up to three times.

Keloid fibroblast proliferation assessment with MTT assay

This technique started with removal of all medium in 96 well plates. Then replaced with new complete medium 200 µL on each well with addition of 50 µL MTT solution 5mg/mL. Then plates was covered with aluminium foil and incubated for 6 hours in incubator CO_2. The medium and MTT solution then removed and replaced by 200 µL DMSO (*dimethyl sulfoxide*) on each wells and shaked. Then 25 µL glycine buffer was added to each wells. The absorbance was measured with multiple reader at 570 nm.

Collagen deposition assessment with sirius red method

Medium from 96 wells plate was removed and the plate was washed with PBS 200 µL on each wells three times. Fixation with Bouin solution for 1 hour, then washed with tap water until yellowish color disappeared, and then plated was dried overnight. Every wells then filled with 200 µL sirius red reagent that was diluted with saturated picric acid, incubated for 1 hour, then removed and washed with 200µL HCl 0,1 N three times until sirius red cleared from the walls and supernatant. 200 µL NaOH 0,5 N was added and incubated for 30 minutes. The absorbance was measured with multiple reader at 550 nm.

Results analysis

After percentage of keloid fibroblast inhibition proliferation and its collagen deposition data was obtained, it is expressed in IC_{50} values using probit regression analysis.

RESULTS AND DISCUSSION

Extract Standardization

Substance: Tagitinin @ 254 nm
Regression via area : Linear
Y = 64.46 + 751.6 * X r = 0.99603 sdv = 5.47

Wincats summary report

Calibration results per Analysis
Sample from vial 1: Sample 1
Result via area

Substance:	Tagitinin
Rf:	0.69
X (average):	1.434 µg
CV (%):	11.521
N:	3
Regression:	Linear

Chromatogram pattern shown by tagitinin C visualized by UV lights (254 and 366 nm) and reagent (visible spot) can be used as a specified marker for the ethanol extract of *T. diversifolia* (figure 1). Tagitinin C standard appeared at 0.32 Rf value, and this value (spot) is also present in the ethanol extract chromatogram.

Tagitinin C concentration in the ethanol extract of *T. diversifolia* was calculated by TLC densitometric, it can be measured the concentration of tagitinin C in ethanol 70%

Table 1. Tagitinin C relative content (%) in ethanolic extract of *T. diversifolia*, calculated by TLC densitometric

No	Name	Sample (µg)	Test vol (µL)	Vol applic(µL)	fp	Area	measurable conc.	Conc. (%)	Average levels
1	Sample 1	50000	10000	10	1000	1000.44	1,245	2.49	
2	Sample 2	50000	10000	10	1000	1230.62	1,552	3.10	
3	Sample 3	50000	10000	10	1000	1196.39	1,506	3.01	2.87

