Effect of *Clinacanthus nutans* Leaf Extract on the Production of TGF-β1 and bFGF in Human Dermal Fibroblasts

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Abstract

A chloroform extract of the leaves of *Clinacanthus nutans*, a medicinal plant, contains purpurin-18 phytyl ester that has the ability to induce fibroblast migration. However, no scientific investigation has been conducted on its effect on the production of proteins related to wound healing.

The aim of this study was to investigate the effect of a *C. nutans* extract on the production of two important growth factors, TGF-β1 and bFGF, by dermal fibroblasts. Methods: A chloroform extract of *C. nutans* was fractionated using a vacuum column chromatography method. Fractions containing a spot with an Rf value of 0.58 (the retention time of authentic purpurin-18 phytyl ester) on TLC plates were used in biological assays. Dermal fibroblasts were exposed to various concentrations of the chloroform extract and fractions from *C. nutans* for 12 or 24 h, and TGF-β1 and bFGF expression at the protein level was measured by enzyme-linked immunosorbent assay (ELISA).

At 12 and 24 h, the TGF-β1 and bFGF protein levels were significantly increased by exposure to the chloroform extract and fractions 5B and 5C compared to the negative control.

Chloroform extracts and fractions of *C. nutans* that contained purpurin 18-phytyl ester increased the protein synthesis of TGF-β1 and bFGF by dermal fibroblasts.

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Introduction

Wound healing is a normal, but very complicated, biological process that involves several phases that overlap with manufacturing and cellular processes of haemostasis, inflammation, proliferation and remodelling.[1,2] Several studies indicate that growth factors play an important role in the cell division, migration, differentiation, enzyme production, and protein expression responsible for wound healing through the stimulation of angiogenesis and cellular proliferation.[3,4] Two key growth factors associated with wound healing are TGF-β1 and bFGF.

TGF-β1 has been shown to activate angiogenesis by stimulating smooth muscle vascular migration.[5] This basic fibroblast growth factor has the ability to regulate the replication and migration of epithelial, endothelial, and fibroblast cells, which participate in collagen production, epithelisation, and neovascularisation, respectively.[6] During wound healing, tissue remodelling allows the replacement of injured tissue, and fibroblasts play an active role in this phase by replenishing damaged tissue through cell proliferation, differentiation and migration. Among these processes, fibroblast migration is a critical biological response during wound healing, as these are the cells responsible for the synthesis of collagen fibres in the connective tissue.[7,8]

Wound healing remains a critical issue, and this has stimulated the search for natural medicines that can promote this process. One of the medicinal plants currently being developed is *Clinacanthus nutans*, a member of the Acanthaceae family. The leaves of this plant have been used as a traditional medicine in Thailand, Indonesia, and Malaysia to treat cancer, herpes, diabetic dysentery, skin rashes, and snake bites. *In vitro* studies have shown that

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ethanolic extracts of *C. nutans* have anti-inflammatory and anticancer activity.[9,10,11] Previous phytochemical studies have identified flavonoids, steroids, triterpenoids, cerebrosides, glycoglycero-lipids, glycerides, sulfur-containing glycosides in these extracts. Among these natural products, glycoglycerolipids and digalactosyl diglycerides exhibit antiviral and anti-HSV activity, respectively.[12] Previous research has revealed that chloroform extracts from *C. nutans* leaves contain purpurin-18 phytyl ester, which shows anti-inflammatory, anti-biofilm, and wound-healing activity. Both the crude chloroform extracts and the isolated purpurin-18 phytyl ester could be potential therapeutic agents for treating gingivitis and inflammations of the mouth (Roeslan *et al*. 2019). The aim of the present study was to investigate the effect of a *C. nutans* leaf chloroform extract and its fractions on the production of two important growth factors, TGF-β1 and bFGF, by dermal fibroblasts.

