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Antioxidant potential of *Shorea robusta* resin-accelerated cutaneous wound healing in streptozotocin-induced diabetic rats

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Abstract
Oxidative stress plays a pivotal role in the development of diabetes complications. It was hypothesized that elimination of reactive oxygen species by the application of herbal extract in the treatment of cutaneous wounds could be an important strategy to improve healing of wounds. The time-dependent and concentration-dependent effects of alcoholic extract of *Shorea robusta* resin (SRE) were evaluated on cutaneous wound healing in diabetic rats. Open excision wounds of 2x2 cm^2 were experimentally created on dorsal region of the diabetic rats. 3, 10 or 30% SRE ointment was applied topically on the wound area twice daily for 19 days, as compared to diabetic control in which only ointment base was applied. Six animals from each group were sacrificed on days 3, 7, 14 and 19 to collect the granulation tissue which was used for the estimation of antioxidant enzyme activity and lipid peroxidation by spectrophotometer. Topical application of SRE markedly decreased the wound size, as compared to that of control group on day 3, 7, 14 and 19 post-wounding. The levels of GSH and activities of SOD and catalase were markedly higher in SRE treated group with concomitant decrease in MDA and NO level on day 3, 7, 14 and 19 as compared to control. The results of this study revealed that SRE treatment caused faster as well as better organized healing of cutaneous wounds in diabetic rats. The quality of the healed tissue was much better than the diabetic control rats. Thus, the SRE has shown great potential in treating wounds in diabetic rats.

Keywords: Diabetes, wound, oxidative stress, antioxidant, *Shorea robusta*

Introduction
India has been frequently referred to as the 'medicinal and aromatic plants garden of the world'. It is generally estimated that more than 6000 plants in India are being used in traditional, folk and herbal medicine [1]. As per WHO estimates, 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs [2]. Diabetes is projected to rise from 171 million to 366 million in 2030 worldwide [3]. This major increase in morbidity and mortality of diabetes is due to the development of both macro- and micro-vascular complications, including failure of the wound healing process. Wound healing is a very orderly and highly controlled process characterized by four distinct but overlapping phases: hemostasis, inflammation, proliferation and remodeling [4]. However, many studies showed that the most common complications involved in diabetes for delayed wound healing are: prolonged inflammatory phase [5], decreased eNOS activity [6], and increased oxidative stress [7]. Medicinal plant based ointment used in folk medicine are reported to have beneficial effect in wound care and healing [8, 9]. Recently, there has been an upsurge of interest in the therapeutic potential of plants as antioxidants, anti-inflammatory and anti-diabetic in reducing tissue injuries associated with metabolic syndrome. Further, in view of the importance of medicinal plants as potential source of cheaper, safer and effective remedies for treating diabetes, the present study is being proposed on an Indian ethnomedicine *Shorea robusta* Gaertn f. (Dipterocarpaceae). Besides the plaster of fumigation, resin is an astringent used therapeutically, in diarrhea, dysentery, and gonorrhea. In addition resin has been reported to possess wound healing [10-12], anti-inflammatory, analgesic [13-16], and microbicidal [17] activities. In view of the above, it was hypothesized that *Shorea robusta resin* might help in cutaneous wound healing, in diabetes where oxidative stress is a major problem.

Materials and methods
Chemicals
Plant material and preparation of extract
Pure resin of *S. robusta* was purchased from a local market of Bhubaneswar, India.

It was processed as described by Yaseen Khan *et al*. (2016) [18]. In short, it was ground into powder then extracted with 70% ethanol under reflux for 72 h using a Soxhlet apparatus. Hydro alcoholic extract was filtered and evaporated to dryness under reduced pressure in rotary vacuum evaporator at 40°C temperature and further kept in vacuum desiccators for complete removal of solvent and the yield of the extract was calculated. Hereafter, extract is to be considered as 'SRE' was stored at 4°C until use.

Animals

Apparently healthy adult male Wistar rats (200-220g) were procured from Laboratory Animal Resource Section, ICAR - Indian Veterinary Research Institute, Izatnagar (U.P.), India. The animals were housed in polypropylene cages and kept for a week, as an acclimatization period with free access to standard feed and water and maintained on a 12 hour dark-to-light cycle in an air controlled room (temperature 22±2°C, humidity 55±5%). The experimental protocols involved in this study were according to the guidelines of CPCSEA. The experimental protocol was approved by the institute animal ethics committee letter vide no. F. 26-1/2015-16/JD(R)/part file dated 16/10/2017.

