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Mixture of Extract *Nerium indicum* Mill and *Tithonia diversifolia* (Hemsley) A. Gray. Inhibits the Migration Activity, Expressions of TGF-β1 and VEGF Keloid Fibroblasts

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Abstract

Nerium indicum Mill and *Tithonia diversifolia* (Hemsley) A. Gray. have been studied as potential anti-**keloid** agents. The mechanism of action of each marker compound, **5α-oleandrin** in *N. indicum* and **tagitinin C** in *T. diversifolia*, have been examined for their ability to inhibit proliferation, collagenation, migration, and TGF-β1 expression of keloid fibroblasts. However, the mixed ethanolic extract of *N. indicum* and *T. diversifolia* has yet to be investigated. Therefore, this study aimed to examine the mechanism of action of the mixed ethanolic extract of *N. indicum* (NiE) and *T. diversifolia* (TdE) against the migration activity, **TGF-β1** and **VEGF** expressions of keloid fibroblasts. The fibroblast migration activity was tested through an in-vitro scratch assay, while TGF-β1 and VEGF expressions were investigated using the Elisa Kits supplied by Bender MedSystems. A mixture of NiE+TdE (8 µg/mL concentration) incubated for 48 hours could optimally inhibit the migration activity compared to the control. The mixture of NiE+TdE at a concentration of 16 µg/mL could suppress TGF-β1 expression compared to the control ($p<0.05$), and such mixture could also significantly inhibit VEGF expressions as opposed to the control ($p<0.05$). The mixed extract of NiE+TdE possessed a mechanism of action to inhibit migration activity, TGF-β1 and VEGF expressions of keloid fibroblasts.

Keywords: Keloid, 5α-oleandrin, tagitinin C, VEGF, TGF-β

INTRODUCTION

One of the cases in the field of dermatology and plastic surgery that attracts attention is keloid. It is a benign fibroproliferative tumor on the dermis layer of the skin in response to excessive wound healing [1]. In developing countries, approximately 100 million patients complain about scarring with 55 million complaints due to elective surgery and 25 million because of other factors [2]. Keloid can make quite an impact on physical, aesthetic, psychological, and social conditions [3].

To date, keloid management has yet to yield satisfying results due to the high recurrence rates [4,5]. A large number of studies have recently been seeking alternatives to keloid treatments form medicinal plants considered capable of curing as well as counteracting adverse drug effects. The plants currently studied for their anti-keloid potential include *Nerium indicum* Mill and *Tithonia diversifolia* Hemsley A. Gray. The compound 5α-oleandrin was successfully isolated from *N. incidum* and potential as an anti-keloid agent [6,7]. The anti-keloid effect of such compound works by suppressing the proliferation, migration activity, and expression of TGF-β1 in keloid fibroblasts [8]. The ethanolic extract of *N. indicum* inhibits keloid fibroblast proliferation with IC_{50} value of 0.458 µg/mL and suppresses collagenation with IC_{50} of 0.055 µg/mL incubated for 72-hours [7]. Decree of the Head of Indonesian National Agency of Drug and Food Control No. HK.00.05.23.3644 dated 9 August 2004 has prohibited the use of *N. indicum* Mill leaves and flowers for dietary supplement materials. While a dietary supplement is always administered orally, the topical preparation of *N. indicum* as an anti-keloid candidate mixed with another potential anti-keloid agent, *T. diversifolia* (Hemsley) A. Gray., needs further developing. Using the Bioassay Guided Isolation (MTT; HeLa cells; IC_{50}: 9.776µg/mL), Tagitinin C was successfully isolated from *T. diversifolia* (Hemsley) A. Gray [9]. Tagitinin C inhibits keloid fibroblast proliferation with IC_{50} value of 0.039 µg/mL in 120 hours of incubation and suppresses collagen accumulation by 44.3% in 120 hours [10]. Tagitinin C restrains not only the migration of keloid fibroblast cells but also the expression of TGF-β1 at a concentration of 0.316 µg/ mL as well as the expression of VEGF optimum at 0.079µg/mL concentration [11,12]. The ethanolic extract of *T. diversifolia* inhibits keloid fibroblast proliferation with IC_{50} value of 3.624 µg/mL and suppresses collagenation with IC_{50} of 2.280 µg/mL in 120 hours of incubation [6]. At concentrations of 20 µg/ml, 10 µg/ml, and 5 µg/mL in a 24-hour incubation, *T. diversifolia* ethanolic extract can inhibit the migration of keloid fibroblasts compared to the control ($p<0.05$). The expressions of TGF-β1 and VEGF inhibited by the extract were also significantly lower than the control ($p<0.05$) [13]. The promising potential of both medicinal plants as anti-keloid agents encourages a further study of the mechanism of action at molecular level (migration activity, TGF-β1 and VEGF expressions) when a mixed extract of *N. indicum* and *T. diversifolia* acts as an anti-keloid agent.

