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Polygonum aviculare L. and its active compounds, quercitrin hydrate, caffeic acid, and rutin, activate the Wnt/β-catenin pathway and induce cutaneous wound healing

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***Polygonum aviculare* L. is a member of the Polygonaceae family of plants, which has been known for its antioxidant and anti-obesity effects. However, the wound healing function of *P. aviculare* extract has not been assessed. In this study, we identified a novel property of *P. aviculare* extract as a Wnt/β-catenin pathway activator based on a screen of 350 plant extracts using HEK293-TOP cells retaining the Wnt/β-catenin signaling reporter gene. *P. aviculare* extract accelerated the migration of HaCaT keratinocytes without showing significant cytotoxicity. Moreover, *P. aviculare* extract efficiently re-epithelized wounds generated on mice. Additionally, ingredients of *P. aviculare* extract, such as quercitrin hydrate, caffeic acid, and rutin, also accelerated the motility of HaCaT keratinocytes with the activation of Wnt/β-catenin signaling. Therefore, based on our findings, *P. aviculare* extract and its active ingredients could be potential therapeutic agents for wound healing. Copyright © 2016 John Wiley & Sons, Ltd.**

Keywords: Polygonum aviculare L; quercitrin hydrate; caffeic acid; rutin; cutaneous wound healing; Wnt/β-catenin pathway.

INTRODUCTION

Wound healing is an interactive process involving a complex cascade of cellular and biochemical actions that restore the structural and functional integrity of the damaged tissues (Olczyk *et al*., 2014). Wound healing is often delayed because of various factors including infection and abnormal bacterial presence (Bowler *et al*., 2001). Therefore, antimicrobial agents have been widely used to prevent the infection of injured tissues and also to defend against infectious bacteria (Burks, 1998). Unfortunately, antimicrobial agents usually cause the formation of a scab or crust over the wound site, which impedes the wound healing process (Lipsky and Hoey, 2009). Moreover, pharmacological antibiotics, which include aminoglycosides and vancomycin, often result in adverse effects such as the aberrant accumulation of cells and organ toxicity (Chu *et al*., 2006). Therefore, better treatment modalities are needed to overcome the limitations and side effects of current therapies.

Recently, various oriental medicinal herbs have been identified for their effectiveness in promoting wound healing without any significant side effects (Goyal *et al*., 2012). The role of herbal medicines in wound management involves disinfection, debridement, and providing a moist environment for the natural healing process (Purna and Babu, 2000). Thus, plant extracts could be relatively safe therapeutic agents that may not cause significant irritation, sensitization, and acute toxicity (Lipsky and Hoey, 2009). Therefore, plant extracts could be ideal therapeutic agents for wound healing.

Cutaneous wound healing is the result of multiple functions of cytokines, growth factors, blood and cellular elements, and the extracellular matrix. Signaling pathways involving several cytokines and growth factors such as Wnt/β-catenin, Notch, and Hedgehog, as well as Ras–extracellular signal-regulated kinases (ERK) pathways, are involved in promoting the wound healing process (Bielefeld *et al*., 2013). The Wnt/β-catenin signaling pathway plays a predominant role in skin injury and scarless repair (Bielefeld *et al*., 2013) and is directly involved in enhancement of wound healing by modulating cell proliferation in the adult epidermis (Lee *et al*., 2012b). Therefore, the Wnt/β-catenin pathway has been an attractive therapeutic target for the development of wound healing agents (Zhang *et al*., 2009). However, efficient natural product-based agents need to be identified and further developed to yield a commercially viable wound healing agent.

We selected *Polygonum aviculare* L. extract as an efficient compound to activate the Wnt/β-catenin signaling pathway following a screen of a library of 350 plant extracts (Cha *et al*., 2014) and evaluated its effects on keratinocyte migration and neo-epidermis formation in wounds of mice. In addition, quercitrin hydrate, caffeic acid, and rutin were characterized as

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ingredients of *P. aviculare* extract that activate the Wnt/β-catenin pathway and play a role in keratinocyte migration. *P. aviculare* extract and its active ingredients could potentially be developed as novel wound healing agents.

MATERIALS AND METHODS

Preparation of plant extract. *Polygonum aviculare* extract was purchased from the Plant Extract Bank (http://extract.pdrc.re.kr; Daejeon, Korea). The extract was obtained from 30–40 g of crude sample mixed with ethanol using solvent extracting equipment (ASE300 Accelerated Solvent Extractor; Dionex Corporation, Sunnyvale, CA, USA) at 45°C and 1500 psi for 20 min and dried (Modul spin 40; Biotron Corporation, Canada) at 40°C for 24 h.

