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ORIGINAL RESEARCH

Antioxidant, Antiinflammation, and Antifibrotic Activity of Ciplukan (*Physalis angulata* L). Extract

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Purpose: *Physalis angulata* Linn. (Ciplukan) is a plant widely used in traditional medicine in subtropical and tropical regions. Most studies focus on its antioxidant and anti-inflammatory activity. Many studies also reported its therapeutic potential for treating cancer, malaria, hepatitis, rheumatism, liver problems, and tumors, but few studies have reported its anti-fibrosis activity. Here, we aimed to investigate the potential of *P. angulata* as an antioxidant and anti-inflammatory that may be correlated with its anti-fibrosis action.

Methods: In our study, we treated 3T3-L1 and TGF-β-induced 3T3-L1 cells with an ethanol extract of *P. angulata*. We then monitored the cell's response, evaluated the antioxidant activity using an MTT assay, and observed the cells' migration using the cell scratch assay. We used RT-PCR to determine the expression of HIF-1α and IL-6 on TGF-β-induced 3T3-L1 cells.

Results: The ethanol extract of *P. angulata* showed antioxidant activity and promoted cell proliferation on 3T3-L1 cells. Interestingly, the extract inhibited the migration of TGF-β-induced 3T3-L1 cells. Further analysis revealed that the extract could inhibit *HIF-1α* expression and suppress *IL-6* expression on TGF-β-induced 3T3-L1 cells.

Conclusion: The ethanol extract of *P. angulata* showed antioxidant and anti-inflammation activities in 3T3-L1 cells. Both activities are associated with the antifibrotic activity of *P. angulata's* ethanol extract.

Keywords: TGF-β, ethanol extract, wound healing, fibroblast cell line

Introduction

Ciplukan has a Latin name *Physalis angulata* Linn. and belongs to the Solanaceae family.[1] Physalis is the fifth-largest Solanaceae genus, containing at least 70 species.[2] *P. angulata* is extensive growth in tropical regions such as Australia, Pacific America, and Asia. Although originally wild and weeds, it has recently been widely cultivated in tropical and subtropical regions.[3] This annual plant has a minuscule stature compared to other Physalis species. The plant has a height of between 15–60 cm. The leaves are oval with long petioles. *P. angulata*'s flowers are bell-shaped and covered by petals, which develop into fruit covers. The fruit is round, juicy, light green (unripe), and turns yellow (ripe).[3]

P. angulata is one of the natural products widely used in traditional medicine. People commonly use this plant for antibacterial, antiparasitic, anti-inflammatory, anti-analgesic, antidiabetic, and antioxidant due to its active properties.[4,5] They benefit from all parts of the plant: roots, flowers, fruits, leaves, and stems. For instance, *P. angulata* has been used for treating malaria and liver dysfunction in Brazil.[6] In the Peru and Indonesia regions, the leaves and fruit of *P. angulata* are also used for itching and postpartum infection.[7,8] Aerial parts of Ciplukan were used for chronic inflammation treatment in Northern Nigeria.[9] People in Nigeria and Ivory Coast have used tea leaves and all parts of it for the treatment of malaria.[10,11] Several

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other diseases such as anti-dermatitis, intestinal worms, abdominal pain, wounds, hepatitis, anemia, urinary tract infections, and tumors are also treated with *P. angulata* as traditional medicine in several countries in the world.[12,13]

In addition, because of its phytochemical content, such as flavonoids, steroids, alkaloids, saponins, and tannins, the development of *P. angulata* for modern medicine is also being carried out.[14] For example, the ethanol extract of Ciplukan leaves reported has high antioxidant activity due to its flavonoid content.[15] Pillai et al also revealed cytotoxic, antimicrobial, and antioxidant activities of ethanolic extract *P. angulata*, which contains alkaloids, glycosides, flavonoids, tannins, and phenolics.[16] This plant's active fractions and compounds are also widely reported to have anti-cancer, immunomodulatory, immunosuppressive, and anti-inflammatory actions.[17] Physalin, Withangulatin, Physagulin, Oleanolic acid, and Myricetin 3-O-neohesperidoside are active compounds successfully isolated from *P. angulate*.[13] Specifically, Physalins B, F, and G were reported as anti-inflammation by inhibiting pro-inflammatory cytokine production.[18] Isolated physalin F from ethanolic extract showed anti-cancer activity on human or animal cell lines.[19] Withangulatin had strong immunosuppressive activity by eliminating T lymphocyte expression and modulating T helper balance.[20]

