

THE ENHANCED EPITHELIALIZATION POTENTIAL OF THE AQUEOUS EXTRACT OF *PRUNELLA VULGARIS* IN THERMALLY INJURED RATS

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Abstract: The aim of the present study was to assess the wound-healing efficiency of the *Prunella vulgaris* extract (PVE) on thermally injured rats. Sixty adult, female rats were divided into four groups, with group I serving as a sham control (without a burn injury). A burn was inflicted on the dorsum of all the rats in the second, third and fourth groups. Group II rats served as the burn-injury control group without any treatment. The burn-injured areas of the rats in the third and fourth groups were treated topically by 1% silver sulfadiazine (SSD) cream or 10% PVE-based cream, respectively. Biochemical (antioxidant, lipid peroxidation, hydroxyproline, mucopolysaccharide), morphological and histopathological evaluations showed the burn-healing efficiency of PVE to be better than that of the SSD group. In conclusion, topical application of PVE enhanced collagen synthesis and antioxidant levels by suppressing lipid peroxidation as well as increasing proliferation of keratinocytes, and thus positively influenced wound-healing.

Key words: *Prunella vulgaris*; burn injury; silver sulfadiazine; histopathology; collagen

INTRODUCTION

Thermal injury is an acute wound result from exposure to heat and chemicals. Burn injuries represent a significant health burden all over the world. Every year, there are 18.1 burn injuries per 100,000 individuals resulting in hospitalization (Kalson et al., 2012). Burn wounds are associated with a significant risk of mortality, as well as significant physical, functional and psychiatric sequela in survivors. Therefore, burn wounds represent a significant effect to patients, health care professionals, and the health care system (Brewster et al., 2013). Wound healing is a complex process during which multiple epithelial and stromal tissue elements interact to reconstitute an injured organ. At the onset of epithelial injury, the activation of multiple signaling pathways initiates a cascade of events that reestablish tissue architecture and barrier

function. The process of tissue regeneration and repair after epithelial injury can be divided into three sequential phases: inflammation, proliferation (epithelialization) and remodeling (Guo and DiPietro, 2010; Gurtner et al., 2008). The common treatment regime for burn wounds would be a topical application of a medicament to enhance wound healing by minimizing free radical generation and inflammatory reaction as well as to prevent opportunistic infections by microbes.

At present, several synthetic drugs are available in the market for treating wounds, but they also cause severe allergic reaction and drug resistance (some bacterial infection), which drew scientists to focus on natural remedies with no or only a few adverse effects (Milovanović et al., 2014). Several studies have shown that plant extracts rich in phytochemicals, such as

phenolic acids, flavonoids, triterpenes, alkaloids and tannins, might positively influence the healing process owing to their strong antioxidant activities by scavenging the highly reactive free radicals, which are involved in delaying the wound-healing process (Manca et al., 2015). In addition, those phytochemicals can also act as anti-inflammatory and antimicrobial agents.

Prunella vulgaris L. (Labiatae) is a perennial herb that has been used in traditional medicine in China and western countries for treating sore throat, fever, for accelerating wound healing as well as for dermatosis and skin allergies. In China, *P. vulgaris* is called 'Xia Ku Cao' and has a long history in therapeutic use as an antipyretic, and more recently for antikeratitis purposes (Kim et al., 2007). It was likewise employed as a major additive component herb in the Chinese traditional functional beverage Guangdong cool tea (Cheung and Zhang, 2008). In South Korea, *P. vulgaris* is applied to patients with goiter, dermatitis and skin allergy (Sun et al., 2005). In addition, it also exhibits antioxidative, anti-allergic, anti-inflammatory and antimicrobial activities (Ryu et al., 2000; Psotova et al., 2003). Some studies also indicated its usefulness in the treatment of atopic dermatitis (Lee et al., 2008) and in providing photoprotection (Vostálová et al., 2010).

Phytochemical studies indicate that *P. vulgaris* is rich in phenolics such as rosmarinic, ellagic, ursolic and caffeic acids and flavonoids (quercetin), saponins and triterpenes (Vostálová et al., 2010). Rosmarinic acid (RA), the main phenolic acid constituent, is known for its wide-ranging biological activities, i.e. antioxidative, anti-inflammatory, antimutagenic, antimicrobial (Skottova et al., 2004; Petersen and Simmonds, 2003). Ursolic acid, a triterpene constituent of PVE, has been described as an effective inhibitor of UVA-modulated expression and activation of metalloproteinase 2, whose increased level is a visible marker of destruction during skin photo-aging (Lee et al., 2003). Hence, the present study was designed to verify the wound-healing activity of PVE attributed to its various phenolic compounds with antioxidant, antimicrobial and anti-inflammatory activity in a thermally injured rat model.

MATERIALS AND METHODS

Plant material

P. vulgaris were collected from Shandong, PR China, in August 2014 and authenticated by Professor Xiao-Ming Xin of the Taishan Medical University in China. A voucher specimen (no. PV23407) was deposited in the Herbarium of the Department of Pharmacology, Taishan Medical University, China.