Induction of diabetes

Initially, rats with fasting blood glucose level in a normal range were selected for the study. Diabetes was induced by single intraperitoneal injection of streptozotocin (60 mg/kg body weight) freshly prepared in citrate buffer (pH 4.5) to overnight starved rats. 2% sucrose solution was given in drinking water for 48 h to alleviate the sudden hypoglycemic phase. Similarly, control group received only equal volume of citrate buffer. After 72 h blood glucose was monitored by tail pricking using digital glucometer (On-Call-Plus, ACON Biotech). Rats having blood glucose levels more than 300 mg/dl were kept for further study and treatment was started after 14 days of observation.

Wound creation

The diabetic rats were anesthetized by ketamine (50 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). Approximately 2x2 cm^2 (400 mm^2) open excision-type wound was created on the dorsal region of the rats to the depth including the panniculus carnosus. Wound was neither dressed nor covered. Animals after recovery from anesthesia were housed individually in properly disinfected cages in the air controlled room at temperature 22±2°C.

Preparation of ointment

Extract ointment of 3, 10 and 30% was prepared using soft white petroleum jelly as vehicle.

Grouping of rats and application of ointment

The animals were randomly divided into 4 groups consisting of 24 animals and each group was further subdivided into 4 subgroups with 6 animals in each group as follows: control (only vehicle was applied topically twice daily for 19 days, SRE 3% (ointment 3% was applied topically twice daily for 19 days), SRE 10% (ointment 10% was applied topically twice daily for 19 days), and SRE 30% (ointment 30% was applied topically twice daily for 19 days).

Measurement of wound closure and collection of tissue

Wound area of each rat was measured on days 0, 3, 7, 14 and 19 post-wounding by tracing using a transparent paper. The area (mm^2) within the boundaries of each tracing was expressed as percent wound contraction, calculated by Wilson's formula as follows: % wound contraction = [(Day 0 wound area – wound area on particular day) / Day 0 wound area] x 100. The granulation tissue was collected immediately after euthanizing the rats with overdose of diethyl ether on days 3, 7, 14, and 19 post-wounding and preserved at −80 °C.

Antioxidant parameters

Super oxide dismutase (SOD) activity

SOD activity was measured by the method of Madesh and Balsubramanium (1998) [19]. It involves the generation of superoxide by pyrogallol auto oxidation and inhibition of superoxide dependent reduction of the tetrazolium dye (MTT) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its formazan. The reaction mixture contained 0.65 ml PBS (pH 7.4), 30 µl MTT (1.25 mM), 10 µl tissue homogenate and 75 µl pyrogallol (100 mM). The mixture was incubated at room temperature for 5 min and the reaction was stopped by adding 0.75 ml of DMSO which solubilizes formazan. The absorbance was read at 570 nm against the blank and the activity was expressed as U/mg protein.

Catalase activity

Catalase activity was assayed as described by Aebi (1984) [20]. Tissue homogenate 0.1 ml was added to 1.9 ml of 50 mM phosphate buffer (1:1.5 v/v of 50 mM KH_2PO_4 and 50 mM Na_2HPO_4 pH 7) in a test tube then content is transferred to cuvette. The reaction was started after adding 1 ml of H_2O_2 (10 mM: 0.1 ml of 30% H_2O_2 was diluted to 100 ml phosphate buffer) directly into cuvette. Optical density was recorded at every 15 sec for 1 min at 240 nm against distilled water (blank). The catalase activity was calculated using milimolar extinction coefficient of H_2O_2 (0.071 mmol cm^{-1}) and the activity was expressed as U/mg protein.

Reduced glutathione (GSH)

GSH level was estimated by the method described by Sedlak and Lindsay (1968) [21]. To 1 ml of tissue homogenate 0.8 ml of distilled water and 0.2 ml of 50% trichloroacetic acid was added and incubated at room temperature for 15 min. Then mixture was centrifuged at 3000 rpm for 15 min. 0.4 ml of supernatant was added to 0.8 ml of 1 M Tris buffer (pH 8.9) followed by 0.2 ml of 5,52 -dithiobis (2-nitrobenzoic acid) (DTNB) (0.01M). The yellow color developed was read immediately (within 5 min) at 412 nm. GSH is expressed as nmol/mg protein. Results were calculated using molar extinction coefficient of chromophore (1.36 x 104 M-1cm-1) and expressed as percentage of control.

