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ORIGINAL PAPER

Asiaticoside suppresses collagen expression and TGF-β/Smad signaling through inducing Smad7 and inhibiting TGF-βRI and TGF-βRII in keloid fibroblasts

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Abstract Asiaticoside (ATS) isolated from the leaves of *Centella asiatica* possesses strong wound-healing properties and reduces scar formation. However, the specific effects of asiaticoside on the formation of keloidal scars remain unknown. In the present study, we evaluated the in vitro effects of asiaticoside on the proliferation, collagen expression, and transforming growth factor (TGF)-β/Smad signaling of keloid-derived fibroblasts. Fibroblasts isolated from keloid tissue and normal skin tissues were treated with asiaticoside at different concentrations. Afterwards, they were subjected to RT-PCR and Western blot analyses. The inhibitory effects of asiaticoside on fibroblast viability were assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Asiaticoside decreased fibroblast proliferation in a time- and dose-dependent manner. It also inhibited type I and type III collagen protein and mRNA expressions. In addition, asiaticoside reduced the expression of both TGF-βRI and TGF-βRII at the transcriptional and translational level. Moreover, it increased the expression of Smad7 protein and mRNA. However, asiaticoside did not influence the expression of Smad2, Smad3, Smad4, phosphorylated Smad2, and phosphorylated Smad3. Taken together, these results suggest that asiaticoside could be of potential use in the treatment and/or prevention of hypertrophic scars and keloids.

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Introduction

Keloids are dermal fibroproliferative disorders secondary to traumatic or surgical injuries in susceptible individuals, characterized by hyperproliferation of secretory and responsive keloid fibroblasts, overproduction of extracellular matrix (ECM), and aberrant cytokine and growth factor activities [27, 28, 31]. Keloid formation severely impairs the quality of life by causing cosmetic and functional deformities, discomfort, and psychological stress [27, 31]. There is an urgent need for a better understanding of the pathogenesis of keloids in order to develop better prevention and treatment approaches.

Although the molecular mechanisms for keloid development are not fully understood, the transforming growth factor-beta (TGF-β) pathway may be involved. TGF-β, especially TGF-β1, plays an important role in wound healing and scar formation [13, 19]. Deep injury of the skin by trauma or surgery initiates TGF-β1 secretion by all major cell types participating in wound repair, especially fibroblasts [18, 19]. Prolonged or impaired healing can lead to unbalanced TGF-β1 activity and excessive scar formation [19]. Three types of receptors are involved in TGF-β signaling (TGF-βRI, TGF-βRII, and TGF-βRIII), with TGF-βRI and TGF-βRII being the serine/threonine kinase receptors, and TGF-βRIII being the ligand to the receptor functioning as a receptor-accessory molecule [4, 19]. After binding to the ligand, type II receptor kinases phosphorylate the GS domain of the type I receptor. Phosphorylated type I receptor activates the down-stream signaling molecules, Smad, which

transduces the signal into the nucleus. Smad proteins are intracellular signaling molecules and mediate TGF-β-inducible transcriptional responses [5, 9, 12, 19]. Smad2 and Smad3 (receptor-regulated Smads, R-Smads) are direct substrates of the TGF-βRI. Once phosphorylated and activated, Smad2 and Smad3 form hetero-oligomeric complexes with Smad4 (common Smads, co-Smads). Then the Smad2/Smad3/Smad4 complex translocates into the nucleus binding target gene promoters and activating transcription. Within the R-Smad family, Smad3 mainly mediates collagen production in dermal fibroblasts stimulated by TGF-β [4]. Smad 7 (an inhibitor Smads) acts as a negative feedback regulator, which can antagonize the activity of the R-Smads. It achieves this by strongly binding to the phosphorylated type I receptor and inhibiting the phosphorylation of R-Smad, thus blocking the TGF-β signaling pathways [5, 12]. Continuous activation or decreased inhibition of the signaling pathway may lead to a persistent autocrine-positive feedback loop that could result in the overproduction of matrix proteins and subsequent fibrosis [4, 19, 30].

At present, there are almost no successful treatment modalities for keloids, including surgical excision, corticosteroid injections, compression therapy, and laser therapy [31]. There is an increasing interest in exploring pharmacological agents, especially derived from natural plants, for keloid therapy. The present study aimed at determining the therapeutic effects of asiaticoside, a natural product, and underlying mechanisms of action, especially the involvement of TGF-β/Smad pathways.

Asiaticoside (ATS) is a saponin component extracted from the medicinal plant *Centella asiatica*, which is used as a remedy in tropical regions. Asiaticoside has been reported to have a variety of biological effects including antioxidant [11], anti-inflammatory [33], and anti-ulcer properties [3, 11]. Asiaticoside has been found to protect β-amyloid-induced neurotoxicity [25], to inhibit nitric oxide synthesis [11], and to induce apoptosis of tumor cells [14]. Asiaticoside also possesses anti-hepatofibrotic effects, due to the reduced expression of prolyl 4-hydroxylase alpha and beta subunits, and TIMP2 [7]. In particular, asiaticoside promotes fibroblast proliferation and collagen synthesis, which facilitates the wound-healing process and reduces scar formation [15, 24]. In this study, we investigated whether asiaticoside treatment could suppress the proliferation of keloid-derived fibroblasts and collagen production. Furthermore, possible inhibitory effects of asiaticoside on TGF-β/Smad signaling were explored. Our results indicate that asiaticoside may be a potential drug candidate for keloid treatment.

Materials and methods

Normal and keloid fibroblast culture

Eight patients who have not received previous treatment for keloids before surgical excision were included in the study. The patients were two males and six females, and ranged in age from 17 to 32 years. The location of keloid lesions were on the earlobe, shoulder and chest. The nature of the keloids was confirmed histologically by hematoxylin and eosin-stained sections of skin tissues. Three skin tissues were obtained from three volunteers (one male and two females, age from 18 to 29 years). The study was approved by the Medical and Ethics Committees of the First Affiliated Hospital, Sun Yat-Sen University, and each patient signed an informed consent before enrolling in the study.