Active compounds of *Polygonum aviculare* extract. Quercetin, quercitrin hydrate, caffeic acid, emodin, and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), and rutin was purchased from Acros (Waltham, MA, USA). These compounds were dissolved in dimethyl sulfoxide (Sigma-Aldrich) for *in vitro* studies.

Cell culture and *in vitro* wound healing assay. Immortalized HaCaT keratinocytes (Lee *et al*., 2012b) and human dermal fibroblasts (provided by Dr J.-K. Hwang at Yonsei University, Seoul, Korea) were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco), 100 mg/mL of penicillin (Gibco), and 100 mg/mL of streptomycin (Gibco), at 37°C in a humidified incubator containing 5% (v/v) CO_2. For the *in vitro* wound healing assay, HaCaT cells or human dermal fibroblast cells were seeded in Dulbecco's modified Eagle's medium containing 10% FBS in triplicate at a density of 400,000 cells/well. Following a 24-h attachment period, the monolayers were carefully scratched with sterile pipette tips and incubated with a medium containing 5% FBS with or without *P. aviculare* extract (1 or 5 μg/mL) or an individual active ingredient (10 μM). After 24 h, cells were harvested, rinsed once with cold phosphate buffered saline (PBS, pH 7.4), fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature, and stained with 2% (w/v) crystal violet. The wound closure area was measured using NIS-Elements imaging software (Nikon, Tokyo, Japan) ($n = 3$).

Transwell migration assay. Transwell migration assays were performed with matrix-coated transwell plates (8-μm pore size; Corning Life Sciences, Lowell, MA, USA). Filters were coated with bovine serum albumin (BSA) (100 μg/mL) and vitrogen and fibronectin (10 μg/mL) in PBS for 1 h at 37°C. HaCaT keratinocytes were seeded onto the filters at a density of 40,000 cells/well and counted to verify equal cell numbers. Different dose (1 or 5 μg/mL) of *P. aviculare* extract was added to the upper and lower compartment period to seeding of the cells. After 24-h incubation, cells in the upper chamber were removed, and cells on the lower surface were fixed with 4% PFA and stained with 2% (w/v) crystal violet. Migrating cells were visualized using a bright-field optical microscope (Nikon TE-200U) and counted at high power magnification ($n = 3$).

β-catenin knockdown by small interfering RNA transfection. The human β-catenin small interfering RNA (siRNA) target sequences are 5′-GAAACGGCTT TCAGTTGAG-3′ and 5′-AAACTACTGTGGACCA CAAGC-3′. The siRNAs were transfected into HaCaT cells or human dermal fibroblast cells using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA) at a final concentration of 100 nM. The cells were scratched, incubated with or without 5 μg/mL of *P. aviculare* extract, and stained as described earlier ($n = 3$).

Luciferase assay. HEK293-TOP cells (Cha *et al*., 2014; 25,000 cells/well) were seeded into 96-well plates and incubated in medium with 10% FBS for 24 h. The cells were incubated for 24 h with or without active ingredients (Sigma). Total cell lysates were extracted with 25 μL 1× reporter lysis buffer (Promega, Madison, WI, USA) per well, and luciferase activities were measured by a microplate luminometer (BMG Labtech, Offenburg, Germany).

Western blot analysis. The cells were washed once with ice-cold PBS and lysed in radio immunoprecipitation assay buffer (Millipore, Bedford, MA, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting was performed with primary antibody against β-catenin (Santa Cruz Biotechnology, Dallas, TX, USA; 1:1000) or α-tubulin (Oncogene Research Products, La Jolla, CA, USA; 1:5000) for 3 h at room temperature. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse (Cell Signaling Technology, Beverly, MA, USA) or anti-rabbit (Bio-Rad, Hercules, CA, USA) antibodies. Proteins were visualized by enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ, USA) using a luminescent image analyzer, LAS-4000 (Fujifilm, Tokyo, Japan).

Immunocytochemistry. The cells were washed once in PBS, fixed with 4% PFA in PBS for 15 min at room temperature, and permeabilized in 0.1% Triton X-100 for 20 min at room temperature. After blocking with 5% BSA for 30 min at room temperature, the cells were incubated with primary antibody against β-catenin (BD Transduction Laboratories, Lexington, KY, USA; 1:100), phalloidin (Molecular Probes, Eugene, OR, USA; 1:200), or collagen I (Abcam, Cambridge, UK; 1:100) overnight at 4°C. The cells were washed with PBS and then incubated with Alexa Fluor 488-conjugated or Alexa Fluor 555-conjugated IgG secondary antibody (Molecular Probes; 1:400) for 1 h at room temperature, counterstained with 4′,6-

diamidino-2-phenylindole (DAPI; Boehringer Mannheim, Germany; 1:5000), and examined under a confocal microscope (LSM510 META; Carl Zeiss, Gottingen, Germany).