MATERIALS AND METHODS

Research Subject

The subject was the subculture of keloid fibroblast cells passage III and V isolated and cultured in the Healthcare

Technology Laboratory of the Department of Dermatology and Venereology.

Research Materials and Equipment

The materials and equipment include mixed ethanolic extract of *N. Indicum* Mill and *T. diversifolia* Hemsley A. Gray. (collection from the Ethnopharmacology Laboratory of the Faculty of Medicine, Public Health, and Nursing of UGM), keloid fibroblast cells (collection from the Healthcare Technology Laboratory of the Department of Dermatology and Venereology), MTT (Sigma), DMEM (Gibco), Fetal Bovine Serum (FBS) (Gibco), Roswell Park Memorial Institute (RPMI) medium 1640 (Sigma), Amphotericin B – Fungizone (Gibco^{TM}), Penicillin – Streptomycin (Gibco-BRL), Ceftriaxone, 0.25% Trypsin-EDTA, 10% Povidone Iodine, 10% Formaldehyde, Phosphate Buffered Saline (PBS), X-Gal (5;Bromo-4-chloro-3-inndoyl-β-Dgalacto-piranoside), Dimethylformamide, Potassium Ferrocyanide, NaCl, MgCl, Citric Acid, Na2HPO4, distilled water (H2O), Methanol (E-Merck), Mitomycin C (10 mg Mitomycin-C Kyowa), sterile laminar airflow hood (NuAire), 75 mm flask, 96-well plate (Nunclon), Pasteur pipette (Brand, 230 nm), 5 ml and 10 ml measuring pipettes (Iwaki, Pyrex), micropipette (Biohit), yellow tip (LP Italiana SPA), blue tip, white tip (LP Italiana SPA), 15 ml and 30 ml tubes (Becton Dickinson, Falcon), reaction tube rack, CO_2 incubator (Galaxy S, RS Biotech), non-CO_2 incubator, sterile Petri dish sized 35 mm (Nunc), 9 cm glass Petri dish (Steriplan), 25 ml flask tube (IWAKI), 1.5 ml Eppendorf tube, gloves, mask, aluminum foil (Klinpak), microscope (Euromex No: 632569), sterilizer, hemocytometer (Neubauer), 0.22 ηm micro-filter (Millex^{TM}), and multi-plate reader.

Research Procedure

Culture of keloid fibroblast subculture cells passage-3 in 96-well plate

The cell suspension required for the research was calculated according to the number of groups and their triplications. Fibroblast culture was harvested and rinsed, and cell suspension was prepared with $2x10^5$/mL medium concentration. Well distribution was designed based on the predefined types, and each well was filled with 200 µl cell suspension and labeled according to the research design. The cells in the 96-well plate were incubated in 5% CO_2 incubator at a temperature of 37ºC for 24 hours.

Preparation of specimen concentration

Each ethanolic extract of *T. diversifolia* and *N.indicum* with each of their own IC_{50} values was weighed for 5.0 mg (extract) and dissolved in 100µL DMSO to obtain a stock solution of 50,000 µg/mL concentrations. A series of specimen doses was then prepared through dilution.