Primary fibroblast cultures were established as described [6]. Specimens were repeatedly washed in sterile Dulbecco's Modified Eagle's Medium (DMEM) supplemented with an antibiotic/antimycotic preparation and then were cut into 5 mm $\times$ 5 mm small pieces. The pieces were incubated in DMEM with 0.5% dispase overnight at 4°C. The epidermis was scraped off, and the dermis was digested in DEME with 0.1% collagenase type I for 4 h at 37°C. Digestion action was quenched by DMEM, and the tissues were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 g/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. When they reached 90% confluence, fibroblasts were subcultured with 0.25% trypsin and 0.04% ethylenediaminetetraacetic acid (EDTA). The cell strains were maintained and stored in liquid nitrogen tanks, and only cells from the forth and fifth passages were analyzed. In the study, we stochastically chose three or four patient-derived fibroblasts for each experiment, and each experiment was repeated three or four times.

Asiaticoside stimulation

Cells were seeded at a density of 2×10^4 cells mL^{-1} into 24-well plates for cell viability, 60-mm plates for RNA and protein analysis in DMEM containing 10% FBS (DMEM/10% FBS). After 24 h, medium was removed and cells were placed in serum-free DMEM. After 48 h, different concentrations of asiaticoside (Shanghai Medical Sales Co., Ltd., Shanghai, China) in DMEM/10% FBS were added simultaneously to cells. Control cells were grown in DMEM/10% FBS without the addition of asiaticoside. At different time points, the fibroblasts were harvested for analysis.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay that inspects the metabolic activity of viable cells. In this study, MTT assay was used to assess the fibroblast proliferative response to asiaticoside treatment at different concentrations and time points.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted and isolated from fibroblast using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg of mRNA by using Superscript™ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT) as primers. β-actin served as an internal control and was used to normalize for variances in input cDNA. The following gene-specific primer pairs were designed using the program, Primer Premier 5.0 software (Table 1): collagen I forward 5′-TGTGCGATGACGTGATCTGTGA-3′, reverse 5′-CTTGGTCGGTGGGTGACTCTG-3′; collagen III forward 5′-CTCTGCTTCATCCCACTATTATTT-3′, reverse 5′-TGCGAGTCCTCCTACTGCTAC-3′; TGF-βRI forward 5′-CGGCGTTACAGTGTTTCT-3′, reverse 5′-TGTC TTTATTGTCTGCTGCTAT-3′; TGF-βRII forward 5′-CG TGAAGAACGACCTAACCT-3′, reverse 5′-AGTTCCCA CCTGCCCAC-3′; Smad2 forward 5′-TTGATGGTCGTCT CCAGGTAT-3′, reverse 5′-GAGGCGGAA GTTCTGTTA GG-3′; Smad3 forward 5′-AACGGGCAGGAGGAGA AATG-3′, reverse 5′-ACAGGCGGCAGTTabAGATGAC A-3′; Smad4 forward 5′-TTCAGGTGGCTGGTCG-3′, reverse 5′-GTTGGGAAAGTTGGCAGT-3′; Smad7 forward 5′-TACCCGATGGATTTTCTCAA-3′, reverse 5′-TC TTCTCCTCCC AGTATGCC-3′; β-actin forward 5′-CA CCAACTGGGACGACA-3′, reverse 5′-GT ACTTGCGC TCAGGAGG-3′.

Table 1 Primer sequence used in this study

	Primer sequence	Size (bp)
Collagen I	F: 5′ TGTGCGATGACGTGATCTGTGA 3′	111
	R: 5′ CTTGGTCGGTGGGTGACTCTG 3′	
Collagen III	F: 5′ CTCTGCTTCATCCCACTATTATTT 3′	470
	R: 5′ TGCGAGTCCTCCTACTGCTAC 3′	
TGF-βRI	F: 5′ CGGCGTTACAGTGTTTCT 3′	483
	R: 5′ TGTCTTTATTGTCTGCTGCTAT 3′	
TGF-βRII	F: 5′ CGTGAAGAACGACCTAACCT 3′	104
	R: 5′ AGTTCCCACCTGCCCAC 3′	
Smad2	F: 5′ TTGATGGTCGTCTCCAGGTAT 3′	245
	R: 5′ GAGGCGGAAGTTCTGTTAGG 3′	
Smad3	F: 5′ AACGGGCAGGAGGAGAAATG 3′	204
	R: 5′ ACAGGCGGCAGTTAGATGACA 3′	
Smad4	F: 5′ TTCAGGTGGCTGGTCG 3′	404
	R: 5′ GTTGGGAAAGTTGGCAGT 3′	
Smad7	F: 5′ TACCCGATGGATTTTCTCAA 3′	171
	R: 5′ TCTTCTCCTCCCAGTATGCC 3′	
β-Actin	F: 5′ CACCAACTGGGACGACA 3′	200
	R: 5′ GTACTTGCGCTCAGGAGG 3′	

Western blot analysis

Western blotting was performed as described in detail previously [6]. Briefly, protein lysates prepared from cultured fibroblasts were separated by an 8 or 10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and subjected to immunoblotting with primary antibodies at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using the ECL chemiluminescence system (Amersham, Piscataway, NJ, USA). Primary antibodies against phospho-Smad2, phospho-Smad3, Smad2, Smad3, and TGF-βRII were obtained from Cell Signal Technology; TGF-βRI, Smad7, and Smad4 from Santa Cruz; Collagen I, Collagen III, and Tubulin from Sigma. Secondary antibodies were from Jackson Company and ECL was from Amersham.

Statistical analysis

The MTT data are presented as the mean ± SD. The RT-PCR and Western blot bands were quantified by densitometry. The mRNA and protein expression were normalized by the levels of β-actin and α-tubulin, respectively. The differences in cell proliferation, mRNA levels, and protein levels were analyzed by SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). Two sets of independent sample data were compared using Student's t test. p Values <0.05 were considered statistically significant.

