Thieme

Ajuga taiwanensis Extract Promotes Wound-healing via Activation of PDGFR/MAPK Pathway

Authors
Wei-Hsiang Hsu[1,2], Jing-Jy Cheng[3,4], Ching-Fen Wu[5], Yun-Lian Lin[1,6]

Affiliations
1 Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan
2 Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taipei, Taiwan
3 National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taipei, Taiwan
4 Program in Clinical Drug Development of Herbal Medicine, College of Pharmacy, Taipei Medical University, Taipei, Taiwan
5 Department of Veterinary Medicine, National Chiayi University, Chiayi City, Taiwan
6 Department of Pharmacy, National Taiwan University, Taipei, Taiwan

Keywords
Ajuga taiwanensis, Lamiaceae, human dermal fibroblasts, wound healing, PDGF receptor

received December 11, 2023
accepted after revision June 5, 2024

Bibliography
Planta Med 2024
DOI 10.1055/a-2378-9274
ISSN 0032-0943

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

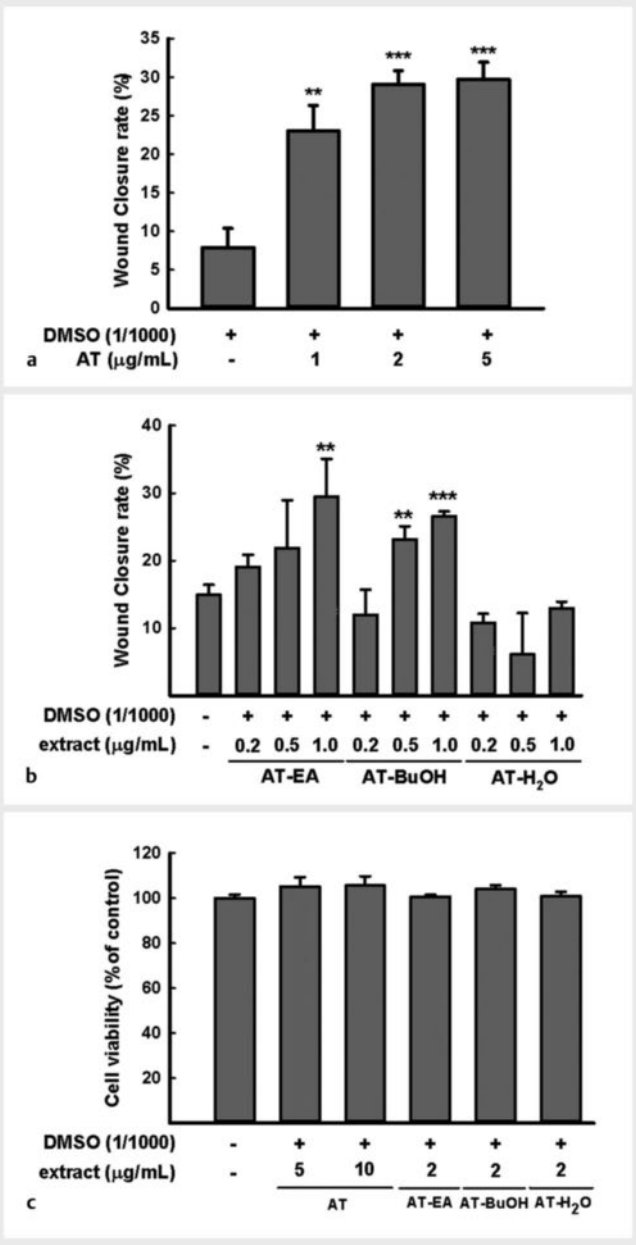
Correspondence
Dr. Yun-Lian Lin
Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University
Hsueh-Shih Road No. 91, 404328 Taichung, Taiwan
Phone: + 886422053366
yllin@mail.cmu.edu.tw

ABSTRACT

Chronic and prolonged wounds are a serious public problem that may severely affect the quality of life and result in psychological pressure. Fibroblasts play a crucial role in the wound process and in skin pathology. Herbal drugs have long been used for wound care worldwide. *Ajuga taiwanensis* (Lamiaceae) is a folk medicine for antipyretics, anti-inflammation, and reducing swelling in Taiwan. This study aimed to investigate the effect of *A. taiwanensis* in wound healing and the underlying mechanisms. Under human dermal fibroblast (HDF) wound-healing activity-guided fractionation, we found that a sub-fraction (AT-M) of *A. taiwanensis* extract (AT) and the major ingredients significantly promoted wound healing and decreased IL-1β and − 6 expressions on HDFs. Furthermore, the fraction of AT-M enhanced wound healing on C57BL/6 mouse skins, increased PDGFR expressions, and activated the PDGFR/MAPK pathway. Taken together, *A. taiwanensis* extracts promote wound healing by the PDGFR pathway and lead to enhanced cell spreading and motility, thereby having a possible beneficial effect on wound healing.

Introduction

The skin tissue is composed of multiple layers of epithelial cells in the upper layer to protect the underlying tissues and organs from mechanical and chemical damage. The underlying connective tissue is mainly composed of fibroblasts and a secreted extracellular matrix and contains neural tissue, vascular tissue, and immune cells, which provide mechanical support and nutrients [1, 2]. They often cause trauma by external factors. Fibroblasts, the major players in the process of scarring, are responsible for collagen deposition and wound contraction, as well as playing a crucial role in skin pathology and tissue repair [3]. Growth factor is helpful for wound healing; it promotes cell proliferation, migration, vascular formation, and other processes that are dysregulated in the process of wound repair. Among them, platelet-derived growth factors (PDGFs) have proven effective in injury management and involve many cellular events in the healing process, including stimulating collagen deposition and angiogenesis, enhancing skin regeneration, inflammatory cell recruitment, and fibroblast proliferation and migration [4–6]. Though researchers make efforts to discover effective interventions on cutaneous wounds, there is still an unmet need with a high priority for the development of an effective treatment [3]. Using medicinal plants for skin wound-healing is a common practice in the domestic medicines of many cultures [7]. Meanwhile, natural products or traditional herbal medicines have been generally accepted by people due to their safety, fewer side effects, and effectiveness. They have been attracting much attention in recent years [8, 9].



▶ **Fig. 1** Wound-healing effect of *A. taiwanensis* extracts on HDFs. HDFs were co-treated with various concentrations of (**a**) *A. taiwanensis* crude extract (AT) and (**b**) three sub-fractions of AT, AT-EA (ethyl acetate (EA)-soluble fraction), AT-BuOH (*n*-butanol (BuOH)-soluble fraction), and AT-H_2O (water (H_2O)-soluble fraction) in 24-well plates. After 8 h culture, images were acquired with a digital fluorescent inverted microscope. Images were processed by Image J and data acquisitions were presented as mean ± SEM of three independent experiments; (**c**) cytotoxicity of crude extract and three sub-fractions on HDFs were measured by MTT assay at 540 nm absorbance. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were compared to the untreated control group.