Observation of fibroblast migration activity

Measurement of cell migration test by in vitro scratch assay method [14], and analyzed by the method used [15]. 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 24, 48, and 72 hours, but every 24 hours microscopic images were taken using inverted microscope. The images obtained from each Well Plate sample were analyzed using imageJ softwere to obtain the percentage of scratch area. The percentage of cell migration is determined by 100% - percentage of scratch area.

Observation of TGF-β1 expression

Assessment of TGF-β1 expression followed the protocol from Bender MedSystems as the producer of Human TGF-β1 Elisa kit. Supernatant was dissolved with a ratio of 1:10 in a buffer essay, in which 20-µL cell supernatant was mixed with 100-µL buffer, resulting in sample solution. A total of 100-µL sample solution was placed in a 96-well plate previously layered with anti-TGF- β1. As much as 50 µL conjugated HRP was added and covered with a plaster. The kit was placed on a shaker and left for 4 hours. The plaster was removed and all the liquid was sucked and rinsed three times with a rinse solution. The result was added with 100-µL TMB substrate and incubated in the dark for 10 minutes. Stop solution was added and stored at a temperature of 4^0C and protected from light for 10 minutes. The result was examined in a plate reader at a 610-650 nm wavelength.

Observation of VEGF expression

VEGF expression was observed by following the protocol provided by Bender MedSystems, the producer of Human VEGF Elisa kit. With a ratio of 1:10, supernatant was dissolved in a buffer essay, where a mixture of 20-µL cell supernatant and 100-µL buffer was prepared to obtain sample solution. In a 96-well plate previously layered with anti-VEGF, 100-µL sample solution was added followed by addition of 50 µL conjugated HRP covered with a plaster. After the kit was left in a shaker for 4 hours, the plaster was detached, and the solution was sucked and rinsed three times. Then, 100-µL TMB substrate was added and incubated for 10 minutes in the dark followed by addition of stop solution stored at 4^0C and kept in the dark for 10 minutes. A plate reader was used to observe the results at a wavelength of 610-650 nm.

Ethics approval of research

This research has received permission from the Ethics Committee Research Faculty of Medicine Public Health and Nursing, University of Gadjah Mada based eligibility letter conduct, with the number KE/FK/0485/EC/2018.

ANALYSIS

Analysis was conducted using One-way ANOVA, a parametric statistical method, since the number of sample groups was more than two. If data was not normally distributed, the Kruskal-Wallis non-parametric analysis could be applied.

The statistical differences between the control group and compound treatment group were analyzed using One-way ANOVA (Analysis of Variance), and to compare several groups, Tukey's post-hoc test was employed with $p<0.05$ for a significant result.

RESULTS AND DISCUSSION

The migration activity with the mixed extract of *N. Indicum* Mill and *T. diversifolia* Hemsley A. Gray. (1:10, w/w) in keloid fibroblasts is described in Figure 1 to 3 and Table 1 to 3.

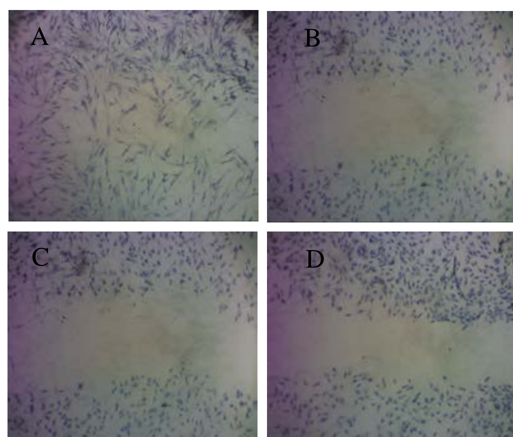

Fig.(1): Migration activity of keloid fibroblasts in 24 hours after the administration of mixed extract of *N. indicum* and *T. diversifolia* (1:10, w/w)