Results

Asiaticoside inhibits keloid fibroblasts proliferation in a time- and dose-dependent manner

To examine whether asiaticoside possesses anti-proliferative properties, normal and keloid fibroblasts were treated with various concentrations of asiaticoside (0, 100, 250, and 500 mg/L) for 5 days, at which point the MTT assay was performed. On day 1, no significant change ($p > 0.05$)

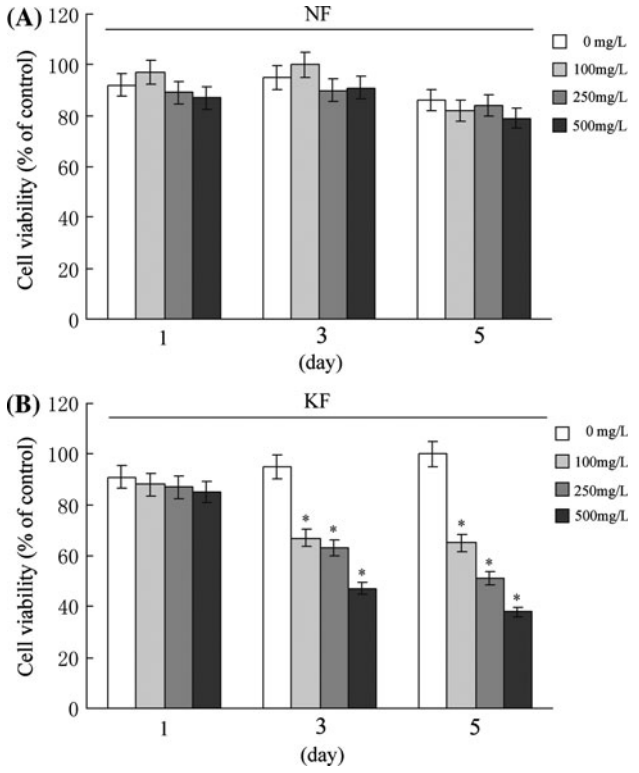

Fig. 1 Asiaticoside inhibited fibroblast proliferation in a time- and dose-dependent manner in keloid fibroblasts. **a** Normal fibroblasts were cultured and treated with different concentrations (100, 250, and 500 mg/L) of asiaticoside in DMEM medium containing 10% fetal bovine serum for 1, 3, and 5 days. **b** keloid fibroblasts were cultured and treated with 250 mg/L of asiaticoside in DMEM medium containing 10% fetal bovine serum for 1, 3, and 5 days. Cell viability was assessed using MTT assay. Untreated fibroblasts were used as control. Data are expressed as the means ± SD of five independent experiments. $*p < 0.05$ compared with the values of control (no asiaticoside)

in keloid fibroblasts proliferation was observed at all concentrations. On day 3, asiaticoside significantly diminished keloid fibroblast proliferation by 28, 30, and 51%, at 100, 250, and 500 mg/L, respectively ($p < 0.05$). On day 5, the reduction of proliferation after asiaticoside treatment was 35, 48, and 63%, at 100, 250, and 500 mg/L, respectively (Fig. 1b). However, the normal fibroblasts' viability after asiaticoside treatment was not significantly decreased between control group and asiaticoside-treated group (Fig. 1a). These data indicate that asiaticoside decreased fibroblast proliferation in a time- and dose-dependent manner.

Asiaticoside decreases the expression of collagen protein and mRNA in keloid fibroblasts

The effects of asiaticoside on the expression of type I and type III collagen in normal and keloid fibroblasts were investigated using RT-PCR analyses and Western blotting. Asiaticoside (100, 250, and 500 mg/L) did not affect the expression of collagen protein and mRNA in normal fibroblasts (Fig. 2a, b). Pretreatment of keloid fibroblasts with asiaticoside (100, 250, and 500 mg/L) significantly reduced the mRNA levels of type I and type III collagen production ($p < 0.05$) (Fig. 2c). Moreover, Western blotting showed that type I and type III collagen protein expressions were correlated with their mRNA levels (Fig. 2d), demonstrating that asiaticoside decreased the expression of collagen.

Asiaticoside has a sustained inhibitory effect of fibroblast proliferation and collagen production in keloid fibroblasts

To examine whether the inhibitory effect of asiaticoside to keloid fibroblasts is reversible, keloid fibroblasts were treated without or with various concentrations of asiaticoside (100, 250, and 500 mg/L). After 72 h, asiaticoside was washed out and fresh DMEM/10% FCS was added. Cell growth recovery was assayed by MTT and type I, III collagen were analyzed by Western blotting after 72 h, as previously described by Mukhopadhyay et al. [26]. It clearly indicated that asiaticoside persistently inhibited fibroblast proliferation (Fig. 3a) and type I and type III collagen production (Fig. 3b).

Asiaticoside reduces the expression of TGF-βRI, TGF-βRII protein, and mRNA in keloid fibroblasts

As TGF-β/Smad signaling pathways perform a significant role in keloid formation and TGF-β receptor binding initiates the signaling cascade, RT-PCR analyses and Western blotting were performed to examine whether asiaticoside reduced the expression of TGF-βRI and TGF-βRII in keloid fibroblasts. Our data showed that at 100, 250, and 500 mg/L, asiaticoside significantly inhibited TGF-βRI and TGF-βRII mRNA, and protein expression in keloid fibroblasts ($p < 0.05$; Fig. 4b, c). Asiaticoside (100, 250, and 500 mg/L) did not affect the expression of TGF-βRI and TGF-βRII protein, and mRNA in normal fibroblasts (Fig. 4a).

Asiaticoside does not influence Smad2, Smad3, Smad4, phosphorylated Smad2, and phosphorylated Smad3 in keloid fibroblasts

Smad proteins act as intracellular signaling mediators of TGF-β. Therefore, mRNA levels and protein expression of Smad2, Smad3, and Smad4, and the level of phosphorylation of Smad2 and Smad3 were investigated in keloid fibroblasts treated without or with asiaticoside (100, 250, and 500 mg/L). It was found that asiaticoside did not have an effect on the mRNA and protein expression of Smad2, Smad3, Smad4 ($p > 0.05$) (Figs. 5a, b). Furthermore, the data also showed that asiaticoside did not regulate

