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Safety and healing efficacy of Sea buckthorn (*Hippophae rhamnoides* L.) seed oil on burn wounds in rats

N.K. Upadhyay [a], R. Kumar [a], S.K. Mandotra [a], R.N. Meena [a], M.S. Siddiqui [b], R.C. Sawhney [a], A. Gupta [a,*]

[a] Department of Biochemical Pharmacology, Defence Institute of Physiology and Allied Sciences, Timarpur, Delhi 110054, India
[b] Department of Toxicology, Jamia Hamdard, Hamdard Nagar, Delhi 110062, India

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ABSTRACT

The present investigation was undertaken to determine the safety and efficacy of supercritical CO_2-extracted *Hippophae rhamnoides* L. (Sea buckthorn) (SBT) seed oil on burn wound model. SBT seed oil was co-administered by two routes at a dose of 2.5 ml/kg body weight (p.o.) and 200 μl (topical) for 7 days on experimental burn wounds in rats. The SBT seed oil augmented the wound healing process as indicated by significant increase in wound contraction, hydroxyproline, hexosamine, DNA and total protein contents in comparison to control and reference control treated with silver sulfadiazine (SS) ointment. Histopathological findings further confirmed the healing potential of SBT seed oil. SBT seed oil treatment up-regulated the expression of matrix metalloproteinases (MMP-2 and 9), collagen type-III and VEGF in granulation tissue. It was observed that SBT seed oil also possesses antioxidant properties as evidenced by significant increase in reduced glutathione (GSH) level and reduced production of reactive oxygen species (ROS) in wound granulation tissue. In acute and sub-acute oral toxicity studies, no adverse effects were observed in any of the groups administered with SBT seed oil. These results suggest that the supercritical CO_2-extracted Sea buckthorn seed oil possesses significant wound healing activity and have no associated toxicity or side effects.

1. Introduction

Burn injury is a global public health issue especially for the developing and undeveloped countries, which lack adequate medical facilities. Burn injury may lead to complications such as long-term disability, prolonged hospitalization, loss of body extremities and even death. The skin is maintained by a discrete architecture of cells and extracellular matrix which, serves as the principle barrier to environmental and infectious agents. Tissue injuries resulting from burns, frost-bite, gunshots etc. disrupt this barrier, triggering a healing process (Arturson, 1995). Wound healing is a body's natural process of regenerating dermal and epidermal tissue. The sequence of events that repairs the damage is categorized into three overlapping phases viz. inflammation, proliferation and tissue remodeling (Singer and Clark, 1999). However, burn is characterized by a hypermetabolic state which compromises the immune system leading to chronic wound healing. Thermal exposure to the body surface causes damage to the skin by membrane destabilization, protein coagulation, associated energy depletion and hypoxia at the cellular level which leads to extensive tissue necrosis. Furthermore, the burn wound is a continuous, severe threat against the rest of the body due to invasion of infectious agents, antigen challenge and repeated additional trauma caused by wound cleaning (Arturson, 1995).

In traditional system of medicine such as Ayurveda, Amchi and Chinese, plants are being used to combat several diseases and pathological conditions. The plants or plants derived products seem to possess moderate efficacy with no or less toxicity as compared to synthetic drugs which make them attractive candidate for drug development research programs. *Hippophae rhamnoides* L. (family Elaeagnaceae; commonly known as Sea buckthorn) (SBT) is a branched and thorny nitrogen-fixing deciduous shrub, native to Europe and Asia growing at a height of 2500–4300 m. The plant has been used extensively in oriental traditional system of medicine for treatment of different diseases for more than 1000 years. All parts of this plant are considered to be rich source of a large number of bioactive substances with high medicinal and nutri-

Abbreviations: DNA, deoxyribonucleic acid; g, gram; kg, kilogram; LDH, lactate dehydrogenase; MMP, matrix metalloproteinase; MCV, mean corpuscular volume; μg, microgram; μl, microliter; mg, milligram; ml, milliliter; min, minute; mm^2, millimeter square; Na^+, sodium; p.o., per os; PAGE, polyacrylamide gel electrophoresis; K^+, potassium; RBC, red blood cells; RO, reverse osmosis; ROS, reactive oxygen species; SBT, Sea buckthorn; SS, silver sulfadiazine; SDS, sodium dodecylsulfate; SE, standard error; SGOT, serum glutamic oxaloacetate transaminase; SGPT, serum glutamic pyruvate transaminase; WBC, white blood cells; VEGF, vascular endothelial growth factor.

* Corresponding author. Tel.: +91 11 23831180; fax: +91 11 23914790.
E-mail address: asheeshgupta2001@gmail.com (A. Gupta).

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tional properties. Sea buckthorn found to have significant antioxidative, antimicrobial, immunomodulatory, cytoprotective, hepatoprotective and tissue regenerative properties (Geetha et al., 2002; Gao et al., 2003; Gupta et al., 2005; Negi et al., 2005). The antioxidative activity of the SBT fruit is due to the presence of high content of vitamin C and E, carotenoids as well as antioxidant enzymes such as various superoxide dismutase isoenzymes (Xing et al., 2002). The SBT seed oil is one of the most versatile natural oil and rich in bioactive substances like carotenoids, tocopherols, omega-3 and omega-6 fatty acids and phytosterols (Basu et al., 2007). Sea buckthorn seed oil is reported to have therapeutic role in atopic dermatitis, gastric ulcers and cardiovascular diseases (Yang et al., 1999; Johansson et al., 2000; Eccleston et al., 2002; Xing et al., 2002; Basu et al., 2007).

In most of the previous investigations, the oils were extracted with organic solvents. Recently, supercritical CO_2-extraction of natural oils has been increasing because of having several advantages over the previous extraction processes. Supercritical CO_2-extraction yields totally solvent free oil, which is free of microbes or their spores. In the present study, supercritical CO_2-extracted SBT seed oil was extracted at low temperature in absence of oxygen, thereby minimizing oxidative and thermal stress. The aim of the present study was to investigate the efficacy of supercritical CO_2-extracted SBT seed oil on experimental full-thickness burn wounds in rats, and its possible mechanism of action. The SBT seed oil is also evaluated for its toxicity (acute and sub-acute), if any, in experimental rats.

