Wound Healing Effect of *Hippophae rhamnoides* L. based Pharmaceutical Preparations

**DR. ASHEESH GUPTA**

Defence Institute of Physiology & Allied Sciences
DRDO, Ministry of Defence, Delhi-110 054, INDIA

Email: asheeshgupta2001@gmail.com
Wound healing is a complex and well-orchestrated process, comprising of three overlapping phases i.e.

- Inflammation
- Granulation tissue formation
- Tissue remodeling

Various growth factors, cellular proteins, cytokines and their receptor play a crucial role in wound healing.
Wound healing proceeds through phases being regulated by changing soluble and matrix factors

cellular dedifferentiation
provisional fibrin clot matrix

keratinocyte migration
fibroblast immigration
angiogenic support
immature matrix (Fn, TnC)

stop im-migration
cellular redifferentiation
return to dermal pauci-cellularity
collagen I production and bundling

pro-motogenic & pro-mitogenic
pro-motogenic & pro-angiogenic

anti-angiogenic & pro-apoptotic
Such a controlled phenomena can be disrupted during pathologic states viz. diabetes, immune disorders, ischemia, venous stasis & injuries viz. burn, frost-bite, bed sore, gun-shot wounds

- **Diabetic wounds:** prolonged inflammation, impaired neo-vascularization, collagen synthesis, increased proteases & defective macrophage function, prone to infection

- **Burn injury:** several complication such as loss of tissue integrity, fluid loss, discomfort, pain, susceptible to infection, scar formation

**Recent approaches:**

- To explore the mechanism of impaired wound healing
- To identify precisely novel healing agents/ dressings/ tissue engineering approach for scar less healing
**Herbal Wound Healer:**

Various plants and plant derived products have been reported to promote the process of wound healing

<table>
<thead>
<tr>
<th>Plant</th>
<th>Scientific Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe vera</td>
<td><em>Tridax procumbens</em></td>
</tr>
<tr>
<td><em>Curcuma longa</em> (Curcumin)</td>
<td><em>Calotropis procera</em></td>
</tr>
<tr>
<td><em>Centella asiatica</em> (Asiaticoside)</td>
<td><em>Cassia fistula</em></td>
</tr>
<tr>
<td><em>Arnebia noblis</em> (Arnebin-1)</td>
<td><em>Hippophae rhamnoides</em></td>
</tr>
<tr>
<td><em>Datura alba</em></td>
<td><em>Rhodiola imbricata</em></td>
</tr>
</tbody>
</table>

Gupta et al., J Ethnopharmacol, 1999
Gupta et al., Int J Lower Ext Wound, 2005
Gupta et al., Mol Cellular Biochem, 2006
Gupta et al., Planta Medica, 2007
Aim of the study

To investigate the wound healing efficacy & possible mechanism of action of *Sea buckthorn (H. rhamnoides L.)* extract
- *H. rhamnoides* (Elaeagnaceae) is a wild shrub, dwarf to tall, branched and thorny nitrogen-fixing deciduous plant, grows in adverse climatic conditions, native to Europe and Asia

- Rich source of bioactive substances: flavonoids, carotenoids, steroids, vitamins, tannins, glycerides of palmitic, stearic, oleic acids

- Traditionally plant has been used extensively in many Asian & European countries to treat skin diseases, gastric ulcers, asthma, lung disorders

- Systematic studies revealed SBT have potent activities viz.

  - Antioxidant
  - Immunomodulatory
  - Anti-stress & Adaptogenic
  - Hepatoprotective
  - Radioprotective
  - Tissue regeneration
Extract Preparations:

• Ethnobotanical Identification:
  SBT-2006 (Voucher specimen)

2. Collection:
  North-West Himalayas (2500-4000 m, amsl)
  (During September)

3. Physico-Chemical Characterization:
  HPLC fingerprinting, Chemical evaluations
Phytochemical Characterization

rich in polyphenols and flavonoids:

Polyphenolic (40.49 mg of gallic acid equi./g dry leaf)
Flavonoids (14.90 mg of rutin equi./g dry leaf)