Animals and *in vivo* wound healing assay. Seven-week-old male C3H mice were purchased from Orient Bio Co. (Gyeonggi-do, Korea) and allowed to adapt to their new environment for 1 week. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center. Animals were maintained under a 12-h light/12-h darkness cycle at 22–25°C in conventional conditions and fed with standard rodent chow and water. To determine the effects of *P. aviculare* extract on wound healing, 8-week-old C3H mice were anesthetized, the dorsal hair was removed using hair clippers, the skin was cleaned with ethanol, and full-thickness incision wounds (diameter = 1.0 cm) were made on the backs of the mice. *P. aviculare* extract and valproic acid (VPA) (500 mM, for positive control) were applied topically until wound closure ($n = 6$). Wound sizes were measured every other day on the assumption that wound depths in each animal are almost constant. The wounded skin tissue was also evaluated by immunohistochemical analysis.

Immunohistochemistry. Tissues were fixed with 4% PFA, and paraffin-embedded tissues were sectioned into 4-mm thickness. The slides were deparaffinized in xylene and rehydrated through a graded alcohol series. For antigen retrieval, the slides were autoclaved in 110-mM sodium citrate buffer. Sections were pre-incubated in PBS and then blocked in PBS containing 5% BSA and 1% goat serum for 30 min at room temperature. The sections were incubated overnight at 4°C with primary antibody against β-catenin (1:100), proliferating cell nuclear antigen (PCNA, Santa Cruz; 1:500), keratin 14 (Covance, Princeton, NJ, USA; 1:500), or collagen I (Abcam; 1:100). The sections were rinsed with PBS and incubated with IgG secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 555 (Molecular Probes; 1:400) for 1 h at room temperature and were counterstained with DAPI (1:5000). Fluorescent signals were visualized on a LSM510 META confocal microscope (Carl Zeiss). For hematoxylin and eosin staining, the sections were stained with hematoxylin for 5 min and with eosin for 1 min. The slides were then dehydrated through a graded alcohol series, cleared in xylene and mounted in Permount (Fisher Scientific, Waltham, MA, USA). The hematoxylin and eosin-stained slides were visualized using a bright-field optical microscope (Nikon TE-200U).

CellTiter-Glo luminescent cell viability assay. HaCaT keratinocytes were plated at a density of 100,000 cells/well of a 24-well plate. Then, cells were treated with dimethyl sulfoxide or *P. aviculare* extract for 72 h. Cell viability was assessed using CellTiter-Glo mixture as recommended by the supplier. Adenosine triphosphate (ATP) was quantified spectrophotometrically at 560 nM using a microplate luminometer (BMG Labtech).

Statistical analysis. Statistical analyses were performed using unpaired two-tailed Student's *t*-test. Asterisks indicate statistically significant differences, with one asterisk indicating p-values <0.05 and two asterisks indicating p-values <0.005.

RESULTS

Polygonum aviculare extract activates the Wnt/β-catenin signaling pathway and enhances the motility of HaCaT keratinocytes

To identify the natural products enhancing wound healing, we tested seven plant extracts (*P. aviculare*, *Thuja orientalis* L., *Pueraria lobata* Ohwi, *Hovenia dulcis* Thunb, *Anemarrhena rhizome*, *Sanguisorbae officinalis* L., and *Persicaria hydropiper*), each of which resulted in a 150% increase in reporter activity compared with control, for their ability to promote the wound healing. *P. aviculare* extract showed the highest (approximately three-fold) enhancement on HaCaT keratinocyte migration among the seven plant extracts tested (Fig. 1A and B), with the highest effect at the 5 μg/mL concentration (Fig. S1A–D). *P. aviculare*-induced wound healing is significantly blocked by using β-catenin siRNA transfection (Fig. S2A and B). Because both keratinocytes and fibroblasts are known to play roles in the cutaneous wound healing process (Werner *et al*., 2007), we also examined the effects of *P. aviculare* on the migration of human dermal fibroblasts. *P. aviculare* extract also most significantly and dose-dependently enhanced the migration of human dermal fibroblasts (Fig. S3A–D). However, migrating effect of *P. aviculare* was significantly reduced by β-catenin knockdown (Fig. S4A and B). β-catenin stabilization and its accumulation in nuclei were also dose-dependently increased by *P. aviculare* extract in both HaCaT keratinocytes (Fig. 1C–E) and human dermal fibroblasts (Fig. S3E). To identify the cellular toxicity of *P. aviculare* extract, we monitored morphological changes in primary neural stem cells, which are highly fragile and sensitive to toxic compounds (Moon *et al*., 2009). The *P. aviculare* extract did not result in any change in the morphology and number of neural stem cells (Fig. S5A), similar to the effect caused by 1 mM VPA, the GSK3β inhibitor inducing hair re-growth via activating Wnt/β-catenin signaling (Lee *et al*., 2012a). In keratinocytes, *P. aviculare* extract did not reveal significant cytotoxicity at concentrations as high as 25 μg/mL (Fig. S5B). Taken together, these results indicate that *P. aviculare* extracts activate the Wnt/β-catenin pathway and stimulate the migration of keratinocytes and fibroblasts without any significant toxicity.