2. Materials and methods

2.1. Experimental animals

Male Sprague-Dawley rats (180 ± 20 g), from the animal colony of the DIPAS, Delhi were used for this study. The animals were maintained under controlled environment at the Institute's animal house at 25 ± 1 °C and 12-h light–dark cycle. The experiments were performed in accordance with the regulations specified by the Institute's Animal Ethical Committee and conform to the national guidelines on the care and use of laboratory animals, India.

2.2. Supercritical CO_2-extraction of SBT seed oil

Well-ripened SBT fruits were collected from the hilly regions of Western Himalayas in the month of September (2007) where the plant grows wildly under natural conditions. Plant material (voucher specimen SBTS 20) was characterized by Dr. O.P. Chaurasia, an ethnobotanist at the Field Research Laboratory, Leh, India. The method of supercritical CO_2-extraction of seed oil has been described earlier (Basu et al., 2007). In brief, supercritical CO_2-extraction of SBT seed oil was carried out using a pilot-model supercritical fluid extraction unit (SFE-2L, Thar Designs, Inc, Pittsburgh, PA, USA) at 60 °C and at 450-bar pressure with a gas flow of 60 g/min for 3 h. The seed oil was collected in the cyclone separator. The oils were stored at 4 °C until used. Fatty acid composition, β-carotene, tocopherols/tocotrienols and sterols in the seed oil were measured and reported previously by our laboratory (Basu et al., 2007).

2.3. Burn wound model

The animals were anesthetized by intra-peritoneal injection of thiopentone (25 mg/kg, i.p.), the dorsal surface of the rat was shaved, and the underlying skin cleaned with 70% ethanol. Full-thickness burn wound was created by using a metal rod (1.5 cm diameter) heated to 85 °C. The temperature of the metal rod was monitored with a fabricated digital computerized multimeter. Hot rod was exposed at the shaved area of the rat for 20 s, resting on its own weight of 30 g. No additional pressure was applied on the hand leaded metal rod. Single burn wound was created on dorsal part of each rat. After 24 h, dead tissues were excised using sterile surgical blade (Priya et al., 2002). Animals were allowed to recover from anesthesia and housed individually in sterile cages.

2.4. Experimental design

In the preliminary screening, the dose-response study of the SBT seed oil was performed to find out the optimal concentration range. The study was designed in two groups; in Group I different doses of SBT seed oil (1.0, 1.5, 2.0, 2.5 and 5.0 ml/kg body weight) were administered via oral route once a day for 7 days. In Group II, to study the localized effect, different doses of SBT seed oil (100, 200 and 400 μl/wound) were applied topically for 7 days. In Group I, SBT seed oil at a dose of 2.5 ml/kg body weight (p.o.) and in Group II, 200 μl/wound (topical) was found to be the most effective in comparison to other respective doses. Hence, for enhanced efficacy SBT seed oil was administered by both the routes at a dose of 2.5 ml/kg body weight (p.o.) and 200 μl (topical) for 7 days in experimental rats. Silver sulfadiazine (SS) cream USP 1% w/w (Ranbaxy Laboratories Ltd., Delhi, India) was used as standard care. The rats of the control group were kept without any treatment under the standard conditions.

2.5. Wound healing potential

2.5.1. Pro-healing parameters

Wound surface area was measured by tracing its contour using a transparent paper on the eighth day before wound excision to determine wound contraction. The area (mm^2) within the boundary was measured planimetrically (Gupta et al., 2007). The granulation tissue excised on eighth post-wound day was used to analyze the biochemical parameters viz. hydroxyproline, hexosamine, DNA and total protein contents (Elson and Morgan, 1933; Lowry et al., 1951; Burton, 1956; Woessner, 1961).

2.5.2. Reactive oxygen species (ROS) and GSH estimations

A 10% homogenate of granulation tissue was prepared in 0.15 M KCl containing 5 mM EDTA. Samples were sonicated and aliquots were withdrawn for estimations of reduced glutathione (GSH) content and ROS generation. Reduced glutathione is estimated by the method of Beutler et al. (1963). The production of free radicals was determined by using DCFH-DA (2,7-dichlorofluorescein diacetate) as described by Cathcart et al. (1983). Protein in the tissue samples was determined by the method of Lowry et al. (1951).

2.5.3. Histological examinations

The granulation tissues were excised on day eight post-wounding and fixed in 10% neutral formalin. A 6 μm thickness sections were stained with hematoxylin–eosin stain and observed for the histopathological changes under microscope.

2.5.4. Morphometric measurements and analysis

All morphometric parameters were done with Image Analyzer (Olympus Microscope BX61) by using image analyzing computer program (Image-Pro Plus 6.2). All histological sections were assessed through the center of the wounds to obtain maximum wound diameter. The measurements were taken three times, by examining the slides in random sequence, blinded to treatment. The thickness of the newly formed epidermis was measured at 1 mm interval, and the mean was calculated. The density of the granulation was evaluated by taking average number of cells in six high power fields (60X objective), midway in the wound bed. We also counted the number of vascular spaces in six high power fields (60X objective), midway in the wound bed. Dermal thickness was determined at the center of each section, vertically, from the surface of granulation tissue to the margin of dermis and subcutis. Eschar where present, was not included in this measurement.

2.5.5. Gelatin zymography

Matrix metalloproteinases (MMPs) expression was studied in the granulation tissues by gelatin zymography assay (Priya et al., 2002). Briefly, granulation tissue was homogenized with Tris-buffer (saline 0.9%, Tris 0.05 M, Triton X-100-0.25% and $CaCl_2$-0.02 M) and centrifuged at 6000 rpm for 30 min. Tissue extract was subjected to 10% SDS–PAGE containing 0.1% SDS and 1 g/L gelatin under non-reducing conditions without prior boiling. After electrophoresis, gels were washed in 2.5% Triton X-100 for 30 min to remove SDS and allow protein to renature, and subsequently immersed in activity buffer (50 mM Tris/HCl, pH 8.0, 5 mM $CaCl_2$, 0.2 M, 0.02% NaN_3) for 16 h at 37 °C. The gels were then stained with 2.5% Coomassie brilliant blue (CBR-250) in methanol, acetic acid, and water (4:1:5) and then destained with methanol, acetic acid, and water (4:1:5). Enzymatic activities were detected as clear bands of gelatin lysis against blue background.