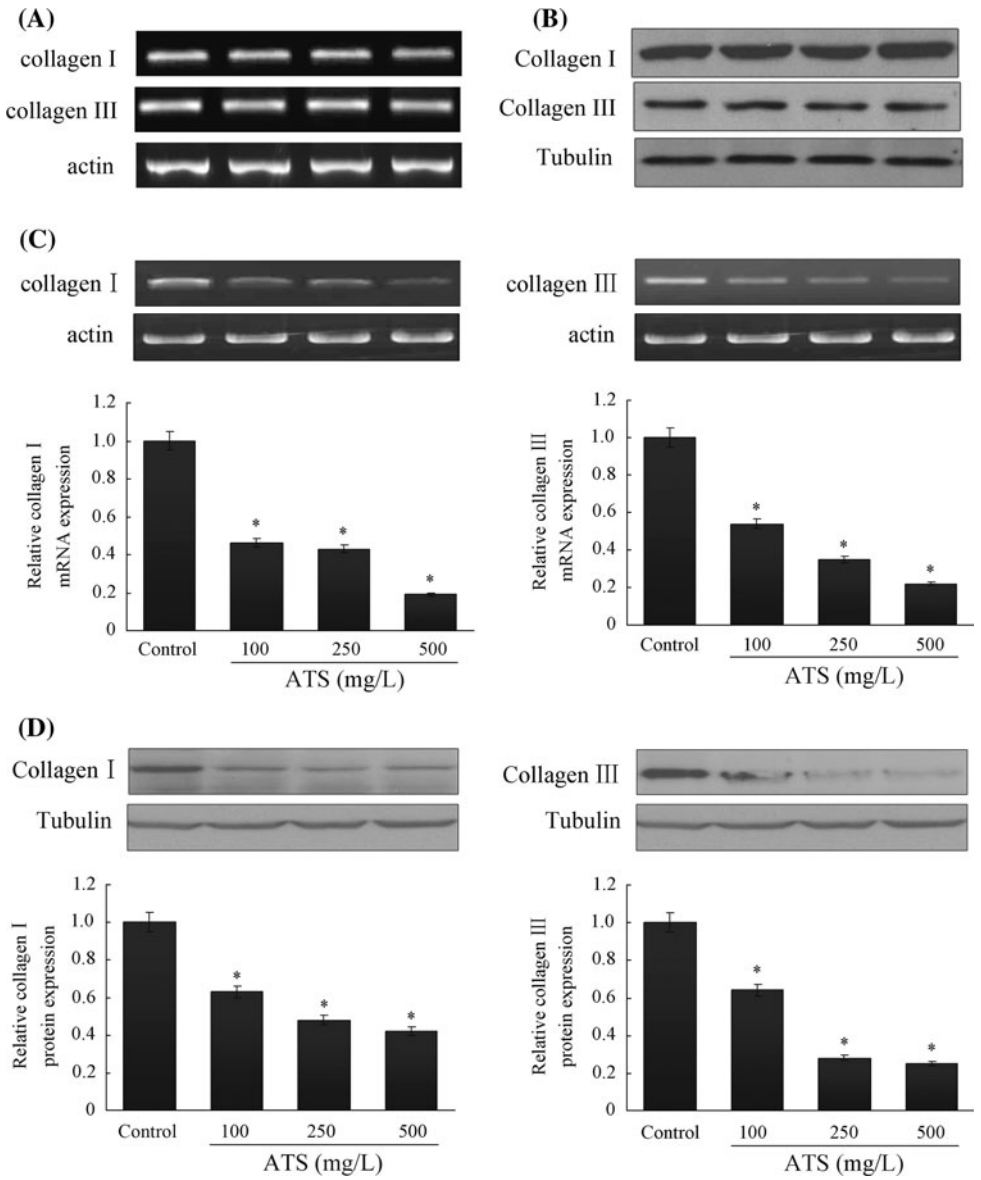

Fig. 2 Asiaticoside reduces the expression of collagen protein and mRNA in keloid fibroblasts. **a** and **b** Normal fibroblasts were cultured and treated without or with different concentrations (100, 250, and 500 mg/L) of asiaticoside in DMEM medium containing 10% fetal bovine serum for 72 h. Total mRNA and cell lysates were prepared and subjected to RT-PCR for collagen I, collagen III or actin (**a**), and to Western blotting with antibody against collagen I, collagen III, or tubulin (**b**), respectively. β-Actin and tubulin were used as a loading control. Experiments were repeated thrice with similar results. **c** and **d** Keloid fibroblasts were cultured and treated without or with different concentrations (100, 250, and 500 mg/L) of asiaticoside in DMEM medium containing 10% fetal bovine serum for 72 h. Total mRNA and cell lysates were extracted from cells and subjected to RT-PCR using specific primers of collagen I, collagen III or actin (**c**) and to Western blotting with antibody against collagen I, collagen III, or tubulin (**d**), respectively. The *graph* is the mean densitometric data showing the level of collagen I and collagen III mRNA normalized to that of actin mRNA, and the level of collagen I and collagen III protein normalized to that of tubulin protein, respectively. $^*p < 0.05$ compared with the values of control (no asiaticoside). Experiments were repeated thrice with similar results

phosphorylated Smad2 and phosphorylated Smad3 ($p > 0.05$; Fig. 5b). At 500 mg/L, asiaticoside slightly inhibited the expression of phosphorylated Smad3, but there were no significant differences compared with the control cells ($p > 0.05$). Taken together, these results demonstrated that Smad2, Smad3, and Smad4 were not involved in asiaticoside-mediated inhibition of fibroblasts proliferation.

Asiaticoside increases the expression of Smad7 in keloid fibroblasts

Inhibitory Smad7 is a significant intracellular antagonist of TGF-β signaling by inhibiting the phosphorylation of R-Smads and co-Smads. Treatment of keloid fibroblasts with asiaticoside (100, 250, and 500 mg/L) significantly

