Triterpenoid saponin-rich fraction of *Centella asiatica* decreases IL-1β and NF-κB, and augments tissue regeneration and excision wound repair

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Abstract: Wound healing is an orderly process driven by numerous cellular mediators that aim to accelerate it. In skin conditions such as leprosy and psoriasis, *Centella asiatica*, which contains high amounts of triterpenoid saponins (TSs), has been reported to have beneficial effects. This study examined the effects of TS fraction of the herb on tissue regeneration and excision wound repair in rat skin. Briefly, TS fraction was prepared from *C. asiatica* and applied on the wound area for 7 consecutive days. The IL-1β, NF-κB, and other biochemical parameters were then estimated, followed by examination of inflammatory cell infiltration, epithelialization, angiogenesis, and general morphology of granulation (wound) tissue by histopathology. Treatment decreased the IL-1β and NF-κB, increased epithelialization and collagen synthesis, and decreased matrix metalloproteinases. Asiatic acid, the active ingredient in the fraction, increased cell viability and inhibited nitrite production in macrophage culture. Taken together, TSs augmented wound healing, which could be due to their effect on cellular mediators involved in the process, thus creating a microenvironment that could promote tissue repair and favor tissue remodeling. The fraction, in addition to its effect on excision wounds in rat skin, is speculated to have a similar role in accidental, surgical, and diabetic wounds.

Key words: *Centella asiatica*, triterpenoid saponins, wound healing, IL-1β, NF-κB, regeneration, repair

1. Introduction  
*Centella asiatica* (Linn.), also known as *'Brahmi'*, is a perennial herbaceous creeper belonging to the family *Umbelliferae*. It has been used in Indian traditional medicine to treat skin conditions such as leprosy and psoriasis, and to improve mental clarity (Somboonwong et al., 2012). The creeper has been included in the Indian pharmacopoeia for its healing property in the treatment of skin diseases such as leprosy, lupus, varicose ulcers, eczema, and psoriasis (Asakawa et al., 1982; Sampson et al., 2001; Coldren et al., 2003; Gohil et al., 2010). Apart from skin diseases, *C. asiatica* is also reported to provide relief in diarrhea, fever, and amenorrhea, and in the treatment of diseases of the female genitourinary tract (Brinkhaus et al., 2000; Gohil et al., 2010). Saponin-containing triterpene acids and their sugar esters, of which asiatic acid, madecassic acid, and asiaticosides are considered the most important, have been identified as substances of therapeutic potential in *C. asiatica* (Somboonwong et al., 2012). Asiatic acid (Figure 1), which is a chief ingredient in the alcoholic fraction (ethanolic extract) of the herb, has been reported to induce the expression of TNFAIP6, a hyaladherin that is involved in extracellular matrix remodeling and in modulating the inflammatory responses in humans (Coldren et al., 2003), thereby increasing collagen synthesis (Somboonwong et al., 2012) (which is a major component in wound healing). The other important ingredient in the extract, asiaticoside, has been reported to have healing properties in gastric ulcers and leprosy (Shukla et al., 1999).

The skin is the largest organ of the human body and is prone to various types of injuries, including surgical and accidental injuries such as burns, which not only increase the chance of infection, but also compromise skin aesthetics and homeostasis. In this study, we demonstrated the healing potential of the triterpenoid saponin-rich fraction (TSF) of *C. asiatica* on excision wounds inflicted on rat skin. The study has implications in the treatment of surgical, accidental, and diabetic wounds, as the fraction is reported to enhance tissue repair and regeneration by modulating the activity of proinflammatory cytokines,
augmenting epithelial cell proliferation, collagen deposition, and tissue remodeling, without the risk of side effects associated with the prolonged use of antimicrobials for wound treatment.

