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Wound Healing Potential of *Hippophae salicifolia* Bark Extract in Rats

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ABSTRACT

Wound healing is a fundamental response to tissue injury that results in restoration of tissue integrity. The present study was undertaken in Sprague Dawley male rats to evaluate the healing efficacy of lyophilized bark extract of H. salicifolia (HS) and compared to reference control (RC)(Povidone Iodine 5% w/v) and placebo control (PC)(Propylene glycol B.P.) using full thickness excision wound model. The optimum concentration of SBT extract for topical application in excised wound healing was found to be 0.25 percent (w/w) on the basis of the percentage wound contraction measurement.. Total of 34 male rats was divided into three groups and topical application of PC, RC and 0.25 percent (w/w) HS bark extract in PG was applied once daily on the 4 mm and 8 mm wounds. Percentage wound contraction measured after 2 days interval revealed that HS group had significantly faster percent wound contraction against RC group on day 2 and against PC group on day 4 and 6. The histological examination of tissues collected on day 2,6,14 further confirmed the superior healing efficacy of the HS in comparison to RC and PC groups. HS group showed faster epithelial bridging, lower PMNL count, higher fibroblast score, higher neo-angiogenesis and collagen score on day 6 while higher remodelling status with respect to the blood vessel, Fibroblast, collagen and epithelial score on day 14. In conclusion, H.salicifolia bark extract revealed significant healing potential in excised wound and positive influence on various phases of wound healing.

Keywords: Hippophae salicifolia bark, Seabuckthorn bark extract; Seabuckthorn bark on wound healing

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INTRODUCTION

Wound healing and tissue repair are complex processes that involve series of dynamic events including clotting, inflammation, re-epithelialization, granulation tissue formation, fibroblast proliferation, collagen synthesis and tissue remodelling. Wound healing may be compromised by various factors like diabetes, radiation exposure, adverse environmental conditions, microbial infection, the presence of reactive oxygen species etc. The process of wound healing is necessary to prevent invasion of damaged tissue by pathogens and to partially or completely reform the damaged tissue.

Alternative and complementary systems of medicine such as Ayurveda, Siddha, Amchi, Chinese and Aromatherapy are being used to combat several pathological conditions using various medicinal plants. Herbal products seem to possess moderate efficacy with little or no toxicity and are less expensive.

Seabuckthorn (SBT) is one among widely known important medicinal plants which has been used by Chinese and Tibetan people as traditional medicine. SBT seed oil has significant wound healing activity in full thickness burns and split thickness harvested wounds [1]. SBT has antiulcerogenic, antioxidant, and wound healing effects [2]. SBT leaves have been found to be effective in wound healing [3]. SBT oil has beneficial effect on skin because it is strong antioxidant and supports wound healing, improves skin elasticity and protect against harmful radiation [4]

Peel of SBT stem contains 5-HT which is rare among plants.It can have important anti-radiation, anti-infection, anti-cancer function and it can promote coagulation by transforming fibrinogen into fibrin [5]. Bark and shoots of SBT have anti-metastatic effects [6]. Proanthocyanidin derived from SBT bark extract enhanced healing of experimentally induced chronic gastric ulceration [7]. The bark of other plants like *Cinnamon, Cassia, Terminalia arjuna, Pinus maritima, Betula alnoides* have shown anti-inflammatory, anti-bacterial, anti-oxidant, hepatoprotective and wound healing properties.

The wound healing efficacy of SBT leaf and fruit extracts has been well studied. However the wound healing efficacy of the SBT bark extract was yet to be investigated. Hence the present study was designed to explore the wound healing efficacy of SBT bark using Povidone Iodine as a reference control for their potential in soft tissue healing using full thickness dermal excision wound model in rats.

MATERIAL AND METHODS

Preparation of bark extract:

The young bark of HS was oven dried and grinded to make a fine powder. The powder was soaked in the water-ethanol mixture (1:1 v/v) for 24 hours at room temperature with intermittent shaking. The supernatant was filtered using a double layer of muslin cloth followed by Whatman filter paper No.1. The filtrate was Rota-evaporated at 68-78°C at 150 rpm. The contents were freeze-dried using lyophilizer for 4-5 hours at - 49°C until the dark brown crystalline form was achieved.

Preparation of topical application using SBT bark extract:

Weighed quantities of SBT bark extract was dissolved in Propylene glycol B.P (Make: LOBA CHEMIE, CAS No. 57-55-6) w/w to prepare different concentrations for topical application.