Aqueous *P. vulgaris* extract

Two hundred g of dried whole plant material was added to 1000 mL of deionized water and incubated at 100°C for 50 min and the resulting aqueous extract was filtered using Whatman filter paper. The residue from the filtration was extracted again by the same procedure. The filtrates obtained were combined and then lyophilized by freeze dryer and stored at -20°C before the study. The final yield of aqueous extract was 28%.

Experimental animals

Sixty female albino Sprague-Dawley (SD) rats procured from the Central Animal House Block, China, weighing 250±20 g were used in the study. The animals were housed at 22±3°C, 55±5% humidity, 12 h light/dark cycles with *ad libitum* access to food and water. Rats were allowed to adapt to their environment conditions for at least 10 days before initiation of the experiment. Animal experiments were conducted according to Institutional Animal Ethics Committee (Reg. No.14/2014/CPCSEA) guidelines and in compliance with the Animal Welfare Act, implementing Animal Welfare Regulations and the principles of the Guide for the Care and Use of Laboratory Animals.

Induction of burn injury

Partial thickness burns were induced on overnight-starved rats under anesthesia by intraperitoneal administration of sodium pentobarbitone ($n=6$ per group), shaving on the dorsum (dorsal side) and burn-

ing with a square steel plate (1.5 cm²) heated at 100°C, for 10 s to create (Haghdoost et al., 2013). Postoperative pain was treated with acetaminophen for three days.

Experimental design

The 60 adult, female SD rats were divided into four groups. Group I served as a negative control group ($n=6$), without any burn injury. Group II served as a burn-injury control group ($n=18$) and did not receive any treatment (positive control). The thermally injured areas of the rats in groups III and IV were treated topically with 0.5 g of 1% silver sulfadiazine (SS) cream ($n=18$) and 0.5 g of 10% (w/w) PVE-based cream ($n=18$), respectively. Six rats each of groups II, III and IV were killed by intraperitoneal administration of sodium pentobarbitone (100 mg/kg bwt) at three different stages (3rd, 7th and 14th day) to assess the progress of wound healing of each treatment group by comparing with group II. The harvested wound was bisected into half, one portion was embedded in paraffin for histological analysis and the other half stored at -80°C for biochemical analyses. The stored wound tissue was homogenized (10%) in 0.02 M potassium-phosphate buffer, pH 7.6 (1:10 w/v) and centrifuged at 3000xg at 4°C. The clear supernatant was used for further analyses.

Wound contraction rate and re-epithelialization

Wound contraction was calculated as a percentage of the original wound size by using wound image. Briefly, at time intervals ranging from 0 through 14 days after wounding, wounds were photographed using a digital camera (20 MP). Digital ImageJ software (NIH, Bethesda, MD) was used to measure the wound size and contraction rate with using the following formula:

$$\left[\frac{\text{Area of the original wound} - \text{Area of the actual wound (on day } n; n = 3, 7 \text{ and } 14 \text{ days)}}{\text{Area of the original wound}} \right] \times 100.$$

The re-epithelialization area of the wound was the area between the wound edge and the newly formed epithelial edge on days 3, 7 and 14.

Tensile strength

The tensile strength of a wound represents the degree of wound-healing processes. A samples of healing skin (from groups II, III and IV), along with normal skin (group I) were excised for tensile strength measurement using a computerized tensiometer (EZTEST I, Shimadzu Corp., Japan). Tensile strength was calculated using the following formula:

$$\begin{aligned} & \text{Tensile strength (N/cm}^2\text{)} \\ & = \text{breaking force (N)/area (cm}^2\text{);} \end{aligned}$$

where area (cm²) = thickness (cm) × width (cm) (Baie and Sheikh, 2000).

Biochemical analysis

Dermal antioxidants and lipid peroxidation

The dermal antioxidant enzymes, i.e. superoxide dismutase (SOD) (Marklund and Marklund, 1974), catalase (CAT) (Sinha et al., 1972), glutathione peroxidase (GPx) (Rotruck et al., 1973) and reduced glutathione content (GSH) (Moron et al., 1979), were estimated according to previously reported methods. The protein content of dermal tissue was determined based on the Biuret reaction of the BCA kit (Thermo, Rockford, IL, USA). The level of lipid peroxides in the dermal tissue was determined by the method of Devasagayam and Tarachand (1987).

Estimation of hydroxyproline

Hydroxyproline content of the tissue sample was estimated by the method of Woessner (1961). The procedure is based on alkaline hydrolysis of the tissue homogenate and subsequent determination of the free hydroxyproline in hydrolyzates. The hydrolyzate was neutralized to pH 7.0 and 1 mL of hydrolyzed tissue sample made up to 2 mL was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by the addition of 3.15 M perchloric acid and color was developed with the addition of p-dimethyl amino benzaldehyde solution at 60°C, cooled under tap water

for 20 min and measured at 557 nm using a spectrophotometer. The hydroxyproline value was extrapolated from the standard curve.