Among the many potentials of *P. angulata*, only a few studies have reported its activity as an anti-fibrotic agent. Given that inflammation and oxidative stress are essential in the pathogenesis of fibrosis, and it has been established that this plant has both antioxidant and anti-inflammatory activity,[21,22] we investigated *P. angulata* potential as anti-fibrosis. In experimental models of cells or tissues, TGF-β is widely used to promote fibrosis.[23,24] Many studies have used the 3T3-L1 cells induced by a profibrotic cytokine, TGF-β, as fibrosis models in vitro.[25,26] Our previous study showed that adjuvant from ethanol extract of *P. angulata* reduced skin fibrosis in scleroderma patients.[27] Previous in vivo studies also revealed the antifibrotic activity of *P. angulata*'s ethyl acetate fraction in rat liver fibrosis induced by CCl4.[28] Our in vitro study with ethanol extract also demonstrated the in-line result that showed their antifibrotic activity through inhibition in *α-SMA*-expressing myofibroblasts.[29] Promising effects of Ciplukan extract on fibrosis-related gene expressions are also shown in the Bleomycin-induced mouse model [ref][30] Therefore, this study focuses on the antioxidant and anti-inflammatory properties that may be correlated with P. angulata's anti-fibrosis action.

Material and Methods

Plant Collection and Extraction

All plant parts, except the roots, were collected from several locations in West Java, Indonesia (Figure 1). The plant was identified using literature by a taxonomist, Joko Kursmoro.[31–33] The Plant Taxonomy Laboratory, the Faculty of Mathematics and Natural Sciences of Universitas Padjadjaran confirmed the accuracy of the taxonomic identification with reference no.106/HB 10112020. The Herbarium of the Plant Taxonomy Laboratory prepared and deposited voucher specimens. The extraction technique is cold maceration. This technique is an extraction process using a solvent with several times stirring at room temperature. We macerated the *P. angulata* herb in 50% ethanol for 3×24 hours. Then, we evaporated the filtrate with a rotary vacuum evaporator (CCA-1100, EYELA, Japan). Furthermore, the condensed extract is freeze-dried into a dry extract.

Materials

3T3-L1 fibroblasts were purchased from the American Type Culture Collection (ATCC). Roswell Park Memorial Institute (RPMI) 1640 Medium, Fetal Bovine Serum (FBS), Penicillin-Streptomycin (PS), and Phosphate Buffer Saline (PBS) solution were supplied by PAN-Biotech. Human transforming growth factor beta-1 (TGF-β), Menadione, N-acetyl-cysteine (NAC), glutathione (GSH), and Dimethylsulfoxide (DMSO) were supplied by Sigma-Aldrich. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay kit and DMSO was obtained from Sigma-Aldrich.

Cell Culture and TGF-β-Treated 3T3-L1 Cells

3T3-L1 fibroblasts were cultured in a rich medium of RPMI 1640 supplemented with 10% FBS and 1% PS. Cells were maintained at 37°C and 5% CO_2 atmosphere. We used 50 ng/mL TGF-β to stimulate 3T3-L1 fibrosis cells for 24 hours of incubation. The medium for fibrosis cells is a starvation medium comprising RPMI 1640 supplemented with 0.1% FBS and 1% PS.

Figure 1 Photographs from the field of *Physalis angulata* Linn.

Proliferation Assay

Cell proliferation and antioxidant activity were conducted using an MTT assay. First, 7.5×10^4 cells were plated in each well of a 96-well plate, then treated with ethanol extracts at different concentrations (10, 100, 500, 750, 1000, 1250, 1500, 2000 µg/mL) and incubated for 24h. To evaluate antioxidant activity, 7.5×10^4 cells in each well of a 96-well plate were treated with menadione (6 µM) and 500 µg/mL extract (or 5 mM GSH or 5 mM NAC) for 24 hours of incubation. Next, MTT reagent was added to each well. Incubation was continued for 4h. Once the formazan crystals are formed, they are dissolved in DMSO. The quantity of formazan crystals was determined at 550 nm using a plate reader (Thermo Scientific® Multiscan EX, Singapore). The graph of absorbance was plotted to determine the 3T3-L1 cell proliferation according to the kit manufacturer's instructions. Experiments were performed in triplicates.

In Vitro Scratch Wound Healing Assay

Cells were grown in 96-well plates up to 90% confluency. Then, we scratched the monolayer of cells using a p20 pipette tip. After washing the cells with PBS, we incubated cells with a transition medium containing the extract (100 µg/mL or 500 µg/mL) for 24h or 48h. The medium with no extract was used as a negative control. We evaluated scratch closure using an inverted microscope (40x magnification), and the scratch area was analyzed using the Image J 1.38 software (NIH, Bethesda, MD, USA).