HPLC fingerprinting & chemical analysis based on marker compounds for its authenticity, purity & consistency of composition in terms of batch-to-batch variation

Peak: (1) Quercetin-3-galactoside (2) Quercetin-3-glucoside (3) Kaempferol (4) Isorhamnetin

< 5% batch-to-batch variation maintained throughout the experiments

Quantitative determination of marker compounds by RP-HPLC (µg/g dry leaf)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quercetin-3-galactoside</th>
<th>Quercetin-3-glucoside</th>
<th>Kaempferol</th>
<th>Isorhamnetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBT-LAQ</td>
<td>1447.66 ± 7.72</td>
<td>105.12 ± 1.79</td>
<td>2.73 ± 0.36</td>
<td>13.53 ± 0.58</td>
</tr>
</tbody>
</table>

Upadhyay & Gupta et al., 2010; FCT
Experimental Models:

**In-vitro**
- Angiogenic potential: Chick chorioallantoic membrane (CAM) model
- Anti-bacterial activity against wound pathogens: Well diffusion assay
- Cytoprotective activity for BHK-cell line (Fibroblast type)

**In-vivo**
- Animal: Male Sprague-Dawley rats (180±20 g)

**Acute Model: Cutaneous Excision Punch Wound**
Transdermal wounds created on pre-shaved dorsal surface of rats

(II) Impaired Model

**Diabetic:** Streptozotocin (50mg/kg, i.p.) & excision wounds were created

**Burn Wound:** created using a metal rod (1.5 cm, dia.) heated to 85 °C, exposed for 20 sec., after 24 hrs. dead tissue was excised using a sterile surgical blade
Assessment of wound healing:

**Physical assay:**  
Wound Contraction

**Pro-healing markers:**  
DNA, Protein, Hexosamine, Hydroxyproline

**Antioxidant Potential:**  
SOD, CAT, GPx, GSH, Vit. C, LPO

**Histological evaluation:**  
H & E; MT Staining, Morphometric analysis

**Differential protein expression:**  
Growth factors/ cellular proteins

**Gelatin zymography:**  
Metrix metalloproteinases
In-Vitro Cytotoxicity Assay

Cell Line: Baby Hamster Kidney (BHK-21) Cell Line
Assay: MTT assay, Trypan blue dye exclusion assay
Conc. tested: 25, 50, 100, 200, 400 microgram/ml

Trypan Blue Dye-exclusion Assay

MTT Assay

Upadhyay & Gupta et al., 2010; FCT
Strong **angiogenic** potential in *in-vitro* CAM model

Significant **anti-bacterial** activity: Growth inhibiting effects on wound pathogens *Pseudomonas aeruginosa, Staphylococcus aureus*

Significant **cytoprotective** activity against H$_2$O$_2$ & HX-XO generated free radicals damage BHK-cell line

*Upadhyay & Gupta et al., 2010; eCAM*
In-vivo studies

Wound area contraction

Visual observations

Gupta et al., 2005; Int J Lower Ext Wounds
Gupta et al., 2006; Mol Cellular Biochem
Effect of SBT Extract on Pro-healing Markers

<table>
<thead>
<tr>
<th>Group</th>
<th>Burn Control</th>
<th>SBT</th>
<th>SSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0% w/w</td>
<td>(1% w/w)</td>
<td></td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>22.80 ± 1.68</td>
<td>29.96 ± 2.37*</td>
<td>25.73 ± 2.02</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.50 ± 0.07</td>
<td>0.71 ± 0.07*</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>Protein</td>
<td>88.74 ± 4.18</td>
<td>120.87 ± 7.77*</td>
<td>105.46 ± 4.16*</td>
</tr>
<tr>
<td>DNA</td>
<td>3.80 ± 0.28</td>
<td>4.09 ± 0.24</td>
<td>3.87 ± 0.23</td>
</tr>
</tbody>
</table>

Value are mean (mg/g tissue wt.) ± SEM; N = 6; * P < 0.05 compared with control. # P < 0.05 compared with silver sulfadiazine (SSD).