Estimation of mucopolysaccharide

One hundred mg of delipidated residue of the tissues (skin) were hydrolyzed with 2 mL of 3N HCl at 80°C for 4 h. The hydrolyzed material was neutralized and used for estimation of hexosamine and hexuronic acid. Hexosamine was estimated by the method of Wagner (1979) and hexuronic acid by the method of Bitter and Muir (1962).

Enzyme-linked immunosorbent assay

Nitric oxide (NO) levels were quantified by the ELISA method using the manufactured protocol (Caymen Chemical, Ann Arbor, MI) in the granulation tissue of experimental rats. The tissue (skin) lysates were prepared by homogenizing frozen wound samples in dounces containing 500 μ L of 0.1 M phosphate buffer (pH 7.4), 1 mM EDTA and 10 μ M indomethacin. The samples were normalized to 1 mg/1 mL.

Histopathological studies

Wounded tissue was stained by the hematoxylin and eosin (H&E) method. Portions of granulation tissues were fixed in 10% formalin. The washed tissues were dehydrated in descending grades of isopropanol and cleared in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5- μ m thicknesses and stained with H&E. The sections were then viewed under a light microscope (Nikon microscope ECLIPSE E400, model 115, Japan) for histopathological changes.

Statistical analysis

The values were expressed as a mean \pm standard deviation (SD). Differences between each group were assessed by one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences) version 21. Post hoc testing was performed for inter-

group comparisons using the least significance difference (LSD) test. *P*-values <0.05 were considered as statistically significant.

RESULTS

Effects of SSD and PVE on wound contraction, epithelialization and tensile strength

Wound contraction and re-epithelialization are the significant characteristics of the healing process. The progression of wound contraction was evaluated by microscopic and macroscopic observations. The effects of SSD and PVE on wound contraction rate in experimental rats are shown in Table 1. On the 3rd and 7th day after wound creation, the wound contraction rate of the SSD- and PVE-treated groups was 27.34, 60.82% and 32.45 and 71.58%, respectively. On the 14th day, the wound contraction rate of the SSD- and PVE-treated groups was 85.76 and 99.74%, respectively. However, the burn-injured group showed the maximum contraction (65.23%) on day 14. The percentage of wound contraction in the SSD- and PVE-treated groups was significantly higher (*P*<0.05 and *P*<0.01) than that of the burn-injured rats.

Fig. 1 depicts the effects of SSD and PVE on the wound re-epithelialization area in experimental rats. During the 3rd and 7th days, the re-epithelialization area concomitantly increased on both treatment groups in comparison with the burn-injured group. The re-epithelialization area on the 14th day was also higher in both treatment groups, but in the PVE-treated group the wound area completely closed without leaving a raw wound behind, which indicates its effec-

Table 1. Effects of SSD and PVE on wound contraction in experimental rats.

| Groups | Days | | |
|--------------|----------------------|----------------------|----------------------|
| | 3 | 7 | 14 |
| Burn-injured | 21.24 \pm 2.29 | 45.23 \pm 5.21 | 65.23 \pm 7.24 |
| SSD-treated | 27.34 \pm 1.78 a* | 60.82 \pm 7.23 a* | 85.76 \pm 9.76 a* |
| PVE-treated | 32.45 \pm 2.24 a** | 71.58 \pm 6.38 a** | 99.74 \pm 9.96 a** |

Values were expressed as mean \pm SD for six rats in each group. Statistical significance (*P* value): ** *P*<0.01, * *P*<0.05 compared with the burn injured group (a).

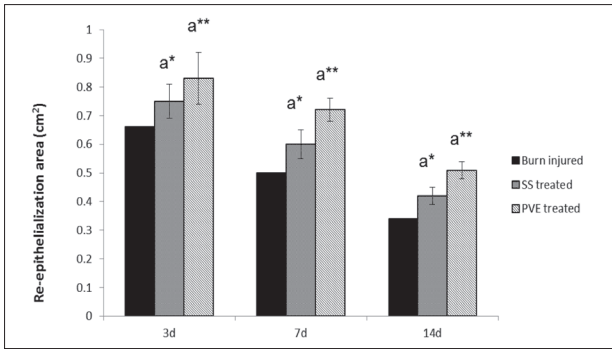


Fig. 1. Effects of SSD and PVE on re-epithelialization area of dermal tissue in experimental rats. Values were expressed as mean±SD for six rats in each group. Statistical significance (*P* value): ***P*<0.01, **P*<0.05 compared with the burn-injured group (b).