2. Materials and methods

2.1. Materials

The sources of biologicals and chemicals used in this study were as follows: the antimouse IgG (whole molecule) peroxidase, Ponceau stain, nonfat milk, d (+) N-acetylglucosamine, deoxyribonucleic acid, gelatin type A from porcine skin, bovine serum albumin, and Griess reagent were obtained from Sigma; the β-actin antibody mAb mouse was obtained from GenScript; the mouse anti-NF-κB p 65 came from Invitrogen; l (+) hydroxyproline and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertazolium bromide (MTT) came from SRL; the unstained protein ladder (3–250 kDa) was supplied by Genei; the prestained protein marker (10–170 kDa) was from Fermentas; Dulbecco's modified Eagle's medium (DMEM) came from Himedia; the fetal bovine serum (FBS) and antibiotic–antimycotic solution came from Gibco; and the asiatic acid standard and oleanolic acid standard came from Aldrich. All other reagents and chemicals used in this study were of the highest analytical grade procured from local commercial sources.

2.2. Procurement of the herb and TSF preparation

The TSF was prepared from the air-dried herb, Centella asiatica, procured from a local commercial supplier of medicinal herbs in Delhi, India. The plant was identified by a taxonomist and assigned a specimen voucher number, BRL/A-11/09; a small sample was kept in the department of biochemistry for future reference. The air-dried plant parts were crushed in a mixer grinder and about 50 g of the powdered plant was extracted with 70% aqueous ethanol in Soxhlet apparatus for 6 h, followed by vacuum evaporation. The material was concentrated using 500 mL of solvent and extracted with an equal volume of n-butanol. The material, which contained TSF, was further evaporated to dryness in a vacuum rotary evaporator and stored in presterilized airtight containers at 4 °C until further use.

2.3. Chemical fingerprinting of TSF

The isolated TSF (section 2.2) was characterized by high performance thin layer chromatography (HPTLC) using a silica gel 60 F254 plate. The solvent system consisted of chloroform, ethyl acetate, and formic acid in a 4:4:1 ratio. The thin layer chromatography (TLC) plate was sprayed with 5% anisaldehyde solution before heating on a hot plate at a constant temperature of 110 °C for 2 min; it was then scanned under computerized CAMAG TLC scanner 3 at 250–500 nm after bringing the plate to room temperature. The HPTLC chromatograms of the TSF and standard asiatic acid are shown in Figure 2.

2.4. Experimental protocol: wound infliction

The excision wound was inflicted on female rats, Wistar strain, weighing 120–180 g, obtained from the animal house facility of the institute. The animals were kept in controlled environmental conditions at 22 ± 3 °C and had free access to commercial pellet diet and water ad libitum. Utmost care was taken to minimize animal suffering and the number of animals in each group was kept at a minimum. In the absence of mortality and signs of toxicity, five animals were used in each group. The study was approved by the animal ethics committee and performed as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (constituted by the Government of India) guidelines for the care and use of animals in experimentation. Briefly, rats were divided randomly into groups (n = 5). The wound (excision) was created on the back of the animal with the help of a sterilized wound cutter, which was produced in our lab. The animals were anesthetized by injecting ketamine hydrochloride, 40 mg/kg of body weight intramuscularly, before inflicting the wound. The size of the wound was 14 mm in diameter, which was equivalent to a wound area of 153.93 mm². Following the infliction of the wound on the animal skin (Figure 3), the animals were allowed to recover from anesthesia and were housed in sterile polypropylene cages. TSF (25 mg/rat) was applied on the wound daily for 7 days. The wound in both treated groups and untreated controls, were left uncovered throughout the experiment. After the completion of 7 days of topical application, the animals were subjected to wound examination and the percentage of reduction in the size of the wound was calculated. Finally, the animals were sacrificed to obtain the tissue for further investigations.

2.5. Preparation of samples for wound analysis

The rats were randomly divided into groups (n = 5) as follows: Group 1, normal (without a wound); Group 2, inflicted with a wound; Group 3, treated with TSF; Group

Figure 1. Structure of asiatic acid.
4, SFM treated, containing 0.5 mg of the active ingredient framycetin sulphate. For the analysis of the wound, the granulation tissue (site of wound) was taken up on the completion of treatment (7 days of topical application of TSF in the experimental group) and subjected to biochemical and histological examination.