Antioxidant activity of SBT

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>SBT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (µg/mg protein)</td>
<td>1.68 ± 0.33</td>
<td>2.16 ± 0.18*</td>
</tr>
<tr>
<td>GST (U/mg protein)</td>
<td>2.04 ± 0.11</td>
<td>2.45 ± 0.20*</td>
</tr>
<tr>
<td>Vitamin C (µg/mg protein)</td>
<td>2.46 ± 0.37</td>
<td>3.60 ± 0.27*</td>
</tr>
<tr>
<td>CAT (U/mg protein)</td>
<td>8.18 ± 0.65</td>
<td>10.03 ± 0.68*</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>1.25 ± 0.20</td>
<td>1.61 ± 0.11*</td>
</tr>
<tr>
<td>MDA (n mol/mg protein)</td>
<td>2.41 ± 0.12</td>
<td>1.79 ± 0.21*</td>
</tr>
</tbody>
</table>

Augments endogenous antioxidants
Reduces LPO levels
**H & E Staining**

Control  
SBT  
Silver-sulfadiazine  

(Scale bar, 100 µm)

**Morphometric Analysis**

? Blood vessel density  
? Epidermal thickness

**MT staining -Collagen**

‘SBT’ treatment showing compact and well-aligned collagen fibers

Control  
SBT  

(Scale bar, 20 µm)

**Upadhyay & Gupta et al., 2010; eCAM**
Burn Wounds
Immunohistochemical Analysis

Up-regulates the expression of
a-SM actin & TGF-β1
Differential expression of growth factors and marker proteins in SBT treated burn wounds

- Enhanced expression of matrix metalloproteinases (MMP-2 & 9) indicate role of ‘SBT-LAQ’ in tissue remodeling phase

- Enhanced expression of VEGF & Collagen Type-III in granulation wound tissue

*Upadhyay & Gupta et al., 2010; eCAM*
‘SBT - WOUND HEALER’

A potent wound healer from natural source

Developed in two dosage forms*:

(i) Ointment based - Acute (incision, excision)
(ii) Hydrogel based wound dressing - Chronic
    (diabetic and burns wounds)


SBT-encapsulated cryogel dressing advantages:

- Maintain moist wound micro-environment
- Controlled and sustained drug release
- Barrier against bacteria
- Oxygen permeability and good handling
### Pro-healing markers in SBT-encapsulated dressing treated wounds

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Hydroxyproline (mg/g tissue wt.)</th>
<th>Hexosamine (mg/g tissue wt.)</th>
<th>DNA (mg/g tissue wt.)</th>
<th>Protein (mg/g tissue wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burn control</td>
<td>20.51 ±0.99</td>
<td>0.53 ±0.026</td>
<td>4.00±0.35</td>
<td>90.64 ±3.98</td>
</tr>
<tr>
<td>Cryogel</td>
<td>25.81 ±1.72*</td>
<td>0.59 ±0.02</td>
<td>4.68±0.22</td>
<td>104.97 ±3.82</td>
</tr>
<tr>
<td>SBT-Cryogel</td>
<td>29.11 ±1.75*</td>
<td>0.68 ±0.02*</td>
<td>5.05±0.20*</td>
<td>123.59 ±4.99*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; N = 6; * P < 0.05 compared with burn control.

### H & E Staining

![H & E Staining](image)
**Dermal Toxicity studies of SBT extract:**

**Animal:** Male Sprague-Dawley rats (180 ± 20 gm)

1. **Dermal Irritation Assay:**
   - OECD Guidelines: 404
   - Observations: erythema/edema
   - Dose: 0.5 gm leaf extract powder

2. **Acute Dermal Toxicity:**
   - OECD Guidelines: 402
   - Limit Dose: 5 gm/Kg body weight
   - Observations: Erythema/Edema score, Mortality, Organ weight/body weight ratio

3. **28-Days Repeated Dermal Toxicity:**
   - OECD Guidelines: 410
   - Limit Dose: 1 gm/Kg body weight
   - Observations: Erythema/Edema score, Organ weight/body weight ratio, Blood clinical biochemistry, Hematological parameters, Gross necropsy & histology
Safety & Dermal Toxicity:

- Safe upto 2g/kg bw for single dermal application
- Dermal irritation studies showed that product is non-irritant via dermal route