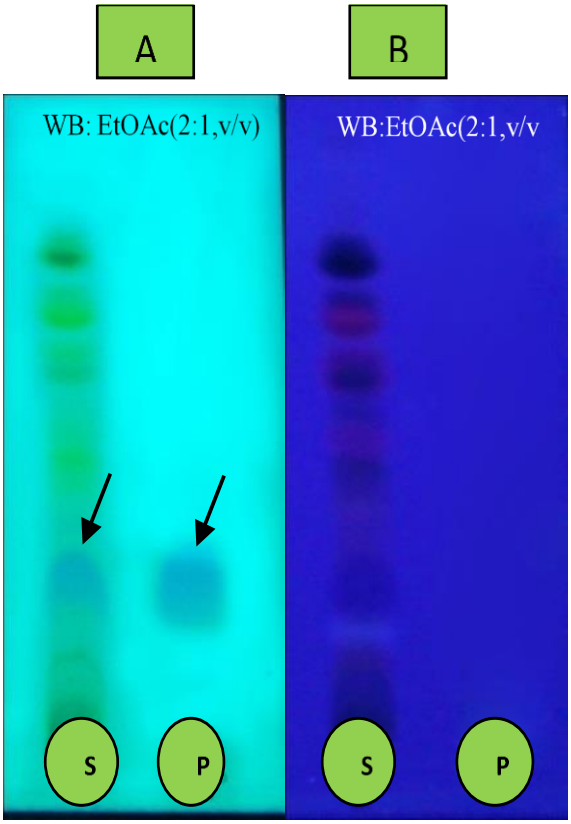

Figure.1. TLC chromatogram of tagitinin C (P) and ethanolic extract of *Tithonia diversifolia* (S) visulaized by UV λ, 254 nm (A), and 366 nm (B).
Note: Position marker tagitinin C at Rf value of 0.32

Table 2. Inhibition effect of ethanolic extract of *T. diversifolia* on keloid fibroblast culture cell proliferation in 72 and 120 hours incubation time

Conc. (µg/mL)	Percentage of average Inhibition (%)	
	72 hrs incubation Mean ± Sd (N=3)	120 hrs incubation Mean ± Sd (N=3)
0.234	10.01 ± 0.16	12.12 ± 0.58
0.468	19.01 ± 0.39	18.11 ± 0.50
0.937	24.34 ± 0.40	24.84 ± 0.21
1.875	28.58 ± 0.35	35.59 ± 1.35
3.75	40.27 ± 1.11	45.10 ± 1.07
7.5	49.23 ± 0.61	50.21 ± 0.78
15	59.13 ± 1.74	74.61 ± 0.93
30	67.30 ± 3.41	98.66 ± 0.57
IC_{50} (µg/mL)	**7.932 ± 1.02**	**3.624 ± 0.74**

extract of *T. diversifolia*, that was relatively valued as 2.87% (Table 1.).

Keloid fibroblast proliferation

The test results for keloid fibroblast proliferation as in Table 2 and figure 2 showed that the percentage of inhibition is directly proportional to the dose given. For 72-hour incubation period at the concentration 0.234 µg/mL, inhibits 10.01± 0.16% fibroblast keloid proliferation. This percentage continues to increase with increasing concentration of standardized ethanolic extract of *T.diversifolia* were used, respectively 0.468; 0.937; 1.875; 3.75; 7.5; 15 3.624 µg/mL with the percentage of inhibition respectively 19.01; 24.34; 28.58; 40.27; 49.23; 59.13%, and the highest was 67.30 ± 3.41% at the concentration of 30 µg/mL. From those percentage inhibition was obtained IC_{50} value, 7.932± 1.02 µg/mL. For 120-hour incubation period at the lowest dose 0.234 µg/mL inhibits 12.12± 0.58% and at the highest dose 30 µg/mL inhibits 98.66± 0.57% and IC_{50} values is 3.624 ± 0.74µg/mL.

Keloid collagen accumulation

For collagen accumulation inhibition as shown in Table 3 and figure 3, at the lowest concentration, 0.234 µg/mL, giving inhibition of collagen synthesis by 20.78± 0.43% for 72-hour incubation period and 31.77± 1.04% for 120-hour incubation period, the percentage of inhibition continues to increase with increasing concentration given. At levels of 0.468; 0.937; 1.875; 3.75; 7.5; 15; 30 µg/mL with each value of the percentage inhibition for 72-hours incubation period 29.02; 35.01; 38.02; 37.65; 49.44; 62.21; 71.29% and amounted to 40.54; 45.41; 48.66; 54.85; 59.23; 61,50; 66.14% for 120-hour incubation period. The decline in the percentage of inhibition obtained only down to 3.75 levels for 72-hour incubation period. From those percentage inhibition, obtained IC_{50} value is 5.498± 1.06 µg/mL for 72-hour incubation period and 2.280± 0.84 µg/mL for 120-hours incubation period.