The constituents of *Polygonum aviculare*, quercitrin hydrate, caffeic acid, and rutin, activate the Wnt/β-catenin signaling pathway and increase the motility of HaCaT keratinocytes

To identify which *P. aviculare* ingredients activate the Wnt/β-catenin pathway and induce the migration of

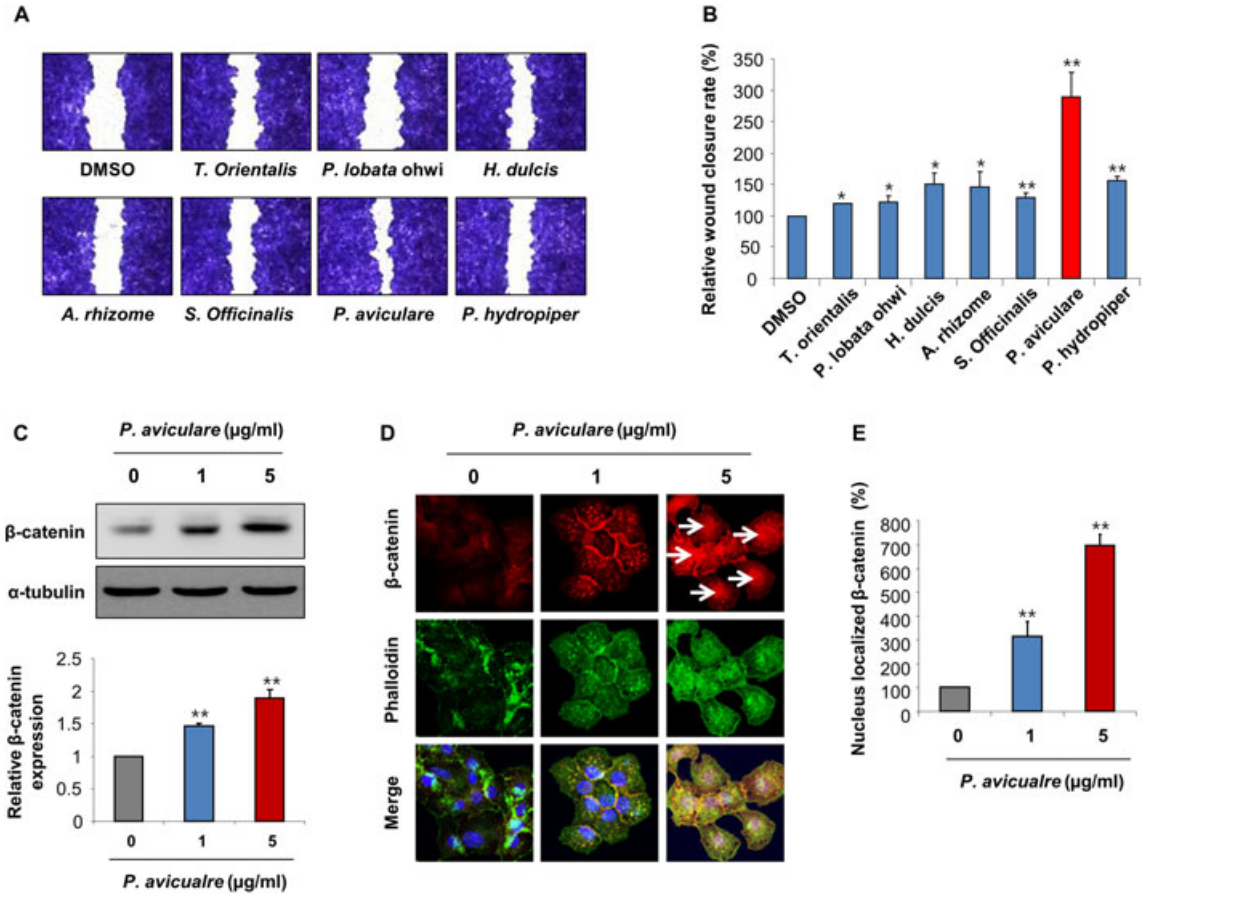

Figure 1. Effects of *Polygonum aviculare* extract on the migration of HaCaT keratinocytes and the activation of β-catenin. (A) HaCaT keratinocytes were grown, and wounds were created evenly with sterile pipette tips. HaCaT keratinocytes were incubated in a medium containing 5% serum with each seven natural plant extracts (5 μg/mL each) or dimethyl sulfoxide (DMSO) (control) for 24 h. Cells were stained with crystal violet for 24 h. (B) The relative wound closure rates shown in Fig. 2A were measured using NIS-Elements imaging software. *P. aviculare* extract, which showed the highest wound closure rate, was marked by a red bar. (C–E) HaCaT keratinocytes were grown and treated for 24 h with *P. aviculare* extract. (C) Whole cell lysates were subjected to western blotting analysis using antibodies against β-catenin or α-tubulin. Lower panel: Relative densitometric ratio of β-catenin protein to α-tubulin is shown. (D) Cells were grown on glass plates and subjected to the immunocytochemical analyses with the anti-β-catenin or phalloidin antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Arrows indicate nuclear-localized β-catenin. (E) Relative percentage of β-catenin localized in cells was measured from staining images of Fig. 1D. (B, C, E) Asterisks denote statistically significant differences as measured by *t*-test with one asterisk indicating $p < 0.05$ and two asterisks indicating $p < 0.005$ ($n = 3$). Original magnification: A, ×40; D, ×635.