2.5.6. SDS–PAGE and Western immunoblotting

Wound tissues were chopped into small pieces and collected in Tris-buffer (50 mM, pH 6.8) containing protease inhibitors, phenyl methyl sulfonyl fluoride (PMSF) and aprotonin (Sigma, St. Louis, MO) at 10 and 2 μg/ml, respectively. Tissues were homogenized in Polytron homogenizer (PT 3100 Switzerland) with four strokes of 15 s each in ice bath. After homogenization, the samples were spun at 3000 *g* at 4 °C and the total protein in the supernatant was measured according to the method of Lowry et al. (1951).

Analysis of total protein in the tissue samples was done by PAGE in 4% (v/v) stacking and 10% (v/v) separation polyacrylamide gels in the presence of SDS, followed by staining with CBR-250 (Gupta et al., 2008). The homogenized tissue samples mixed in a sample buffer containing 1% SDS, 2% 2-mercaptoethanol and 10% glycerol were heat reduced in a boiling water bath, where as the gel and running buffer contained 0.1 and 0.2% SDS, respectively. All other conditions were same as described previously by Gupta et al. (2008). The molecular weight was determined using standard protein markers (Broad range 200–6.9 kDa, BIO-RAD).

After electrophoretic separation of tissue proteins by SDS–PAGE, the proteins were electro-transferred on to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h in Tris-buffered saline with 0.1% Tween-20 (TBST, pH 7.5) containing 5% milk protein. After incubation with rabbit polyclonal primary antibody (VEGF, Santa Cruz Biotechnology, CA) and mouse monoclonal primary antibody (Collagen Type-III, Sigma, St. Louis, MO) for 2 h, the blots were washed extensively with TBST. Primary antibodies were revealed via incubation with alkaline phosphatase-conjugated secondary antibody, goat-anti-rabbit and goat-anti-mouse (Santa Cruz Biotechnology, CA), respectively for 1 h. The blots then were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma, St. Louis, MO).

2.6. Safety evaluation of SBT seed oil

2.6.1. Acute oral toxicity

Acute toxicity studies have been carried out to investigate the safety aspects of the SBT seed oil. Healthy adult rats of either sex, fasted overnight, were fed with four different doses of SBT seed oil (2.5, 5.0, 7.5 and 10.0 ml/kg body weight, p.o.). After single administration of SBT seed oil, the animals were provided with food and water immediately and closely observed in their cages for any mortality and signs of severe toxic symptoms such as hypo-activity, piloerection, anorexia, salivation, diarrhea, syncope, muscle cramping, convulsions, if any, for 24 h and daily for next 14 days.

2.6.2. Sub-acute oral toxicity

The repeated dose oral toxicity study was further sub-divided into two studies. In study I, the SBT seed oil was administered orally to rats at two different doses, 2.5 ml/kg and 5.0 ml/kg, once a day, for 14 days. Control rats were given orally equal volume of RO water, once a day, for 14 day.

In study II, SBT seed oil was given orally to determine toxicity, if any, after long-term administration of maximal effective dose of SBT seed oil. Rats were given daily, maximal effective dose (2.5 ml/kg body weight) of SBT seed oil, orally, for 28 days. Control rats received equal volume of RO water once daily for 28 days. For each study body weight of the animals was recorded daily. Organ weight/body weight ratios were determined of all the rats in each group. Food and water were freely available to the animals during the experiment. After 14 and 28 days of drug treatment, animals were fasted overnight and for clinical chemistry blood samples were obtained from orbital sinus (Riley, 1960) using capillary tubes (with and without heparin as per requirement) under mild ether anesthesia. Blood for hematology studies was collected into tubes containing ethylene-diamine-tetra-acetic acid (EDTA) as an anticoagulant.

After animals were sacrificed, vital organs (kidney, liver, spleen, adrenal, testis, heart and lung) were carefully dissected out, cleaned of the adhering connective tissues, blotted and accurately weighed. Ratio of each organ to body weight was determined. Fasting blood glucose was estimated using blood glucose meter (Bayer Diagnostics India Ltd., Baroda, Gujarat, India). In whole blood lactate dehydrogenase (LDH) activity was determined (Kornberg, 1969). Serum SGOT (King, 1965), SGPT (Wroblewski and LaDue, 1956), alkaline phosphatase (Lowry et al., 1954), bilirubin (Malloy and Evelyn, 1937), creatinine (Bonsnes and Taussky, 1945), total cholesterol (CHOD-PAP Diagnostic kits, Cat. Number E 98118; Velon Pharmacal. Pvt. Ltd., Mumbai, India), triglycerides (GPO-PAP Diagnostic kits, Cat. Number E 96388; Velon Pharmacal. Pvt. Ltd., Mumbai, India), total serum protein (Lowry et al., 1951) and serum electrolytes (Na^+, K^+) using Stat Profile Phox Plus Blood-Gas Analyzer (Nova Biomedical, Waltham, MA 02454-9141, USA) were estimated. Hemoglobin and other hematological parameters were determined in control and SBT seed oil treated animals using semi-automated micro cell counter Sysmex F-820 (TOA Medical Electronics Corporation Ltd., Kobe, Japan). All the vital organs (Kidney, heart, adrenal, testes, spleen, liver, and lung) of the animals were taken out to study histopathological change, if any. After fixation, tissues were routinely processed, embedded in paraffin, cut in microtome setting of 6 μm, mounted on glass slides, stained with hematoxylin and eosin and examined by light microscopy.