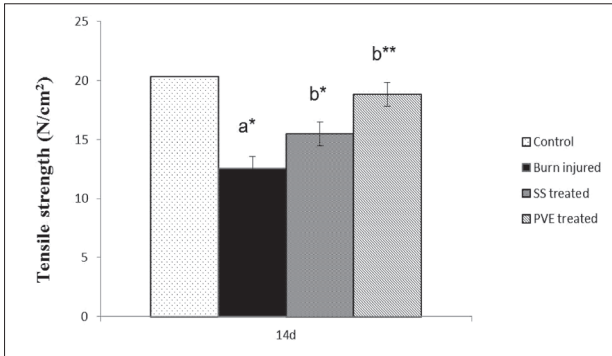


Fig. 2. Effects of SSD and PVE on tensile strength (14th day) in dermal tissue of experimental rats. Values were expressed as mean±SD for six rats in each group. Statistical significance (*P* value): ***P*<0.01, **P*<0.05 compared with the control (a) and burn-injured group (b).

tive wound-healing ability when compared with the SSD group, which still showed some wound area had yet to heal. The order of the re-epithelialization rate is PVE>SSD>burn-injured group.

Fig. 2 shows the effect of SSD and PVE on tensile strength in the dermal tissue of experimental rats. The tensile strengths of the burn-injured rats were substantially lower (*P*<0.05) than those of the negative control group. A significant increase in tensile strength was observed in the SSD- and PVE-treated group (*P*<0.05 and *P*<0.01, respectively) compared with the burn-injured group. Our previous results of wound contraction, re-epithelialization rate and tensile strength demonstrated similar positive results of the enhanced wound-healing ability of PVE.

Table 2. Effects of SSD and PVE on dermal antioxidant in experimental rats.

| Groups | Parameters | Day 3 | Day 7 | Day 14 |
|-------------------|--|--------------|---------------|---------------|
| Control (No burn) | SOD (U/mg of protein) | 1.35±0.13 | 1.41±0.22 | 1.37±0.14 |
| Burn-injured | | 1.65±0.18 a* | 1.48±0.16 | 1.12±0.15 a* |
| SSD-treated | | 1.98±0.19 | 2.82±0.29 b** | 1.84±0.13 b* |
| PVE-treated | | 2.05±0.22 b* | 3.57±0.41 b** | 1.63±0.19 b* |
| Control (No burn) | CAT (µm H ₂ O ₂ /min/mg protein) | 3.47±0.35 | 3.43±0.39 | 3.45±0.29 |
| Burn-injured | | 3.76±0.44 | 2.95±0.28 a* | 2.89±0.22 a* |
| SSD-treated | | 3.92±0.22 | 4.35±0.45 b* | 3.69±0.36 b* |
| PVE-treated | | 4.01±0.38 b* | 5.07±0.68 b** | 3.54±0.41 b* |
| Control (No burn) | GPx (nmole GSH utilized/min/mg/protein) | 3.56±0.43 | 3.52±0.26 | 3.56±0.29 |
| Burn-injured | | 3.93±0.39 a* | 3.01±0.39 a* | 2.34±0.35 a** |
| SSD-treated | | 4.05±0.42 | 4.62±0.48 b* | 3.91±0.36 b* |
| PVE-treated | | 4.17±0.34 | 4.85±0.51 b** | 3.69±0.43 b* |
| Control (No burn) | Reduced glutathione (µg/mg protein) | 3.55±0.22 | 3.51±0.34 | 3.56±0.30 |
| Burn-injured | | 3.97±0.45 a* | 3.31±0.37 | 2.72±0.28 a* |
| SSD-treated | | 4.39±0.28 b* | 4.48±0.45 b* | 3.42±0.35 b* |
| PVE-treated | | 4.28±0.33 | 5.13±0.49 b** | 4.07±0.41 b** |

Values were expressed as mean±SD for six rats in each group. Statistical significance (*P* value): ***P*<0.01, **P*<0.05 compared with the control (a) and burn-injured group (b).

Effect of SSD and PVE on dermal antioxidants

The effects of SSD and PVE on dermal antioxidants in experimental rats are shown in Table 2. Increasing trends in the levels of antioxidants were observed in the SSD- and PVE-treated group on the 3rd and 7th days, but the slight reduction on 14th day especially in PVE group, was also observed. Thermal wounds treated with SSD (*P*<0.05) and PVE (*P*<0.01) revealed a maximum antioxidant activity on the 7th day compared to control and burn-injured rats.

Effects of SSD and PVE on lipid peroxidation

The effects of SSD and PVE on lipid peroxidation (LPO) in the dermal tissue of experimental rats are shown in Fig. 3. LPO levels were substantially elevated on days 3 and 7 in the burn-injured rats, but started to decline after treatment with SS and PVE. The levels of malondialdehyde (MDA) were restored to normal (*P*<0.01) in the PVE-treated rats on the 14th day, whereas they were significantly reduced (*P*<0.05) in the SSD-treated rats on the 14th day. In the case of the burn-injured group, the levels of MDA did not revert back to near normal till the 14th day due to non-medication.