Malondialdehyde (MDA)

Lipid per oxidation in tissue homogenate was estimated by thiobarbituric acid reactive substances (TBARS) method of Buege and Aust (1978) [22]. In short, 0.1 ml of serum in 200 mM Tris-HCl buffer, pH 7.5 was treated with 2 ml of (1:1:1) TBA-TCA-HCl reagent (TBA 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min and cooled then centrifuged at 2000 rpm for 10 min. The absorbance of clear supernatant was measured at 535 nm against the blank. Concentration was calculated using molar extinction coefficient of MDA which is 1.56 X 10^5 M^{-1} cm^{-1} and expressed as nmol/mg protein.

Nitric oxide (NO)

Nitrite measurement in the serum is an indirect indicator of NO production [23]. Briefly, it was measured in tissue

homogenate (100 µl) taking equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). After 10 min incubation at room temperature, the absorbance was measured at 550 nm in a microplate reader (SpectraMax Multi-mode). The nitrite concentration was determined from extrapolation of the calibration curve was made with standard sodium nitrite (0-100 µM).

Statistical analysis

Results were expressed as mean ± S.E. The Statistical significance was analyzed by applying one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the GraphPad Prism v5.03 software program (San Diego, California USA) and the difference between the control and treatment groups were considered statistically significant at $p<0.05$.

Results

The gross appearance of wound was found better in SRE-treated groups in a concentration and time-dependent manner, which was evidenced by early formation and shedding of scab. Well formed and thick red granulation tissues were also distinguishable in SRE-treated rats compared to control from day 7 onwards and healing was observed on day 19 in a particular group (Fig. 1A). Control and treated rats on different days (3, 7, 14, and 19) revealed greater wound closure in diabetic treated, compared to diabetic control. The percent closure of wound area in control and diabetic treated on the same days shown a significant increase percent closure in diabetic treated groups on days 7, 14, and 19, as compared to diabetic control group (Fig 1B).

Result exhibited the activities of SOD and catalase and also levels of GSH, MDA and NO in the granulation tissues of diabetic rats treated with various concentrations of SRE, as compared to vehicle treated diabetic control rats are shown (Fig. 2, 3). SRE treatment at particularly 10% and 30% significantly increased the SOD and catalase enzyme activity. Reduced glutathione content was found to be significantly higher on day 14 and 19. The MDA and NO levels were decreased from day 3 onwards as compared to respective control.