2.6. Biochemical estimations

Hydroxyproline and hexosamine were determined according to the procedure described by Neuman and Logan (1950). Briefly, the skin tissue was dried in an oven at 60 °C for 12 h, homogenized (Heidolph DIAX900) in ultrapure water, and hydrolyzed by autoclaving in the presence of 2.5 N NaOH at 120 °C for 20 min, followed by the addition of CuSO₄ (0.01 M), NaOH (2.5 N), and H₂O₂ (6%) to the hydrolyzed sample. The sample was kept at 80 °C in a water bath for 5 min, and then chilled in ice cold water before adding conc. H₂SO₄ (3 N) and freshly prepared para-dimethylaminobenzaldehyde (PDMAB). Finally, the mixture was incubated at 70 °C for 10 min and the color of the supernatant was recorded at 540 nm. For hexosamine estimation, acetyl acetone was added to the hydrolyzed tissue and the tube was boiled for 40 min. The tube was cooled and ethanol and freshly prepared PDMAB were added to it. The mixture was vortexed and incubated at 70 °C in a water bath for 10 min. The pink color of the solution was read at 530 nm. The protein in the sample was estimated by the method described by Lowry et al. (1951).

2.7. Gelatin zymography for matrix metalloproteinases (MMPs)

Briefly, tissue was homogenized in trisphosphate buffer (50 mM, pH 7.5) containing 0.01 M CaCl₂ and 0.25%
triton X-100. The homogenate was centrifuged for 15 min at 8000 rpm and 4 °C. The supernatant was aspirated and further centrifuged at 50,000 rpm for 15 min at 4 °C for analysis. The protein content of the sample was measured by Lowry’s method. For zymography, gelatin substrate gels were prepared by incorporating gelatin (1 mg/mL) in 10% polyacrylamide. The protein (50 µg) was mixed with 2X sample buffer and the sample was loaded onto the gels without boiling. Following electrophoresis under nonreducing conditions, the gel was washed for 30 min at room temperature in 2.5% triton X-100 and subsequently incubated twice for 30 min at room temperature in a developing buffer consisting of 1 mM CaCl₂ in 0.1 M tris-HCl (pH 7.5). The gel was then incubated for 18–20 h at 37 °C in the developing buffer. Gels were stained with CBR 250 (Coomassie brilliant blue R250) for 2 h and destained in 10% acetic acid, 45% methanol, and 45% ultrapure water. A clear zone of lysis, which appeared against a blue background, represented MMPs. MMP 2 and 9 were identified with the help of a molecular weight marker loaded in a separate lane.

2.8. Western blot analysis
A small piece of the tissue taken from the site of the wound was homogenized in the nuclear fraction extraction buffer containing 20 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mM MgCl₂, 0.3 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 20% glycerol, 1 mM dithiothreitol (DTT) and 10 µL/mL protease inhibitor (Sigma). Tissue was homogenized in a Polytron homogenizer with four strokes of 15 s each in ice bath. After homogenization, the sample was allowed to swell on ice for 30 min and then centrifuged at 20,000 × g for 15 min at 4 °C. The total protein in the supernatant was measured according to Lowry's method and analyzed by PAGE, with 4% (v/v) stacking and 10% (v/v) separation gels in the presence of Nonreducing conditions, the gel was washed for 30 min at room temperature in 2.5% triton X-100 and subsequently incubated twice for 30 min at room temperature in a developing buffer consisting of 1 mM CaCl₂ in 0.1 M tris-HCl (pH 7.5). The gel was then incubated for 18–20 h at 37 °C in the developing buffer. Gels were stained with CBR 250 (Coomassie brilliant blue R250) for 2 h and destained in 10% acetic acid, 45% methanol, and 45% ultrapure water. A clear zone of lysis, which appeared against a blue background, represented MMPs. MMP 2 and 9 were identified with the help of a molecular weight marker loaded in a separate lane.

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2.9. Cytokine analysis
The proinflammatory cytokines, IL-1β and TNF-α, were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. Briefly, the ELISA plate was coated with a coating buffer. The plate was kept at 4 °C overnight. Serum and standards were added to a 96-well plate coated with antibody and incubated at 4 °C overnight. Each well was washed with the washing buffer and HRP conjugated detection antibody was added to each well; the plate was incubated at room temperature and washed once again. The plate was incubated with streptavidin solution at room temperature followed by TMB one step substrate reagent at room temperature. The reaction was stopped by 2 M H₂SO₄. The optical density of the solution was taken at 450 nm. The sample concentration was interpolated from standard curves. Results were expressed as pg/mL.