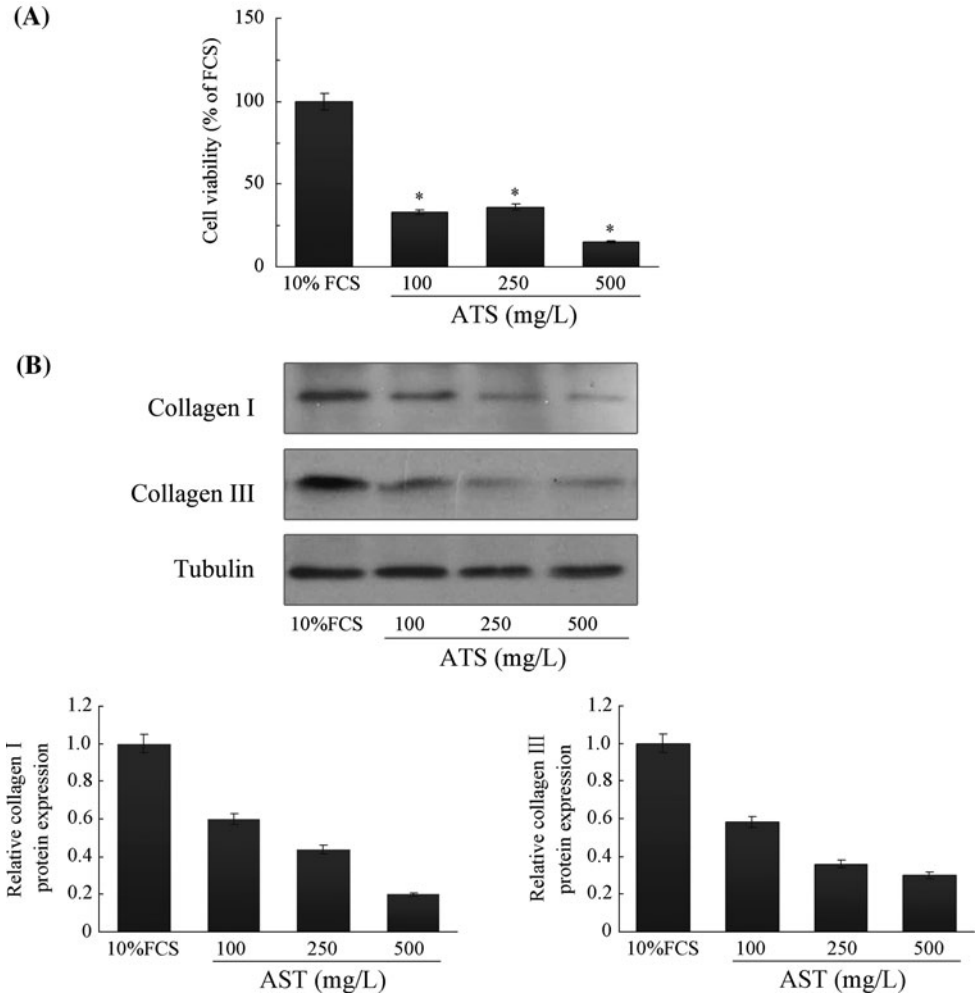

Fig. 3 Asiaticoside has a sustained inhibitory effect of fibroblast proliferation and collagen production in keloid fibroblasts. Keloid fibroblasts were treated without or with different concentrations (100, 250, and 500 mg/L) of asiaticoside followed by replenishments with fresh growth medium containing 10% fetal bovine serum. **a** MTT assay was done after 72 h. Untreated fibroblasts were used as control. Data are expressed as the means ± SD of four independent experiments (*$p < 0.05$ vs. control). **b** Cell lysates were extracted from cells and subjected to Western blotting with antibody against collagen I, collagen III, or tubulin, respectively. The *graph* is the mean densitometric data showing the level of collagen I and collagen III protein normalized to that of tubulin protein, respectively. *$p < 0.05$ compared with the values of control (no asiaticoside). Experiments were repeated thrice with similar results

upregulated the expression of Smad7 mRNA by 1.96, 2.15, and 2.36, respectively ($p < 0.05$; Fig. 5e). The expression of Smad7 protein was also increased ($p < 0.05$; Fig. 5f). However, Asiaticoside (100, 250, and 500 mg/L) did not affect the expression of Smad7 protein and mRNA in normal fibroblasts (Fig. 5c, d).

Discussion

Keloid scars occur as a result of a pathological wound-healing process, characterized by excess collagen deposition and hyperproliferation of fibroblasts. In the present study, we investigated the inhibitory effects of asiaticoside on keloid fibroblasts. Our major results indicate that asiaticoside inhibited type I and type III collagen expression and keloid fibroblast proliferation in a time- and dose-dependent manner. In addition, it was observed that asiaticoside persistently inhibited fibroblast proliferation and type I and type III collagen production even after 72 h of asiaticoside removal and cells were replenished with growth medium.

The reduction in collagen expression caused by asiaticoside could be attributed to its effects on TGF-β Smad signaling. There is overwhelming evidence showing that TGF-β plays a key role in wound healing. However, the overproduction of TGF-β can lead to fibroproliferative disorders and excessive scar formation [4, 18, 19]. TGF-β exerts its effects via the TGF-β receptors. Chin et al. [4] comparatively studied the levels of TGF-β receptors in keloid fibroblasts and normal dermal fibroblasts, showing that there is an increased expression of TGF-β receptors I and II in keloid fibroblasts. Smads are intracellular signaling molecules of the TGF-β pathway which mediate collagen production stimulated by TGF-β by inducing the transcription of a2(I) procollagen gene (COL1A2) [1, 2]. Several studies have suggested that Smad3 is overexpressed and over phosphorylated in keloid fibroblasts compared with normal fibroblasts, suggesting that it is involved in keloid pathogenesis pathogenesis [1, 2, 4]. In addition, the expression of Smad7 is reduced, which prevents the inhibition of TGF-β signaling [17, 32].

Due to the close relationship between TGF-β signaling and the production of collagen, blocking TGF-β signaling