2.6.3. Acute dermal irritation assay

A primary skin irritation study was conducted with albino rabbit to determine the irritation potential of the SBT seed oil. Each animal was treated with 0.5 ml of SBT seed oil and applied to the skin of one flank using a gauze patch. The patch was held in place with a semi-occlusive bandage for 4 h, after which the patch was removed and skin cleaned of residual SBT seed oil. Skin reactions and irritation effects were assessed at approximately 1, 24, 48, and 72 h after the removal of the dressings. Adjacent areas of untreated skin from each animal served as controls. Erythema and edema were scored on a scale of 0–4, with 0 showing no effect and 4 representing severe erythema or edema.

2.7. Statistical analysis

Data are expressed as mean + SE and statistical significance between experimental and control values was analyzed by ANOVA followed by Dunnett's test using Graph Pad Prism 2.01 (Graph Pad Software Inc.). A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Wound healing potential

3.1.1. Augmented pro-healing parameters

Untreated burn wounds showed edema, whereas wounds treated with SBT seed oil showed reduced or no oedema (Fig. 1). There was increase rate of wound contraction in SBT seed oil treated burn wounds at 4 and 7 day post-wounding as compared with the reference control treated with silver sulfadiazine and untreated control burn wounds (Fig. 2). The SBT seed oil treatment significantly increased the DNA (34%), total protein (31%), hydroxyproline (24%) and hexosamine content (28%) in the granulation tissues of experimental rats (Fig. 3A–D). However silver sulfadiazine treated burn wounds showed increase in hydroxyproline and total protein content comparable with that of SBT seed oil treated burn wounds but

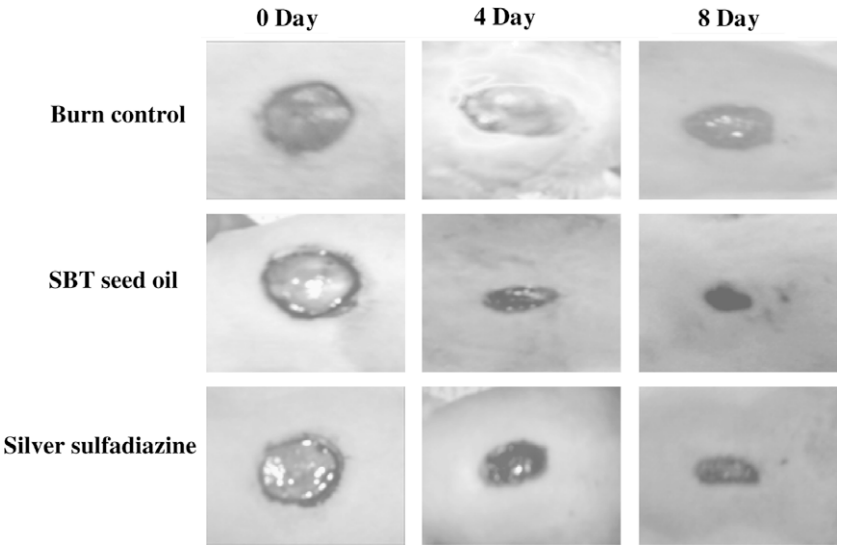

Fig. 1. Photomicrographs showing no, absence inflammation and faster wound contraction in Sea buckthorn seed oil treated burn wounds as compared to untreated control burn wounds.

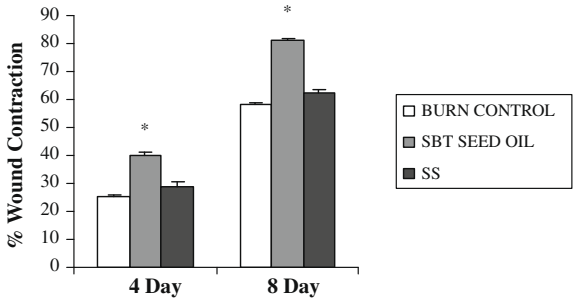

Fig. 2. Percent wound contraction in Sea buckthorn seed oil treated and untreated burn wounds after 7 days of treatment. Values are mean ± SE of six rats. $^{*}P < 0.05$ compared with untreated burn wound.

rate of wound contraction, DNA and hexosamine level were significantly lesser as compared to SBT seed oil treated burn wounds (Fig. 3A–D).

3.1.2. Reactive oxygen species (ROS) and GSH

Fig. 4 shows GSH and ROS generation in granulation tissue of experimental and control burn wounds. A significant increase in GSH content by 22% was observed in SBT seed oil treated wounds, whereas the ROS production decreased significantly by 29% as compared to untreated control burn wounds.

3.1.3. Histological examinations

The histological examinations showed that tissue regeneration was much better in the SBT seed oil treated burn wounds. Granulation tissue of SBT seed oil treated wounds showed reduced congestion, edema and polymorphonuclear leukocytes (PMNLs) infiltration, which was present in untreated control burn wounds. The SBT seed oil and silver sulfadiazine treated burn wound showed complete epithelialization with increased fibroblast and collagen deposition in the dermis. SBT seed oil treated burn wounds also showed well developed blood vessel formation at the wound site in comparison to untreated control burn wounds (Fig. 5).

3.1.4. Morphometric analysis

Granulation tissue is composed of new blood vessels, collagen and several cell types including fibroblasts, myofibroblasts and macrophages. The migration and the proliferation of cells into the wound bed promote wound healing by secreting various growth factors and extracellular matrix proteins. The morphomet-