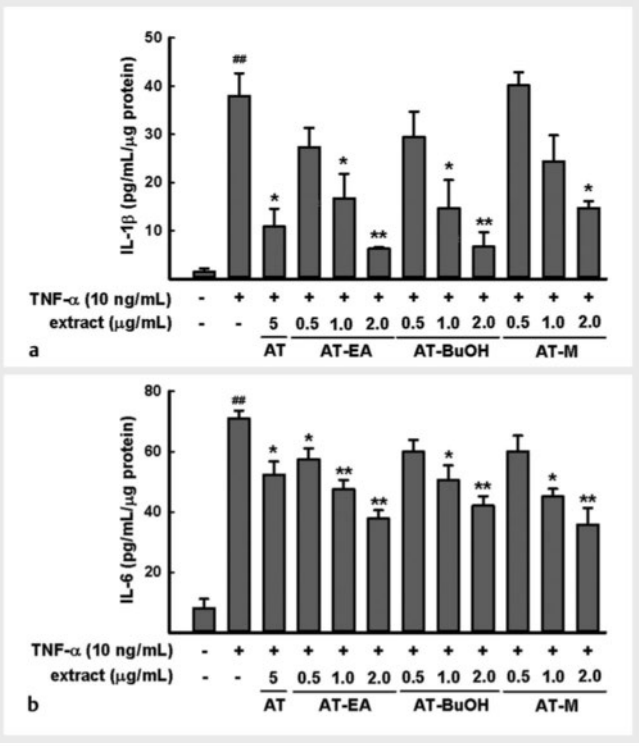
A. taiwanensis Nakai *ex* Murata (Lamiaceae) is an indigenous species in Taiwan and grows in plains and in low- and middle-altitude mountainous areas [10]. The aerial parts of *A. taiwanensis* are used in the folk medicine of Taiwan to "clear heat", "detoxify", and reduce swelling [11]. Several bioactivities, such as anti-inflammation [12], enzyme inhibition [13, 14], anti-senescence [15], and anti-bacterial activity [16], have been reported in *A. taiwanensis*. Different kinds of active ingredients, including phytoecdysteroids, neo-clerodane diterpenoids, phenolic compounds, iridoid glycosides, flavonoids, and ergosterol-5,8-endoperoxide [17–19], have been isolated from the genus *Ajuga*. However, the effect on wound healing remains to be explored. The aim of this study is to investigate the effect of *A. taiwanensis* in wound healing and the underlying mechanisms.

Results

In this study, we characterized the extracts and the active constituents from *A. taiwanensis* by wound healing assays *in vitro* and *in vivo*. We found the crude extract (AT, 20.07% based on dried herbs) could promote human dermal fibroblast (HDF) wound healing in a concentration-dependent manner and with significant effect at 1, 2, and 5 μg/mL (▶ **Fig. 1a**). Furthermore, we tested the sub-fractions of AT-H_2O (water-soluble fraction, 12.55% based on dried herbs), AT-EA (ethyl acetate-soluble fraction, 4.75% based on dried herbs), and AT-BuOH (*n*-butanol-soluble fraction, 3.24% based on dried herbs). The results showed that the active fractions, AT-EA and AT-BuOH, had better efficiency for wound healing in a concentration-dependent manner (▶ **Fig. 1b**), and all of them were without significant cytotoxicity in HDFs (▶ **Fig. 1c**).

Proinflammatory cytokines are among the first factors to be produced in response to skin wounds, and they regulate the functions of immune cells in epithelialization. Pro-inflammatory cytokines, mainly including tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, and IL-17, participate in the inflammation phase of wound healing through activating downstream cascades [20]. Overproduction of proinflammatory cytokines may lead to prolonged inflammation and wound healing [20]. Thus, we tested whether AT extracts could decrease cytokine secretion in TNF-α-induced inflammation. The effect of AT on pro-inflammatory cytokine secretion was examined. The results showed that stimulation with TNF-α significantly enhanced IL-1β and IL-6 secretions from HDFs. Treatments of HDFs with crude extract (AT), AT-EA, AT-BuOH, and AT-M (a mixture of AT-EA and AT-BuOH) significantly suppressed TNF-α-induced IL-1β (▶ **Fig. 2a**) and IL-6 (▶ **Fig. 2b**) secretions and in a concentration-dependent effect on AT-EA, AT-BuOH, and AT-M, respectively.

Under the wound-healing activity guided screening, the active fractions, AT-EA and AT-BuOH, were further purified over silica gel and Sephadex LH-20 column chromatography to get five major compounds (**1**–**5**): chlorogenic acid (**1**) [21], 8-*O*-acetylharpagide (**2**) [16], ajugalactone (**3**) [22], cyasterone (**4**) [22], ajugacumbin F (**5**) [23] (▶ **Fig. 3a**), and pheophytin-a [24]. Their structures were mainly characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS) and compared with the published spectroscopic data. Because the activities of AT-EA and AT-BuOH are similar (▶ **Fig. 1** and **2**), we therefore mixed AT-EA and AT-BuOH together as AT-M for the Western blotting and animal experiments. The chemical profile of AT-M by HPLC is shown in ▶ **Fig. 3b**



▶ **Fig. 2** Effect of *A. taiwanensis* extracts on the secretion of pro-inflammatory cytokines in TNF-α-induced HDFs. The secretion levels of IL-1β (**a**) and IL-6 (**b**) with *A. taiwanensis* extracts were assessed by an ELISA kit after HDFs were co-treated with TNF-α only or with TNF-α and different concentrations of AT (crude extract) or AT-EA (EA-soluble fraction) or AT-BuOH (BuOH-soluble fraction) or AT-M (mixture of EA and BuOH fractions). The data are expressed as mean ± SEM of three independent experiments. ##$p < 0.01$ compared with control group. *$p < 0.05$, **$p < 0.01$ compared with only TNF-α treated group.

and **c**. Compound **2** is the major component. The content of **2** is about 1% in AT-M.