Cell Migration (Scratch) Assay

Cells were grown in 96-well plates up to 90% confluency. After the culture medium was discarded, a scratch was made on the monolayer of cells using a p20 pipette tip. The plates were rinsed with PBS and then incubated with a medium containing TGF-β (10 ng/mL) and *P. angulata* extracts (100 and 500 µg/mL). Medium with TGF-β was used as a control. Cells were incubated for 24 h and 48 h. The cell migration was observed using a light microscope (40x magnification), and the scratch area was analyzed using the Image J 1.38 software (NIH, Bethesda, MD, USA).

RT-PCR Analysis

After treatment with TGF-β and *Physalis angulata* extract, total RNA was isolated from the cells using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Then, mRNA expression was determined by real-time PCR using SensiFAST™ SYBR® No-ROX One-Step Kit (Meridian Biosciences) and specific sequence primer in Agilent AriaMX PCR System (Agilent Technologies, USA). The primers were synthesized by Integrated DNA Technologies (IDT, Singapore). The nucleotide sequences (5′ to 3′) of the forward primers and reverse primers are GAPDH, 5′-CAAGATCATCAGCAATGCCTCC −3′ (sense), and 5′-GCCATCACGCCACAGTTTCC −3′ (antisense); HIF1α, 5′- GTA ATG CTC CCC TCA CCC AAC −3′ (sense) and 5′- GTG CAG GGT CAG CAC TAC TTC −3′ (antisense),[34] IL-6, 5′-AGTGG CTAAG GACCA AGACC-3′ (sense) and 5′-TCTGA CCACA GTGAG GAATG-3′ (antisense).[35] The PCR conditions were as follows: 45°C for 10 min for reverse transcription, 95°C for 2 min, 40 cycles of 95°C for 5 s, and 60°C for 20s. Data were analyzed by the 2-ΔΔCT method, using the housekeeping gene GAPDH as the internal control.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using Microsoft Excel Office 2010 software. Differences between the two groups were analyzed by unpaired two-tailed Student's *t*-tests. A p-value less than 0.05 was considered significant. Levels of significance are indicated in the Figures and text as appropriate.

Results

P.Angulata's Ethanol Extract Inhibits Oxidative Stress and Accelerates Wound Healing on 3T3-L1 Cells

In this study, we first treated 3T3-L1 fibroblast cells with various extract concentrations to ensure that the concentration did not become toxic, which could affect cell migration. The result showed that the extract did not disturb cell viability until 500 µg/mL of treatment. We monitored starting at 750 µg/mL of extract, and cells lost the viability of 45% (Figure 2). Then, we evaluate the antioxidant activity of 500 µg/mL extract on fibroblast 3T3-L1 cells. Here, we induced cells with menadione, widely used to induce cellular stress oxidative[36,37] In parallel, we co-treated cells with the extract or other extracellular antioxidants, NAC and GSH. The results showed that the extract has been able to provide protection against menadione toxicity and reduce oxidative stress. Treatment with 500 µg/mL ethanol extract for 24h significantly increased cell viability. The extract had a similar antioxidant effect to N-acetyl-cysteine (NAC) and glutathione (GSH) as oxidizing radical scavenger agents (Figure 3).

Then, we monitored the wound healing response of 3T3-L1 fibroblast cells using a cell scratch test in the absence or presence of the extract. An in vitro scratch assay has often been used in previous studies to measure the progression of wound closure.[38,39] Fibroblast migration is a crucial step in the wound-healing process.[40] Therefore, we evaluate the effect of the *P. angulata* extract on cell migration (Figure 4A). Here, we calculated the percentages of area closure for comparison. After 24h, treatment with 500 µg/mL of the extract can reduce the wound area (0.23 fold) compared to the control (0.38 fold). Moreover, the effect of the extract on scratch closure was statistically significant (Figure 4B).

P.Angulata's Ethanol Extract Inhibited the Migration of TGF-β-Induced 3T3-L1 Fibrosis Cells

After showing the wound-healing activity of *P. angulata* to 3T3-L1 cells and our knowledge of their antifibrotic effect on TGF-B-induced fibrosis in 3T3-L1 cells,[25] we investigated the wound-healing activity of the extract to fibrosis cells (Figure 5A). Then, we scratched TGF-β induced-3T3-L1 fibrosis cells and treated them with non-toxic concentrations of *P. angulata* to cells (100 or 500 µg/mL), for 24h or 48h incubation. The result demonstrated mild migration of cells observed in treated cells and progressive migration in control. ImageJ software analysis showed a significant decrease in migration cells on 48h incubation of 100 µg/mL extract (0,56-fold) and 500 ug/mL (0,65-fold) compared to the control (0,26-fold). We observed similar results on 24h treatment of 500 µg/mL extract (0,88-fold) compared to the control (0,60-fold) (Figure 5B).