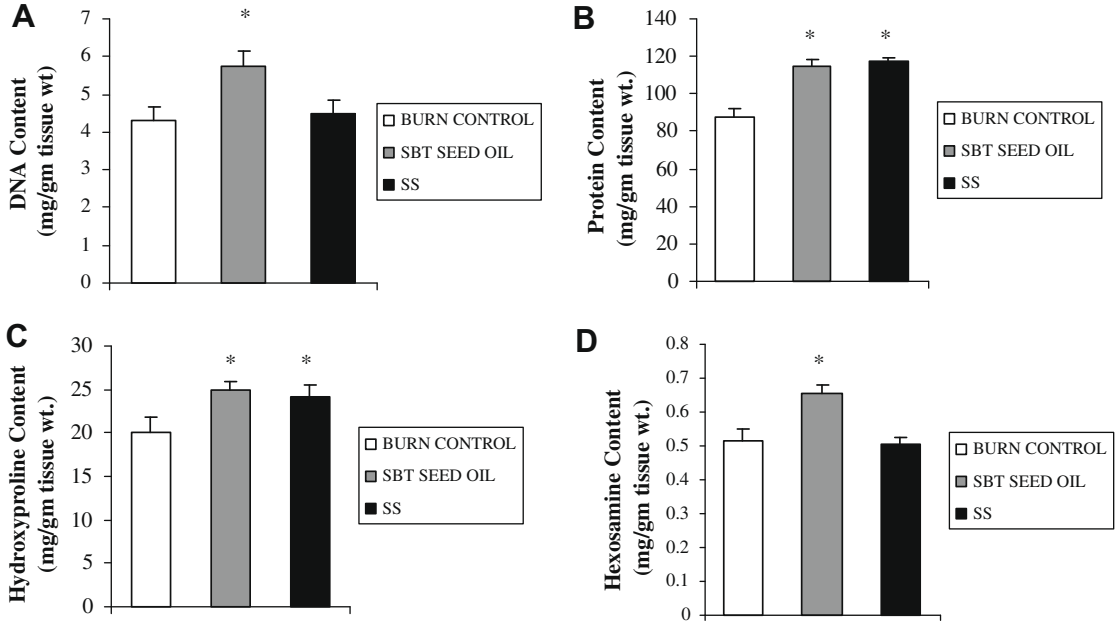

Fig. 3. Pro-healing parameters in Sea buckthorn seed oil treated and untreated burn wounds after 7 days of treatment (A) DNA, (B) total protein, (C) hydroxyproline, and (D) hexosamine content. Values are mean ± SE of six rats. $^{*}P < 0.05$ compared with untreated burn wound.

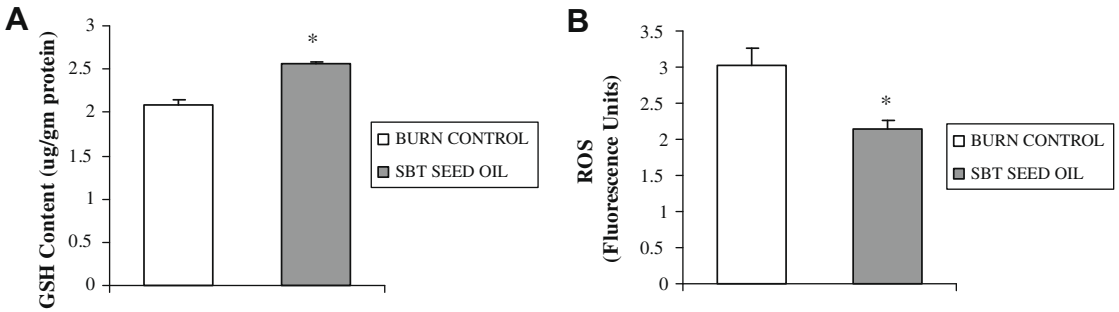

Fig. 4. (A) Reduced glutathione (GSH) levels, and (B) reactive oxygen species (ROS) generation in Sea buckthorn seed oil treated and untreated burn wounds after 7 days of treatment. Values are mean ± SE of six rats. $^{*}P < 0.05$ compared with untreated burn wound.

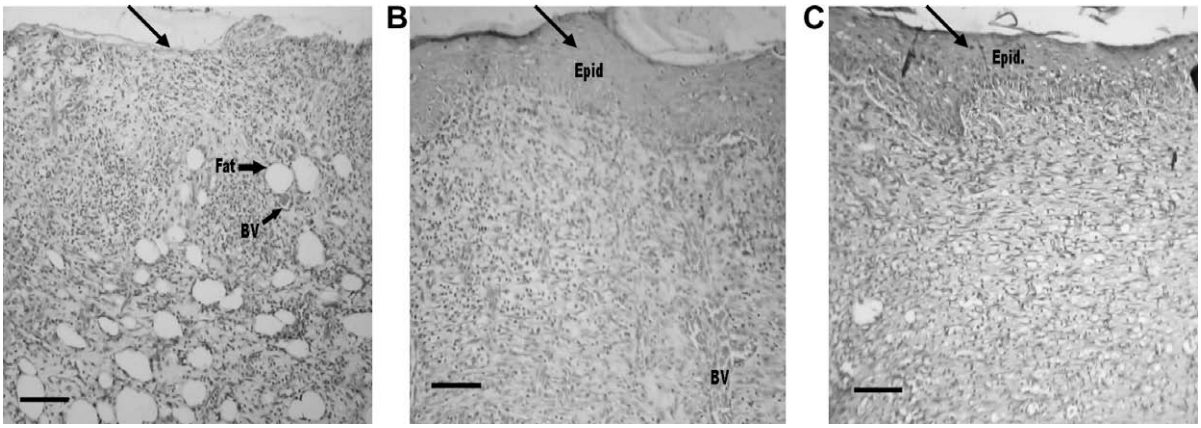

Fig. 5. Histopathological changes of the skin wound section of (A) untreated burn control rats on eighth post-wound day showing non-epithelialized (←) wound surface with slight edema and congestion. Skin wound section of (B) SBT seed oil, and (C) silver sulfadiazine treated burn wounds showing wound surface with well-organized thick epithelium (←) and significant fibroblast and collagen deposition in deeper dermis. Neovascularization (BV) is well developed in SBT seed oil treated burn wounds. Scale bar 100 μm.

ric analyses of the histological sections of the wound section showed that the SBT seed oil treatment resulted in increased granulation tissue density (37%) when compared to untreated burn controls (Fig. 6C). SBT seed oil treatment also resulted in the formation of augmented epidermis (52%) and dermis (28%) as compared with untreated burn control (Fig. 6B and C). We have investigated whether the healing wounds showed any increase in angiogenesis by counting the blood vessels in the dermis. Vessel counts showed a significant increase (2 fold) in the number of blood vessels in SBT seed oil treated wounds on the 8th post-wounding day (Fig. 6D).