2.10. Histopathology of the granulation tissue
Briefly, the granulated tissue (wound site) was cut into a few millimeter thick pieces, fixed in buffered formalin (10%), and processed as per the standard procedure for the preparation of histopathological slides. The sections were stained with hematoxylin–eosin (HE) and Masson’s trichrome (MT) stains for light microscopic analysis.

2.11. Cell viability and nitrite assays
The cell viability and nitrite assays were performed on RAW 264.7 macrophage cells in the presence of different concentrations of asiatic acid. Cell viability was determined as per the procedure described by Yun et al. (2008). The cell line, RAW 264.7, was obtained from the National Centre for Cell Science (NCCS), Pune. Cells were grown at 37 °C in DMEM supplemented with 10% FBS and antibiotic–antimycotic solution (1.5%) in an atmosphere of 5% CO₂ (humidified). Cells were first seeded in culture media in a 96-well plate at a concentration of 1 × 10⁴ cells per well for 24 h, and then with different concentrations (40, 80, or 160 µM) of asiatic acid. The media were removed after 24 h and cells were incubated with MTT (5 mg/mL) for 45 min. The MTT was removed and purple formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO); the optical density of the solution was taken after 2 h at 570 nm.

Nitrite, which accumulated in the culture medium, was measured as an indicator of nitric oxide production. Cells were first seeded in phenol-free culture media in a 24-well plate at a concentration of 2 × 10⁴ cells/well for 24 h. After 24 h of incubation, the cells were incubated with different concentrations (40, 80, or 160 µM) of asiatic acid and challenged with lipopolysaccharide (LPS) (1 µg/mL) after an hour. A few wells were treated with LPS alone as well. After 24 h, media were removed in a microcentrifuge tube and centrifuged at 3000 rpm for 5 min to settle down any cells or precipitate. Supernatant was used to determine the concentration of nitrite by the Griess reagent system. Briefly, Griess reagent (0.4 mg/mL) was added to the supernatant in dark and, after 10 min, absorbance was taken at 540 nm using a microplate reader. Fresh culture
medium was used as a blank in all experiments. The amount of nitrite in the sample was measured with the help of a standard curve prepared by sodium nitrite serial dilution.

2.12. Statistical analysis
Data were analyzed using one-way ANOVA and subjected to Dunnett’s post hoc test for multiple comparisons using GraphPad InStat Version 3.10 package. Differences in means between paired observations were accepted as significant at \( P < 0.05 \).

3. Results
3.1. HPTLC fingerprint analysis of TSF
The chemical fingerprint of TSF by HPTLC revealed the presence of triterpenoid asiatic acid in the sample, which was identified by running asiatic acid standard, as shown in Figure 2. The other saponins as reported to be commonly present in *C. asiatica* included asiaticoside, madecosside, and madecassic acid (Singh et al., 1969).

3.2. Assessment of healing efficacy of TSF: effect on wound closure and biochemical parameters
Wound healing efficacy of TSF was determined by measuring the contraction of the wound and other general parameters (Table) on day 7. The size of the wound decreased considerably in TSF treated rats. There was an almost 90% reduction in wound size in TSF rats when compared with the untreated wounds (80%). In the Soframycin (SFM) treated group, the reduction in wound size was 85.59%, i.e. less than the value reported in the TSF rats. The hydroxyproline content of the granulated tissue in the TSF rats was 27.94 ± 2.04 mg/g, which was considerably more than that observed in the untreated control group (20.99 ± 3.46). In the normal skin tissue, the hydroxyproline content was 48.80 ± 0.32 mg/g. Similarly, hexosamine, which decreased in the untreated group, 22.12 ± 0.06 vs. 37.78 ± 1.00 mg% in normal skin tissue, increased to 24.21 ± 0.44 mg% in the TSF rats. The hexosamine content, however, was more, 27.78 ± 0.58 mg%, in the SFM rats, in comparison to the TSF treated rats. In the TSF rats, the protein content increased considerably (75.70 ± 2.44 mg/g), which was more than the value reported in the SFM group (69.40 ± 2.23 mg/g). The protein content of the normal rat skin is reported to be 98.98 ± 1.70 mg/g, which decreased to 41.72 ± 1.48 mg/g in the untreated wounds.