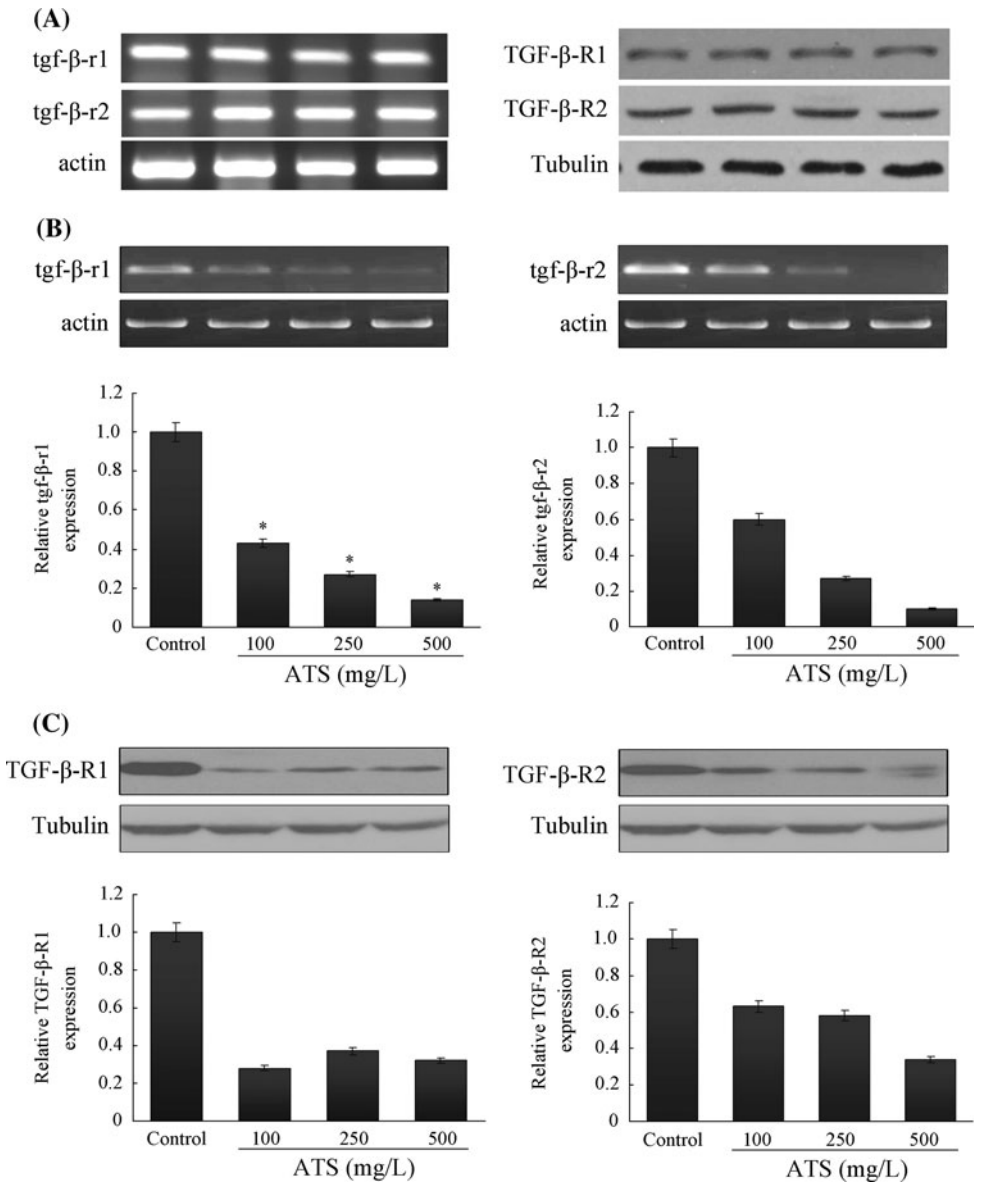

Fig. 4 Asiaticoside reduces the expression of TGF-βRI, TGF-βRII protein and mRNA in keloid fibroblasts. **a** Normal fibroblasts were cultured and treated without or with different concentrations (100, 250, and 500 mg/L) of asiaticoside in DMEM medium containing 10% fetal bovine serum for 72 h. Total mRNA and cell lysates were prepared and subjected to RT-PCR for TGF-βRI, TGF-βRII or actin and to Western blotting with antibody against TGF-βRI, TGF-βRII, or tubulin, respectively. β-Actin and tubulin were used as a loading control. Experiments were repeated thrice with similar results. **b** and **c** Keloid fibroblasts were cultured and treated without or with different concentrations (100, 250, and 500 mg/L) of asiaticoside in DMEM medium containing 10% fetal bovine serum for 72 h. **b** Total mRNA was extracted from cells and subjected to RT-PCR using specific primers of TGF-βRI, TGF-βRII or actin, respectively. The *graph* is the mean densitometric data showing the level of TGF-βRI, TGF-βRII mRNA normalized to that of actin mRNA, respectively. $^*p < 0.05$ compared with the values of control (no asiaticoside). Experiments were repeated thrice with similar results. **c** Cell lysates were prepared and subjected to Western blotting with antibody against TGF-βRI, TGF-βRII or tubulin, respectively. The *graph* is the mean densitometric data showing the level of TGF-βRI, TGF-βRII protein normalized to that of tubulin protein, respectively. $^*p < 0.05$ compared with the values of control (no asiaticoside). Experiments were repeated thrice with similar results

has the potential of repressing fibroblast proliferation and collagen synthesis, thereby preventing the formation of keloids. TGF-β receptor ligands, TGF-β receptors, and Smads represent potential drug targets for the treatment of this fibrotic disorder. Recently, several studies have demonstrated that strategies targeted to antagonize TGF-β Smad signaling are effective in reducing wound scarring [21]. Neutralizing antibodies to TGF-β1 and TGF-β2 applied in rat incision wounds during the early phase has been shown to reduce scarring [22]. Other approaches used to antagonize the actions of TGF-β include TGF-β-binding proteins, such as decorin and biglycan [16], TGF-β