3.1.5. Gelatin zymography

MMP-2 and 9 were the major matrix metalloproteinases observed in the present study. Gelatin zymography analysis of the granulation tissue after 7 days of SBT seed oil treatment showed increased expression of both MMP-2 and 9 in comparison to the untreated control burn wounds (Fig. 7A).

3.1.6. SDS–PAGE and Western blotting

SDS–PAGE analysis of granulation tissue after 7 days treatment with SBT seed oil in experimental rats showed differential expression of some proteins as compared to the untreated control burn wounds (Fig. 7B). Western blot analysis showed an up-regulated expression of VEGF and collagen type-III in SBT seed oil treated wounds as compared to untreated control burn wounds (Fig. 7C).

3.2. Safety studies

3.2.1. Acute toxicity studies

The acute toxicity studies showed that the SBT seed oil was safe up to a maximum dose of 10 ml/kg body weight of the animal. No adverse effects were observed in any of the groups, treated, with single oral dose of SBT seed oil upto 10.0 ml/kg body weight within 24 h of treatment and even after 14 days of drug treatment.

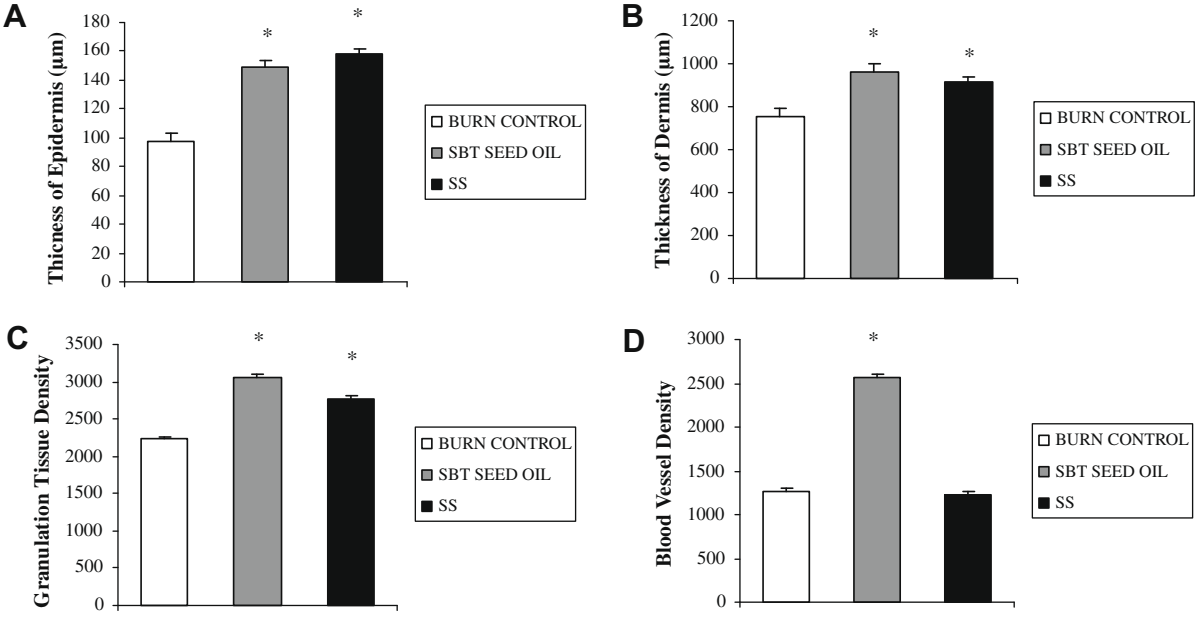

Fig. 6. Morphometric analysis of the histological sections showing (A) thickness of the epidermis, (B) thickness of the dermis, (C) granulation tissue density, and (D) blood vessel density of Sea buckthorn seed oil treated and untreated burn wounds after 7 days of treatment (Image-Pro Plus 6.2 Version). Values are mean ± SE of six rats. $^{*}P < 0.05$ compared with untreated burn wound.

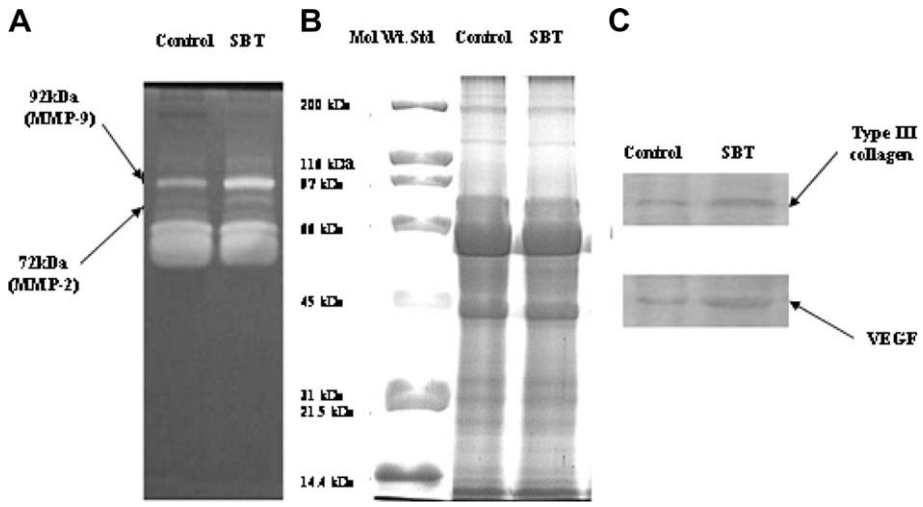

Fig. 7. (A) Matrix metalloproteinase expression by gelatin zymography (10% SDS–PAGE, 1 g/l gelatin) in SBT seed oil treated and untreated burn wound tissue of experimental rats after 7 days of treatment. (B) Expression of standard marker proteins (200–6.9 kDa, BIO-RAD, 10% SDS–PAGE). (C) VEGF and collagen type-III analyzed by Western blot in SBT seed oil treated and untreated wound tissue of experimental rats after 7 days of treatment.