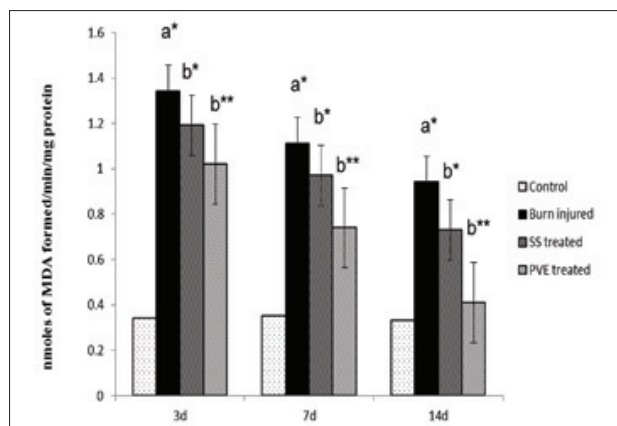


Fig. 3. Effects of SSD and PVE on lipid peroxidation (LPO) in dermal tissue of experimental rats. Values were expressed as mean±SD for six rats in each group. Statistical significance (*P* value): ***P*<0.01, **P*<0.05 compared with the control (a) and burn-injured group (b).

Table 3. Effects of SSD and PVE on dermal hydroxyproline, hexosamine and hexuronic acid in experimental rats

| Groups | Parameters | Day 3 | Day 7 | Day 14 |
|-------------------|----------------------------------|--------------|---------------|--------------|
| Control (No burn) | Hydroxyproline (mg/100mg tissue) | 3.13±0.36 | 3.15±0.23 | 3.12±0.43 |
| Burn-injured | | 3.19±0.32 | 3.28±0.32 a* | 3.37±0.44 a* |
| SS-treated | | 3.41±0.27 b* | 3.57±0.33 b* | 3.45±0.36 b* |
| PVE-treated | | 3.67±0.43 b* | 3.98±0.39 b** | 3.51±0.52 b* |
| Control (No burn) | Hexosamine (mg/g wet tissue) | 6.57±0.54 | 6.52±0.58 | 6.49±0.66 |
| Burn-injured | | 6.67±0.45 | 6.75±0.43 a* | 6.45±0.96 |
| SS-treated | | 6.79±0.48 | 6.92±0.75 b* | 6.82±0.71 b* |
| PVE-treated | | 6.83±0.62 | 7.03±0.73 b** | 6.76±0.80 b* |
| Control (No burn) | Hexuronic acid (mg/g wet tissue) | 8.27±0.86 | 8.28±0.53 | 8.29±0.91 |
| Burn-injured | | 8.24±0.53 | 8.21±0.61 | 8.24±0.70 |
| SS-treated | | 8.26±0.48 | 8.40±0.72 | 8.32±0.69 |
| PVE-treated | | 8.24±0.74 | 8.48±0.56 b* | 8.38±0.61 |

Values were expressed as mean±SD for six rats in each group. Statistical significance (*P* value): ***P*<0.01, **P*<0.05 compared with the control (a) and burn-injured group (b).

Effects of SSD and PVE on dermal hydroxyproline, hexosamine and hexuronic acid

The collagen in granulation tissue was estimated by the breakdown of collagen into hydroxyproline, which is an index of collagen turnover. Table 3 demonstrates the effects of SSD and PVE on dermal hydroxyproline, hexosamine and hexuronic acid in experimental rats. The levels of dermal hydroxyproline, hexosamine and hexuronic acid were significantly elevated in SSD- and

PVE-treated animals (*P*<0.05 on 7th day and *P*<0.01 on 3rd and 7th day, respectively), but declined slightly on the 14th day, when compared with the burn-injured and negative control groups. No significant changes were noted between the negative control groups.

Effects of SSD and PVE on nitric oxide (NO)

Fig. 4 shows the effects of SSD and PVE on nitric oxide (NO) in the dermal tissue of experimental rats. Thermally injured rats showed a substantial reduction (*P*<0.01) in the levels of NO when compared with negative control rats on the 3rd, 7th and 14th days. Treatment with SSD (*P*<0.05) and PVE (*P*<0.01) displaying a marked elevation in the levels of NO were noted on the 3rd, 7th and 14th days when compared with burn-injured rats.