human keratinocytes as well as dermal fibroblasts, we listed its ingredients (quercetin, quercitrin hydrate, emodin, catechol, caffeic acid, gallic acid, and rutin) and tested their capabilities in accelerating the migration of human keratinocytes and dermal fibroblasts. Similar to *P. aviculare* extract, quercitrin hydrate, caffeic acid, and rutin also critically accelerated the migration of keratinocytes (Figs 2A and B and S6A and B) and dermal fibroblasts (Fig. S7A and B). Of these ingredients, quercitrin hydrate, caffeic acid, and rutin, which stimulated wound closure, specifically increased the level of β-catenin (Fig. 2C). Quercitrin hydrate, caffeic acid, and rutin did not cause cytotoxicity (Fig. S8). Therefore, we chose these three active ingredients (Fig. S9A–C) to further characterize Wnt/β-catenin pathway activation during the wound healing process. These active ingredients increased TOPflash activity dose-dependently in HEK293 reporter cells (Fig. 2D). The role of three active ingredients in the activation of the Wnt/β-catenin signaling was further confirmed by the induction and nuclear localization of β-catenin in keratinocytes (Fig. 2E). Moreover, stress fibers and cortical networking were increased by all these three active ingredients, as shown by phalloidin straining (Fig. 2E). β-catenin and collagen I were also increased in dermal fibroblasts treated with all these three active ingredients (Fig. S7C and D).

Polygonum aviculare extract promotes cutaneous wound healing *in vivo*

To investigate the therapeutic potential of *P. aviculare* extract on cutaneous wounds, we created full-thickness 1.0-cm^2 dorsal wounds on the backs of mice after shaving and topically applied vehicle, *P. aviculare* extract, or VPA (Lee *et al.*, 2012). *P. aviculare* extract accelerated the wound healing process, and partial re-epithelialization and scab attachment were observed in mice treated with 1 mg/mL *P. aviculare* extract. On the contrary, a complete re-epithelialization of wounds was observed in mice treated with 5 mg/mL of *P. aviculare* extract for 14 days. The effectiveness of 5 mg/mL *P. aviculare* extract on wound healing was similar to that acquired by the application of 500 mM VPA (Fig. 3A and B).