3.2.2. Sub-acute oral toxicity

The 2.5 ml/kg and 5.0 ml/kg body weight SBT seed oil doses treated animals gained body weight well up to 14 days in comparison to one another and control animals. Table 1 shows the organ weight/body weight ratio of animals treated with 2.5 ml/kg and 5.0 ml/kg body weight drug dose. There was no significant change in the organ weight/body weight ratios of rats, in comparison to control animals. Table 2, shows the biochemical and hematological parameters of animals orally treated with 2.5 ml/kg and 5.0 ml/kg body weight of SBT seed oil. There was no significant change in any studied parameter, in comparison to control animals. The 2.5 ml/kg body weight SBT seed oil dose (maximal effective dose) treated animals gained body weight well up to 28 days in comparison to control animals. Table 3 shows organ weight/body weight ratio

Table 1
Effects of sub-acute Sea buckthorn seed oil oral administration (2.5 ml/kg and 5.0 ml/kg body weight for 14 days) on the organ weight/body weight ratio of rats.

Organ	Control	2.5 ml/kg	5.0 ml/kg
Liver $\times 10^{-3}$	28.58 ± 1.04	27.87 ± 0.82	30.08 ± 0.81
Heart $\times 10^{-3}$	3.45 ± 0.07	3.43 ± 0.12	3.66 ± 0.12
Kidney $\times 10^{-3}$	4.23 ± 0.16	4.11 ± 0.08	4.05 ± 0.17
Spleen $\times 10^{-3}$	2.22 ± 0.10	2.20 ± 0.09	2.14 ± 0.14
Testis $\times 10^{-3}$	4.55 ± 0.27	4.75 ± 0.19	4.41 ± 0.18
Adrenal $\times 10^{-5}$	9.25 ± 0.64	9.41 ± 0.59	9.47 ± 0.40
Lung $\times 10^{-3}$	4.90 ± 0.41	5.05 ± 0.46	4.87 ± 0.22

All the values are mean ± SE of eight rats in each group.

Table 2
Effects of sub-acute Sea buckthorn seed oil, oral administration (2.5 ml/kg and 5.0 ml/kg body weight for 14 days) on the biochemical and hematological parameters of rats.

Parameters	Control	2.5 ml/kg	5.0 ml/kg
Creatinine (mg/dl)	0.60 ± 0.01	0.63 ± 0.02	0.59 ± 0.02
Sodium (meq/l)	144.72 ± 3.35	150.64 ± 4.19	147.75 ± 2.27
Potassium (meq/l)	4.94 ± 0.43	5.18 ± 0.48	5.18 ± 0.33
Cholesterol (mg/dl)	82.21 ± 2.73	83.16 ± 2.93	82.04 ± 3.24
Triglyceride (mg/dl)	60.74 ± 1.67	63.21 ± 2.17	60.11 ± 1.43
Direct bilirubin (mg/dl)	0.37 ± 0.01	0.37 ± 0.02	0.35 ± 0.01
Alkaline phosphatase (IU)	7.83 ± 0.30	7.71 ± 0.50	8.08 ± 0.37
SGOT (IU)	29.19 ± 1.95	29.31 ± 2.37	31.83 ± 1.73
SGPT (IU)	7.46 ± 0.50	7.41 ± 0.29	7.16 ± 0.46
LDH (nmol/mg protein)	10.16 ± 0.82	10.26 ± 0.53	9.99 ± 0.48
Blood glucose (mg%)	93.41 ± 5.03	95.94 ± 4.78	93.34 ± 5.20
Protein (g/dl)	8.70 ± 0.74	8.91 ± 0.71	8.90 ± 0.80
WBC ($\times 10^3$ μl)	4.90 ± 0.40	5.06 ± 0.42	4.82 ± 0.20
RBC ($\times 10^6$ μl)	7.34 ± 0.51	7.42 ± 0.26	7.11 ± 0.40
Hemoglobin (g%)	14.18 ± 0.79	14.42 ± 0.51	14.39 ± 1.00
Hematocrit (%)	43.75 ± 1.06	45.95 ± 0.99	43.98 ± 0.90
MCV (fl)	59.36 ± 1.68	60.71 ± 2.51	58.99 ± 1.55
Platelets (10^3 μl)	752.57 ± 22.34	744.23 ± 28.34	756.95 ± 37.35

All the values are mean ± SE of eight rats in each group.

Table 3
Effects of sub-acute Sea buckthorn seed oil, oral administration (2.5 ml/kg body weight for 28 days) on the organ weight/body weight ratio of rats.

Organ	Control	2.5 ml/kg
Lung $\times 10^{-3}$	4.79 ± 0.23	4.71 ± 0.20
Liver $\times 10^{-3}$	29.77 ± 1.08	30.13 ± 0.96
Heart $\times 10^{-3}$	3.62 ± 0.07	3.87 ± 0.10
Kidney $\times 10^{-3}$	3.98 ± 0.15	3.93 ± 0.08
Spleen $\times 10^{-3}$	2.07 ± 0.08	2.20 ± 0.08
Testis $\times 10^{-3}$	4.65 ± 0.23	4.62 ± 0.31
Adrenal $\times 10^{-5}$	9.32 ± 0.41	9.39 ± 0.53

All the values are mean ± SE of eight rats in each group.

Table 4
Effects of sub-acute Sea buckthorn seed oil, oral administration (2.5 ml/kg body weight for 14 days) on the biochemical and hematological parameters of rats.