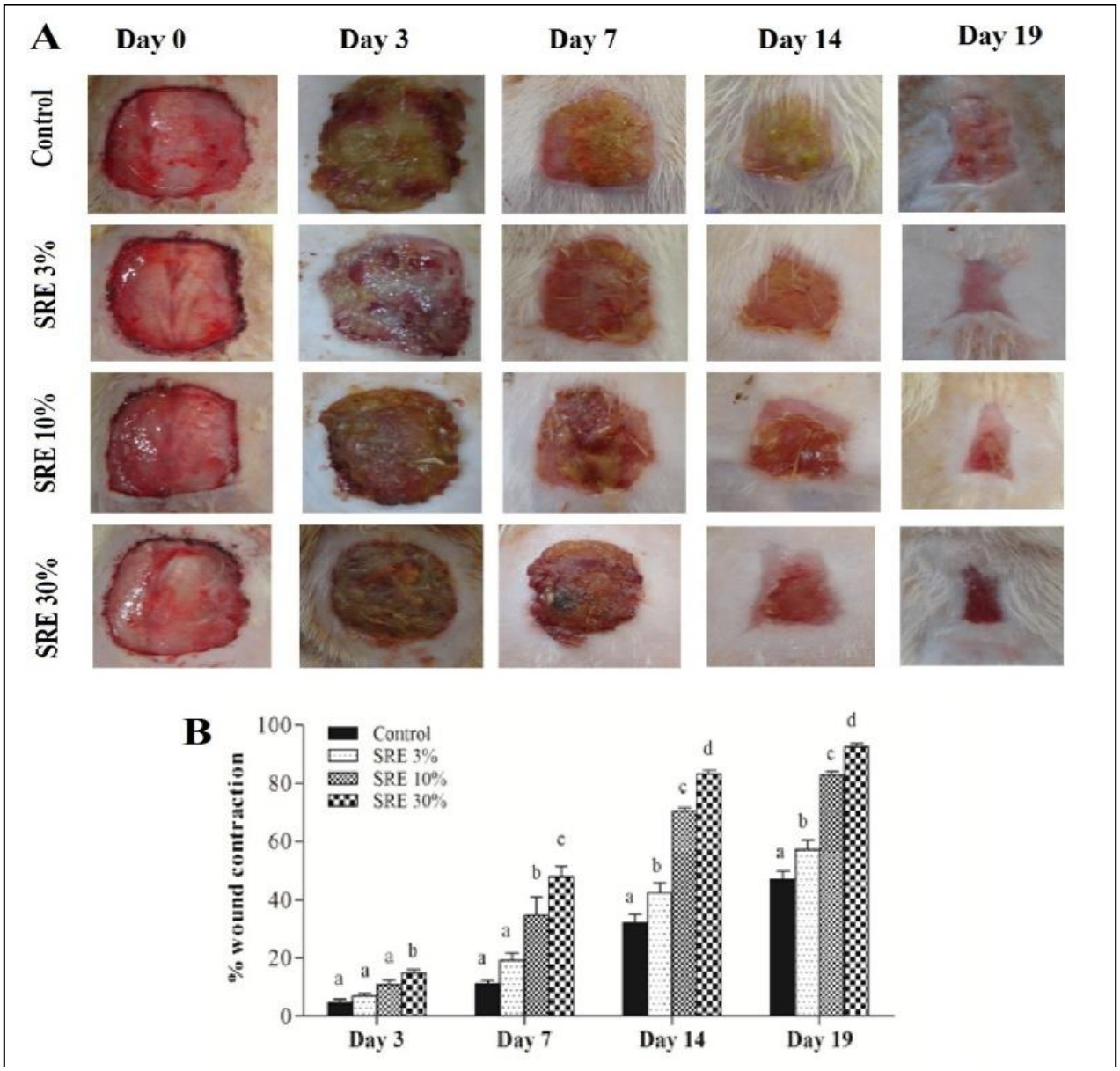

Fig. 1: Representative gross photographs of wounds on day 3, 7, 14 and 19 post-wounding in diabetic rats (A). Comparison of percent closure of wound area on different days (B). Data are expressed as mean ± SE (n=6). Values bearing different superscripts (a, b, c, d) differ significantly ($p<0.05$) on the same day.

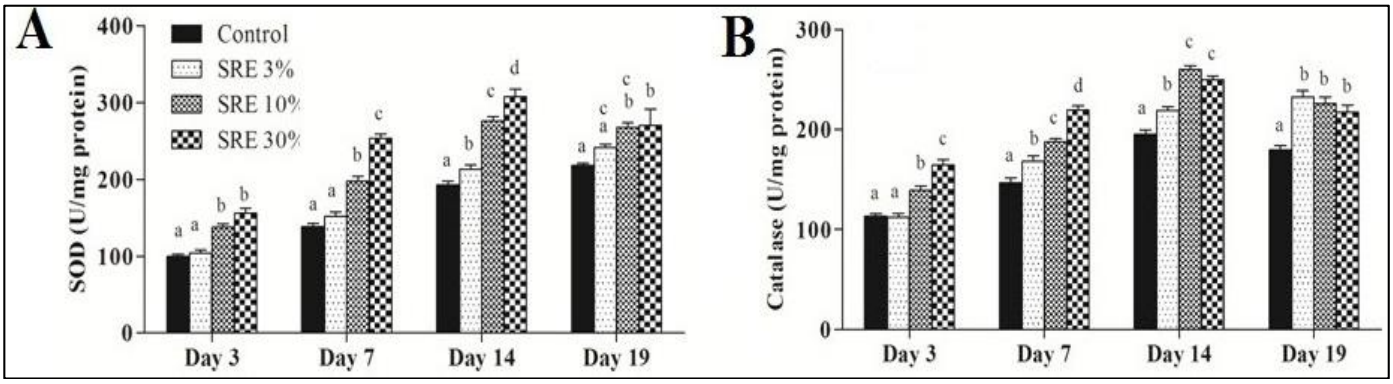

Fig. 2: Effect of topical application of SRE on the activities of SOD (A) and catalase (B) in granulation tissue on days 3, 7, 14 and 19 post-wounding in diabetic rats. Data are expressed as mean ± SE (n=6). Different superscripts (a, b, c, d) differ significantly (p<0.05) on the same day.