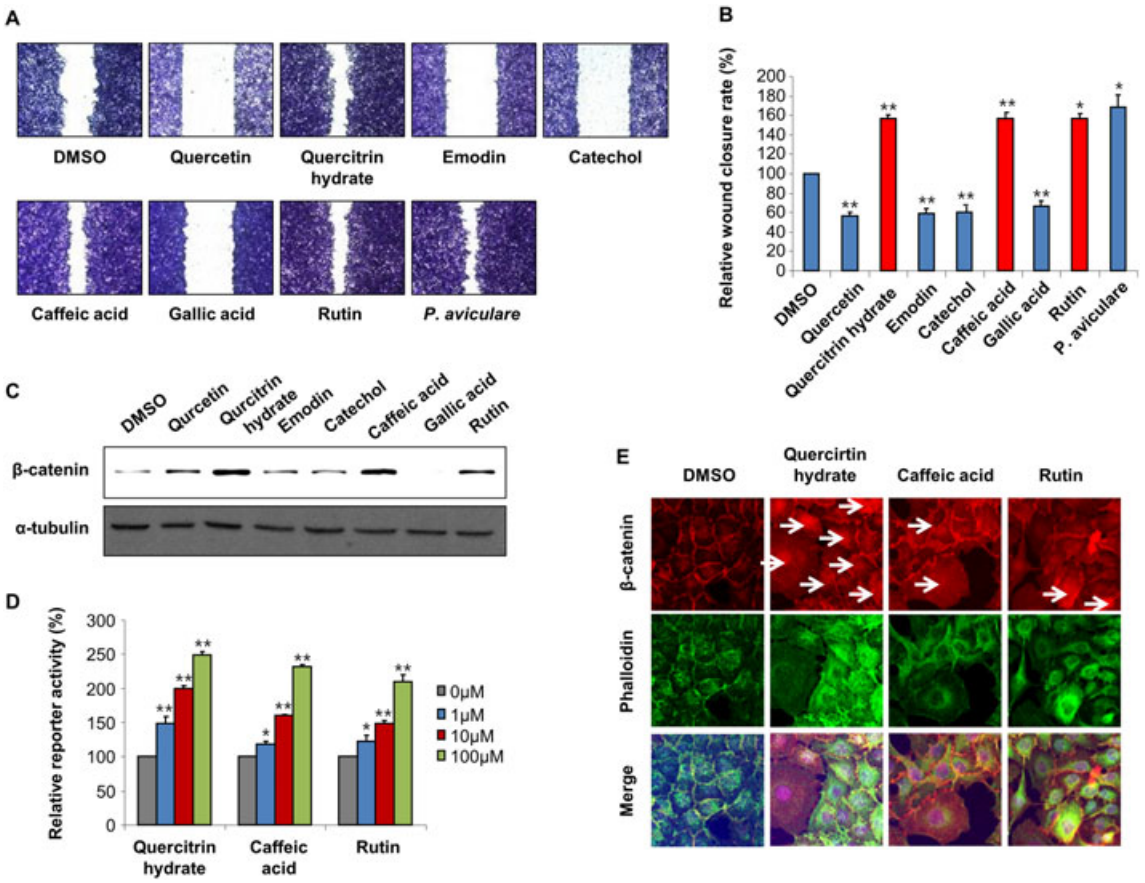

Figure 2. Effects of active ingredients of *Polygonum aviculare* extract on migration of HaCaT keratinocytes and β-catenin activation. (A–C, E) HaCaT keratinocytes were grown as in Fig. 1. (A) After creating a scratch as described in Fig. 1A, HaCaT keratinocytes were treated with each of the seven ingredients (10 μM each) and *P. aviculare* extract (5 μg/mL) for 24 h, and then cells were stained with crystal violet. (B) The relative wound closure rates were measured as described in Fig. 1B. Of the seven ingredients tested, three active ingredients (quercitrin hydrate, caffeic acid, and rutin), which enhanced wound closure rate, were marked by red bars. (C) Western blotting analyses for whole cell lysates were performed using antibodies against β-catenin or α-tubulin. (D) HEK TOP cells were treated with each of these three ingredients of *P. aviculare*, and luciferase activities were measured as described in the Materials and Methods section ($n = 3$). (E) Immunocytochemical analyses were performed with anti-β-catenin or phalloidin followed by Alexa Fluor 488-conjugated or Alexa Fluor 555-conjugated IgG secondary antibodies, respectively. Arrows indicate nuclear-localized β-catenin. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. (B, D) Asterisks denote statistically significant differences as measured by *t*-test with one asterisk indicating $p < 0.05$ and two asterisks indicating $p < 0.005$ ($n = 3$). Original magnification: A, ×40; B, ×635. DMSO, dimethyl sulfoxide.