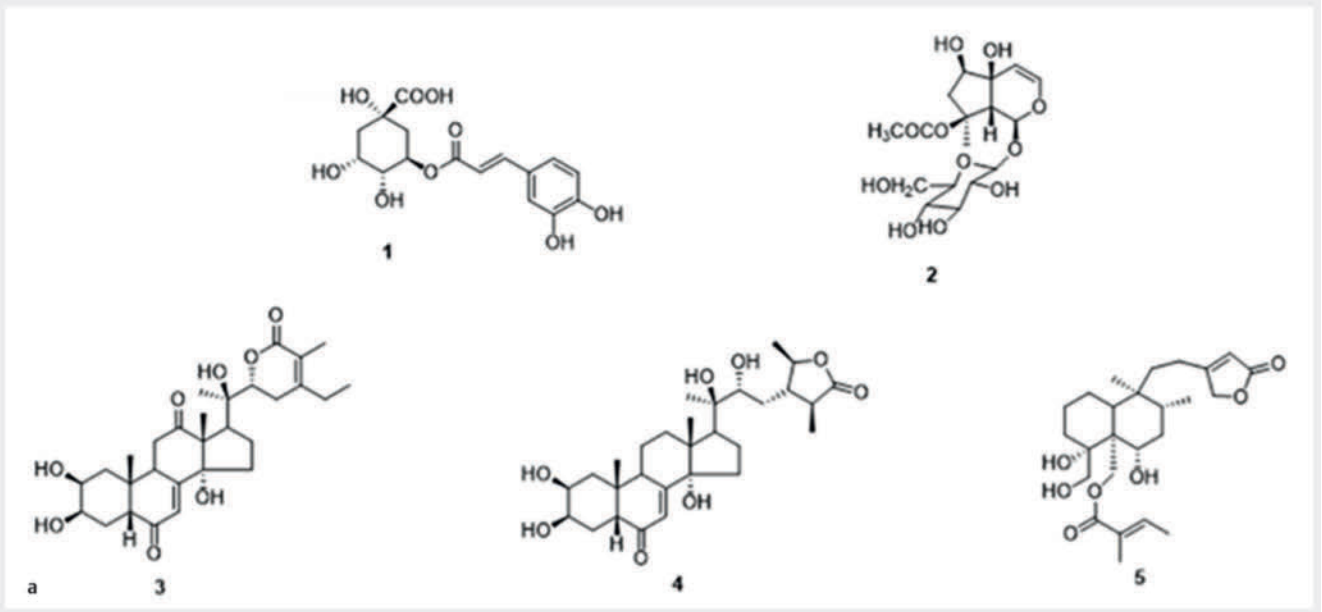
All of the isolated constituents from AT-M were tested for wound-healing activity in HDFs. In addition to ajugalactone (**3**), chlorogenic acid (**1**), 8-*O*-acetylharpagide (**2**), cyasterone (**4**), and ajugacumbin F (**5**) significantly promoted wound healing in HDFs at 10 μM (▶ **Fig. 4a**). Of them, 8-*O*-acetylharpagide (**2**) and cyasterone (**4**) have better efficiency than ajugacumbin F (**5**) (▶ **Fig. 4a**). In addition, these compounds have no cytotoxicity in HDFs (▶ **Fig. 4b**). Moreover, we investigated whether these compounds could affect proinflammatory cytokine secretion in HDFs. The results showed that treatment of HDF cells with the isolated compounds (10 μM) significantly suppressed TNF-α-induced IL-1β (▶ **Fig. 5a**) and IL-6 production (▶ **Fig. 5b**). Furthermore, AT-M compounds **2** and **4** were selected for the following study.

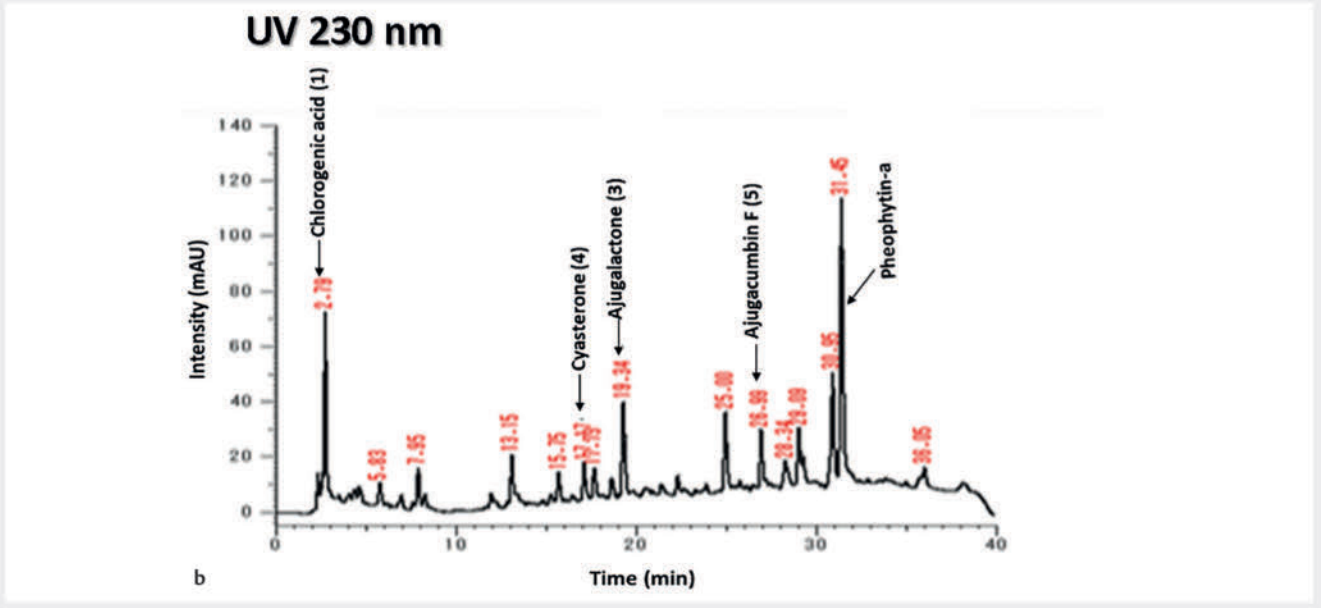
The expression levels of FGF-2 and PDGF-BB/PDGFR-β can accurately reflect the progression of wound healing, and these molecules are important targets for research on the effects of drugs on wound healing [5]. We found that with PDGF treatment (positive control), the protein expressions of p–ERK/ERK, p–AKT/**AKT**, and p–PDGFR-β significantly increased, which is closely associated with wound repair. The pure compounds 8-*O*-acetylharpagide (**2**), cyasterone (**4**), and *Bletilla striata* polysaccharide (BS) (as positive control, ▶ **Fig. 6a**) could also significantly induce p–PPDGFR-β and p–ERK/AKT expression increase, but compounds **2** and **4** have no significant effect on p–AKT/AKT expression (▶ **Fig. 6a**). Moreover, PDGFR-β was knocked down by siRNA-reduced p-ERK expression, and AT-M compounds **2** and **4** treatments could not reverse PDGFR-β and p-ERK/AKT expressions (▶ **Fig. 6b**). In addition, we found PDGF treatment markedly suppressed E-cadherin and promoted β-catenin expressions. AT-M, 8-*O*-acetylharpagide (**2**), and cyasterone (**4**) could also inhibit *E*-cadherin expression and increase β-catenin expression; however, BS could increase β-catenin expression only (▶ **Fig. 7**) [5, 25].