Parameters	Control	2.5 ml/kg
Creatinine (mg/dl)	0.61 ± 0.02	0.62 ± 0.01
Sodium (meq/l)	146.32 ± 2.08	144.96 ± 1.95
Potassium (meq/l)	5.04 ± 0.36	5.02 ± 0.32
Cholesterol (mg/dl)	80.96 ± 2.19	80.91 ± 0.94
Triglyceride (mg/dl)	61.15 ± 1.45	62.86 ± 1.31
Direct bilirubin (mg/dl)	0.36 ± 0.02	0.38 ± 0.02
Alkaline phosphatase (IU)	8.01 ± 0.13	7.84 ± 0.42
SGOT (IU)	31.83 ± 2.35	31.97 ± 2.27
SGPT (IU)	7.88 ± 0.40	7.58 ± 0.53
LDH (nmol/mg protein)	10.25 ± 0.64	10.07 ± 0.45
Blood glucose (mg%)	93.14 ± 4.91	90.62 ± 5.67
Protein (g/dl)	9.10 ± 0.51	9.19 ± 0.62
WBC ($\times 10^3$ μl)	4.80 ± 0.22	4.70 ± 0.17
RBC ($\times 10^6$ μl)	7.71 ± 0.34	7.66 ± 0.63
Hemoglobin (g%)	14.59 ± 0.93	14.75 ± 0.94
Hematocrit (%)	44.43 ± 1.43	44.51 ± 0.72
MCV (fl)	59.11 ± 1.48	60.05 ± 1.38
Platelets (10^3 μl)	780.91 ± 40.29	746.13 ± 41.27

All the values are mean ± SE of eight rats in each group.

of rats treated with 2.5 ml/kg body weight drug dose for 28 days. Table 4 shows the results on the biochemical and hematological parameters of animals orally treated with 2.5 ml/kg maximal effective dose for wound healing efficacy, once daily for 28 days. There was no significant change in any studied parameter of experimental rats, in comparison to control. The kidney, liver, spleen, adrenal, testis, heart and lung of rats treated with 2.5 ml/kg and 5.0 ml/kg body weight doses for 14 days and with 2.5 ml/kg body weight dose for 28 days showed a normal histological appearance.

3.2.3. Acute dermal irritation assay

No irritation was observed following the 4 h dermal exposure of SBT seed oil over the skin of the test animals.

4. Discussion

In the present study, no experimental rats administered with supercritical CO_2-extracted Sea buckthorn seed oil were died or showed any adverse effects in acute and sub-acute oral toxicity studies. Wound healing is a highly ordered and well coordinated process that involves inflammation, granulation tissue formation, fibrogenesis, neovascularization, wound contraction and resurfacing of the wound defect with the epithelium. Inflammation plays an important role in fighting infection and inducing the proliferation phase necessary for healing. However, inflammation can lead to tissue damage if it lasts too long. In the present study, untreated burn wounds showed edema reflecting persistent inflammation, whereas wounds treated with seed oil showed reduced or no edema. This can be correlated with the faster contraction of the wound size. The increased rate of wound contraction in SBT seed oil treated wounds might be attributed to increased proliferation and transformation of fibroblast cells into myofibroblasts (Gupta et al., 2007; Priya et al., 2002).

The process of post-inflammatory wound healing is characterized by a complex, multicomponent cascade of biosynthetic and degradative reactions that direct underlying cell–cell and cell–extracellular matrix interactions. Collagen is a major extracellular matrix protein which confers strength and integrity to the tissue matrix and plays an important role in homeostasis and in epithelialization at the later phase of healing (Singer and Clark, 1999). Hydroxyproline is a marker of collagen content. The increased level of hydroxyproline and hexosamine in SBT seed oil treated burn wounds provide the strength to the regenerated tissue. Enhanced expression of collagen type-III as revealed through Western blotting signifies the increased collagen biosynthesis in SBT seed oil treated burn wounds.

MMPs are a family of zinc endopeptidases which, play a key role in regulating repair processes, i.e., eliminate damaged protein, destroy the provisional extracellular matrix, facilitate migration to the center of the wound, remodel the granulation tissue, probably control angiogenesis and also regulate the activity of some growth factors. In the present study, gelatin zymography revealed the increased expression of MMP-2 and 9 in treated rats. It suggests that SBT seed oil played an important role in tissue remodeling phase of wound healing (Jessica and Barbara, 2006).

Granulation tissue is composed of new blood vessels, collagen, and several cell types including fibroblasts, myofibroblasts, and macrophages. The increased level of granulation tissue, DNA and total protein content in treated burn wounds reflects the mitogenic potential of the SBT seed oil. VEGF appears to be a key factor in tissue repair that involves neovascularization as well as enhanced vascular permeability. VEGF improves angiogenesis during wound healing by stimulating the migration of endothelial cells through the extracellular matrix. Angiogenesis during wound repair serves the dual function of providing the nutrients demanded by the healing tissues and contributing to structural repair through the formation of granulation tissue (Singer and Clark, 1999). In the present study, SBT seed oil was found to increase angiogenesis as evidenced by the enhanced expression of VEGF. Histological evaluation also revealed increased blood vessels formation in the granulation tissue of SBT seed oil treated burn wounds.

In biological systems, a major endogenous thiol antioxidant is GSH. The SBT seed oil treated wounds exhibited an increase in GSH content, which might be correlated with the reduced reactive oxygen species (ROS) generation at the wound site. Thus, it appears that the SBT seed oil mediated wound healing activity could be due to its antioxidant property as one of the factors.

The SBT seed oil is very rich in polyunsaturated fatty acids (PUFA) like oleic acid (30%), linoleic acid (26%) and linolenic acid (18%) (Basu et al., 2007). The omega-3 fatty acids can increase pro-inflammatory cytokine production at wound sites and thus reported to have therapeutic potential in cutaneous wound healing (McDaniel et al., 2008). Tocopherols, β-carotene and carotenoids are other compounds present in SBT seed oil that could accelerate the wound healing process (Gerber and Erdman, 1982; Musalmah et al., 2002).

In conclusion, the present study suggests that supercritical CO_2-extracted Sea buckthorn seed oil has significant wound healing activity in full-thickness burn wounds and found to be safe for use. The wound healing potential of Sea buckthorn seed oil might be due to presence of omega-3 and omega-6 fatty acids, tocopherols and carotenoids.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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