Materials and methods

Plant material preparation, extraction and fractionation

Fresh leaves of *C. nutans* were collected in Indonesia, and the specimens were authenticated by the Indonesian Institute of Science. The *C. nutans* leaf powder (182 g) was sequentially extracted in a Soxhlet extractor at 55°C, first with hexane and then with chloroform (Merck, USA), using a method described previously with some modification.[13] The solvent was then evaporated in a rotary evaporator (Buchi, Switzerland) at 30°C to generate hexane (7.53 g) and chloroform (4.01 g) extracts. The chloroform extract was fractionated using the column vacuum chromatograph method. The extract was loaded onto a column packed with silica gel 60 and eluted with a hexane-ethyl acetate gradient (9:1 to 1:9 v/v). This method generated multiple fractions; these were later combined based on their similar components, as determined by TLC analysis, resulting in 6 fractions (F1–F6). The fractions were combined based on their content of a compound with a retention factor (Rf) value of 0.58, the known retention time of the purpurin-18 phytyl ester.[13] F5 was selected for further fractionation and generated 5 new fractions (F5A, F5B, F5C, F5D and F5E). Of these fractions, F5A, F5B and F5C TLC showed a high intensity of the Rf = 0.58 spot, so these three fractions were chosen for further protein assays. The scheme of the extraction is shown in Figure 1.

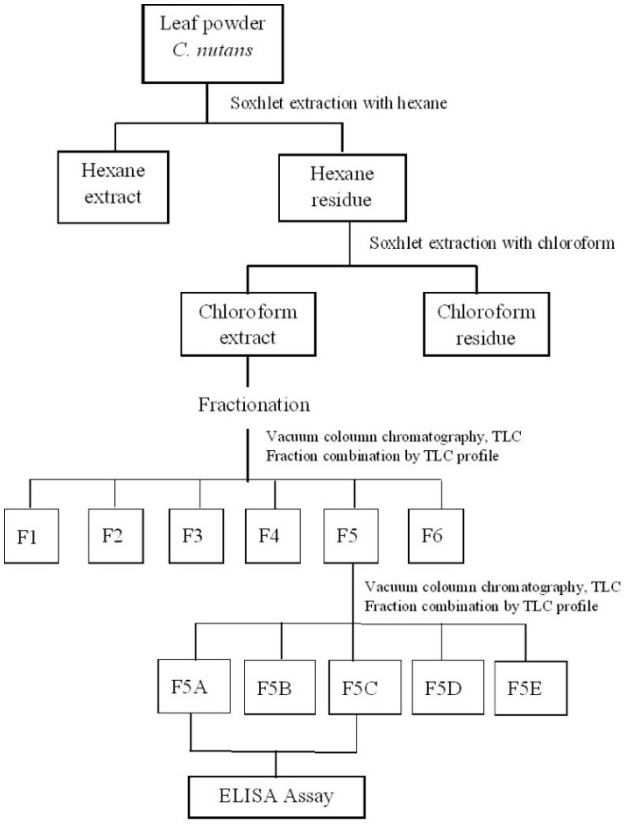

Figure 1. Scheme of the extraction and fractionation of *Clinacanthus nutans* leaves. The leaf powder was sequentially extracted with hexane and chloroform in a Soxhlet extractor. The chloroform extract was fractionated by column vacuum chromatography using a hexane-ethyl acetate gradient (9:1 to 1:9) as the eluting solvent. Each eluted fraction was analysed by TLC using hexane:ethyl acetate (7:3) solvent as the mobile phase. After combination of fractions with similar profiles, 6 fractions were obtained. Based on the TLC profile, fraction 5 contained a spot with an Rf value of 0.58, so this fraction was further fractionated. The second fractionation yielded 5 fractions (F5A-F5E). Based on the TLC profile, F5A, F5B and F5C contained spots with an Rf value of 0.58. These three fractions were then used in the ELISA assay.

Analytical TLC

Thin-layer chromatography was performed on crude extracts and extract fractions using pre-coated silica gel 60F-254 with 0.2 mm layer thickness (ALUGRAM Xtra SIL G/UV254;

Macherey-Nagel, Germany). Capillary tubes were used to apply two microliters of the solutions onto the plates, developed with a mobile phase of hexane:ethyl acetate (7:3, v/v) solvent, dried, and visualised under UV 366 nm.