Animal models:

Sprague Dawley rats procured from NIPER (Mohali, Punjab) were bred to F1 generation progeny for use in the present study. Male rats more than 8 weeks old and weighing 200±20 grams body weights were used. All the experimental procedures were performed according to guidelines of IAEC.

Experimental design:

Excision wound model:

The rats were anaesthetized using an intraperitoneal injection of a combination of Ketamine @60-70 mg/kg and Xylazine@5mg/kg body weight. A midline incision of 12-16 mm was made for inserting spatula under the skin. Biopsy punch of 4mm and 8 mm size were applied to make a wound on the skin on both sides and the midline incision was sutured with silk.

Dose Standardization:

The rats were placed in 7 groups with one male rat in each group. Four excision wounds of 8mm diameter were created on each rat. A range of various doses of SBT (0%, 0.25%, 0.5%, 1%, 2%, 2.5% and 5 %) were tested for optimization of the dose. A total of 4 reading was taken from each rat and dose was decided on the basis of percent wound contraction as observed on day 0, 3, 6 and 8 [3,8]. The wound area was measured using images of wound area along with the scale.

Wound healing potential of H. *salicifolia*

The wound healing potential of HS was evaluated and compared with Placebo control (PC) i.e. Propylene Glycol and Reference control (RC) i.e. Povidone Iodine. The experimental group and their details are shown in table 1. The rats were sacrificed on day 2nd, 6th and 14th post surgery. The wound area was measured every alternate day from day zero till sacrifice.

Histopathological and histochemical analysis:

The rats from each group were euthanized by using Halothane gas chamber for collection of tissue from wound area on day 2nd, 6th and 14th post surgery. The tissues were processed by paraffin embedding technique for histological evaluation using routine Hematoxylin-Eosin (Basic staining) and Van-Gieson staining (Collagen staining). Histological assessment was done using the semi-quantitative method of Gal et al. (2008) to evaluate microscopic changes in wound area viz. re-epithelialization, polymorphonuclear leucocytes (PMNL), fibroblasts, neo-angiogenesis and collagen [9] as shown in table 2.

Statistical analysis:

Gross percent contraction data:

The data of percent wound contraction was analyzed using parametric one way ANOVA followed by Tukey-Kramer multiple comparison tests.

Histological score data analysis:

Statistical difference between the means of groups was analyzed using non-parametric ANOVA followed by Kruskal-Wallis test.

RESULTS

Dose Standardization:

The study of the optimum concentration of SBT extract for wound healing in rats revealed that rats treated with 0.25% extract showed a faster reduction in wound area on day 3, 6 and 8 in comparison to placebo control group. Thus 0.25% w/w lyophilized bark extracts of HS were used as treatment and were compared with placebo control (Propylene Glycol) and reference control (5% w/v Povidone Iodine) in a subsequent study.

Wound Healing Efficacy

Gross Observations:

Day 2 post surgery:

HS group showed significantly higher percent wound contraction in comparison to PC group.

Day 4 post surgery:

HS group showed significantly higher percent wound contraction as compared to PC group.

Day 6 post surgery and onwards, there was no significant difference in percent wound contraction of wound among groups. These findings are shown in Table 3.

Histopathological and Histochemical Analysis:

Day 2 post surgery:

There was no significant difference in percent epithelialization, fibroblast proliferation and PMNL infiltration among groups.

Day 6 post surgery:

HS groups showed significantly higher mean thickness of epithelium in comparison to RC and PC group which is an indication of earlier bridging of wound gap. Both HS and RC group showed significantly lower PMNL infiltration as compared to PC group. The neo-angiogenesis was significantly higher in HS group as compared to PC group. The fibroblast proliferation in HS and RC group was significantly higher than PC group. The synthesis of collagen in HS group was higher in comparison to PC and RC group.

Day 14 post surgery:

The epithelial wound gap was bridged in all cases and the epithelial score was given on basis of keratinization status of the epithelium. The epithelial score of HS group was significantly higher than PC group. The thickness of epithelium is an indicator of remodeling status of epithelium, where thinner epithelium at day 14 suggested higher maturity and advance remodeling status. The HS group showed significantly thinner epithelium in comparison to RC group. The PMNL were few in number and there was no significant difference among groups. The HS group showed significantly lower blood vessel count in comparison to PC and RC group which is an indicator of advanced stage of remodeling. The fibroblast score was given on basis of arrangement and organization of fibroblast in remodeling tissue and lower fibroblast score suggested a higher stage of remodeling. The fibroblast score of HS group was significantly lower than PC group. The collagen score of HS group was higher than PC group suggestive of advance stage of remodeling. These findings are presented in Table number 4, 5, 6 and 7.