Polygonum aviculare extract activates β-catenin and induces wound healing *in vivo*

To further characterize the role of Wnt/β-catenin signaling on re-epithelialization following treatment with *P. aviculare* extract, we evaluated β-catenin levels and wound healing markers. The level of β-catenin was dose-dependently increased in wound tissues by the application of *P. aviculare* extract, as shown by VPA treatment (Fig. 4A). The increase in β-catenin was confirmed by immunocytochemical analyses of the neo-epidermal tissues treated with *P. aviculare* extract (Fig. 4B). Moreover, PCNA, a proliferation marker, was also upregulated by treatment with *P. aviculare* extract in a dose-dependent manner compared with the control group, especially in the neo-epidermis area (Fig. 4B). Accordingly, the expression levels of both keratin 14, a terminally differentiated marker of keratinocytes, and collagen I, a marker of myofibroblast differentiation, were increased in *P. aviculare* extract-treated wounded tissues especially at the newly formed epidermis (Fig. 4B). Taken together, we found that *P. aviculare* extract accelerates wound healing and is correlated with the activation of the Wnt/β-catenin signaling pathway.

DISCUSSION

Polygonum aviculare, a member of the Polygonaceae family, has been traditionally used for therapeutic purposes because of its antioxidant and anti-obesity properties (Salama and Marraiki, 2010). In addition, *P. aviculare* extract contains active phytochemicals such as tannins, saponins, flavonoids, alkaloids, and sesquiterpenes with various biological activities including anti-inflammation, antioxidant, anticancer, and antibiotic (Salama and Marraiki, 2010).

In this study, we identified and characterized the acceleration of wound healing by *P. aviculare* extract via the activation of Wnt/β-catenin signaling. We found that *P. aviculare* extract induces wound healing, with increased stress fiber and myofibroblast differentiation in keratinocytes and dermal fibroblasts. In addition, three active ingredients in *P. aviculare*, quercitrin hydrate, caffeic acid, and rutin, similarly promoted the migration of keratinocytes and dermal fibroblasts, accompanied by activation of the Wnt/β-catenin pathway.

In the normal wound healing process, the re-epithelialization stage includes covering the wound