Notes:
A: Keloid fibroblasts in the medium
B, C, D: Keloid fibroblasts + extract of NiE + TdE
B: Concentration at 2 x IC_{50}; C: 1 x IC_{50}; D: 1/2 x IC_{50}

Table (1): Average number of fibroblast cells in a migration (using ImageJ) after the administration of mixed extract of NiE + TdE (1:10, w/w) incubated for 24 hours

Ni + TD	Number of Cells	
	Ni + TD	Control
16 µg/ml	118	191
8 µg/ml	155	191
4 µg/ml	166	191

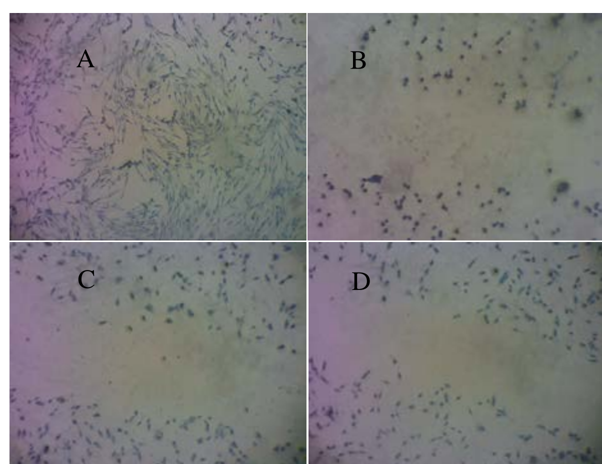

Fig.(2): Migration activity of keloid fibroblasts in 48 hours after the administration of mixed extract of *N. indicum* and *T. diversifolia* (1:10, w/w)

Notes:
A: Keloid fibroblasts in the medium
B, C, D: Keloid fibroblasts + extract of NiE + TdE
B: Concentration at 2 x IC_{50}; C: 1 x IC_{50}; D: 1/2 x IC_{50}

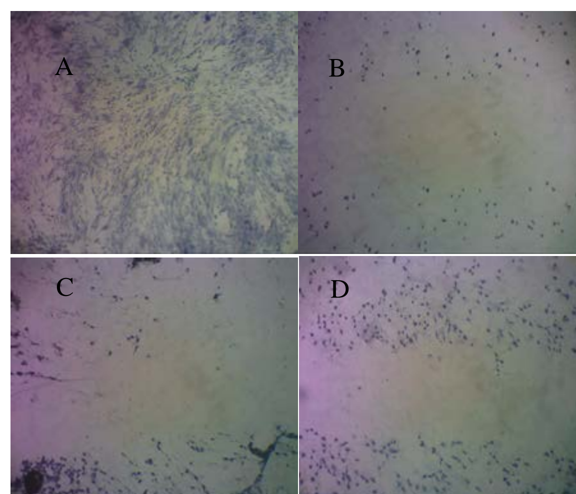

Fig.(3): Migration activity of keloid fibroblasts in 72 hours after the administration of mixed extract of *N. indicum* and *T. diversifolia* (1:10, w/w)

Notes:
A: Keloid fibroblasts in the medium
B, C, D: Keloid fibroblasts + extract of NiE + TdE
B: Concentration at 2 x IC_{50}; C: 1 x IC_{50}; D: 1/2 x IC_{50}

Table (2): Average number of fibroblast cells in a migration (using ImageJ) after the administration of mixed extract of NiE + TdE (1:10, w/w) incubated for 48 hours

Ni + TD	Number of Cells	
	Ni + TD	Control
16 µg/mll	116	302
8 µg/ml	152	302
4 µg/ml	165	302

Table (3): Average number of fibroblast cells in a migration (using ImageJ) after the administration of mixed extract of NiE + TdE (1:10, w/w) incubated for 72 hours

Ni + TD	Number of Cells	
	Ni + TD	Control
16 µg/ml	70	386
8 µg/ml	112	386
4 µg/ml	155	386