DISCUSSION

Collagen plays an important role in the healing of wounds as it is a principal component of connective tissues and provides a structural framework and strength to healing tissues. HS groups on day 6 showed the higher synthesis of collagen and higher mean thickness of epithelium in comparison to RC group. HS group in comparison to PC group showed higher collagen synthesis on day 6 and higher stage of collagen remodelling on day 14.

The neovascularization represents an essential component in wound healing due to its fundamental impact from the very beginning after skin injury until the end of the wound remodelling [10]. Delayed or aberrant re-vascularization contributes to the etiology of chronic wounds [11]. As the collagen matures during remodeling, the blood vessels are progressively compressed reducing their perimeter, so that the endothelial cells undergo apoptosis [12]. HS groups showed lower blood vessel count on day 14 in comparison to RC group indicating higher remodeling status. HS group when compared to PC group, showed higher neo-angiogenesis on day 6 and lower blood vessel count at day 14.

Fibroblasts are believed to play a key role in wound contraction by exerting tension on surrounding E.C.M proteins such as collagen to stabilize the contraction [13]. HS group showed higher fibroblast proliferation against PC group on day 6 and day 14.

The PMNL infiltration is necessary to protect exposed body surface from pathogens, but the release of free radicals like ROS are responsible for damage to tissue and delay in wound healing process [14]. Wound healing is accelerated by preparations having anti-inflammatory and free radical scavenging activity [8,14]. HS group on day 6 showed lower PMNL count in comparison to PC group.

The faster bridging of wound gap is very important for the process of wound healing and it is estimated on basis of per cent epithelialization and thickness of bridged epithelium. On day 6, the bridged epithelium is immature and thicker epithelium represents early bridging, while on day 14 mature and thinner epithelium reflects the higher phase of remodelling. HS group on day 6 showed the higher mean thickness of epithelium in comparison to PC group thus representing early bridging and faster healing.

Table 1: Experimental design showing number of rats sacrificed on different days post excision surgery under different treatments

Group	Treatment	Day 2	Day 6	Day 14	Total
PC	Propylene Glycol	4	3	4	11
RC	Povidone Iodine (5%w/v)	4	4	3	11
HS	*H.salicifolia* (0.25% w/w)	4	4	4	12

(where PC: Placebo control (Propylene glycol); RC: Reference control (Povidone Iodine); HS: *H.salicifolia)*

Table 2: Histopathological criteria for evaluation of the histological sections for comparison between groups.

Grade scale	Epithelialization	PMNL	Fibroblast	New Vessels	Collagen
0	Thickening of cut edges	Absent	Absent	Absent	Absent
1	Migration of cells (<50%)	Few-mild ST/GT	Mild ST	Mild ST	Minimal GT
2	Migration of cells (>50%)	Mild DL/GT	Mild GT	Mild GT	Mild GT
3	Bridging the excision	Moderate DL/GT	Moderate GT	Moderate GT	Moderate GT
4	Keratinization	Marked DL/GT	Marked GT	Marked GT	Marked GT

(Where ST: Surrounding tissue; GT: Granulation tissue; DL: Demarcation line)

Table 3: Percent wound contraction ± SE of excised wounds in Sprague Dowley rats following application of various treatments measured at different days post excision

Group	DAY 2	DAY 4	DAY 6	DAY 8	DAY 10	DAY 12	DAY14
PC	38.97±4.6 ^{b}	43.04±5.89 ^{a}	64.32±3.37 ^{a}	78.19±3.49^{a}	91.74±2.65 ^{a}	97.72±1.23 ^{a}	99.25±0.54^{a}
RC	44.68±3.3^{ab}	45.37±2.85^{ab}	61.7±3.2^{a}	70.52±4.89 ^{a}	88.36±3.66 ^{a}	90.61±7.35 ^{a}	98.17±1.65 ^{a}
HS	53.71±4.3 ^{a}	58.19±3.59 ^{b}	72.68±3.22 ^{a}	88.81±4.1 ^{a}	95.65±3.53 ^{a}	97.77±2.22 ^{a}	99.72±0.28 ^{a}

(Where Values are Mean± Standard error of per cent wound contraction calculated as {1-(Wound area at nth day/wound area at 0th day)}* 100. Values having superscripts in common do not differ significantly at 5 per cent level when compared within column. PC: Placebo control (Propylene glycol); RC: Reference control (Povidone Iodine); HS: *H.salicifolia)*

Table 4 : Semi-quantitative histological score of changes in healing wounds in Sprague Dawley rats after various treatments at day 2 post excision.