Effects of SSD and PVE on histological changes

The effects of SSD and PVE on histological changes in the dermal tissue of experimental rats are shown in Fig. 5. Transection of granulated tissue of negative control rats showed a normal architecture of the dermis and epidermal layer with proper distribution of collagen fibers without any infiltration (H&E, 100X) (5A). Transection of burn-injured (untreated) rats on days 3 and 7 showed an irregular pattern of collagen fiber distribution with ulceration and infiltration of inflammatory cells (5B and C), whereas on the 14th day the presence of immature collagen fibers and less inflammatory cells (5D) were noted. Transection of the SSD-treated group showed an irregular pattern of collagen fibers with ulceration and infiltration of inflammatory cells on the 3rd day (5E), whereas less ulceration and infiltration with moderate collagen fiber were noted on the 7th and 14th days (5F and G). Transection of the PVE-treated group showed mild ulceration with inflammatory cells on the 3rd day (5H), whereas few infiltrations were noted on the 7th day (5I). On the 14th day, a uniform accumulation of collagen fibers with prominent bundles consisting of a moderate number of fibroblast without any inflammatory cells (5J) was seen, similar to that of the negative control group. A close examination of granulation

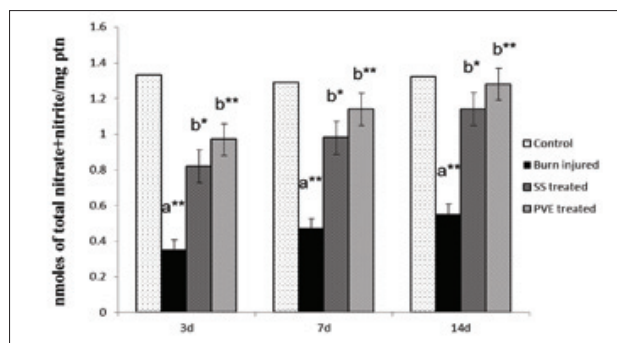


Fig. 4. Effects of SSD and PVE on nitric oxide (NO) in dermal tissue of experimental rats. Values were expressed as mean \pm SD for six rats in each group. Statistical significance (P value): ** P <0.01, * P <0.05 compared with the control (a) and burn-injured group (b).

tissue sections revealed that tissue regeneration was much faster in the treated groups, especially the PVE-treated group, in comparison to untreated rats.

DISCUSSION

The healing of a skin burn is a multistep process involving several complex phases such as hemorrhage, inflammation, granulation tissue formation, re-epithelialization and the late remodeling phase of repair

(Kim et al., 2009). Management of skin burns often represents a difficult therapeutic challenge for physicians: a successful treatment should lead to physiological function recovery, relief from symptoms and an aesthetic improvement of skin lesions. Silver sulfadiazine (SSD) is the topical agent of choice in severe burns and is used almost universally today in preference to compounds such as silver nitrate and mafenide acetate and hence we used SSD as a standard for comparing with PVE. Preliminary phytochemical analyses were done, which revealed the presence of phenolic (10.34 ± 0.81 mg GAE/bwt), flavonoid (6.12 ± 0.35 mg QE/bwt), saponin (0.41 ± 0.02 mg/bwt) and alkaloid (3.45 ± 0.24 mg/wt) contents.

The wound contraction and re-epithelialization rate of the PVE-treated group was significantly higher than that of the standard SSD in that it completely closed the wound area without leaving a raw wound behind, displaying its efficacious epithelialization rate on the 14th day. PVE treatment increased the rate of re-epithelialization by stimulating the proliferation of keratinocytes. However, on day 14 the SSD and burn-injured (positive control) groups showed some unclosed (healed) area of the wound, indicating the

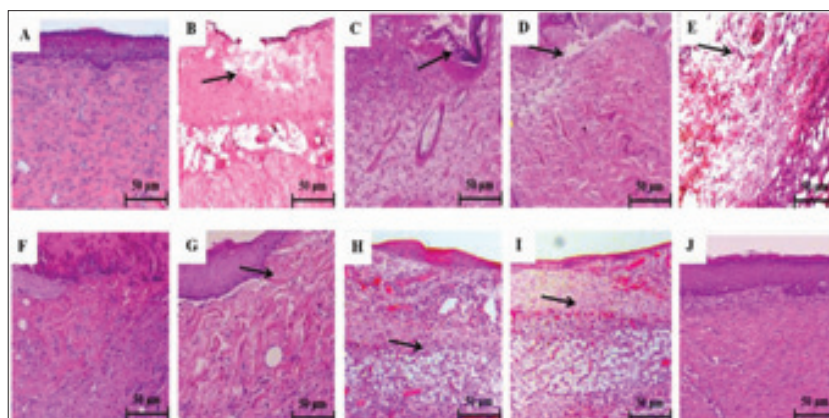


Fig. 5. Effects of SSD and PVE on histological changes in dermal tissue of experimental rats. Transection of granulated tissue of control rats showed normal architecture of dermis and epidermal layer with proper distribution of collagen fibers without any infiltration (H&E, 100X) (5A). Transection of burn-injured (untreated) rats on 3rd and 7th days showed irregular pattern of collagen fibers with ulceration and infiltration of inflammatory cells (5B and C), whereas on day 14, the presence of immature collagen fibers and less inflammatory cells (5D) was seen. Transection of SSD group showed irregular pattern of collagen fibers with ulceration and infiltration of inflammatory cells on the 3rd day (5E), whereas less ulceration and infiltration with moderate collagen fiber were noted on the 7th and 14th days (5F and G). Transection of PVE group showed mild ulceration with inflammatory cells on the 3rd day (5H), whereas few infiltrations were noted on the 7th day (5I). On the 14th day, a uniform accumulation of collagen fibers with prominent bundles consisting of moderate number of fibroblast without any inflammatory cells (5J) was noted, similar to that of control group (H&E, 100X) (Scale bar-50 μ m).