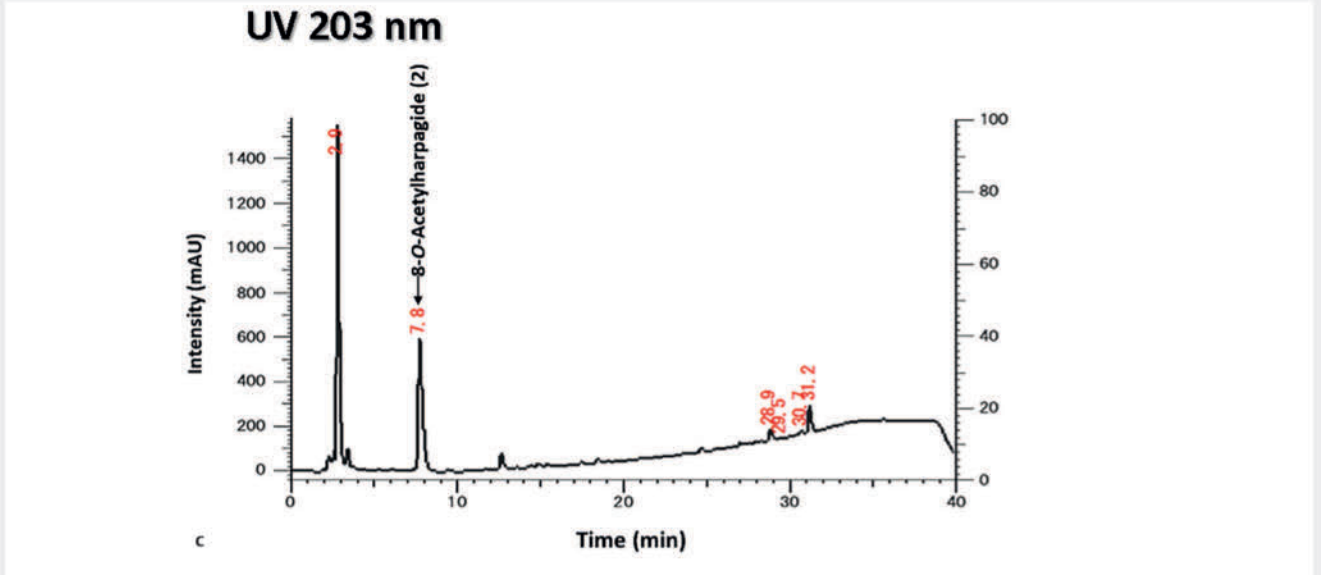
Based on the results of the above ▶ **Fig. 1** and **Fig. 2**, we knew AT-EA and AT-BuOH were the active fractions. The mixture of AT-EA and AT-BuOH (AT-M) was used to determine whether AT-M could improve skin wound healing. A wound was produced by a 5 mm punch on mice as described in Methods [25]. Carbopol cream with or without AT-M (25%) was applied on the mouse wound area every day for one week. Commercially available Winsolve-B Ointment was used as a positive control. As ▶ **Fig. 8** shows, wound closure significantly improved after treatment of 25% AT-M in Carbopol cream as compared to the control (▶ **Fig. 8a**). The percentage of wound closure was measured and showed significant differences of wound-healing rate between control and AT-M group at days 5 and 7 (▶ **Fig. 8b**). At day 7, the percentage of wound area was 24.3% for control, 11.2% for AT-M, and 15.3% for the Winsolve-B ointment group and better than positive control. Histological sections of wound area were evaluated for hematoxylin–eosin stains and collagen production using masson's trichrome stain on day 7. The stain produces red keratin and muscle fibers, blue or green collagen, light red or pink cytoplasm, and dark brown to black cell nuclei. AT-M significantly increased collagen production (blue) as compared with control. The muscle fiber seemed to be more obvious in the control group than in the AT-M group. This might be due to the wound still not healing in the control group (▶ **Fig. 8c**). All these results indicated a significant improvement in wound closure after AT-M treatment.

Discussion and Conclusion

Wounds are physical injuries associated with surgery, falling, heat, infectious disease, or underlying pathological conditions of tissue that interrupt normal tissue functions. Wound repairing is a natural physiological reaction to tissue injury and a complex process with many dynamic phases to restore the cellular structure of the damaged tissues back to their initial healthy status. Wound healing involves a complex interplay between numerous cell types, cytokines, mediators, and the vascular system [26]. In Persian medicine, the genus *Ajuga (Kamaphytus)* is used for treating jaundice, joint pain, gout, amenorrhea, sciatica, and wound healing [27]. In this study, we verified that crude extract AT, AT-M (mixture of AT-EA and AT-BuOH fractions of AT), and two major components, 8-*O*-acetylharpagide (**2**) and cyasterone (**4**), could accelerate wound healing via PDGFBB/PDGFR regulation. In addition, these compounds could also regulate pro-inflammatory cytokine release in the progress of wound healing.







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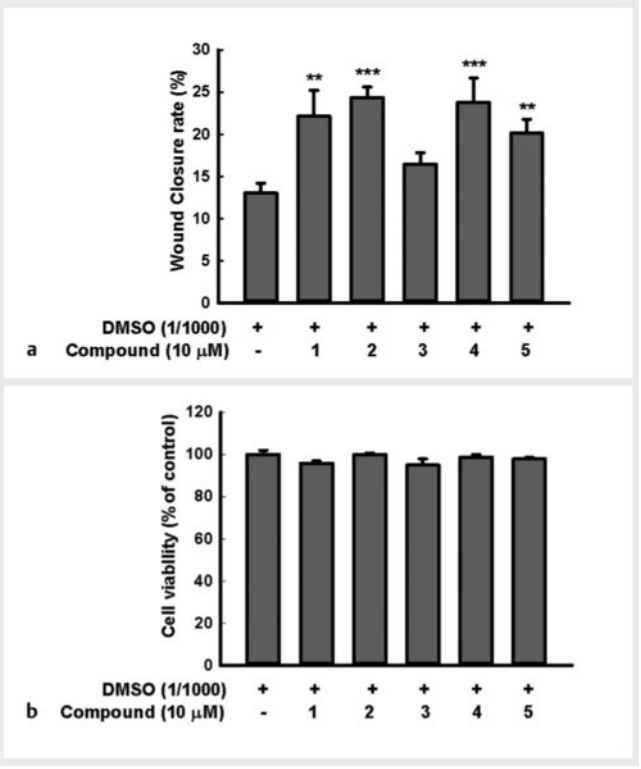
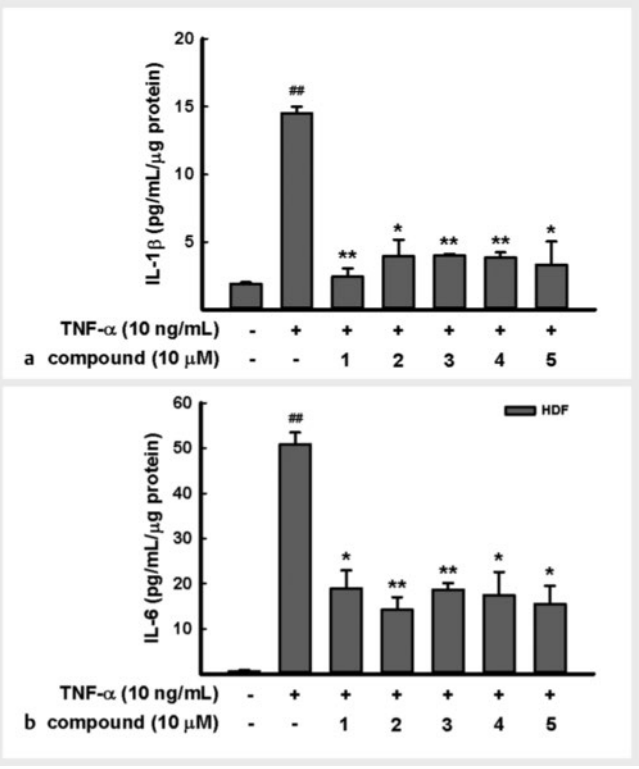
▶ **Fig. 3** Structures isolated from AT-EA and ET-BuOH and chemical profile of crude extract AT-M by HPLC. **a** Structures of compounds isolated from AT-EA and ET-BuOH. **b** Chemical profile of AT-M by HPLC with a reverse-phase column (RP-18 end capped, 5 µm, 150 × 4.6 mm), and using a mobile phase of solvent A: 0.2 % H_3PO_4 in H_2O and solvent B: methanol; with a program: 0–5 min, 70 % A; 5–15 min, 70–40 % A; 15–25 min, 40–20 % A, and 5 min, 20 % A; flow rate: 1 mL/min; inject volume: 10 µL; detector: UV 230 (**b**) and 203 (**c**) nm. **1:** chlorogenic acid; **2:** 8-*O*-acetylharpagide; **3:** ajugalactone; **4:** cyasterone; **5:** ajugacumbin F.