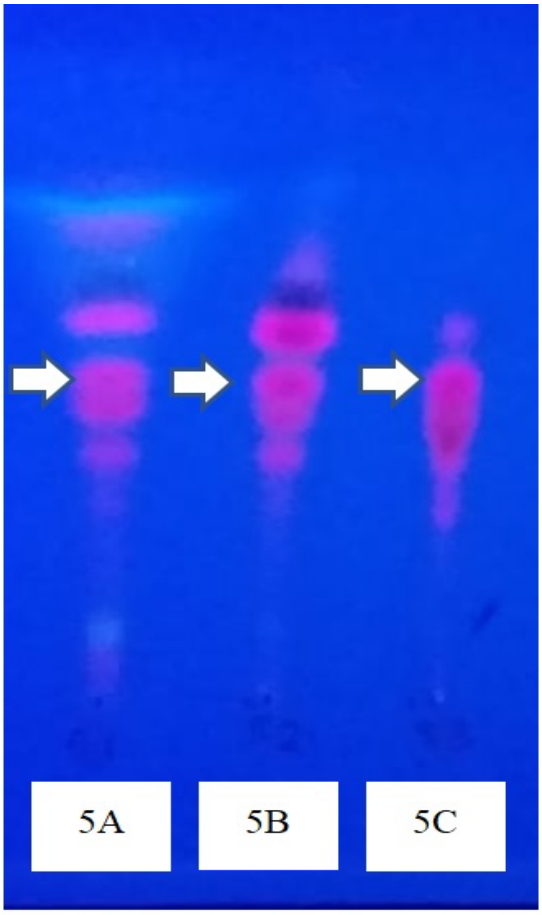

Figure 2. Chromatographic profiles of fractions from *Clinacanthus nutans* chloroform extracts detected by exposure to UV366. These three fractions contain purpurin-18 phytyl ester with an Rf value of 0.58 (white arrow), and were tested on fibroblasts.

Cell culture

Human dermal fibroblasts were a gift from Dr. Indra Kusuma from Faculty of Medicine, YARSI University, Jakarta, Indonesia. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA), supplemented with 10% foetal bovine serum (Gibco, USA) and 250 IU/mL penicillin (Invitrogen, USA), under standard culture conditions at 37°C in a 5% CO_2 humidified incubator. The medium was changed regularly, and the cells were sub-cultured every 3–4 days or after reaching 80% confluence in the culture flasks.