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>2 gm/kg (bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>78.8±3.8</td>
<td>78.8±3.4</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>56.7±1.9</td>
<td>53.8±4.8</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.67±0.05</td>
<td>0.68±0.04</td>
</tr>
<tr>
<td>Direct Bilirubin (mg/dl)</td>
<td>0.39±0.08</td>
<td>0.42±0.1</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU)</td>
<td>7.8±0.3</td>
<td>8.1±0.55</td>
</tr>
<tr>
<td>SGOT (IU)</td>
<td>28.7±2.8</td>
<td>30.6±3.8</td>
</tr>
<tr>
<td>SGPT (IU)</td>
<td>7.9±0.5</td>
<td>7.8±0.8</td>
</tr>
<tr>
<td>LDH (nmol/mg protein)</td>
<td>10.8±0.8</td>
<td>11.2±0.9</td>
</tr>
<tr>
<td>Blood glucose (mg%)</td>
<td>89.7±6.9</td>
<td>92.3±5.8</td>
</tr>
<tr>
<td>Protein (g/dl)</td>
<td>8.8±0.7</td>
<td>7.5±0.4</td>
</tr>
<tr>
<td>Sodium (meq/l)</td>
<td>140.6±2.7</td>
<td>138.8±4.6</td>
</tr>
<tr>
<td>Potassium (meq/l)</td>
<td>5.7±0.03</td>
<td>4.8±0.03</td>
</tr>
<tr>
<td>WBC (×10³ µl)</td>
<td>7.8±0.4</td>
<td>8.3±0.6</td>
</tr>
<tr>
<td>RBC (×10⁶ µl)</td>
<td>6.6±0.3</td>
<td>6.8±0.4</td>
</tr>
<tr>
<td>Hemoglobin (g%)</td>
<td>14.1±0.4</td>
<td>14.8±0.7</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47.2±0.8</td>
<td>48.6±1.8</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>58.9±1.0</td>
<td>56.4±1.8</td>
</tr>
<tr>
<td>Platelets (10³ µ/l)</td>
<td>778.8±23.8</td>
<td>755.3±13.2</td>
</tr>
</tbody>
</table>

Single dose dermal toxicity

(Organ/body weight ratio)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>2 gm/kg (bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver ×10⁻³</td>
<td>30.5±1.4</td>
<td>30.1±0.8</td>
</tr>
<tr>
<td>Heart ×10⁻³</td>
<td>3.5±0.1</td>
<td>3.74±0.07</td>
</tr>
<tr>
<td>Kidney ×10⁻³</td>
<td>3.7±0.08</td>
<td>3.6±0.09</td>
</tr>
<tr>
<td>Spleen ×10⁻³</td>
<td>1.89±0.07</td>
<td>1.93±0.13</td>
</tr>
<tr>
<td>Testis ×10⁻³</td>
<td>4.8±0.17</td>
<td>4.9±0.29</td>
</tr>
<tr>
<td>Adrenal ×10⁻³</td>
<td>9.5±0.3</td>
<td>8.9±0.4</td>
</tr>
<tr>
<td>Lung ×10⁻³</td>
<td>4.8±0.3</td>
<td>5.1±0.20</td>
</tr>
</tbody>
</table>
### Repeated dose dermal toxicity study (28-Days)

(Organ/body weight ratio)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>1 gm/kg (bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver ( \times 10^{-3} )</td>
<td>23.3±0.4</td>
<td>30.1±0.8</td>
</tr>
<tr>
<td>Heart ( \times 10^{-3} )</td>
<td>3.2±0.08</td>
<td>3.1±0.3</td>
</tr>
<tr>
<td>Kidney ( \times 10^{-3} )</td>
<td>3.1±0.06</td>
<td>3.3±0.11</td>
</tr>
<tr>
<td>Spleen ( \times 10^{-3} )</td>
<td>1.9±0.06</td>
<td>1.8±0.03</td>
</tr>
<tr>
<td>Testis ( \times 10^{-3} )</td>
<td>4.7±0.12</td>
<td>4.6±0.01</td>
</tr>
<tr>
<td>Adrenal ( \times 10^{-3} )</td>
<td>8.4±0.6</td>
<td>8.0±0.5</td>
</tr>
<tr>
<td>Lung ( \times 10^{-3} )</td>
<td>5.4±0.1</td>
<td>5.2±0.2</td>
</tr>
</tbody>
</table>