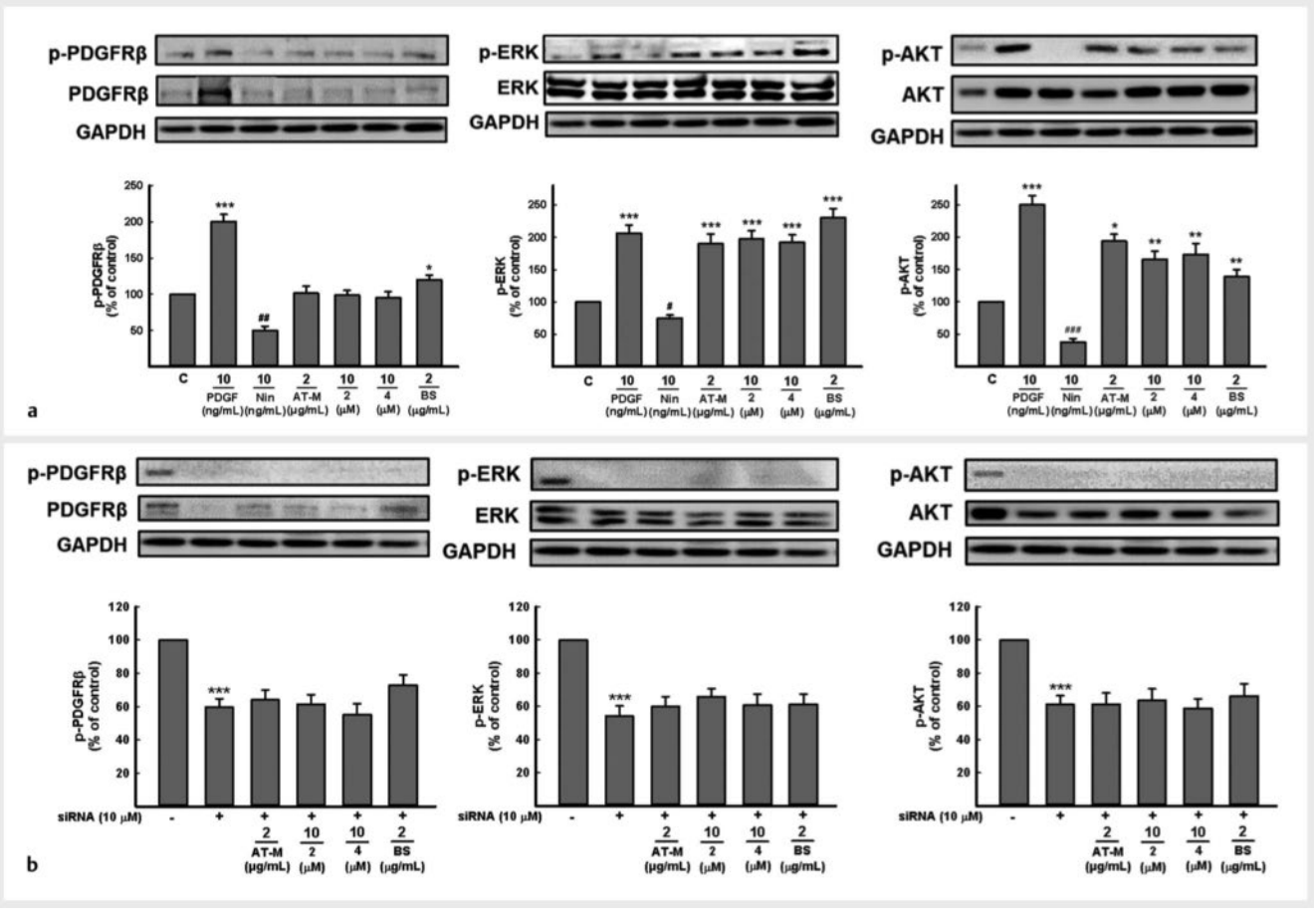
▶ **Fig. 4** Wound-healing effect of isolated pure compounds from *A. taiwanensis* extract on HDFs. **a** A wound-healing assay was evaluated by co-treatment of isolated pure compounds at 10 µM. **b** Cell viability on HDFs. **1:** chlorogenic acid; **2:** 8-*O*-acetylharpagide; **3:** ajugalactone; **4:** cyasterone; **5:** ajugacumbin F. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control group.



▶ **Fig. 5** Effect the isolated pure compounds on the secretion of proinflammatory cytokines in TNF-α-induced HDFs. The secretion levels of IL-1β (**a**) and IL-6 (**b**) were assessed by ELISA kit after HDFs were co-treated with TNF-α only or TNF-α and isolated pure compounds at the indicated concentrations for 24 h incubation. **1:** chlorogenic acid; **2:** 8-*O*-acetylharpagide; **3:** ajugalactone, **4:** cyasterone; **5:** ajugacumbin F. The data are expressed as mean ± SEM of three independent experiments. $^{\#\#}p < 0.01$ compared with control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with only TNF-α treated group.

As the body's largest organ, skin has an extraordinary characteristic of natural regeneration through wound healing [28]. Winsolve-B ointment (as a positive control in animal studies), which contains the mixture of three principle ingredients, namely bacitracin, neomycin, and polymyxin B sulfate, has been formulated in hydrogel for the treatment of wounds. One of the major components, polymyxin B sulfate, showed effectiveness in increasing wound healing and accelerated the time of healing [29]. The other two major components, bacitracin and neomycin, worked by inhibiting the bacterial cell wall synthesis, preventing bacterial growth by stopping the production of essential protein in the bacterial cells and killing the bacteria to avoid wound infections [30]. In comparison to a partially purified *A. taiwanensis* extract (AT-M) used in the present study, the results demonstrated that AT-M exerted complete wound healing in about seven days and had better efficiency in wound healing than the Winsolve-B ointment at days 5 and 7 (▶ **Fig. 8b**).