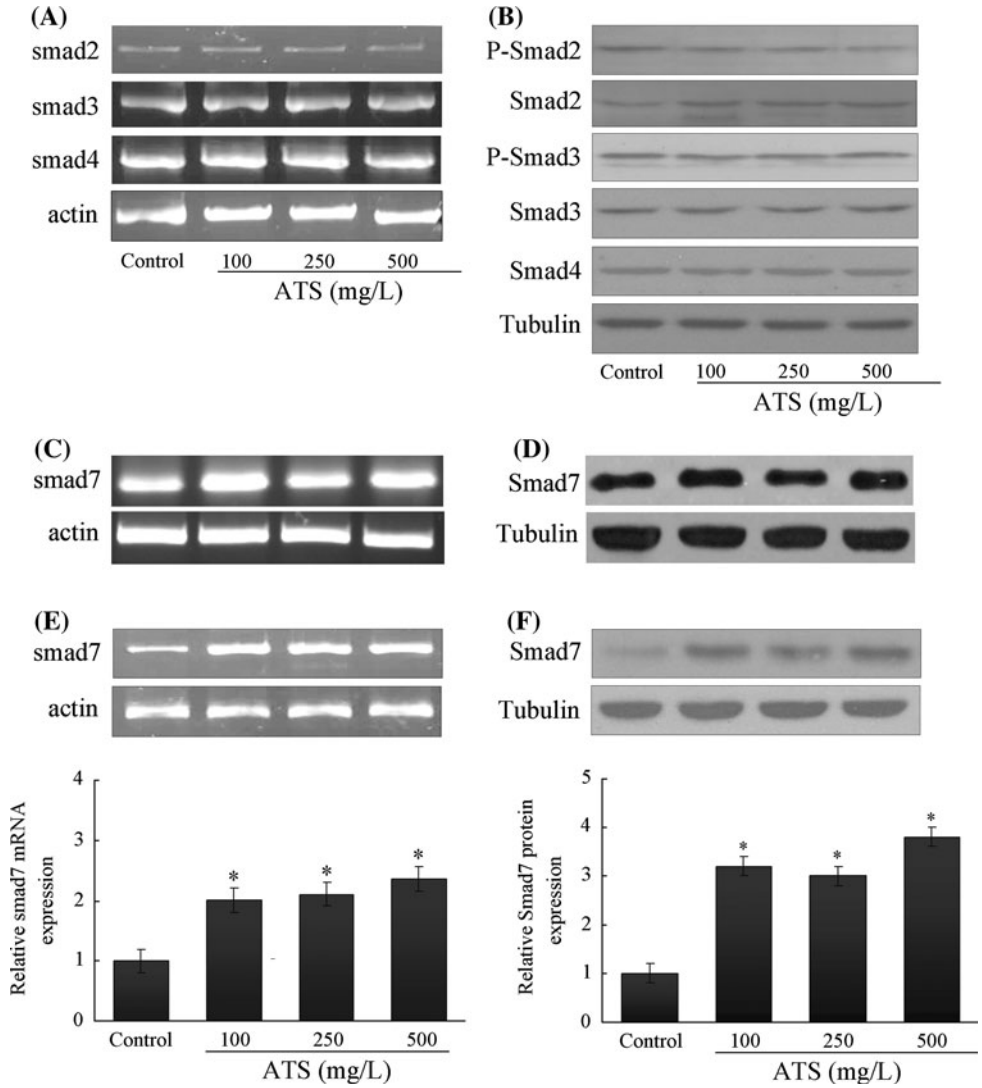

Fig. 5 Asiaticoside increases the expression of Smad7. However, asiaticoside does not influence Smad2, Smad3, Smad4, phosphorylated Smad2, and phosphorylated Smad3. Normal and keloid fibroblasts were cultured and treated without or with different concentrations (100, 250, and 500 mg/L) of asiaticoside in DMEM medium containing 10% fetal bovine serum for 72 h. **a** Total mRNA was extracted from keloid fibroblasts and subjected to RT-PCR using specific primers of Smad2, Smad3, Smad4 or actin, respectively. Experiments were repeated thrice with similar results. **b** Cell lysates from keloid fibroblasts were prepared and subjected to Western blotting with antibody against Smad2, p-Smad2, Smad3, p-Smad3, Smad4, or tubulin. Experiments were repeated thrice with similar results. **c** and **d** Total mRNA and cell lysates from normal fibroblasts were prepared and subjected to RT-PCR for Smad7 or actin (**c**), and to Western blotting with antibody against Smad7 or tubulin (**d**), respectively. **e**, **f** Total mRNA and cell lysates from keloid fibroblasts were prepared and subjected to RT-PCR for Smad7 or actin (**e**), and to Western blotting with antibody against Smad7 or tubulin (**f**), respectively. The *graph* is the mean densitometric data showing the level of Smad7 mRNA normalized to that of actin mRNA, and the level of Smad7 protein normalized to that of tubulin protein, respectively. $*p < 0.05$ compared with the values of control (no asiaticoside). Experiments were repeated thrice with similar results

activation inhibitor like mannose-6-phosphate (M6P) [21], and dominant negative TGF-β receptors (TGF-βR) [10]. In addition, several gene therapeutic approaches have used the overexpression of Smad7 to successfully inhibit the pro-fibrogenic effects of TGF-β [8, 29]. Our study showed that asiaticoside inhibited the protein and mRNA expressions of TGF-βRI and TGF-βRII and increased the protein and mRNA expression of Smad7 in a dose-dependent manner. However, asiaticoside did not alter Smad2, Smad3, Smad4, phosphorylated Smad2, and phosphorylated Smad3 levels. It is generally known that reduction of TGF-βRI expression may lead to the reduction of phosphorylated R-Smads (Smad2/3). In addition, Smad7 is known to play its inhibitory effect by competing with R-Smads for the binding to TGF-βRI, this also leads to decreased levels of Smad2/3 phosphorylation. However, in our study, asiaticoside just slightly inhibited the expression of phosphorylated Smad3 at 500 mg/L; there were no significant

differences compared with the control cells ($p > 0.05$). The specific mechanism must be studied further.

Another important observation of the present study is that asiaticoside inhibited keloid fibroblast proliferation in a time- and dose-dependent manner (Fig. 1). Although the precise mechanism by which asiaticoside reduces keloid fibroblast proliferation remains to be determined, previous studies have shown that asiaticoside can induce apoptosis of some tumor cells [14, 20]. Moreover, several studies have revealed that the regulation of apoptosis and proliferation of fibroblasts are altered in keloids. Keloid fibroblasts show a lower rate of apoptosis than normal fibroblasts [23]. Thus, we speculate that the ability of asiaticoside to inhibit keloid fibroblast proliferation may also be related to cytotoxicity. Previous studies have revealed that asiaticoside increases collagen synthesis in vitro and extracellular matrix accumulation in vivo [15, 24]. It enhances tensile strength in wound tissue, and accelerates the wound healing process. Therefore, we presume that asiaticoside is likely to have a dual role by promoting wound healing and preventing scar formation. However, the precise mechanism is unclear, and further studies are needed to answer this question.

In summary, the results from the present study provide evidences supporting that asiaticoside inhibits keloid-derived fibroblast proliferation and collagen synthesis. Our data show that asiaticoside can negatively regulate the expression of both TGF-βRI and TGF-βRII and increased the expression of Smad7, thereby altering fibroblast proliferation and collagen production. Overall, asiaticoside appears to have the potential to prevent keloid formation and excessive scaring.