Fig. 1 and Table (**1**) show that, during the 24-hour incubation after the mixed extract of NiE + TdE (1:1, w/w) at a concentration of 16 µg/mL was administered, the inhibition of keloid fibroblast migration reached the highest level compared to the other concentrations. Since a large number of cells died at such concentration, the number of cells entering the scratch was much less than the cells that almost fully filled the control. Meanwhile, the mixed extract of NiE + TdE at 8 µg/mL concentration incubated for 48 hours could also inhibit the migration activity more vividly than the 24-hour incubation. In addition, the extract with 72-hour incubation clearly suppressed the migration activity as a large number of cells had died possibly due to a lack of nutrition (**Fig.3** and Table **3**). The observation results in **Fig. 3** and Table **(3)** indicate that the growth of fibroblasts during the Wound Healing and Scratch Assay was inhibited by the treatment given in the form of

administration of *N.indicum* and *T.diversifolia* mixed extract (1:10, w/w). The control group with cells receiving no treatment had a more significant number of cells compared to the treatment group.

Cell migration is an essential process to stimulate the synthesis of new extracellular matrix, thereby contributing to a wound-healing process. A previous study by [16], found that the fibroblast taken and cultured from keloid tissue indicated an increasing migration activity. Another study from [17], showed production differences in the keloid fibroblast-derived paracrine factors between perilesional keloid fibroblasts and intralesional keloid fibroblasts.

Observation of TGF-β1 expression of NiE + TdE mixed extract

Table **(4):** Average TGF-β1 expression after the administration of the mixed extract of NiE+TdE (1:10, w/w) between groups

Keloid Control	Concentration ± SD		
	4 µg/mL	8 µg/mL	16 µg/mL
19.23±9.76	12.21±8.29	12.8±5.15	10.14±5.49

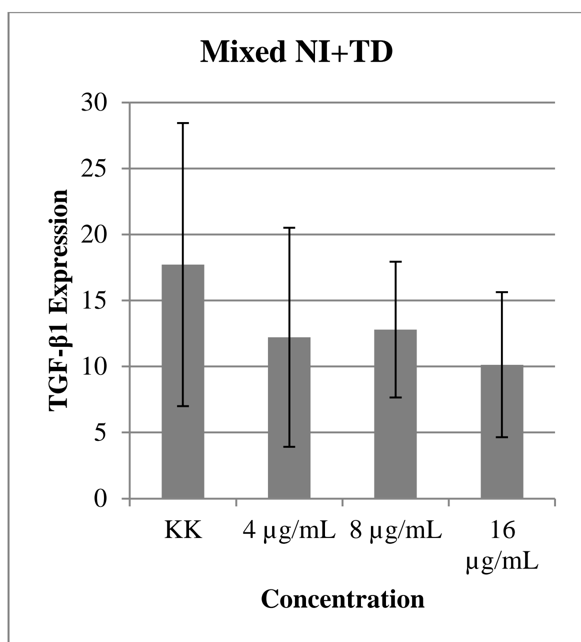

Fig.(4): Graph of TGF-β1 expression with the mixed extract of NiE+TdE (1:1, w/w) compared to the control

Fig. 4 shows that the administration of the mixed extract of NiE+TdE (1:10, w/w) at 4 µg/mL, 8 µg/mL, and 16 µg/mL concentrations inhibited TGF-β1 expression in keloid fibroblasts. At a concentration of 16 µg/ mL, the mixed NiE+TdE could significantly suppress TGF-β1 expression compared to the control ($p<0.05$).