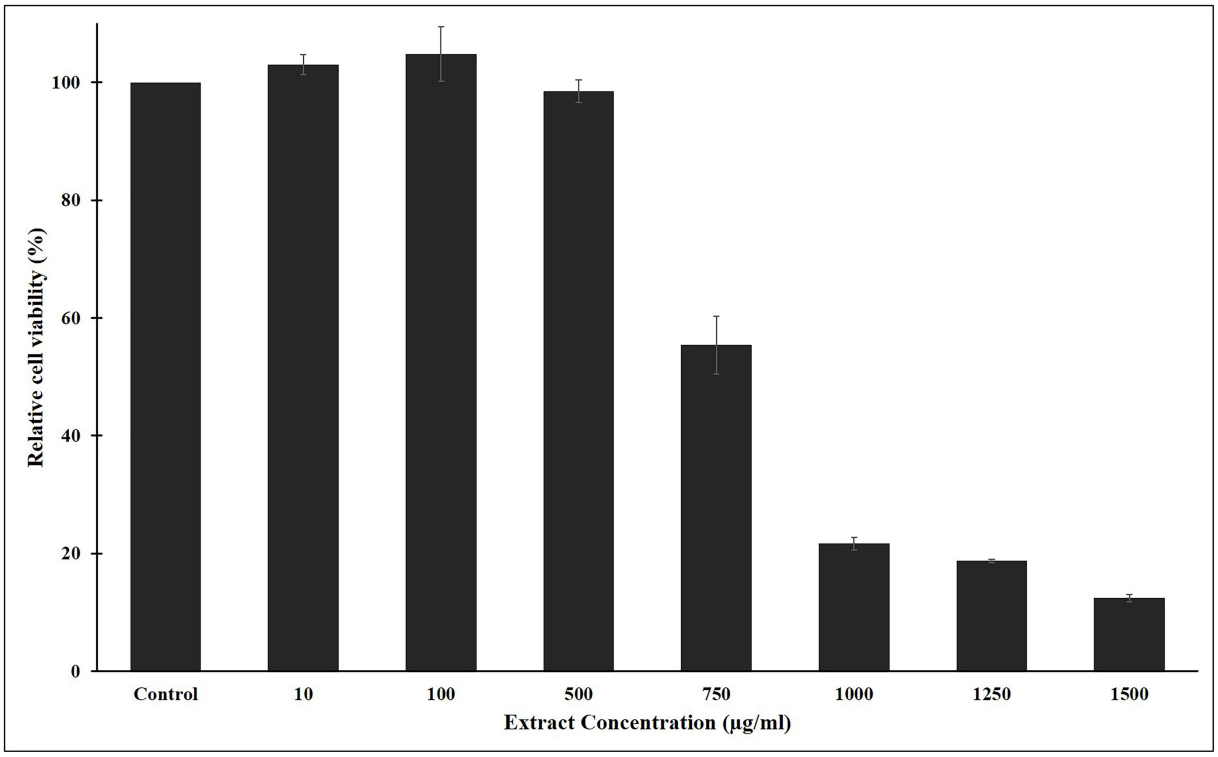

Figure 2 Relative cell viability as affected by 24 h treatment in complete medium with *P. angulata* extract 10, 100, 500, 750, 1000, 1250, and 1500 µg/mL or the solvent (control). Cell viability was then evaluated by MTT assay and expressed as a percentage of untreated cells. Values represent the mean ±SD of three independent experiments.