re-epithelialization in these groups was not so significant when compared to the PVE-treated group. The enhanced rate of wound contraction and reduction in healing time may be due to enhanced epithelialization (Priya et al., 2002). Skin tensile strength is determined by the amount and quality of synthesized collagen, as well as degradation of preformed collagen (Chen et al., 2012). The tensile strengths of the SSD and PVE groups were substantially higher than that of the burn-injured group due to increased wound contraction rate, increased re-epithelialization area as well as regulation of the collagen disposition. The results of wound contraction, re-epithelialization rate and tensile strengths demonstrated similar positive outcomes, which probably influence the wound-healing ability of PVE.

Free radicals are the major contributor to poor healing ability, and to enhance the wound-healing process antioxidants play a vital role. It is well established that wounds initiate inflammation that in turn induces the production of free radicals by phagocytic cells such as neutrophils and macrophages. Overproduction of these reactive oxygen species (ROS) results in oxidative stress, thereby causing cytotoxicity and delayed wound healing. Free radicals will contribute to the cellular damage in the zone of metastasis, which leads to necrosis and conversion of superficial wounds into deeper wounds by triggering inflammation and promoting premature apoptosis in the extracellular matrix (Rai et al., 2005). Therefore, the elimination of ROS could be an important strategy in the healing of chronic wounds.

Flavonoids and polyphenols are known to lower the excessive production of protease and reactive oxygen species, often formed in the injured site to protect cells and extracellular matrix from oxidative damage by preventing or slowing down the progress of cell necrosis and its neoplastic transformation (Surh, 2003; Amin et al., 2009). The damage inflicted on the skin results in the production of ROS and also in a reduction in various enzymatic and non-enzymatic free radical scavengers, thereby hindering the healing process (Serarslan et al., 2007). Antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) break down free

radicals to accelerate wound healing. Treatment with SSD and PVE showed an increased antioxidant activity compared with negative control and burn-injured rats. Previously it was reported that rosmarinic acid (RA), the main phenolic acid constituent, owing to its hydroxyl group, can effectively scavenge free radicals (Skottova et al., 2004). PVEs are rich in flavonoids (quercetin) and saponins, which might also act as good antioxidant agents.

Lipid peroxidation is an oxidative deterioration of polyunsaturated fatty acids in cell membranes by excessive production of free radicals, which contribute to cellular damage. Lipid peroxidation levels were elevated in the burn-injured group due to an increased generation of free radicals. Similarly, several studies have reported increased levels of lipid peroxidation following burn injury (Sener et al., 2005; Toklu et al., 2006). In this study, PVE-treated rats exhibited showed decreased lipid peroxidation levels in comparison to burn-injured rats. Thus, the above results indicate that due to the antioxidant activity of PVE, lipid peroxidation product levels were significantly decreased, which probably enhanced the healing activity. It is well documented that during burn injury the levels of free radicals are elevated with reduced antioxidant activity, thus delaying the wound-healing process. PVE is rich in phytochemicals (RA, UA, quercetin) and able to quench the free radical generation and therefore endorse the wound-healing process by improving wound contraction and re-epithelialization rates (Khaled-Khodja et al., 2014).

Collagen is a major protein of the extracellular matrix and is the component that ultimately contributes to skin (tensile) strength and integrity. The breakdown of collagen liberates hydroxyproline and its peptides. Therefore, the estimation of hydroxyproline is used as an index to assess wound-healing potency (Manjunatha et al., 2007). An increasing trend in hydroxyproline concentration was noted in the SSD- and PVE-treated groups on days 3 and 7, as compared with the control, pointing to rapid collagen turnover (collagen synthesis), which indirectly influenced the healing process. Our results were in contrast with Priya et al. (2002) who reported elevated hydroxyproline

(collagen) levels at various stages of wound-healing processes. Based on the findings of this study, PVE can act as a chemoattractant for fibroblasts and stimulates fibroblast proliferation, which in turn increases the synthesis of collagen in thermally injured skin. In addition, it can effectively suppress lipid peroxidation and thereby protect the cells from damage and thus increase the viability of collagen fibrils. Collagen is the major protein component of connective tissue and is responsible for the tissue strength, and increased tensile strengths were observed as a characteristic of the healing activity of PVE.