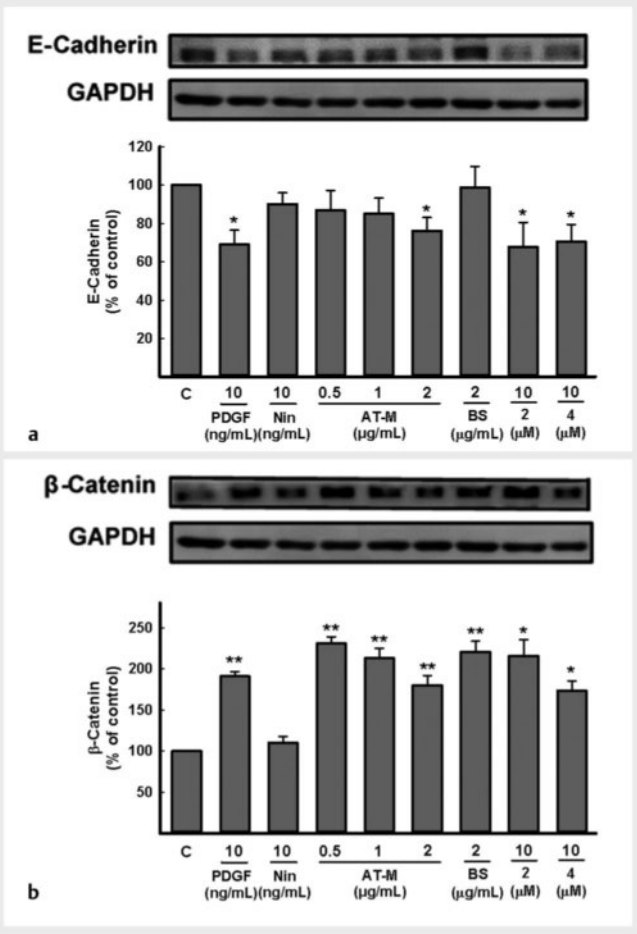
Furthermore, HDFs are the main cell types directly involved in dermal wound healing [6]. Therefore, we focused on the effect of AT-M and its pure compounds on HDFs first. Platelet-derived growth factor (PDGF) plays an important role in wound healing and has been approved for the treatment of wounds related to diabetes mellitus [31]. Previous studies have shown that the levels of angiogenic growth factors such as FGF, PDGF and VEGF and their respective receptors are downregulated in nonhealing chronic wounds [32, 33]. Recent studies also suggest that when Wnt and β-catenin are increased, epidermal cell proliferation, differentiation, and migration are enhanced, and wound healing is



▶ **Fig. 6** Effect of AT-M and isolated pure compound on PDGFBB/PDGFR pathway in HDFs. Representative images of Western blotting and quantification analysis of p-PDGFR*β*, p–AKT, and p-ERK proteins expression in HDFs under without (C: control) or with the treatments of (**a**) PDGF (positive control), Nintedanib (PDGFR inhibitor, a negative control), AT-M, 8-*O*-acetylharpagide (**2**), cyasterone (**4**), and BS (as positive control); (**b**) siRNA only, siRNA with 8-*O*-acetylharpagide (**2**), cyasterone (**4**), and BS pre-treatments, respectively. The data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group.

accelerated as well [34, 35]. Therefore, it is presumed that activation of angiogenic factor receptors along with their downstream pathways may represent a promising novel approach for the treatment of nonhealing chronic wounds and promotion of tissue repair [36]. Furthermore, key components of wound healing are re-epithelialization and epithelial-mesenchymal transition (EMT), which are the movement or migration of the epithelial sheets under stimulation of injury signals. Wound healing and EMT share central signaling pathways [37]. The common growth factors indispensable for both processes include PDGF, FGF, EGF, HGF, and TGF-*β* [37, 38]. PDGF altered the expression levels of *E*-cadherin and *N*-cadherin, two master regulators of EMT [38], as well as upregulates MMP2/MMP9 expression and induces EMT to promote cell migration. PDGFR and its ligands expressed in cells lead to an autocrine loop that fuels the activation of downstream PI3K/Akt, MAPK, and STAT pathways, in addition to the maintenance of EMT and enhanced metastasis [6, 39]. Meanwhile, the expression of E-cadherin is downregulated, and the expression of N-cadherin, vimentin, slug, and snail is upregulated [38, 40]. To further clarify the effectiveness of our treatment in healing wounds, the PDGFBB/PDGFR pathway was detected in this study. We exhibited that AT-M could enhance the activity of the PDGFBB/PDGFR pathway significantly. But the purified compounds from AT-M, 8-*O*-acetylharpagide (**2**), and cyasterone (**4**) could induce the PDGF-BB/PDGFR/MAPK pathway only. AT-M, 8-*O*-acetylharpagide (**2**), and cyasterone (**4**) might induce EMT via E-cadherin downregulation, *β*-catenin upregulation, and the MAPK signaling pathway. In addition, wound healing relies on interactions between various growth factors and cytokines that regulate cellular responses [20, 41], and diabetic wound healing is significantly slow compared to non-diabetic wounds mainly due to a prolonged inflammation phase [42]. The upregulation of IL-1*β* and IL-6 levels under TNF-*α* treatment, which mimic the inflammatory conditions, was inhibited by AT-M, 8-*O*-acetylharpagide (**2**), and cyasterone (**4**) (▶ **Fig. 5 a** and **b**).

There are several limitations in the present study. First, the molecular mechanisms of the proangiogenic effect of AT-M, 8-*O*-acetylharpagide (**2**), and cyasterone (**4**) have not been fully explored. Here, we found that AT-M, 8-*O*-acetylharpagide, and cyasterone mediated PDGFBB/PDGFR/MAPK signaling to promote wound



▶ **Fig. 7** Effect of AT-M and isolated pure compound on E-cadherin and *β*-catenin in HDFs. Representative images of Western blotting and quantification analysis of E-cadherin and *β*-catenin proteins expressions in HDFs under without (C: control) or with treatments of PDGF (positive control), Nintedanib (PDGFR inhibitor, as negative control), AT-M, 8-*O*-acetylharpagide (**2**) and cyasterone (**4**). The data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group.

healing. However, many other pathways or miRNAs, such as Nrf/Keap1, miRNA-21–5 p/RASA1, HIF-1α/VEGF/VEGFR2, the FGF pathway, miRNA-497, miRNA-21–3 p, and miRNA-31–5 p have also been reported as critical factors in regulating wound healing [36, 43–45], which may be involved in the actions of AT-M, 8-*O*-acetylharpagide (**2**), and cyasterone (**4**). Second, angiogenesis and wound healing have an intricate and close causal relationship [46]. Future studies should carefully investigate the detailed molecular vascular events of AT-M, 8-*O*-acetylharpagide, and cyasterone in angiogenesis. Third, our study only used one model to explore enhancing the wound-healing effect of *A. taiwanensis* extracts and a different animal model is needed to investigate this issue in the future.