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References

1. Chen SJ, Yuan W, Lo S, Trojanowska M, Varga J (2000) Interaction of smad3 with a proximal smad-binding element of the human alpha2(I) procollagen gene promoter required for transcriptional activation by TGF-beta. J Cell Physiol 183:381–392
2. Chen SJ, Yuan W, Mori Y, Levenson A, Trojanowska M, Varga J (1999) Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. J Invest Dermatol 112:49–57
3. Cheng CL, Guo JS, Luk J, Koo MWL (2004) The healing effects of Centella extract and asiaticoside on acetic acid induced gastric ulcers in rats. Life Sci 74:2237–2249
4. Chin GS, Liu W, Peled Z, Lee TY, Steinbrech DS, Hsu M, Longaker MT (2001) Differential expression of transforming growth factor-beta receptors I and II and activation of Smad 3 in keloid fibroblasts. Plast Reconstr Surg 108:423–429
5. Derynck R, Zhang YE (2003) Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577–584
6. Dienus K, Bayat A, Gilmore BF, Seifert O (2010) Increased expression of fibroblast activation protein-alpha in keloid fibroblasts: implications for development of a novel treatment option. Arch Dermatol Res 302(10):725–731
7. Dong MS, Jung SH, Kim HJ, Kim JR, Zhao LX, Lee ES, Lee EJ, Yi JB, Lee N, Cho YB, Kwak WJ, Park YI (2004) Structure-related cytotoxicity and anti-hepatofibric effect of asiatic acid derivatives in rat hepatic stellate cell-line, HSC-T6. Arch Pharm Res 27:512–517
8. Dooley S, Hamzavi J, Breitkopf K, Wiercinska E, Said HM, Lorenzen J, Ten Dijke P, Gressner AM (2003) Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. Gastroenterology 125:178–191
9. Goumans MJ, Mummery C (2000) Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. Int J Dev Biol 44:253–265
10. Gressner AM, Weiskirchen R (2003) The tightrope of therapeutic suppression of active transforming growth factor-beta: high enough to fall deeply? J Hepatol 39:856–859
11. Guo JS, Cheng CL, Koo MWL (2004) Inhibitory effects of *Centella asiatica* water extract and asiaticoside on inducible nitric oxide synthase during gastric ulcer healing in rats. Planta Med 70:1150–1154
12. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Wrana JL et al (1997) The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. Cell 89:1165–1173
13. Hsu YC, Chen MJ, Yu YM, Ko SY, Chang CC (2010) Suppression of TGF-β1/SMAD pathway and extracellular matrix production in primary keloid fibroblasts by curcuminoids: its potential therapeutic use in the chemoprevention of keloid. Arch Dermatol Res 302(10):717–724
14. Huang Y-H, Zhang S-H, Zhen R-X, Xu X-D, Zhen Y-S (2004) Asiaticoside inducing apoptosis of tumor cells and enhancing anti-tumor activity of vincristine. Ai Zheng 23:1599–1604
15. Kimura Y, Sumiyoshi M, Samukawa K-I, Satake N, Sakanaka M (2008) Facilitating action of asiaticoside at low doses on burn wound repair and its mechanism. Eur J Pharmacol 584:415–423
16. Kolb M, Margetts PJ, Sime PJ, Gauldie J (2001) Proteoglycans decorin and biglycan differentially modulate TGF-beta-mediated fibrotic responses in the lung. Am J Physiol Lung Cell Mol Physiol 280:L1327–L1334
17. Kopp J, Preis E, Said H, Hafemann B, Wickert L, Gressner AM, Pallua N, Dooley S (2005) Abrogation of transforming growth factor-beta signaling by SMAD7 inhibits collagen gel contraction of human dermal fibroblasts. J Biol Chem 280:21570–21576
18. Kryger ZB, Sisco M, Roy NK, Lu L, Rosenberg D, Mustoe TA (2007) Temporal expression of the transforming growth factor-Beta pathway in the rabbit ear model of wound healing and scarring. J Am Coll Surg 205:78–88
19. Leask A, Abraham DJ (2004) TGF-beta signaling and the fibrotic response. FASEB J 18:816–827
20. Lee YS, Jin D-Q, Kwon EJ, Park SH, Lee E-S, Jeong TC, Nam DH, Huh K, Kim J-A (2002) Asiatic acid, a triterpene, induces apoptosis through intracellular Ca^{2+} release and enhanced expression of p53 in HepG2 human hepatoma cells. Cancer Lett 186:83–91
21. Liu W, Wang DR, Cao YL (2004) TGF-beta: a fibrotic factor in wound scarring and a potential target for anti-scarring gene therapy. Curr Gene Ther 4:123–136
22. Lu L, Saulis AS, Liu WR, Roy NK, Chao JD, Ledbetter S, Mustoe TA (2005) The temporal effects of anti-TGF-β1, 2, and 3

monoclonal antibody on wound healing and hypertrophic scar formation. J Am Coll Surg 201:391–397
23. Luo S, Benathan M, Raffoul W, Panizzon RG, Egloff DV (2001) Abnormal balance between proliferation and apoptotic cell death in fibroblasts derived from keloid lesions. Plast Reconstr Surg 107:87–96
24. Maquart FX, Chastang F, Simeon A, Birembaut P, Gillery P, Wegrowski Y (1999) Triterpenes from *Centella asiatica* stimulate extracellular matrix accumulation in rat experimental wounds. Eur J Dermatol 9:289–296
25. Mook-Jung I, Shin JE, Yun SH, Huh K, Koh JY, Park HK, Jew SS, Jung MW (1999) Protective effects of asiaticoside derivatives against beta-amyloid neurotoxicity. J Neurosci Res 58:417–425
26. Mukhopadhyay A, Khoo A, Cheong HH, Chan SY, Aalami O, Lim IJ, Phan TT (2007) Targeting of Sp1 transcription factor: a novel therapeutic approach for keloids, an in vitro analysis. Exp Dermatol 16:1023–1031
27. Seifert O, Mrowietz U (2009) Keloid scarring: bench and bedside. Arch Dermatol Res 301(4):259–272
28. Shih B, Bayat A (2010) Genetics of keloid scarring. Arch Dermatol Res 302(5):319–339
29. Terada Y, Hanada S, Nakao A, Kuwahara M, Sasaki S, Marumo F (2002) Gene transfer of Smad7 using electroporation of adenovirus prevents renal fibrosis in post-obstructed kidney. Kidney Int 61:S94–S98
30. Verrecchia F, Mauviel A (2002) Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. J Invest Dermatol 118:211–215
31. Wolfram D, Tzankov A, Pülzl P, Piza-Katzer H (2009) Hypertrophic scars and keloids—a review of their pathophysiology, risk factors, and therapeutic management. Dermatol Surg 35:171–181
32. Yu H, Bock O, Bayat A, Ferguson MWJ, Mrowietz U (2006) Decreased expression of inhibitory SMAD6 and SMAD7 in keloid scarring. J Plast Reconstr Aesthet Surg 59:221–229
33. Yun K-J, Kim J-Y, Kim J-B, Lee K-W, Jeong S-Y, Park H-J, Jung H-J, Cho Y-W, Yun K, Lee K-T (2008) Inhibition of LPS-induced NO and PGE2 production by asiatic acid via NF-kappa B inactivation in RAW 264.7 macrophages: possible involvement of the IKK and MAPK pathways. Int Immunopharmacol 8:431–441