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Achillea asiatica extract and its active compounds induce cutaneous wound healing

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ABSTRACT

Ethnopharmacological relevance: *Achillea asiatica* Serg. is a perennial herb belonging to the Asteraceae family that has long been traditionally used to treat acute intestinal and stomach inflammation, persistent fever, ulcers, wounds, and rheumatism.
Aim of the study: We investigated the effect of *A. asiatica* extract (AAE) on cutaneous wound healing.
Materials and methods: To assess the effect of AAE on wounds, an incisional Sprague-Dawley (SD) rat model was topically treated with AAE for 2 weeks. HaCaT keratinocytes, Hs68 dermal fibroblasts, and RAW 264.7 macrophages were used for in vitro experiments. After treatment with AAE, cell viability, cell migration, and production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) were investigated. mRNA expression of collagen type I and III and inflammatory cytokines was measured by RT-PCR. The effect of AAE on activation of β-catenin and other markers was determined by Western blot analysis.
Results: AAE treatment significantly increased epithelialization and accelerated wound healing in SD rats. Meanwhile, AAE and its active compounds reduced NO and PGE_2 release and mRNA expression of inflammatory cytokines in RAW 264.7 macrophages, reflecting anti-inflammatory activity. Furthermore, AAE and its constituents stimulated collagen expression in Hs68 fibroblasts by activating transforming growth factor-β and stimulated keratinocyte differentiation and motility by inducing β-catenin, Akt, and keratinocyte differentiation markers.
Conclusions: AAE improves skin wounds in SD rats and supports keratinocyte development.

1. Introduction

Wound healing is a highly dynamic and complex process that involves multiple types of cells, extracellular matrix (ECM), and soluble molecules (Lee et al., 2012a, 2012b; Wells et al., 2016). Wound healing comprises several phases, beginning with hemostasis, followed by an inflammation-induced early phase and then provisional matrix build-up and scar remodeling after re-epithelialization (Werner et al., 2007). The NFκB, phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt), and Wnt/β-catenin signaling pathways play crucial role in various physiological processes involved in healing wounded tissue.

The bioactive molecule nitric oxide (NO) is involved in inflammation and stimulates vascular cells (Isenberg et al., 2005). NO produced by inducible nitrogen oxide synthase (iNOS) regulates cell proliferation, wound contraction, and collagen formation during wound healing (Witte and Barbul, 2002). Prostaglandin E_2 (PGE_2) is one of the most common inflammatory mediators, is synthesized from arachidonic acid by cyclooxygenase (COX), and exerts its activity through four receptor subtypes (EP1–

Abbreviations: AAE, *A. asiatica* extract; SD, Sprague-Dawley; NO, nitric oxide; PGE_2, prostaglandin E_2; ECM, extracellular matrix; PI3K/Akt, phosphatidylinositol-3 kinase/protein kinase B; iNOS, inducible nitrogen oxide synthase; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; TGF-β, transforming growth factor beta; LPS, lipopolysaccharide; EA, ethyl acetate; BuOH, *n*-butanol; ACN, acetonitrile; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; IKK, IκB kinase

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EP4) to participate in several processes, including inflammation and pain sensation (Kawahara et al., 2015). Meanwhile, NO, produced by iNOS, PGE_2, and the inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) play a major role in the inflammation process during wound healing (Eubank and Marsh, 2010). However, excessive and/or prolonged inflammation is detrimental to wound healing and leads to various fibrotic problems including hypertrophic scarring, which can be disfiguring, and non-healing chronic wounds (Röhl et al., 2015).

β-catenin is an upstream regulatory molecule that mediates transcription of cofactors, such as T cell factors/lymphoid enhancer factors, to induce gene expression in a cell type-specific manner and is an important mediator of skin tissue proliferation including development of hair follicles, which contain differentiated keratinocytes (Ito et al., 2007). The Wnt/β-catenin signaling pathway plays a major role in skin injury and scarless repair (Bielefeld et al., 2013) and is directly involved in enhancement of wound healing by activating proliferation of adult dermal fibroblasts and keratinocytes (Lee et al., 2012a, 2012b). Besides skin development, this pathway is also important in cancer progression and fibroproliferative diseases (Liu et al., 2012).

The extracellular signal-regulated kinase (ERK) and PI3K/Akt signaling pathways participate in various processes during wound healing, especially keratinocyte differentiation and development (Iversen et al., 2005) and corneal wound healing (Xu and Yu, 2011). Several growth factors such as fibroblast growth factor and epidermal growth factor (EGF) induce or augment mitogen-activated protein kinase (MAPK) signaling pathways. For example, keratinocyte growth factor induces ERK1/2 and PI3K/Akt signaling and is involved in wound healing (Sharma et al., 2003). Therefore, the ERK1/2 and Akt signaling pathways may regulate several processes during wound healing and be helpful for the treatment of chronic wounds (Eckert et al., 2002).

Epidermal-mesenchymal communication is critical for crosstalk between keratinocytes and fibroblasts in skin morphogenesis to develop and maintain adult skin structures during the re-epithelialization phase. Communication is mostly mediated by secreted cytokines and other factors, which establish a paracrine loop between these cells (Ghahary and Ghaffari, 2007; Werner et al., 2007). Transforming growth factor beta (TGF-β) exerts cell-specific activity via binding to TGF-β receptor I and TGF-β receptor II heterodimers, which induce phosphorylation of the downstream targets Smad 2 and 3. This complex translocate into the nucleus where it modulates gene expression (Verrecchia and Mauviel, 2002; Leask, 2004). Indeed, TGF-β signaling plays a role in many events associated with wound healing, including proliferation of fibroblasts (Puolakkainen et al., 1995) and synthesis of ECM components such as collagen I and fibronectin (Varga et al., 1987; Hocevar et al., 1999).