Group	Epithelial score	PMNL	Fibroblast	Per cent Epithelialization
PC	0.87± 0.09 ^{a}	3.94± 0.06 ^{a}	0.75± 0.12 ^{a}	22.97± 5.03 ^{a}
RC	0.93± 0.06 ^{a}	3.74± 0.12 ^{a}	0.6± 0.16 ^{a}	22.26± 4.8 ^{a}
HS	0.8± 0.10 ^{a}	3.70± 0.11 ^{a}	1.18± 0.15 ^{a}	23.11± 3.68 ^{a}

(Where Values are Mean± Standard error of histological score. Values having superscripts in common do not differ significantly at 5 per cent level when compared within column. PC: Placebo control (Propylene glycol); RC: Reference control (Povidone Iodine); HS: *H.salicifolia)*

Table 5: Semi-quantitative histological score of changes in healing wounds in Sprague Dowley rats after various treatments at day 6 post excision.

Group	Epithelial Score	PMNL	Blood Vessels	Fibroblast	Collagen	Per cent Epithelialization
PC	1.55±0.24^{a}	2.82 ±0.12 ^{a}	2.36 ±0.27 ^{a}	3 ±0.13 ^{a}	1.09 ±0.09 ^{a}	76.31 ±16 ^{a}
RC	2 ±0.23 ^{a}	1.75 ±0.14 ^{b}	3.31 ±0.15 ^{ab}	3.63 ±0.12 ^{ab}	1.18 ±0.10 ^{a}	79.54 ±12 ^{a}
HS	1.8 ±0.26 ^{b}	1.75 ±0.28 ^{b}	3.81 ±0.10 ^{b}	3.81 ±0.10 ^{b}	1.88 ±0.08 ^{b}	70.06 ±11 ^{a}

(Where Values are Mean± Standard error of histological score. Values having superscripts in common do not differ significantly at 5 per cent level when compared within column. PC: Placebo control (Propylene glycol); RC: Reference control (Povidone Iodine); HS: *H.salicifolia)*

Table 6 : Semi-quantitative histological score of changes in healing wounds in Sprague Dowley rats after various treatments at day 14 post excision.

Group	Epithelial score	Blood vessels	Fibroblast	Collagen
PC	3.25±0.13^{a}	2.25±0.21 ^{b}	2.92±0.08 ^{b}	3.17±0.11 ^{b}
RC	3.67±0.14 ^{ab}	2.08±0.17 ^{b}	2.58±0.14 ^{ab}	3.42±0.14 ^{ab}
HS	4±0 ^{b}	1.13±0.09 ^{a}	2±0.13 ^{a}	3.73±0.11 ^{a}

(Where Values are Mean± Standard error of histological score. Values having superscripts in common do not differ significantly at 5 per cent level when compared within column. PC: Placebo control (Propylene glycol); RC: Reference control (Povidone Iodine); HS: *H.salicifolia)*

Table 7: Mean ±SE thickness of epithelium of healing wounds in Sprague Dawley rats after various treatment at day 6 and day 14.

Group	Day 6 (Proliferation) (µ)	Day 14 (Maturation) (µ)
PC	40.39±7.15 [b]	39.66±4.68 [ab]
RC	35.36±3.79 [b]	40.67±2.04 [b]
HS	62.67±4.79 [a]	30.3±1.77 [a]

(Where, PC: Placebo control (Propylene glycol); RC: Reference control (Povidone Iodine); HS: *H.salicifolia)*

SUMMARY AND CONCLUSION

On basis of percent wound contraction data HS group showed higher early wound contraction in comparison to PC group. Histologically, on day 6 HS group showed the higher mean thickness of the epithelium, higher collagen synthesis in comparison to RC group and milder inflammation, higher neo-angiogenesis and higher fibroblast proliferation in comparison to PC group. On day 14, HS group showed thinner epithelium, lower blood vessel count in comparison to RC group and high epithelial score, low blood vessel count, high fibroblast and collagen remodelling in comparison to PC group.

Thus, it may be concluded that the HS bark extract promotes healing by reducing inflammation, promoting neo-angiogenesis, fibroblast proliferation and collagen synthesis during the phase of proliferation and faster remodeling of epithelium and granulation tissue during the phase of remodeling. The wound healing potential of HS bark is significantly higher than RC group.

CONFLICT OF INTEREST: All authors mentioned in this manuscript, do not share any conflict of interest.

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