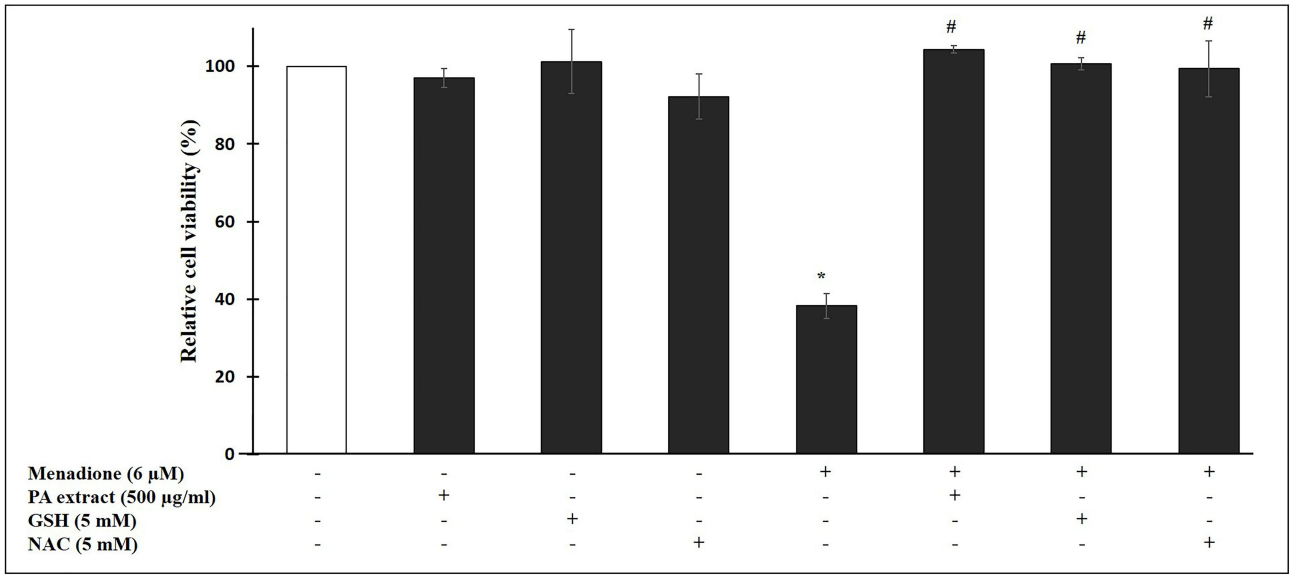

Figure 3 Effects of *P. angulata*'s extract on Menadione toxicity on 3T3-L1 cells. Cells were treated with stress agent Menadione (6 µM), Menadione (6 µM) + 500 µg/mL extract (or 5 mM GSH, or 5 mM NAC), for 24 hours incubation. We used medium with solvent (or GSH, or NAC, or extract) as negative controls. Cell viability was then determined by MTT assay. Values represent the mean ±SD of three independent experiments. * Significantly different compared with that of the control (complete medium) ($p < 0.05$). # Significantly different compared to that of 6 µM Menadione ($p < 0.05$).