TGF-β signaling is the main pathway that determines cellular behavior in both homeostasis and pathological conditions, including keloid formation. The activity of TGF-β signaling in fibroblast cells leads to phenotype modification, thereby strengthening the ability to proliferate and migrate or invade. An increased TGF-β activity in fibroblast cells can enhance the capability to stimulate tissue remodeling, thus increasing keloid tissue growth. Suppression of TGF-β pathway is an indicator of treatment success for keloid [18]. On the surface of keloid tissue there is often a higher suppression mechanism of the surrounding tissue, this is influenced by the expression of TGF-β1 which can regulate the expression of Smooth muscle actin (SMA) protein. TGF-β1 increases cell rigidity through interactions between TGF-β1 receptors and the SMA axis so that they can be targeted for keloid therapy [19]. A therapy with TGF-β1 improves the stimulation of protein synthesis in normal skin fibroblast but not affecting keloid fibroblast. This indicates that the regulation of TGF-β signaling in fibroblast keloid tissue experiences a transformation. Biosynthesis of fibronectin in keloid fibroblast increases more rapidly than normal fibroblast by involving a mechanism of transcription and enhancing the production of extracellular matrix in keloid fibroblast with the modification of TGF-β regulation program [20].

Observation of VEGF expression of NiE + TdE mixed extract

Table (5): Average VEGF expression after the administration of NiE+TdE mixed extract (1:10, w/w) among groups

Keloid Control	Concentration ± SD		
	4 µg/mL	8 µg/mL	16 µg/mL
69.55±41.66	67.41±39	37.96±17.26	25.2±18.44

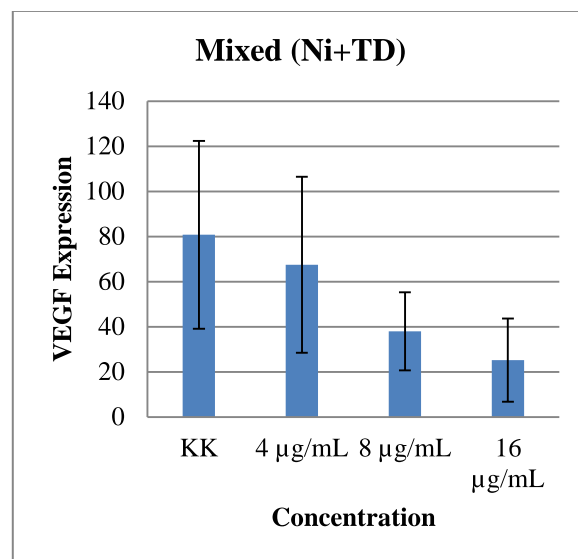

Fig.(5): Graph of VEGF expression with the mixed extract of NiE+TdE (1:1, w/w) compared to the control

Fig. 5 describes the inhibition of VEGF expression in the keloid fibroblast group compared to the normal fibroblast group. The mixed extract of NiE+TdE (1:10, w/w) at a concentration of 16 µg/mL for keloid fibroblast showed a significantly different result from the control group ($p<0.05$). The NiE+TdE mixed extract affected VEGF expression by reducing the supernatant VEGF as opposed to the control group.

VEGF is a proangiogenesis cytokine, which pathologically plays an important role in a normal condition as well as during wound healing [21]. It is a type of angiogenic

peptides with different isoforms. VEGF is also a vascular permeability factor that promotes neo-vascularization as well as cell growth [22,23]. Proliferation in keloid tissue is caused by escalation of exogenous and endogenous VEGF in keloid fibroblasts. Exogenous VEGF will increase with the support of VEGF receptor, while increased endogenous VEGF corresponds to the rising VEGF transcription factor in the tissue of keloid fibroblast cells [24]. VEGF as a pro-angiogenic growth factor enhances keloid tissue angiogenesis, leading to chronic inflammation and increasing persistent fibroblast proliferation.

CONCLUSION

The mixed extract of NiE+TdE (1:10, w/w) at a concentration of 8 µg/mL could optimally inhibit the migration of keloid fibroblast in 48 hours of incubation compared to the control. At 16 µg/mL concentration, NiE+TdE mixed extract could significantly suppress TGF-β1 expression as opposed to the control ($p<0.05$). The same concentration of NiE+TdE mixed extract could also significantly inhibit VEGF expression when compared to the control ($p<0.05$).

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