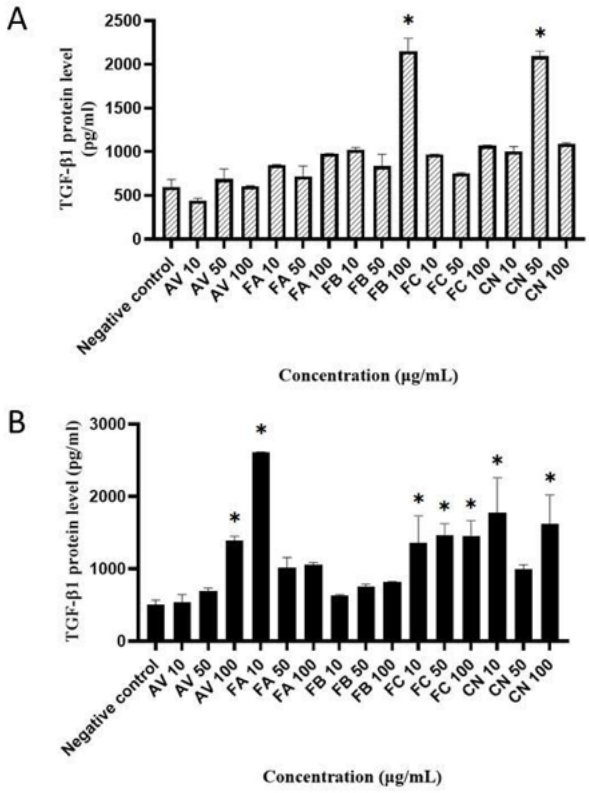

Figure 3. TGF-β1 expressions in fibroblast exposed to crude extracts and fractions from *Clinacanthus nutans*. TGF-β1 production was determined by ELISA of fibroblast culture supernatants after a 12 h (A), and 24 h (B) incubation with various concentrations of the extracts or fractions. *Aloe vera* (AV) was used as a positive control. * indicates a statistically significant difference compared to the negative control ($p < 0.05$).

Analysis of TGF-β1 and bFGF protein expression by ELISA

Dermal fibroblast cells were grown in 96-well plates (10^5 cell/well) and exposed to *C. nutans* solutions at 10, 50 and 100 mg/mL. The culture supernatant was then collected at 12 and 24 h and frozen at -20°C. The TGF-b1 and bFGF levels from the cell culture supernatants were measured with ELISA kits (Raybiotech, Norcross, USA) according to the manufacturer's instructions. In brief, 100 µL of standard and sample were added to each cell culture supernatant in appropriate wells. The wells were covered and incubated at room temperature for 2.5 h. The solution was discarded, and the cells were washed 4 times with 200 µL of wash solution (1×) per well. Then, 100 µL of 1× prepared biotinylated antibody were added to

each well. The plate was incubated at room temperature for 1 h, then the solution was discarded and the cells were washed 4 times with 1× 200 µL of wash solution per well. After washing, 100 µL of prepared streptavidin solution was added to each well and incubated for 45 min at room temperature. The solution was discarded, and the cells were washed five times with 1 × 200 µL of wash solution per well. Then, 100 µL of TMB One-Step Substrate Reagent was added to each well and incubated at room temperature for 30 min in the dark. After addition of 50 µL of stop solution, the absorbance was read immediately at 450 nm. The results were expressed as TGF-b1 and bFGF levels in pg per mL of cell culture supernatant.

Statistical analysis

The data were reported as the means ± standard deviation of the mean. Significant differences were determined using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., USA). Differences were considered significant at $P < 0.05$.

Results

TLC analysis

Thin-layer chromatography was performed to identify fractions containing the compound with an Rf value of 0.58 (purpurin-18 phytyl ester). The TLC profiles of the fractions used for bioassay are shown in Figure 2.

TGF-β1 and bFGF protein analysis

TGF-β1 and bFGF secretions were evaluated by incubating dermal fibroblasts in the presence of various concentrations of the crude extracts and fractions of *C. nutans* leaves. The culture supernatants were collected at 12 and 24 h for quantification of protein levels. At 12 h, cells treated with fraction 5B (concentration 100 µg/mL) and the crude extract (concentration 50 µg/mL) showed a significantly higher release of TGF-β1 protein compared to the negative control. At 24 h, cells treated with fraction 5C (all concentrations), the crude extract (concentrations of 10 µg/mL and 100 µg/mL) showed statistically significant increased release of TGF-β1 protein compared to the negative control (Figure 3). The release of bFGF protein at 12 h was significantly greater from cells treated with fraction 5B (concentration 50 µg/mL) and fraction 5C (concentration 50 and 100 µg/mL) compared to the negative control. At 24 h, treatment with *aloe vera* (concentration 100 µg/mL), fraction 5B (concentration 10 and 50 µg/mL), fraction 5C (concentration 50 and 100 µg/mL), and the *C. nutans* crude extract (all concentrations) significantly increased the release of bFGF protein from the treated fibroblasts compared to the negative control (Figure 4).