In conclusion, we provide evidence that *A. taiwanensis* extracts significantly facilitate cutaneous wound healing in mice, and the underlying mechanism may be their activation of function properties of fibroblasts in the wounds, as *A. taiwanensis* extracts can enhance migration of fibroblasts *in vitro*. Activating PDGFBB/PDGFR/MAPK signaling is the mediator of the *A. taiwanensis* extract's effect. Our findings suggest that *A. taiwanensis* extract may represent a novel therapeutic agent for skin wounds.

Materials and Methods

Chemicals

Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). All chemicals were obtained from Sigma-Aldrich/Merck (St. Louis, MO, USA).

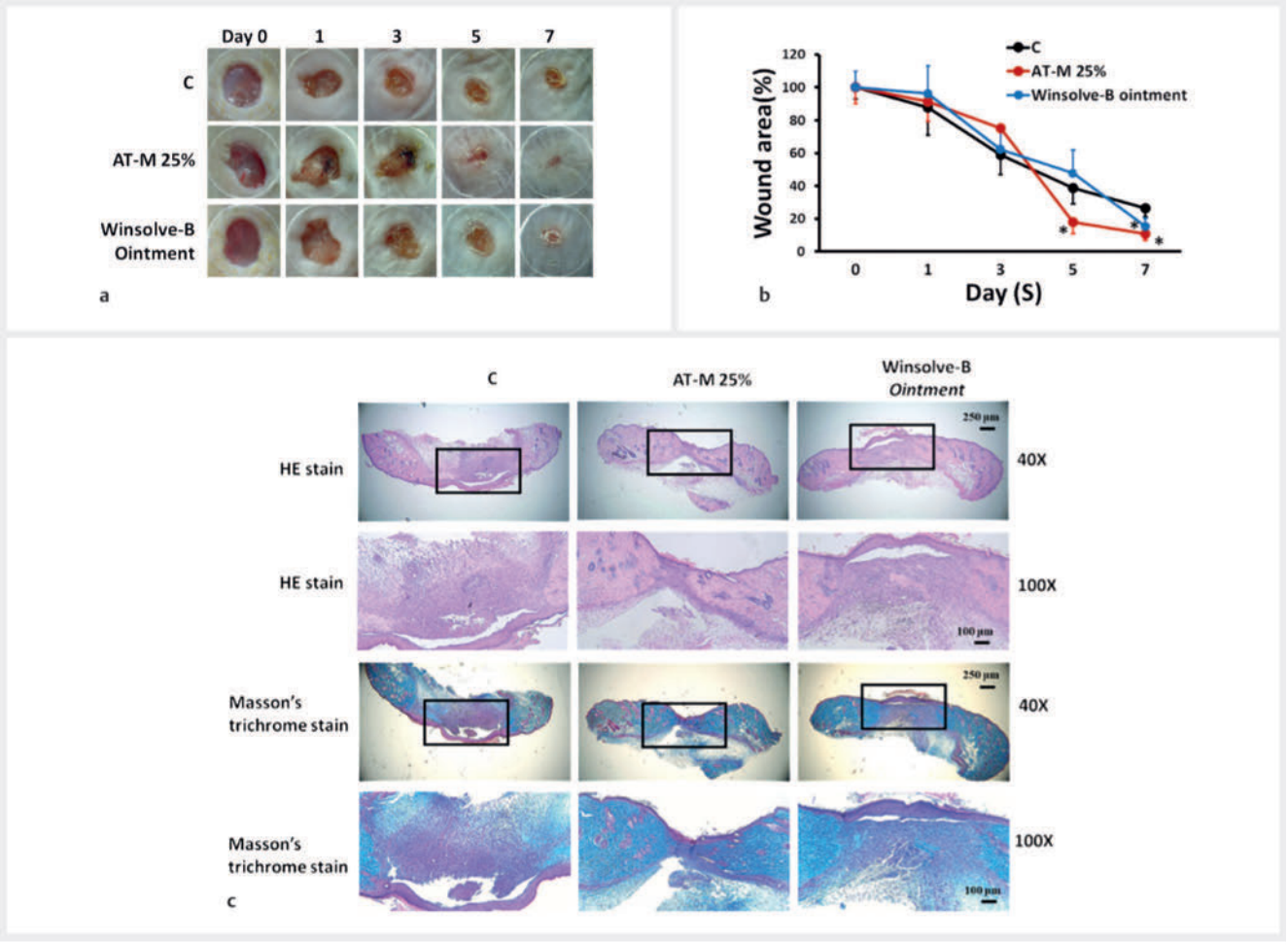
Preparation of the extracts of *A. taiwanensis*

The dried aerial part of *A. taiwanensis* (600 g) was purchased from a local herb retailer in Taipei, Taiwan, and taxonomically authenticated by Shu-Fen Cheng, a senior technical specialist of the Herbarium of National Taiwan University, Taipei, Taiwan, and deposited in the Herbarium of National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taiwan (NHP-00515). The pulverized aerial parts were extracted with 95% ethanol (2×), then 80% ethanol (1×) at 60 °C overnight. The combined extract was concentrated under reduced pressure to give a crude extract (AT, 120.4 g, 20.07% on dried herbal weight). The crude extract was partitioned successively between water and ethyl acetate (EA) and *n*-butanol (BuOH, *v*/*v*, 1 : 1 each), respectively, to give ethyl acetate- (AT-EA, 28.5 g), *n*- butanol- (AT-BuOH, 19.44 g), and water- (AT-H_2O, 75.30 g) soluble fractions. Under the wound-healing activity guided screening, the active fractions, AT-EA and –BuOH, parts of both fractions, were further purified over silica gel and Sephadex LH-20 (GE Healthcare Bio-sciences AB, Uppsala, Sweden) column chromatography to yield five major compounds (**1**–**5**): chlorogenic acid (**1**) [21], 8-*O*-acetylharpagide (**2**) [16], ajugalactone (**3**) [22], cyasterone (**4**) [22], ajugacumbin F (**5**) [23] (▶ **Fig. 3 a**), and pheophytin-a [24]. Their structures were identified mainly by nuclear magnetic resonance (NMR) and mass spectrometry (MS) and compared with the published data. Each extract and pure compound was dissolved in dimethyl sulfoxide (DMSO) as a stock solution before use.