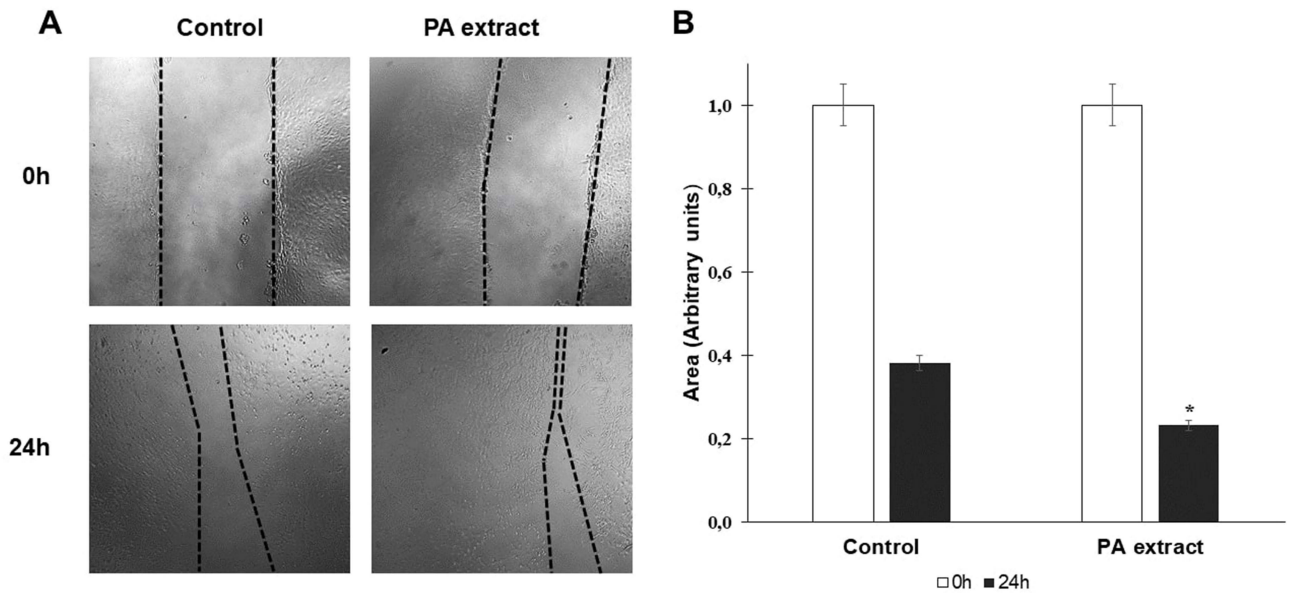

Figure 4 Effect of *P. angulata* extract on the scratch closure in fibroblast 3T3-L1 cells. (**A**) Representative optical images of 3T3-L1 fibroblast cells migration after scratch time 0 and 24h of treatment in complete medium (control) and 500 µg/mL extract. We monitored scratch closure using an inverted microscope (40x magnification); (**B**) The uncovered area that was affected by *P. angulata*'s extract. The data was obtained by using Image J 1.38 software and were expressed as the mean ± standard deviation (x ± s, n = 6). * Significantly different compared to 0h.

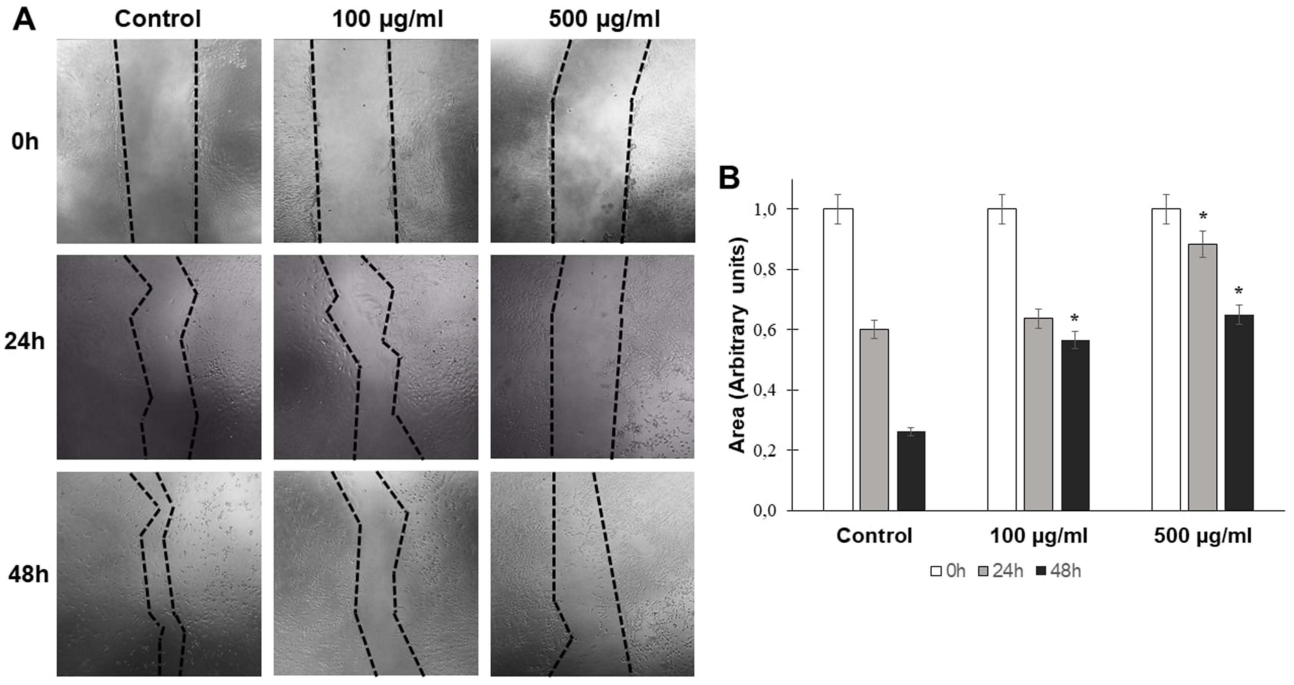

Figure 5 Effect of *P. angulata* extract on the scratch closure in 3T3-L1 fibrosis cells induced by TGF-β. (**A**) Representative optical images of fibrosis cells migration after scratch time 0, 24h, and 48 h of treatment in fibrosis 3T3-L1 cells (control) and extract-treated fibrosis cells (100 µg/mL, 500 µg/mL). We monitored scratch closure using an inverted microscope (40x magnification); (**B**) The uncovered area that was affected by *P. angulata*'s extract. The data was obtained by using Image J 1.38 software and were expressed as the mean ± standard deviation (x ± s, n = 6). * Significantly different compared to control.