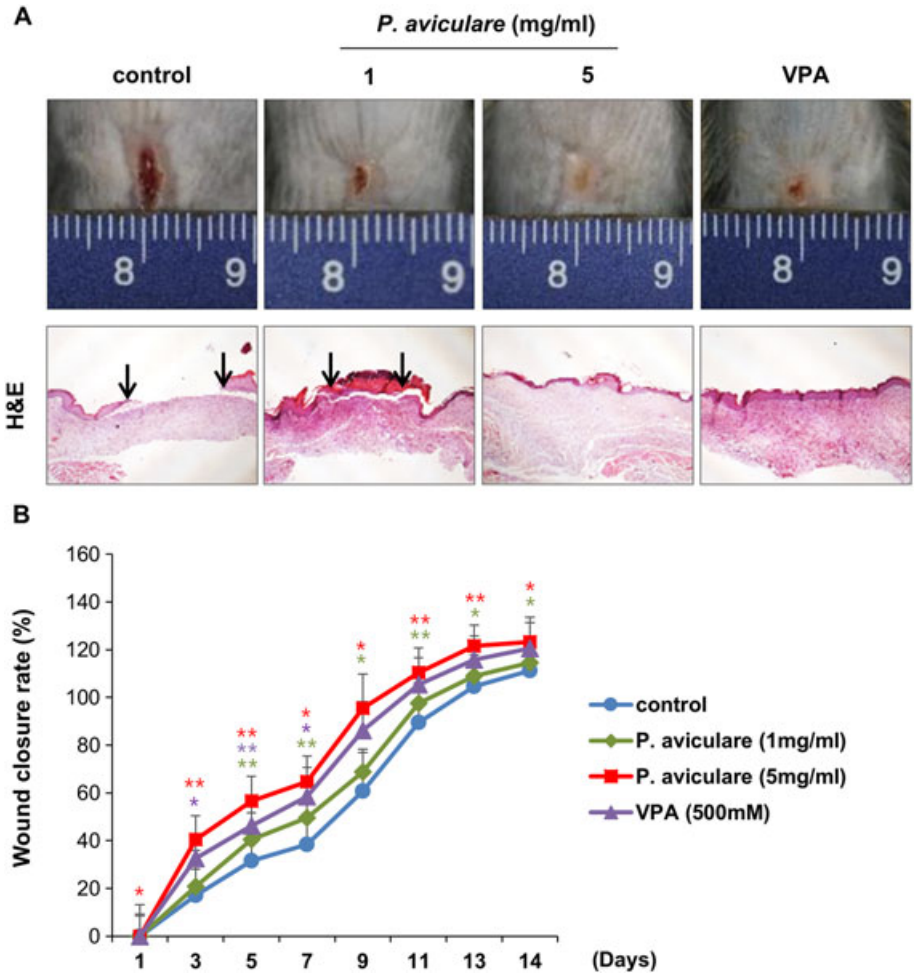

Figure 3. Effects of *Polygonum aviculare* extract on cutaneous wound healing. Full-thickness skin incisions (diameter = 1.0 cm) were made on the back of 8-week-old male C3H mice, and *P. aviculare* extract or valproic acid (VPA) (500 mM) were topically applied daily on the wounds for 14 days. (A) Representative gross images of wounds at 14 days and representative hematoxylin and eosin (H&E) staining (lower panel). Arrows indicate wound edges. (B) Wound sizes were measured every 2 days after creating the wound. Asterisks denote statistically significant differences as measured by *t*-test with one asterisk indicating $p < 0.05$ and two asterisks indicating $p < 0.005$ ($n = 3$). Original magnification: A, ×40.

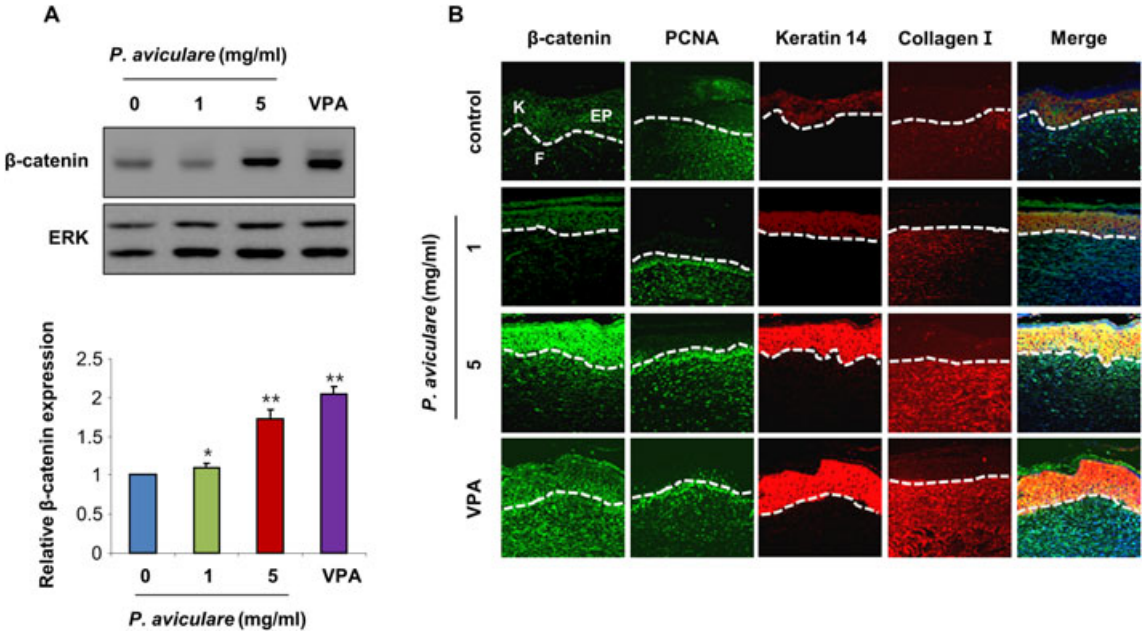

Figure 4. Effects of *Polygonum aviculare* extract on β-catenin expression, proliferation, and wound healing markers in cutaneous wound healing. Wounded tissues were excised from C3H mice at 14 days post-wounding and subjected to immunohistochemical analysis. (A) Wounded tissue extracts were subjected to western blot analysis using the anti-β-catenin antibody. The protein levels were quantified as described in Fig. 1C. Asterisks denote statistically significant differences as measured by *t*-test with one asterisk indicating $p < 0.05$ and two asterisks indicating $p < 0.005$. (B) Immunohistological analysis of β-catenin, PCNA, keratin 14, or collagen I in the control and *P. aviculare*-treated wounded tissues. Original magnification: ×200. VPA, valproic acid; ERK,; EP, epidermis.

surface with a layer of epithelium, which involves the differentiation, proliferation, and migration of epidermal keratinocytes (Bellavia *et al*., 2014). *P. aviculare* extract efficiently accelerated the re-epithelialization of wounds in the murine model. Moreover, *P. aviculare* extract showed a similar efficacies as VPA, which was recently characterized as an efficient wound healing agent functioning via the Wnt/β-catenin pathway (Lee *et al*., 2012), in neo-epidermis formation and the fibroblast–myofibroblast transition in the wound.

Considering the relative safety of natural products and their phenolic or flavonoid ingredients (Lee *et al*., 2011), *P. aviculare* and its active components identified in this study can be used to develop novel pharmaceutical products for the treatment of wounds.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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