Achillea asiatica Serg. (*Achillea millefolium* var. *manshurica* Kitam, Mongolian yarrow) is a perennial herb with rhizomes that is distributed over Central Asia, Russia, Mongolia, and China. *A. asiatica* has been widely used to treat persistent fever, inflammatory diseases, enterogastritis, and wounds in traditional Mongolian medicine (Sanchir et al., 2003; Ligaa et al., 2005). *A. asiatica* extract (AAE) influences gastric secretion (Slipchenko, 1994), promotes healing of chronic ulcers (Pitschmann et al., 2013), and elicits anthelmintic effects (Nemeth and Bernath, 2008). *Achillea millefolium* (yarrow), a same species with *Achillea asiatica,* has been extensively used against inflammatory disorders and skin wound healing in traditional medicine (Tadić et al., 2017). However, studies of AAE on wound-healing activity are lacking. Therefore, we aimed to elucidate the molecular mechanisms underlying the wound-healing effect of AAE and its constituents using in vitro and in vivo models.

2. Material and methods

2.1. Reagents and chemicals

All chemicals and compounds used in this study were analytical grade. Dulbecco's modified Eagle medium (DMEM), penicillin, and fetal bovine serum (FBS) were obtained from Hyclone (South Logan, UT, USA). Monoclonal antibodies against Smad2/3, phospho-Smad2, (Ser465/467)/Smad3 (423/425), β-catenin, Akt, phospho-Akt (Tyr525/526), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2), and β-actin were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Antibodies against keratinocyte differentiation markers (filaggrin, loricrin, and involucrin) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipopolysaccharide (LPS) (*Escherichia coli*, serotype 0111:B4) and all other chemicals were obtained from Sigma Chemicals (St Louis, MO, USA).

2.2. Plant materials, extraction, and isolation of compounds

A. asiatica raw material was collected from near Ulaanbaatar, Mongolia. Voucher specimens were identified by Dr. C. Sanchir (Institute of Botany, Mongolian Academy of Sciences) and a voucher specimen (M-010) placed in the Flora and Plant Systematic Laboratory (Institute of Botany, Mongolian Academy of Sciences).

The dried aerial part of *A. asiatica* was extracted with pure ethanol for one week at room temperature, and the solvent was evaporated under reduced pressure to yield 70 g of ethanol extract. Then, the extract was soaked in 1 L of distilled water and sequentially partitioned with the organic solvents n-hexane, ethyl acetate (EA), and *n*-butanol (BuOH).

The BuOH fraction was inserted into an RP-18 column and eluted with a methanol gradient (40–60%), generating five fractions. Fraction 2 was separated using a Sephadex LH-20 column (Φ 3 × 60 cm) in 70% methanol and then eluted with a chloroform:methanol:water (CMW) mixture (from 5:1:0.1 to 1:1:0.1) in a silica gel column (Φ 3.5 × 40 cm) to isolate compound **1**. Compound **2** was isolated using 70% methanol in Sephadex LH-20 column chromatography (Φ 4 × 40 cm), followed by semi-preparative high-performance liquid chromatography (HPLC) of Fraction 4 of the BuOH fraction (15% acetonitrile (ACN), 35 min, 10 ml/min, 254 nm). Fraction 5 was subjected to open column chromatography with absorbents (RP-18 column, Φ 3.5 × 40 cm), and elution was performed with a methanol gradient (40–50%). Finally, separation was performed using pure methanol in a Sephadex LH-20 column (Φ 3 × 60 cm) to isolate compound **3**.

The EA fraction was purified by silica gel open column chromatography (Φ 6×50 cm) and eluted with a CMW mixture (from 20:1:0.1 to 1:1:0.1) to generate ten fractions. Furthermore, the methanol-soluble parts of Fraction 5 and Fraction 7 were subjected to chromatography with a silica gel column in a CMW mixture (15:1:0.1) to yield compound **4** and compound **5**, respectively. Crystals formed by the EA fraction were subjected to further fractionation by semi-preparative HPLC with a YMC J'sphere ODS column (Φ 20 × 250 mm, 4 μm, 23% ACN, 25 min, 10 ml/min, 254 nm) and purified to isolate compound **6**.

2.3. HPLC profiling

HPLC analysis of AAE and its compounds was performed using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA), which consisted of a quaternary pump, a UV detector, and an autosampler. A YMC Hydrosphere ODS analytical column (4.6 × 250 mm, S-5 μm) was used. A gradient system of 0.1% formic acid prepared in water (A) and 0.1% formic acid prepared in ACN (B) was used as the mobile phase as follows: 10% B (0–5 min), 10–90% B (5–35 min), and 90% B (35–40 min). Detection was performed at 254 nm. The injection volume was 10 μl, and the flow rate was set to 1 ml/min. The HPLC chromatogram is displayed in Fig. 1.

2.4. Cell culture and cell viability assay

The human dermal fibroblast cell line Hs68, the human keratino-