Mucopolysaccharides (MPS) are a major component of the extra cellular matrix of the skin, joints, eyes and many other tissues and organs. MPS are the first components of the extracellular matrix to be synthesized during wound healing, and they form the scaffold for collagen and elastin deposition. MPS can protect granulating tissue from oxygen free radical damage and thereby stimulate wound healing (Trowbridge and Gallo, 2002). Uronic acid is one of the main glycosaminoglycan present in wounds, attracting fibroblast and stimulating collagen synthesis by providing more fluid, which facilitates greater cell mobility, early remodeling and helps the wound to heal without scar formation (Hu et al., 2003). Hexosamine and hexuronic acid content increases in the early stages of wound healing and indicates that the fibroblasts have actively synthesized with mucopolysaccharides on which the collagen can be laid (Karthikeyan and Rani, 2003).

Both hexosamine and hexuronic acid were elevated in the PVE-treated rats, but minimal changes were observed in the SSD group on days 3 and 7 when compared with negative control and burn-injured rats. A similar study by Murthy et al. (2013) reported increased hexosamine and hexuronic acid content in *Bacopa monniera*-treated rats, which may contribute to rapid wound healing. The observed increase in hexosamine and hexuronic acid levels in the PVE group may accelerate the wound-healing process. A slight decline in the levels of hydroxyproline, hexosamine and hexuronic acid were noted on day 14, when compared with the burn-injured and negative control groups, and is an indication of the wound-healing

process reaching the final phase (re-epithelialization is almost completed). Suriyamoorthy et al (2014), using *Acacia caesia* in wound treatment, corroborate our findings with increased levels of these components during the early stages of wound healing followed by restoring to near normal.

Nitric oxide (NO) is an intercellular signaling molecule, and an efficient, balanced production of it plays an important role in burn healing. The highly valuable effect of bioavailable NO is ascribed to the scavenging of superoxide, as the major component of oxidative stress. NO also has a beneficial effect on angiogenesis, inflammation, matrix deposition, and remodeling (Soneja et al., 2005). Furthermore, NO has a regulatory role in vascular endothelial growth factor (VEGF) through the wound-healing process. The levels of NO in burn-injured rats were substantially reduced as time passed owing to increased ROS generation. With PVE treatment, the levels of NO were markedly elevated on days 3, 7 and 14 when compared with burn-injured rats and the SSD group. Flavonoids and polyphenols present in PVE could favor endothelial cell and fibroblast proliferation and migration. Therefore, they are capable of leading to the formation of new blood vessels and capillaries, regenerating new dermal tissue and remodeling the newly formed tissue by stimulating collagen fiber production (Paolino et al., 2012).

Our biochemical results correlated well with the histological findings. Transection of granulated tissue of negative control rats showed a normal architecture of the dermis and epidermal layer with proper distribution of collagen fibers without any infiltration (H&E, 100X) (5A). Transection of burn-injured (untreated) rats on days 3 and 7 showed an irregular pattern of collagen fibers with ulceration and infiltration of inflammatory cells (5B and C), whereas on the 14th day the presence of immature collagen fibers and less inflammatory cells (5D) were noted. Transection of the SSD group showed an irregular pattern of collagen fibers with ulceration and infiltration of inflammatory cells on the 3rd day (5E), whereas less ulceration and infiltration with moderate collagen fiber were noted on the 7th and 14th days (5F and G). Transection of the PVE group showed mild ulceration with inflammatory

cells on the 3rd day (5H), whereas few infiltrations were noted on the 7th day (5I). On the 14th day, a uniform accumulation of collagen fibers with prominent bundles consisting of moderate number of fibroblast without any inflammatory cells (5J) was observed, similar to that of the negative control group. Our results are in correlation with other studies reporting that treatment with plant extracts led to an increase in collagen fibers and increased proliferation fibroblast and keratinocytes (Manjunatha et al., 2007; Somboonwong et al., 2012). No pathological changes were noted in any of the negative control groups throughout the study.

PVE might promote fibroblast proliferation by activation of Akt /mTOR signaling. Activated mTOR stimulates the protein synthesis required for cell growth and metabolism via phosphorylation of 4EBP1 and p70S6K. Moreover, activation of the PI3K/AKT and mTOR/p70S6K signaling pathways regulates thrombin-induced retinal pigment epithelium (RPE) cell proliferation. Therefore, activation of AKT/mTOR signaling promotes cyclin D1 protein translation, leading to cell proliferation, which could be the major mechanism involved in acemannan-promoted wound repair (Xing et al., 2015), as well as in our model of burn injury.

CONCLUSION

Our results indicate that the topical application of PVE, owing to its high content of various phytochemicals, might accelerate the various phases of wound healing by reducing lipid peroxidation or enhancing antioxidants to halt or slowdown the onset of cell necrosis and apoptosis, thereby increasing the viability of collagen fibrils by increasing the strength of collagen fibers (tensile strength) as well as upregulating proliferation of keratinocytes, fibroblast (re-epithelialization), extracellular matrix formation (MPS) and lowering inflammatory response on the final stage of healing. Further studies are required to reveal the molecular mechanism behind PVEs wound-healing activities.

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