3.3. Histopathology of the wound tissue
The changes in tissue histopathology were graded as per the grading system adopted by Nayak (2006). As shown in Figure 4a, an organized epithelization and granulation tissue arrangement (2.75/3) was evident in the TSF rats. Inflammatory cell infiltration, edema, and congestion were much less in the TSF treated rats as compared with the untreated rats. Collagen deposition and fibroblast deposition were moderate, 2 on a scale of 0–3, in the TSF rats. This was somewhat similar to what was observed in the SFM treated rats. The organization and maturation of collagen and fibroblast in dermis was also determined by MT staining (Figure 4b). Collagen deposition was abundant in the TSF rats when compared with other groups.

Table. Parameters associated with wound healing.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal animal skin (without wound)</th>
<th>Untreated excision wound</th>
<th>TSF treated</th>
<th>Treated with Soframycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound closure (%)</td>
<td>-</td>
<td>80.44 ± 2.06</td>
<td>88.80 ± 2.21*</td>
<td>85.59 ± 1.39*</td>
</tr>
<tr>
<td>Hydroxyproline (mg/g)</td>
<td>48.80 ± 0.32*</td>
<td>20.99 ± 3.46</td>
<td>27.94 ± 2.04*</td>
<td>25.25 ± 5.33*</td>
</tr>
<tr>
<td>Hexosamine (mg%)</td>
<td>37.78 ± 1.00*</td>
<td>22.12 ± 0.06</td>
<td>24.21 ± 0.44*</td>
<td>27.78 ± 0.58*</td>
</tr>
<tr>
<td>Protein (mg/g)</td>
<td>98.98 ± 1.70*</td>
<td>41.72 ± 1.48</td>
<td>75.70 ± 2.44*</td>
<td>69.40 ± 2.23*</td>
</tr>
<tr>
<td>Collagen/Fibroblast (MT)</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>Epithelization</td>
<td>3</td>
<td>0.25</td>
<td>2.75</td>
<td>1</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>3</td>
<td>1.75</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>0</td>
<td>1.75</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Fibroblast (HE)</td>
<td>3</td>
<td>1.5</td>
<td>1.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Collagen, epithelization, angiogenesis, inflammatory cells, and fibroblast were measured by histopathology on a scale of 0–3, where 0 means none, 1 is slight, 2 is moderate, and 3 is abundant. *Values represent mean ± SD (n = 5); P < 0.05 when compared with the untreated control is considered significant. TSF: triterpenoid saponin-rich fraction.
3.4. MMPs
MMPs play a role in each phase of wound healing and remodeling of the granulation tissue. There was a reduced expression of MMP 2 and MMP 9 (Figure 5) in treated rats, suggesting wound maturation.

3.5. Cytokines and transcription factor, NF-κB
TSF decreased the expression of IL-1β, which increased significantly in the wound tissue (Figure 6). The level of TNF-α, however, was more in the TSF treated rats when compared with the wound tissue (Figure 6). NF-κB decreased in TSF treated skin (wound) tissue (Figure 7). In SFM treated animals, the reduction in NF-κB was less, indicating a better response in the presence of TSF.

3.6. Cell viability and nitrite assay
The cell viability increased in the presence of asiatic acid at 40 and 80 µM concentrations with respect to the control group (Figure 8). Viability, however, decreased at 160 µM (data not shown), indicating some cytotoxicity at higher concentrations. Asiatic acid inhibited nitrite production at 40 or 80 µM, but increased the (nitrite) level at 160 µM (data not shown).