DISCUSSION

Tithonia diversifolia (Hemsley) A. Gray contains sesquiterpene lactones, which among others consists of tagitinin A, a natural bioflavonoid, tagitinin C ($C_{11}H_{16}O_5$), and falvonoid hispudin. Besides that the leaves and flowers of *T. diversifolia* also contains the essential oils like α-pinene (32.9%), β-caryophyllene (20.8%), germacrene D (12.6%), β-pinene (10.9%) and 1, 8-cineole (9.1%). Germacrene D (20.3%), β-caryophyllene (20.1%) and bicyclogermacrene (8.0%)[9]. Isolates of *T. diversifolia* is in some previous studies are known to have a lot of potential as a drug. Its use as a drug has been tested, such as chemoprotective on cancer, anti-malarial, and anti-

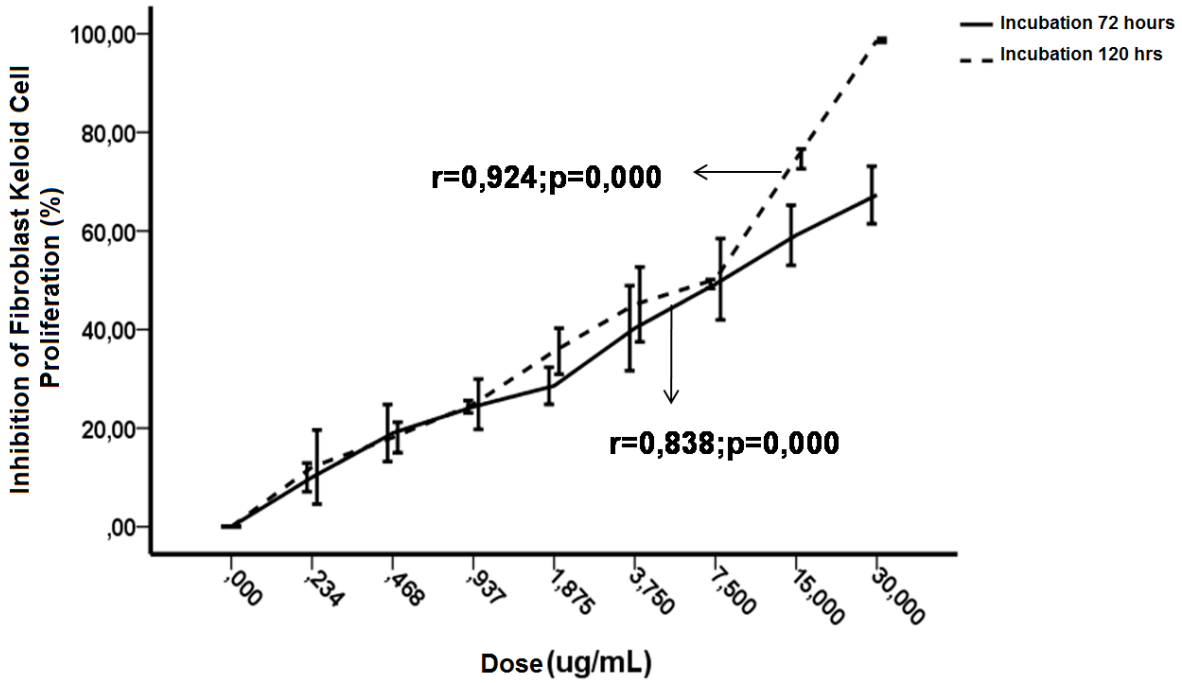

Figure 2: Effect of ethanolic extract of *T. diversifolia* in inhibition of keloid fibroblast culture cells proliferation.