#### Biochemical and Hematological parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>1 gm/kg (bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>69.7±2.9</td>
<td>72.4±3.0</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>63.7±1.7</td>
<td>61.8±4.8</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.59±0.04</td>
<td>0.57±0.03</td>
</tr>
<tr>
<td>Direct Bilirubin (mg/dl)</td>
<td>0.35±0.04</td>
<td>0.38±0.08</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU)</td>
<td>7.4±0.4</td>
<td>7.1±0.5</td>
</tr>
<tr>
<td>SGOT (IU)</td>
<td>28.3±2.7</td>
<td>29.5±1.3</td>
</tr>
<tr>
<td>SGPT (IU)</td>
<td>6.9±0.5</td>
<td>6.8±0.3</td>
</tr>
<tr>
<td>LDH (nmol/mg protein)</td>
<td>10.4±0.6</td>
<td>10.3±0.5</td>
</tr>
<tr>
<td>Blood glucose (mg%)</td>
<td>92.6±3.9</td>
<td>95.3±4.2</td>
</tr>
<tr>
<td>Protein (g/dl)</td>
<td>8.6±0.4</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>Sodium (meq/l)</td>
<td>138.7±2.2</td>
<td>128.5±3.6</td>
</tr>
<tr>
<td>Potassium (meq/l)</td>
<td>6.2±0.04</td>
<td>6.7±0.03</td>
</tr>
<tr>
<td>WBC ( \times 10^3 ) ( \mu l )</td>
<td>7.7±0.6</td>
<td>7.9±0.5</td>
</tr>
<tr>
<td>RBC ( \times 10^6 ) ( \mu l )</td>
<td>6.7±0.2</td>
<td>6.8±0.5</td>
</tr>
<tr>
<td>Hemoglobin (g%)</td>
<td>15.4±0.3</td>
<td>14.3±0.6</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>49.4±0.5</td>
<td>47.6±2.8</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>56.9±1.7</td>
<td>55.7±2.8</td>
</tr>
<tr>
<td>Platelets ( \times 10^3 ) ( \mu l )</td>
<td>698.8±34.8</td>
<td>718.3±24.2</td>
</tr>
</tbody>
</table>

*Upadhyay et al., 2009; FCT*
Salient findings

- **Possesses significant healing potential for acute & chronic burns wounds**

- **Augments healing by**
  - accelerating wound contraction & re-epithelialization,
  - improving collagen synthesis and stabilization
  - Mitogenic & angiogenic potential
  - Enhance expression of growth factors & cellular proteins

- **Probably by**
  - Modulating the levels of VEGF & TGF-β1
  - Scavenging ROS & augmenting level of endogenous antioxidants
  - Increasing neovascularization

- **Rich in quercetin derivatives which could be one of the factors contributing to the wound healing potential of SBT-LAQ**

  - Safety & toxicological studies (OECD Guidelines) showed safe use for dermal application
Possible mechanisms by which SBT extract enhances wound healing process

- Induced endogenous antioxidants
- Increased cell proliferation
- Anti-inflammatory response
- Increased angiogenesis
- Free radical scavenging
- Reduced lipid per-oxidation & protein oxidation
- Enhanced collagen accumulation & ECM
- Anti-microbial properties
Publications:

- SBT extract  
  *Int J Low Extrem Wounds* 4: 88-92, 2005

- SBT flavone (from fruit pulp)  

- Poly-herbal formulation (PHF) including SBT  
  - SBT supercritical CO2- extracted seed oil  

- SBT extract for burn wound treatment  
  *eCAM* 73: 774-77, 2010
  - Antioxidant, anti-bacterial activity & phytochemical, HPLC analysis of SBT extracts  

Patent:

- SBT incorporated hydrogel based wound dressing for burns wound healing  
  *Patent file number – 837/DEL/2009*
THANKS