4. Discussion
The disruption in skin integrity, or the infliction of a wound, may lead to a number of complications, including infection and altered homeostasis. Wound healing deploys a series of unique cascading functions that mainly consist of hemostasis and regeneration (Schilling, 1968). Hemostasis, being the first stage of acute wound healing, involves the formation of a provisional wound matrix within a few hours after injury (Reinke and Sorg, 2012), followed by the inflammatory phase, leading to the recruitment of neutrophils and then monocytes. The regeneration phase includes transformation and proliferation, where the proliferation phase mainly focuses on healing, leading to wound recovery, granulation tissue formation, and restoration of the vascular network. Inflammation, which usually peaks around day 2 of injury, is followed by the proliferation phase, which peaks around day 4, and remodeling, which peaks around day 20 of the injury (Palagummi et al., 2014). In the process, the skin restores its cellular structures and tissue layers in the damaged area, resulting in the formation of granulation tissue, which is characterized by high protein content. The reduction in the area of the wound is a characteristic feature of healing. Although it continues throughout the healing, the reduction is most prominent in the final phase, i.e. the maturation phase. In this study, we observed a significant increase in protein content of the wound tissue treated with TSF. The protein content of the wound tissue in TSF rats was 76 mg/g of tissue, which was significantly higher when compared with the untreated (42 mg/g) or SFM treated (69 mg/g) wounds. There was also a significant reduction in the time taken for wound closure in the TSF treated rats. As shown in the Table, on day 7 of the treatment, wound closure was 89% when compared with the untreated wounds (80%). The reduction in the SFM treated rats was 85.59%, which was less than the value observed in the TSF rats.
Figure 5. Gelatin zymography for matrix metalloproteinases. NS: Normal skin tissue; WT: Wound tissue; TSF-CA: Triterpenoid saponin-rich fraction of *C. asiatica*; SF: Soframycin treated rats; and M: Marker proteins (170–10 kDa). Animals were treated for 7 days. Zymography was performed in 10% SDS containing 1 g/L gelatin.

Figure 6. IL-1β and TNF-α in the wound tissue after the treatment (topical application) of *Centella asiatica*. NS: Normal skin tissue; WT: Wound tissue; TSF-CA: Triterpenoid saponin-rich fraction of *C. asiatica*; SF: Soframycin treated rats. *Values represent mean ± SD (n = 5); P < 0.05 is considered significant, when compared with the WT.

Figure 7. NF-κB p65 in the wound tissue and control rats. NS: Normal skin tissue; WT: Wound tissue; TSF-CA: Triterpenoid saponin-rich fraction of *C. asiatica*; SF: Soframycin treated rats. The β-actin was used as a loading control.
The rate of epidermal closure depends on a number of parameters, including age and sex. In females, for example, estrogen has been reported to affect the process of healing by regulating a variety of genes associated with regeneration, matrix production, epidermal function, and the genes primarily associated with inflammation (Hardman and Ashcroft, 2008). The higher rates of healing and regeneration reported in this study in both control and treated rats can be attributed to the age and sex of the animals. The contraction in TSF treated wounds was statistically significant (P < 0.0001) when compared with the untreated wounds, indicating the role of the drug in augmenting the healing process.

The granulation (wound) tissue is primarily composed of fibroblasts, collagen, and new small blood vessels. Collagen is a major component of the skin tissue, and strengthens and supports the extracellular matrix. Earlier studies have reported a rapid synthesis of collagen postwounding (Hayouni et al., 2011). Hydroxylation of proline (by prolyl hydroxylase) is the first step in collagen biosynthesis (Shukla et al., 1999). The amount of hydroxylated proline (OHPro) has been used as an index for tissue collagen (Kumar et al., 2006). In this study, OHPro content of the wound tissue measured on day 7 of inflicting the wound decreased to less than half of the value reported in normal skin tissue. TSF caused a significant increase in OHPro. The response was better than the one reported with SFM (the standard control drug used in this study). The TSF treated rats further showed an increase in hexosamine, which also acts as a ground substratum for the synthesis of a new extracellular matrix. Increase in hexosamine has been reported in the early stages of wound healing, following which its normal level is restored (Shukla et al., 1998).

Histopathology of the wound tissue corroborated the biochemical findings with regard to an increase in collagen in the TSF treated wounds. On a scale of 3, the collagen in the TSF treated rats was 2. There was also a remarkable effect of TSF on epithelization of the wound tissue. TSF induced a marked increase in epithelization (2.75) when compared with the untreated wounds (0.25) or the wounds treated with SFM on a scale of 3, as shown in the Table. The fibroblast cells in the SFM treated rats were, however, more when compared with the TSF treated rats. There was no
significant difference observed with regard to angiogenesis in either the TSF or SFM treated rats, indicating that neither of the drugs promoted the formation of new blood vessels.