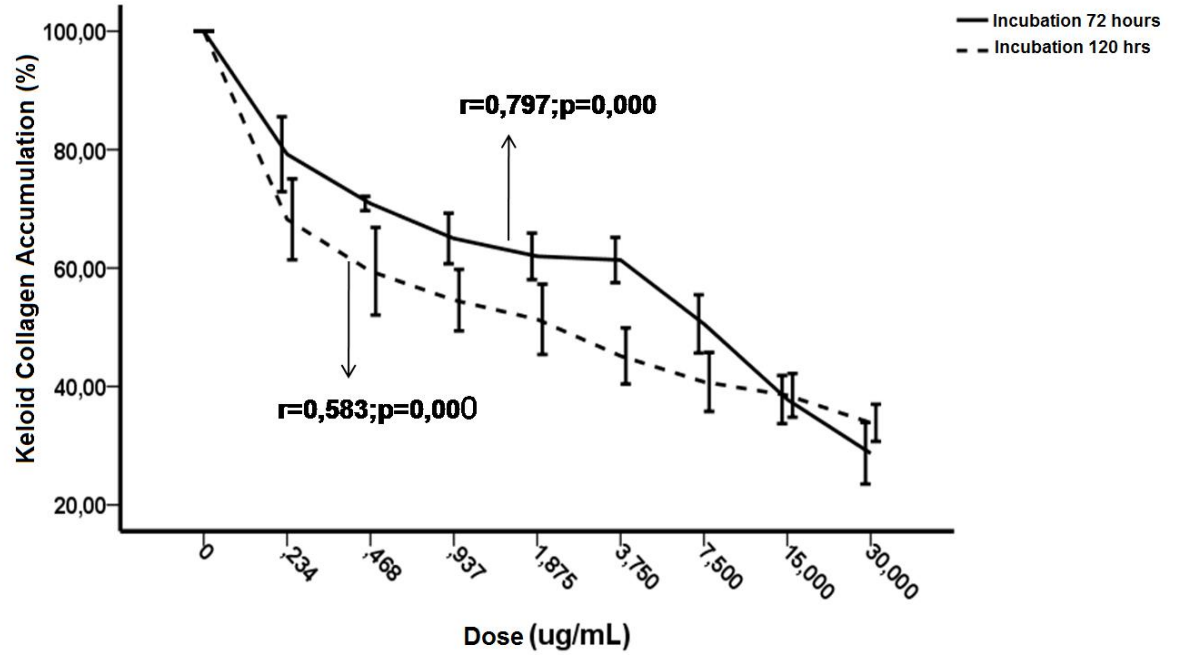

Figure 3: Effect of ethanolic extract of *T. diversifolia* in inhibition of keloid collagen deposition

inflammatory[10]. Using Bioassay-guided isolation (cytotoxic test , HeLa cells), Tagitinin C was isolated from the leaves of T. diversifolia with IC50 values, 9.776 ± 0.98µg/mL[20].Tagitinin C were isolated from the leaves of *T.diversifolia* is one that acts as a major sesquiterpenoid anti-proliferative. Methanolic extract of *T.diversifolia* and tagitinin C have cytotoxic activity against human hepatoma cells Hep-G2 with IC_{50} values respectively by 40.0 ± 2.0 and 2.0 ± 0.1 µg/mL[11]. This study was conducted to determine antifibrotic activity of standardized ethanolic extract of *T.diversifolia* and determine the value of Fifty Percent Inhibitory Concentration, ie the concentration of a compound that inhibits cell growth by 50%. A compound that displayes anti-fibrotic activity is the compound that has an inhibitory effect fibroblast proliferation, collagen synthesis which causes a decrease in collagen pile on living cells, and increasing inhibition at each increasing doses. In general, the percentage of collagen accumulation inhibition by standardized ethanolic extract of *T.diversifolia* has increased along with the addition of a given concentration of the extract. Based on the criteria of cytotoxicity activity for crude extracts established by American National

Table 3: Effect of ethanolic extract of *T. diversifolia* in inhibition of keloid collagen deposition in 72 hours and 120 hours incubation time