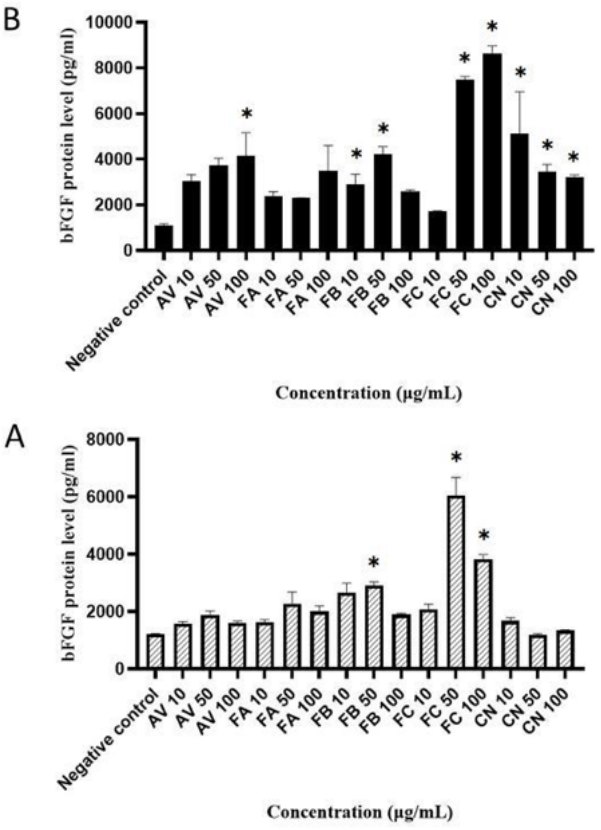

Figure 4. bFGF expression in fibroblasts exposed to crude extracts and fractions of *Clinacanthus nutans*. The bFGF production was determined by ELISA of fibroblast culture supernatants after a 12 h (A), and 24 h (B) incubation with various concentrations with various concentrations of the extracts or fractions. *Aloe vera* (AV) was used as a positive control. * indicates a statistically significant difference compared to the negative control ($p < 0.05$).

Discussion

In this study, powdered leaf material from *C. nutans* was extracted using a Soxhlet extraction method. The main advantage of

Soxhlet extraction is that it requires only a clean warm solvent for extraction of the solids placed in the thimble and is a continuous, easy-to-use and inexpensive process. This continuous process can increase the extraction efficiency compared to simply heating the solid in a flask containing the solvent. Conversely, the disadvantages of the Soxhlet extraction method are that heat-resistant compounds may be lost during the process, the extraction takes a long time and it requires a large volume of solvent.[14,15]

The solvents used in this study for extraction were hexane and chloroform. Hexane is a nonpolar solvent, so hexane can dissolve nonpolar compounds, whereas chloroform is a semi-polar solvent so it can dissolve semi-polar compounds. The advantage of sequential extraction with hexane and then chloroform is that the nonpolar compounds have already been extracted by hexane, so they do not interfere with extraction of the semi-polar compounds by chloroform. In previous research, all crude extracts showed activity in a wound healing scratch test, but the chloroform extract showed the strongest activity. This is the reason why the chloroform extract was chosen for further isolation. The difference in solvents in the extraction can affect the final extracted content of the desired compound due to differences in polarity of the solvents.[16,17]

The resulting extract can be further purified by the fractionation process; in this case, the fractionation method was vacuum liquid column chromatography. The column is filled with a solid adsorbent as the fixed phase and a solvent is used as the mobile phase. The sample to be fractionated is run into the column and then eluted with the mobile phase. The separation of the components depends on the degree of polarity of the mobile phase and the compounds contained in the mixture. In this case, silica gel was used as the absorbent or fixed phase, and various solvent ratios of hexane and ethyl acetate (9:1 to 1:9) were applied as the mobile phase, using a vacuum pump to facilitate and speed up the elution.

The active constituents of *C. nutans* have been studied extensively. Chlorophyll compounds are the only elements of *C. nutans* that have been reported to have beneficial activities, such as antiviral, anti-inflammatory and anti-biofilm activities.[13,18] One previous study showed that purpurin-18 phytyl ester is the compound responsible for wound healing activity and that it had an Rf value on TLC of 0.58.[13] In line with the previous research, the purpurin-18 phytyl ester in our TLC analysis was found in the *C. nutans* chloroform extract fractions 5A, 5B and 5C, and showed the appropriate Rf value, colour characteristics and TLC profile. These fractions were therefore deemed appropriate for use in the fibroblast experiments.