The number of inflammatory cells increases in the wound tissue (Hayouni et al., 2011; Nayak et al., 2011). Upon the infliction of a wound, the physiological and gross molecular events that take place in the wounded tissue include a primary clot formation, which occurs within 1–3 days, followed by epidermal edges activation and early inflammatory response characterized by neutrophil accumulation at the wound gap (Braiman-Wiksman et al., 2007). Four to 7 days postwounding is marked by 60%–70% epidermal wound closure, having features like scab formation due to the migration of the epidermal edges and the proliferation of the early granulation tissue. All this is followed by tissue remodeling and differentiation to enable full recovery of the skin tissue and the restoration of skin aesthetics. In all groups in this study, the least number of inflammatory cells were reported in the TSF treated wounds, indicating an involvement of inflammatory mediators (IMs) such as interleukin (IL)-1β. The proinflammatory cytokines function at low concentrations and modulate the wound healing directly or indirectly due to stimulation of neutrophils, macrophages, fibroblast chemotaxis, growth of fibroblasts and keratinocytes, and the reorganization of the extracellular matrix during the proliferative stage (Guo and Di Pietro, 2010). The infliction of a wound causes an increase in IL-1β and TNF-α. In the present study, we observed that TSF decreased the expression of IL-1β. The level of TNF-α, however, increased in the TSF treated rats. The increase can be attributed to the ability of IL-1β to augment TNF-α-mediated inflammatory responses (Saperstein et al., 2009). Moreover, the TNF-α is chemotactic for macrophages and activates these cells in vitro (Riches et al., 1996) in the proliferative stage of healing. An increase in TNF-α, therefore, is important in inducing earlier inflammation and prompt removal of pathogens invading the wound. IL-1β, on the other hand, is reported to be a part of a proinflammatory positive feedback loop that sustains a persistent proinflammatory wound macrophage phenotype, which contributes to impaired wound healing. Blocking the IL-1β has been reported to improve healing (Mirza et al., 2013). The TSF in our study appears to target the IL-1β pathway and, therefore, represents a new therapeutic approach in improving healing. NF-κB is a regulator of IL-1β secretion (Coggswell et al., 1994; Greten et al., 2007). The present study reports an increase in NF-κB, which was significantly brought down after the treatment of with TSF. SFM did not decrease the expression level of NF-κB, indicating that TSF accelerated the healing of the wound by targeting the pathways involving the transcription factor NF-κB. We also measured the release of nitric oxide by LPS-primed macrophages in this study in the presence or absence of asiatic acid, a key ingredient in TSF. In vitro experiments conducted on RAW cell culture showed that asiatic acid inhibited the release of nitric oxide. It caused an inhibition in the level of nitric oxide at 40 and 80 µM concentrations; the nitrite concentration, however, increased at 160 µM (data not shown). Asiatic acid, when compared with the untreated controls, also caused an increase in cell viability at 40 and 80 µM, although viability decreased slightly (95%) at 160 µM (data not shown).

The normal wound healing is an orderly sequence of events passing through the inflammatory, proliferation, and remodeling/maturation phases. During the maturation of the wound, the extracellular matrix undergoes certain changes, followed by scar formation, which marks the physiological endpoint of healing. The endpoint is also directly proportional to the extent of inflammatory processes occurring throughout the healing. The MMPs, which have an important role in tissue remodeling, eliminate damaged protein, destroy the provisional extracellular matrix, facilitate its migration to the center of the wound, remodel the granulation tissue, regulate the activity of some growth factors, and probably control angiogenesis (Gill and Parks, 2008). In our study, MMPs, MMP 2 and MMP 9, decreased in the wound tissue treated with TSF. MMP 2 and 9 regulate the matrix proteins turnover by degrading basement membrane proteins, which include collagen IV and V, gelatin, elastin, and fibronectin (Swarnakar et al., 2005). The amount of active MMP 9 is inversely correlated with the wound closure rate. That is, high levels of active MMP 9 correlate with lower wound closure rates (Henshaw et al., 2015). Studies have shown that MMP 9 activity is an important contributor in delaying healing (Gibson et al., 2009). In the untreated wound, we observed a significantly high level of MMP 9, suggesting delayed healing. The level reduced considerably in the granulation tissue of rats treated with TSF. The increase can be correlated with the increase in TNF-α in the TSF group. TNF has been reported to stimulate the activation of MMP (pro-MMP2) in human skin through NF-κB mediated induction of MT1-MMP (Han et al., 2001).