Conc. (µg/mL)	Percentage of average collagen accumulation inhibition (%)	
	72 hrs incubation Mean ± Sd (N=3)	120 hrs incubation Mean ± Sd (N=3)
0,234	20,78 ± 0.43	31,77 ± 1.04
0,468	29,02 ± 1.28	40,54 ± 0.53
0,937	35,01 ± 0.66	45,41 ± 0.91
1,875	38,02 ± 0.59	48,66 ± 0.74
3,75	37,65 ± 1.72	54,85 ± 0.35
7,5	49,44 ± 2.53	59,23 ± 0.39
15	62,21 ± 0.87	61,50 ± 1.24
30	71,29 ± 0.42	66,14 ± 1.49
IC_{50} (µg/mL)	**5.498 ± 1.06**	**2.280 ± 0.84**

Cancer Institute (NCI) is an IC_{50} value of <30 μg/mL in preliminary assay, and for other substance < 20 μg/mL[12,13]. A study reported that the standardized ethanolic extract of *T.diversifolia* has cytotoxic activity on WiDr cells with IC_{50} 53.19 µg/ml[14]. A standardized ethanolic extract of *T.diversifolia* in this study were obtained IC_{50} 7.932± 1.02 µg/mL for 72-hour incubation period and 3.624±0.74 µg/ mL for 120-hour incubation period. Similarly, the inhibition of collagen stack having IC_{50} value of 5.498± 1.06 µg/mL for72-hour incubation period and 2,280±0.84 µg/mL at 120-hour incubation period. So it can be said that the standardized ethanolic extract of *T.diversifolia* has cytotoxic properties which inhibits fibroblast proliferation and collagen synthesis in keloid actively so it's concluded the potential material to be developed as an anti-keloid[15]. That is greater than that of the IC_{50} value in this study, is due to the use of standardized ethanolic extract of *T.diversifolia* as cytotoxic require larger doses than its use as an anti-fibrotic agent. The cytotoxic agents commonly used in treatment of cancer and anti-fibrotic agent commonly used in keloid treatment or the treatment that aims to prevent fibrosis. As noted above, one of the major content of *T.diversifolia* is tagitinin C. This substance is known to have inhibitory activity on the cell proliferation. Previous study indicated that tagitinin C is active against cells proliferation through the termination phase of cell mitosis, particularly in sub-G1 phase and S phase arrest[16]. Research on the effects antifibrotic Tagitinin C isolates from the leave of Tithonia diversifolia (Hemsley) A. Gray in keloid fibroblasts has been done with the result that tagitinin C can inhibit the viability of keloid fibroblasts with IC_{50} 0.122 µg/mL (72h) and 0.039 µg/mL (120 h), whereas mitomycin C IC_{50} 0.120 µg/mL (72h) and IC_{50} of 0.100 µg/mL (120h). At IC_{50} dose of tagitinin C on keloid collagen deposition 53.1% (72h) and 44.3% (120h), whereas the IC_{50} dose of mitomycin C on keloid collagen deposition 60.4% (72h) and 52.1% (120h). At a doses of ≤ 2µg/mL tagitinin C is not toxic on normal fibroblasts[17]. Tagitinin C concentration in the ethanol extract turns out to be 2.87% (Tabel 1.) based on the TLC densitometric data, using Tagitinin C as internal standard. Tagitinin C levels in this extract is considered large amount, it is evident that the activity is also high, for keloid fibroblast proliferation IC50 of 7.932 mg/mL (72 hours) and 3.624 mg/mL (120 hours), whereas inhibition on keloid collagen deposition is IC50 5.498 mg/mL (72 hours) and 2.280 mg/mL (120 hours)[18,19]. Therefore, we conclude that the ethanol 70% of *T. diversifolia* is potential to be developed as antikeloid in the future although some additional data such as toxicity and clinical study.

CONCLUSION

Standardized ethanol extract of *T. diversifolia* inhibit keloid fibroblast culture proliferation, based on concentration given and incubation time at the lowest IC_{50} value (3.624± 0.74 µg/mL) in 120 hours of incubation time, also inhibit collagen deposition at the lowest IC_{50} value (2.280 ± 0.84 µg/mL) in 120 hours incubation time.

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