Furthermore, the remaining active fractions, AT-EA and AT-BuOH, were mixed together (AT-M) for animal experiments. The chemical profile of the mixture was analyzed by high-performance liquid chromatography (HPLC) with a reverse-phase column (RP-18 end capped, 5 μm, 150 × 4.6 mm). Chromatographic analysis was performed by a mobile phase of solvent A: 0.2% H_3PO_4 in H_2O, solvent B: methanol with a program of 70% A for 5 min, 70–40% A for 15 min, 40–20% A for 10 min, and 20% A for 5 min. The solvent flow rate was set at 1 mL/min. Inject volume was 10 μL. The column oven was maintained at 40 °C and detected at UV 230 and 203 nm.

Cell culture

Human dermal fibroblasts (HDFs) were purchased from American Type Culture Collection (ATCC) and cultured in Fibroblast Basal medium (ATCC PCS-201-030) with Fibroblast Growth Kit-Low serum (ATCC PCS-201-041) containing 2% FBS, 2 mM L-glutamate, and 50 U/ml penicillin. Cells were incubated in a humidified incu-



▶ **Fig. 8** Effect of AT-M and on mouse cutaneous wound closure in C57BL/6 mice. **a** Images of wounds closure on days 0, 1, 3, 5, and 7 after treatment with control (**c**) and 25% AT-M cream. Winsolve-B ointment was used as a positive control. The circle outside each wound area is 0.5 cm. **b** Percentage of wound area as compared with Day 0 of each treatment was calculated. *$p < 0.05$ compared to the untreated control group at the same time using t-test. **c** Microscopic image of collagen of cutaneous wounds stained with HE stain and Masson's trichrome stain on skin at day 7 after treatment with control or 25% AT-M. Data are representative results from 3 separate experiments.

bator with 5% CO_2 at 37 °C and passaged when they reached 80% confluence.

Wound-healing assay

HDFs (1×10^4 cells) were seeded in 24-well with a Cultur-Insert (ibidi Cat. No. 80209) overnight. Then, the Cultur-Insert were removed to make a 500 ± 100 µm wound area in each well, and a fresh medium was replaced with or without various concentrations of drugs. After an 8 h culture, images in the 24-well plate were acquired with a digital fluorescent inverted microscope at 100× magnification, respectively, and Image J was used for image processing and data acquisition [25].

Cell viability

Cell viability (5×10^3 cells) in a 96-well plate was evaluated by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and the absorbance was measured at 540 nm in a microplate reader.

Measurement of IL-1β and IL-6 cytokines

HDFs in a 24-well plate (1×10^5/well) were cultured overnight, then a fresh medium was replaced with or without TNF-α (10 ng/mL) and TNF-α + at various concentrations of extract or pure compounds or *Bletilla striata* polysaccharide (BS, 5 µg/mL as positive control) [25]. After 24 h culture, the inflammatory cytokines IL-1β and IL-6 in supernatant were analyzed by using commercially available ELISA kits according to the manufacturer's instructions. The total amount of IL-1β and IL-6 in the cultured medium was normalized to the total amount of protein in the viable cell pellets. The samples were analyzed in duplicate and repeated at least three times.

Western blotting

The Western blotting analyses were performed as those previously mentioned [21]: 1×10^6 HDFs (in 6-well) without and with PDGF (10 ng/mL), compounds **2** and **4** (10 µM), AT-M (10 µg/mL), BS (as positive control, 2 µg/mL) were cultured for 30 min. After

removing the culture medium, lysis buffer was added and sonicated; the cell lysate was harvested by centrifugation (12 000 g, 10 min). For the PDGFR-β siRNA (10 µg/mL) experiment, siRNA was added after drug pretreatments for 30 min, respectively. The cells were further cultured for 24 h. After the cells were harvested, equal amounts of cell lysates (20 µg) were separated by polyacrylamide gel electrophoresis (PAGE) and subsequently electroblotted onto Immobilon polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skim milk for 1 h at room temperature and then incubated with the first antibody (anti-PDGFRβ, anti-p-PDGFRβ, anti-ERK, anti-p-ERK, anti-AKT, anti-p-AKT, anti-E-cadherin, and anti-β-catenin) at 4 °C overnight and with the second antibody at room temperature for 1 h. Protein expression was visualized by using a Western blot ECL substrate. The optical density of the immunoreactive bands was visualized with Fujifilm LAS4000 luminescent image analysis system and quantified by using Image J.

Animal experiments

Cream gel preparation

One gram Carbopol 940 was solved in 80 g water, stirred and solubilized; then 0.1 N NaOH was added to adjust the pH value to 7.0. The final amount was adjusted to 100 ml. The blank cream gel was formed. Testing components were added into the blank cream gel at concentrations of 25% for further evaluation [25].

Animal wound model and wound-healing measurement

The 8-week-old male C57BL/6 mice were purchased from BioLASCO Taiwan Co. Three mice for each group were used [25]. The mice were fed under normal condition with a 25 ± 2 °C and humidity of 55 ± 5%. After shaving the dorsal hair and cleaning the exposed skin with 70% ethanol, four full-thickness wounds were produced using a 5 mm punch. Histological section was prepared for their pathological analysis. The application of the ointment was performed every day at the same time. The wounds were observed at days 0, 1, 3, 5, and 7 post-surgery. All surgical procedures were approved by the Institutional Animal Care and Use Committee in National Research Institute of Chinese Medicine (approval No. 110–369–1, approved on 16/04/2021 and valid from 01/08/2021 to 31/07/2024). Wound size was measured by digital photographs taken by an Olympus digital camera. The percentage of wound closure was calculated [47].

Then, animals were sacrificed at predetermined time points three and seven days after infliction of the wound; the animals were euthanized with an overdose inhalation of 2% isoflurane. The skin wound was removed, washed and immediately put into 4% paraformaldehyde at 4 °C for 3–4 h and, then, directed to the histological process. Samples were sectioned into 5 µm sections and stained with hematoxylin–eosin and Masson's trichrome. The tissues were examined by a light microscope [47, 48].

Statistical analysis

IBM SPSS v23.0 was used for all data analyses. Data were compared using two-way analysis of variant (ANOVA) or Student's *t* test. Descriptive statistics are presented as mean ± standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

Contributors' Statement

Investigation, methodology, data curation and draft: W. H. Hsu; animal experiment: J. J. Cheng; data curation: C. F.Wu; conceptualization, supervision, review and editing: Y. L.Lin. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Funding Information

This work was supported by AXR Pharma Inc., Taipei City 112, Taiwan (CMU No. 107426CD) and China Medical University, Taichung 404328, Taiwan (CMU109-MF-93) to Y. L. L., and MOHW112-NRICM-D-325-000300 to J. J. C.

Conflict of Interest

The authors declare that they have no conflict of interest.

Disclosure

Dr. Wei-Hsiang Hsu's current address is the Institute for Cancer Research, Shenzhen Bay Laboratory, Shenzhen, China; Spot Biosystems Ltd., Haining, Jiaxing, Zhejiang, China; Spot Biosystems Ltd., Palo Alto, CA, USA; Spot Biosystems Ltd., Haining, Jiaxing, Zhejiang, China; Spot Biosystems Ltd., Palo Alto, CA, USA.

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