Epithelial cell proliferation in the process of healing in TSF treated rats was an important finding in this study, indicating the potential of the drug in accidental, surgical, and diabetic wound. In its initial stages (0–7 days), wound contraction and healing depend on a number of factors, including blood clotting, epidermal edge activation, early inflammation, epidermal edge migration, keratinocyte migration, and granulation tissue proliferation. Reepithelialization is the basis for subsequent healing stages.
like fibroblast migration, followed by the granulation tissue formation and collagen synthesis (Bráiman-Wiksman et al., 2007). The early epidermal migration is preceded by inflammatory cell infiltration of neutrophils, macrophages, and lymphocytes (Guo and Di Pietro, 2010). Studies have linked fibroblast proliferation to early granulation tissue formation and completion of reepithelialization, signifying the importance of epithelialization in the early stages of healing (Andresen et al., 1997).

Compounds of herbal origin such as silymarin (obtained from \textit{Silybum marianum}) have been reported to increase epithelialization and decrease inflammation; however, silymarin, unlike TSF, did not show a significant effect on wound contraction, collagenization, or OHPro level (Sharifi et al., 2012). As discussed above, the increase in epithelialization in the presence of TSF may be attributed to its effect on IL-1β and NF-kB, which led us to speculate on the effect of TSs in the healing of diabetic wounds. In diabetes, the impairment of acute wound healing is reported to be mainly due to hypoxia and insufficient angiogenesis (Tandara and Mustoe, 2004). Hypoxia amplifies the early inflammatory response, leading to an increase in reactive oxygen species, and thus prolonging the repair (Woo et al., 2007). In our study, we found that the drug decreased IL-1β, an inflammatory mediator in the initial stages of wound healing, and also NF-kB, which thereby accelerated the approach to the proliferative stage of healing, as reported by others (Greten et al., 2007).

Considering the emergence of the IL-1β pathway as a possible target of triterpenoid saponins in early wound repair, we speculated about the role of the drug (TSF) in the repair of diabetic wounds.

Phytomedicine remedies possess significant pharmacological effects. These remedies are popular amongst the general population in regions all over the world. A number of phytotherapeutic agents, including \textit{Aloe vera}, \textit{Mimosa}, \textit{Echinacea}, grape vine, chamomile, ginseng, green tea, jojoba, tea tree oil, rosemary, lemon, soybean, comfrey, papaya, oat, garlic, ginkgo, olive oil, and ocmium have been reported for their wound healing properties (Pazyar et al., 2014). Triterpenoids (de León et al., 2005) and flavanones (Tsuchiya et al., 1996) in various substances of herbal origin have been reported to promote healing mainly due to their astringent and antimicrobial properties (Singh et al., 2006), which appear to be responsible for wound contraction and an increased rate of epithelization. The healing property of \textit{Cecropia peltata} (Nayak, 2006) and \textit{P. lanceolata} (Nayak et al., 2005) has been attributed to the presence of triterpenoids. The healing effect of \textit{C. asiatica} may also be subsequent to the astringent and antimicrobial effect. Various other properties of the saponins in the plant extract, such as TSF, may also contribute to its healing potential. Saponins have earlier been reported to prevent UVA-mediated photoaging, glutamate-induced neurotoxicity, and also for their antiulcer and antioxidant activities, in addition to their antihepatofibric activity and cytotoxic effects at high concentration (Shukla et al., 1999; Yun et al., 2008). The observed effect of TSF in this study may be attributed to the effects of saponins on human physiology, particularly on some of the mediators of inflammation and innate immunity.

Taken together, the results of this study indicate that the triterpenoid saponin-rich fraction of \textit{C. asiatica} accelerates healing by targeting the pathways involved in inflammation and innate immunity. The drug increased epithelization and collagen synthesis, and decreased matrix metalloproteinase expression in the granulation tissue, thus promoting regeneration in accidental, surgical, and diabetic wounds.

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References


