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

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INTRODUCTION

- Wound healing is a complex and well-orchestrated process, comprising of three overlapping phases i.e.

  Inflammation

  Granulation tissue formation

  Tissue remodeling

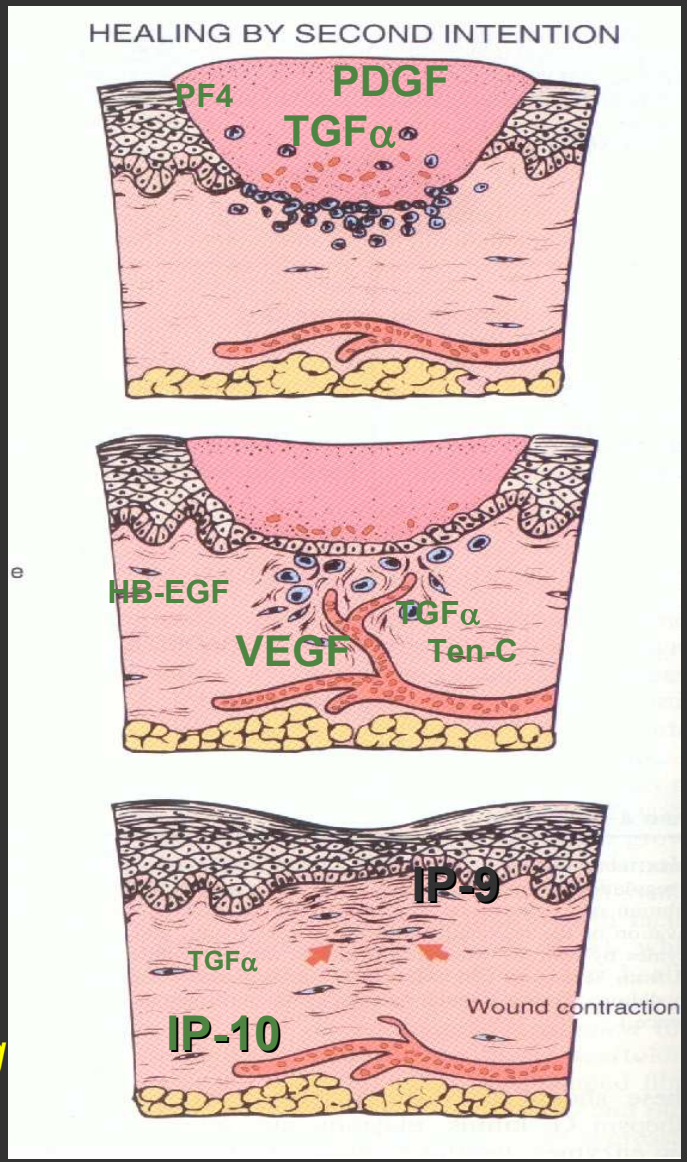
- Various growth factors, cellular proteins, cytokines and their receptor play a crucial role in wound healing

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

Aloe vera

Curcuma longa (Curcumin)

Centella asiatica (Asiaticoside)

Arnebia noblis (Arnebin-1)

Datura alba

Tridax procumbens

Calotropis procera

Cassia fistula

Hippophae rhamnoides

Rhodiola imbricata

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)

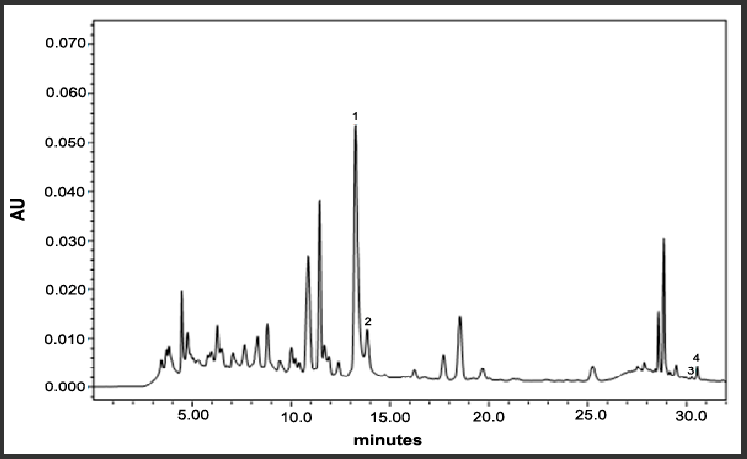
3. Physico-Chemical Characterization:

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 authencity, 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)

Sample	*Quercetin-3-galactoside*	*Quercetin-3-glucoside*	*Kaempferol*	*Isorhamnetin*
SBT-LAQ	1447.66 ± 7.72	105.12 ± 1.79	2.73 ± 0.36	13.53 ± 0.58

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)

⑩ 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 ^{0}C, exposed for 20 sec. , after 24 hrs. dead tissue was excised using a sterile surgical blade