P.Angulata's Ethanol Extract Suppresses IL-6 and HIF-1α Genes Expression on TGF-β-Induced 3T3-L1 Fibrosis Cells

Interleukin-6 (IL-6) regulates the inflammatory phase and wound healing. In response to injury such as fibrosis, IL-6 signaling governs the immune response and switches to a reparative environment.[41,42] To determine the anti-

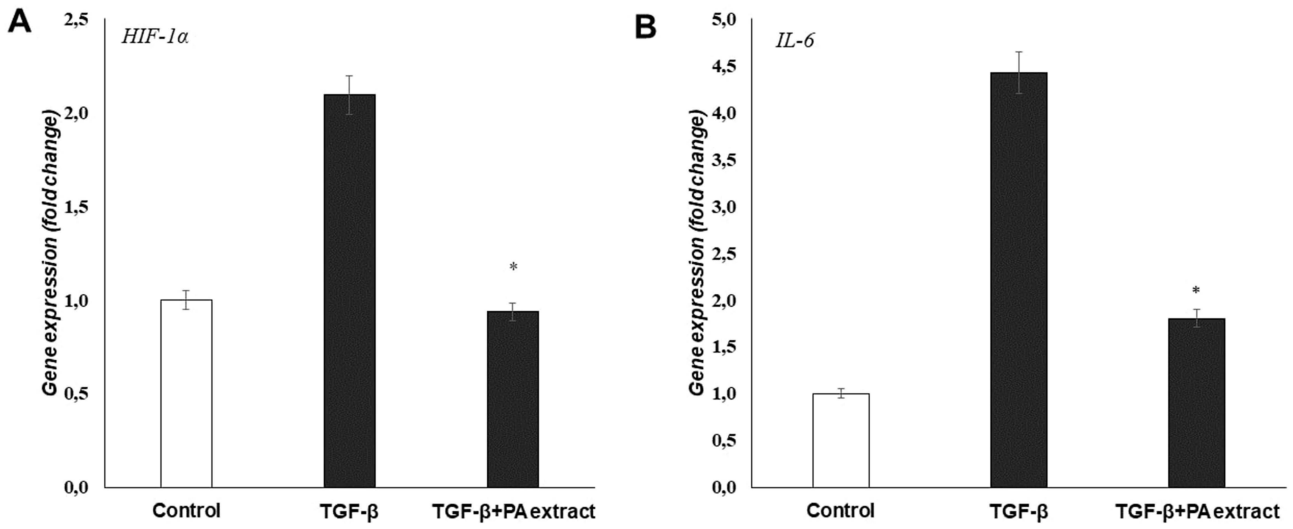

Figure 6 RT-PCR analysis of TGF- β-induced-3T3-L1 fibrosis cells' response to *P. angulata*'s extract treatment. Cells were treated only with the starvation medium (control), TGF-β (20 ng/mL), and *P. angulata*'s extract (500 µg/mL) for 24h incubation. The mRNA level of *IL-6* or *HIF-1α* in treated cells was measured using real time PCR. GAPDH was used as the internal control gene. Data represent the mean ± SEM (n = 3). * Significantly different compared to 20 ng/mL of TGF-β ($p < 0.05$). (**A**) Effects of *P. angulata*'s extract on IL-6 mRNA expression in TGF-β-induced 3T3-L1 cells; (**B**) Effects of *P. angulata*'s extract on *HIF-1α* mRNA expression in TGF-β-induced 3T3-L1 cells.

inflammatory effects of the *P. angulata* extract, we evaluated the expression level of the *IL-6* gene in cells. RT-PCR analysis showed an elevated level of *IL-6* after inducing TGF-*β* on 3T3-L1 fibroblast cells. However, the *IL-6* level was decreased significantly after we treated fibrosis cells with 500 µg/mL extract (Figure 6A). These results demonstrated the anti-inflammatory properties of *P. angulata*.

Furthermore, we investigated *HIF-1α* gene expression, a hypoxia regulatory protein that plays an essential role in regulating the process of inflammatory and oxidative stress in hypoxia. While confirming that the extracts showed their good ability as an antioxidant, we investigated whether the oxidative stress involved in the antifibrotic activity of *P. angulata*'s extracts through the expression of the *HIF-1α* gene. We used 500 µg/mL extract to treat TGF-β-induced-3T3-L1 fibrosis cells. The result showed that the extract significantly reduced the *HIF-1α* level in fibrosis cells induced by TGF-β (Figure 6B). It indicated that the oxidative stress pathway is associated with the antifibrotic activity of *P. angulata*.