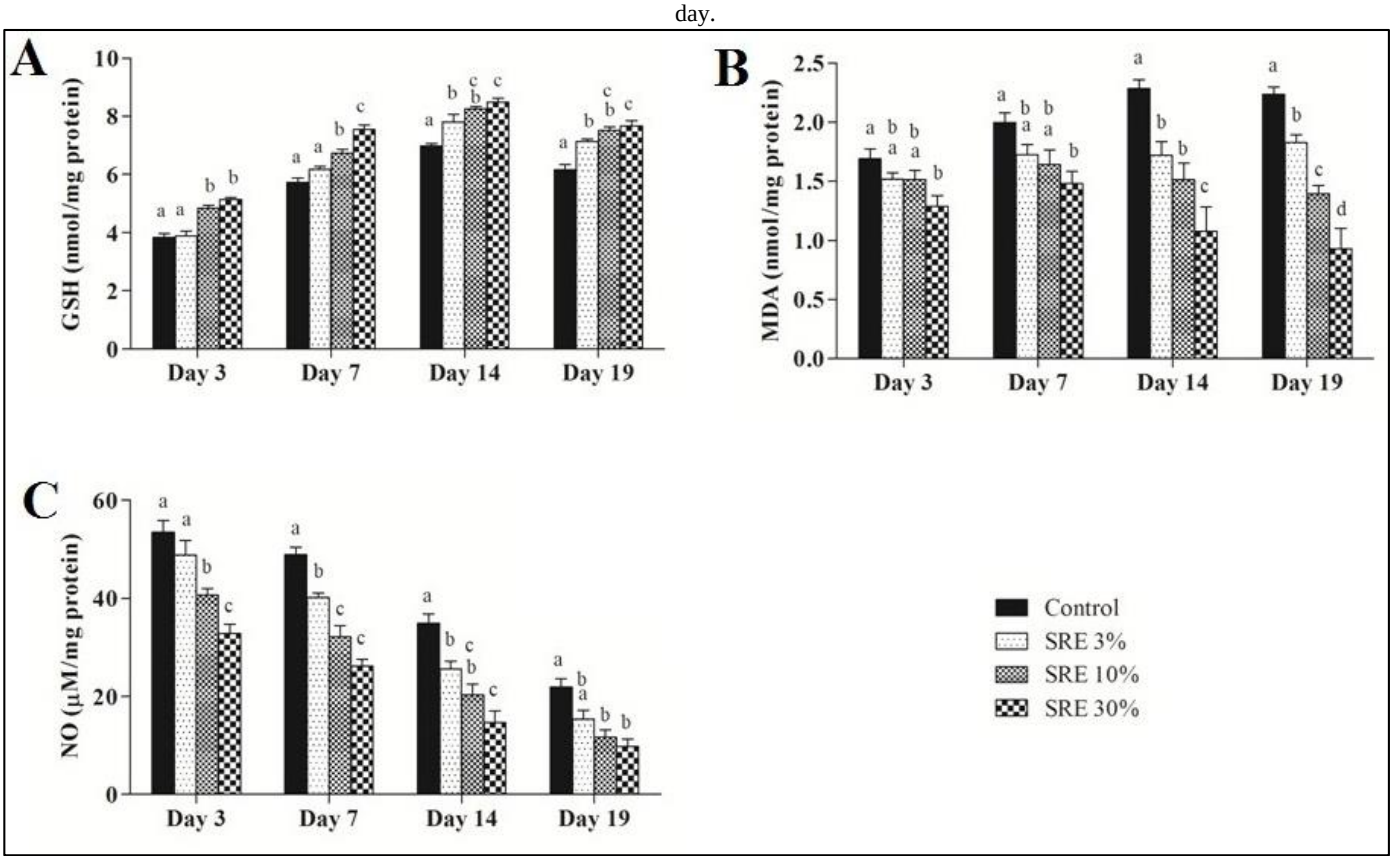

Fig. 3: Effect of topical application of SRE on the levels of GSH (A), MDA (B) and NO (C) in granulation tissue on days 3, 7, 14 and 19 post-wounding in diabetic rats. Data are expressed as mean ± SE (n=6). Different superscripts (a, b, c, d) differ significantly (p<0.05) on the same day