The proliferation, differentiation, and migration of fibroblasts are key roles in wound healing.[19] Fibroblasts proliferate and migrate to the wound area, synthesise extracellular matrix, and express thick actin spindles during their transition into myofibroblasts.[20] TGF-β1 and bFGF are important cytokines produced by fibroblasts and are responsible for cell division, cell differentiation, protein expression and enzyme production. Fibroblasts have the ability to promote wound healing through the stimulation of angiogenic factors and by cellular proliferation that influences the production and degradation of extracellular matrix through chemotactic roles.[21]

Previous study showed that Aloe vera enhanced oral mucosa wound healing in vivo by increasing TGFβ-1.[22] Another previous study showed that *Aloe vera* A previous study comparing the expression of TGF-β1 and bFGF in mouse embryo fibroblasts treated for 12 or 24 h with various concentrations of *Aloe vera* (AV) gel at concentrations of 50, 100 and 150 g/mL showed increased expression of TGF-β1 and bFGF at 12 h but decreased expression at 24 h.[23] These results differ from those of the present study, where the expression of bFGF following treatment of fibroblasts with *C. nutans* extract or fractions for 24 h was significantly higher than after a 12 h treatment. By contrast, the TGF-β1 expression by fibroblasts showed only a slight and not statistically significant increase after a 24 h exposure to *C. nutans* extract or fractions.

The expression of TGF-β1 increased significantly following 12 h exposure to fraction B at a concentration of 100 g/mL or the crude extract at all concentrations. The expression of TGF-β1 was significantly increased after 24 h exposure to fraction A (10 g/mL), fraction C (10, 50 and 100 g/mL) and the crude extract of *C. nutans* (10 and 100 g/mL). The results for TGF-β1 after 12 and 24 h exposure to the crude extract of *C. nutans* alone showed a significant increase and probably reflects a response to

compounds other than purpurin-18 phytyl ester that are present in the crude extract of *C. nutans* and can also increase the expression of TGF-β1 and bFGF. Another possibility is that a synergistic effect occurs between the compounds in the crude extract of *C. nutans* and activates this extract. Notably, the present results indicate that these compounds from *C. nutans* leaves are more active than the compounds contained in *Aloe vera* extracts. Fraction A, from the TLC analysis, showed a thick, dense material with the Rf value of 0.58, indicating the possibility of the presence of compounds other than purpurin-18 phytyl ester in fraction A. This could explain why both fraction A and the crude extract of *C. nutans* showed increases in protein expression at 12 and 24 h.

The expression of bFGF at 12 h was increased by exposure of the fibroblasts to fraction B at a concentration 50 µg/mL and fraction C at concentrations of 50 and 100 µg/mL. However, the bFGF expression at 24 h increased in response to the *Aloe vera* positive control (100 g/mL), fraction B (50 and 100 µg/mL), fraction C (50 and 100 g/mL) and the crude extract of *C. nutans* (10, 50 and 100 µg/mL). Fractions B and C had higher activity than fraction A and probably reflects the thicker density of fractions B and C on the TLC plates at an Rf value of 0.58, indicating a higher concentration of purpurin-18 phytyl ester than in fraction A. Fractions A, B and C are sub-fractions that have been fractionated with hexane (nonpolar) and ethyl acetate (semi-polar) eluents in various ratios, so the compounds contained in them are purer than in the crude extracts.

Conclusions

Our study revealed that chloroform extracts and fractions of *C. nutans* that contained purpurin-18 phytyl ester could increase the protein production of TGF-β1 and bFGF in dermal fibroblasts. *Clinacanthus nutans* may therefore be a potential medicinal plant for accelerating wound healing. However, further study is still required to confirm this speculation.

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Declaration of Interest

The authors report no conflict of interest.

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