Discussion

Fibrosis is the accumulation of excess extracellular matrix (ECM) components, eventually leading to organ malfunction and death if it is highly progressive. Idiopathic pulmonary and hepatic fibrosis (IPF) is the most common and lethal form. Meanwhile, skin fibrosis causes disabilities, including hair loss, subcutaneous atrophy, and irreversible structural and functional impairment. The mechanism of fibrosis is believed to be inflammation and vascular injury at an early stage, followed by fibroblast activation and myofibroblast formation such as α-SMA, which leads to excessive ECM components such as tenascin-c, fibronectin, and collagens in fibrotic organs, which in turn disrupts homeostasis and architecture of cells and tissues.[43–45]

Therapies to treat fibrosis still pose challenges, including side effects, high costs, and resistance, which result in disease progression and higher morbidity.[46,47] Many studies have been conducted to find new antifibrosis agents that suppress cell proliferation and ECM synthesis, thereby improving patients' quality of life.[48] Some antioxidants derived from plants have been evaluated for their antifibrotic activity, considering that plants have been an important source of medicine for years.[49,50] To our knowledge, our study is the first study to report the antifibrosis activity of the plant *P. angulata*. We previously reported that this plant can alleviate skin fibrosis in scleroderma patients, able to improve fibrosis in rat livers, CCl_4-induced fibrosis, and significantly reduce *α-SMA* gene in TGF-β induced-3T3-L1 fibrosis cells, an indicator gene for fibrotic active myofibroblasts.[27–29] Many reports also show the activity of antifibrosis candidates through the α-SMA pathway, even being used as an important target in drug development.[48,51,52]

Antioxidant agents, especially from plants, are often reported to provide benefits in treating fibrosis related to their flavonoid content.[49] Researchers monitored that the antioxidants derived from medicinal plants could suppress reactive oxygen species (ROS), activate antioxidant defense, inhibit ECM gene expression, and protect fibrosis in an animal model.[53–55] In 3T3-L1 cells, *P. angulata* also showed action as an antioxidant. The antioxidant activity affects the accelerated wound healing of fibroblast cells. This result is in line with other studies that reported *P. angulata* leaf extract to have wound-healing activity in vivo, which supports using this plant as a wound-healing drug.[56] Another important finding is that its antioxidant activity also applies in fibrosis cells by reducing *HIF-1α* gene expression, a key role in response to hypoxic conditions and associated with oxidative stress and inflammation. Interestingly, this ability effectively inhibited the proliferation and migration of fibrosis cells. Inhibition of cell migration of other antifibrotic agents has been reported by other groups, which also induce fibrosis in cells with TGF-β.[48,57,58] These results agree with recent studies suggesting that fibrosis results from abnormal wound healing in response to the alveolar epithelium microinjury.[59–61] Thus, *P. angulata* plays a role in deactivating cells, inhibiting cell migration, and decreasing expression of α-SMA in TGF-β-induced-3T3-L1 fibrosis cells. It also explains the clinical improvement of skin fibrosis in patients receiving *P. angulata* extract, which we reported previously.[27]

In many organs, the inflammatory state is reported to play an essential role in triggering fibrosis.[44] Hence, controlling inflammation is crucial to antifibrosis drug discovery. In this present study, we showed the protective effect of *P. angulata* on inflammation in fibrosis cells. Our data indicated that TGF-β activated proinflammatory factor *IL-6* gene expression in 3T3 cells. Meanwhile, the extract suppressed *IL-6* gene expression on TGF-β-induced 3T3-L1 fibrosis cells. These results demonstrated that *P. angulata* plays a role in alleviating inflammation that protects cells against fibrosis. Thus, agents that inhibit ROS, inflammation, or myofibroblast formation would be promising as antifibrosis candidates, as demonstrated by *P. angulata*. Evaluation of the levels of ECM proteins, including tenascin-c, fibronectin, or collagen, could be performed in further studies to understand the factors contributing to the inhibition of fibrosis by *P. angulata*.

Conclusion

In this study, we presented that *P. angulata* reduced oxidative stress and accelerated wound healing in 3T3-L1 cells. Interestingly, the extract could inhibit *HIF-1α* and *IL-6* gene expression in TGF-β-induced fibrosis cell models. Such activities decrease fibrosis cell migration, possibly benefiting patients' skin repair. In summary, our results suggested that the antioxidant and antiinflammation activities of *P. angulata* are involved in its anti-fibrosis mechanism.

Acknowledgments

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Author Contributions

HL, SE, ER, and SD initiated the conception and design of the study. HL and SE developed the design and analyzed the results. SD, HL, and SE supervised the work. HL and AL prepared the first draft of the manuscript. HL, SE, ER, AL, AZ, RH, YW, EH, MB, and SD reviewed and edited the manuscript. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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