Discussion

In the present study, we observed a progressive decrease in the wound area in a concentration- dependent manner in SRE treated wounds. Our results showed a significant increase in per cent wound closure in SRE-treated groups from day 7 to 19, as compared to control. The results indicate marked acceleration of wound healing process after SRE application. Diabetes mellitus is one major complicating factor for normal wound healing. Impaired healing of diabetic wound is caused by prolongation of inflammatory phase and neutrophil infiltration [24]. This leads to overproduction of ROS through glucose autoxidation [25, 26], which enhances cellular damage and has been implicated in the pathogenesis of diabetes mellitus [27, 28] in animals and humans [29]. According to Gumieniczek *et al.* (2002) [30], higher glucose concentration causes a depression in natural antioxidant defense agents such as glutathione or vitamin C. The increase in the levels of ROS and free radicals cause damage to the biological structures such as cell membrane, genetic material and enzymes. SRE increases superoxide dismutase (SOD) level, which catalyzes the dismutation of O^{-2} into oxygen and H_2O_2, thus, decreases ROS generation and oxidative stress [31]. In the present study, an increase in lipid peroxidation (MDA) with concomitant decrease in GSH, SOD and CAT was observed in the granulation tissues of control rats. Malondialdehyde (MDA) production is an index of lipid peroxidation [32]. The production of free radicals increases the peroxidation of lipid molecules. In the present study, lipid peroxidation is significantly lower in SRE-treated group, as compared to control, which indicates that SRE prevents lipid peroxidation by scavenging free radicals and thus, curtails the undue prolongation of the inflammatory phase of wound healing. It is also similar to our previous findings reported in diabetic neuropathy rat model [16]. Reduced glutathione (GSH) is a major non protein thiol antioxidant compound present in living organisms, which serves a significant role in antioxidant defense mechanism [33]. In this study, there was a significant increase in the level of GSH in SRE treated group

compared to control. Similar observations have been reported earlier [34]. GSH acts as an effective antioxidant, protecting the cellular components from oxidative damage caused by ROS [35]. GSH primarily prevents the oxidation of water soluble proteins and is the main factor in detoxification and antioxidant systems, providing a defense against free radicals and cytotoxins. Enzymatic antioxidant mechanisms play an important role in scavenging free radicals (ROS). Catalase is a heme containing enzyme catalyzing detoxification of H_2O_2 to water and oxygen [33]. The inhibition of catalase activity as a result of STZ-induced hyperglycemia was reported earlier [36]. In this experiment a significantly increased catalase activity was observed in SRE-treated rats revealed that accumulation of excessive H_2O_2 in wound tissues due to enhanced activity of SOD might be properly neutralized by catalase [37, 31, 16]. SOD is considered to be an inducible key enzyme and its activity depends on superoxide concentration in the biological system [38]. A significant increase in the levels of SOD, as observed in the present study, might be due to the adaptive nature of enzyme against increased oxidative stress and tissue damage in diabetic rats. Similar observations were found by Govan *et al*. (1997) [39]. SOD catalyzes the dismutation of superoxide into oxygen and H_2O_2, thus decreases ROS generation and oxidative stress [40, 31]. Increased activities of SOD in SRE-treated rats scavenge superoxide radical and protect the tissue damage by free radicals [41, 34]. Similar results were observed in earlier findings [31, 16]. Thus, SRE possesses antioxidant and free radical scavenging property, as well as enhancing the activities of other antioxidants, such as superoxide dismutase, catalase and reduced glutathione. In conclusion the results suggest that *S. robusta* resin might improve the deranged cutaneous wound healing process by reducing oxidative stress or ROS in diabetic wound.

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