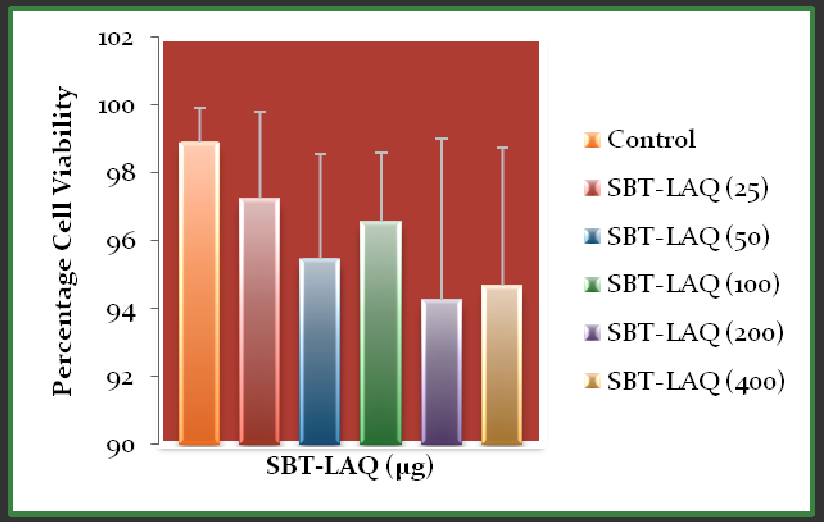
- **Dose-Dependent Study:** Various doses (0.5-10.0%, w/w)
- **Treatment Schedule:** Ointment applied topically, twice daily, for 7 days
- **Reference Control :** Silver Sulfadiazine, Povidone-Iodine

Assessment of wound healing:

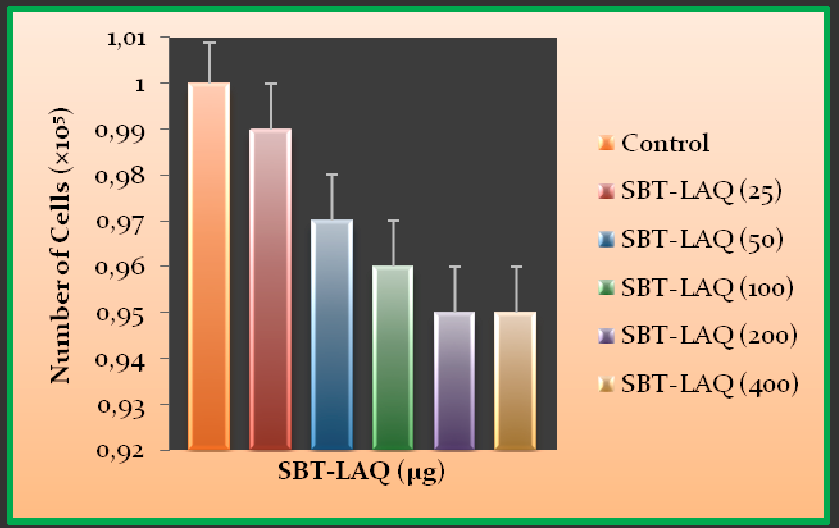
Physical assay:	Wound Contraction
Pro-healing markers:	DNA, Protein, Hexosamine, Hydroxyproline
Antioxidant Potential:	SOD, CAT, GPx, GSH, Vit. C, LPO
Histological evaluation:	H & E; MT Staining, Morphometric analysis
Differential protein expression:	Growth factors/ cellular proteins
Gelatin zymography:	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

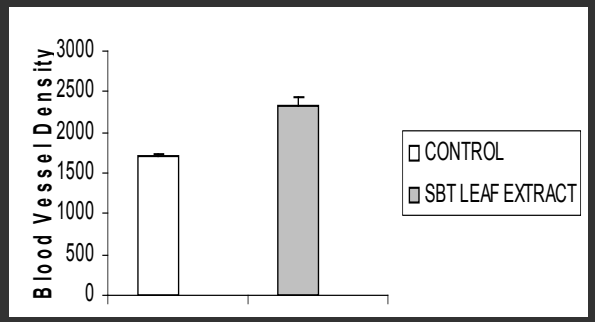


MTT Assay

Upadhyay & Gupta et al., 2010; FCT

❖Strong angiogenic potential in *in-vitro* CAM model

Control SBT Extract



Control SBT

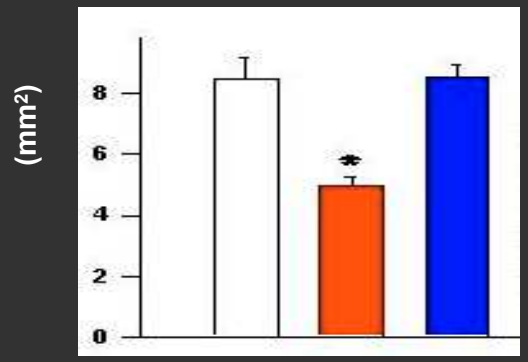
❖Significant anti-bacterial activity: Growth inhibiting effects on wound pathogens *Pseudomonas aureginosa, Staphylococcus aureus*

❖Significant cytoprotective activity against H_2O_2 & HX-XO generated free radicals damage BHK-cell line

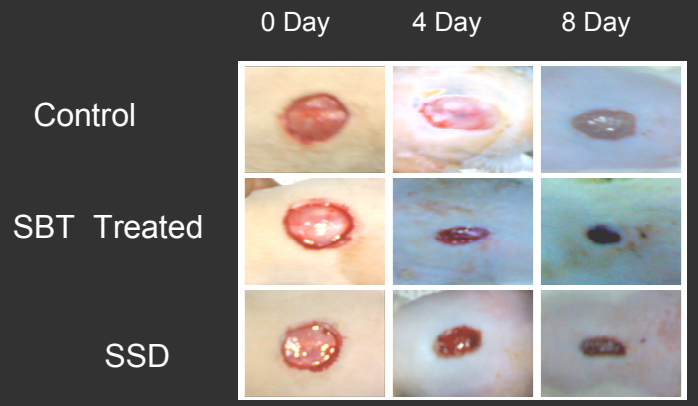
Upadhyay & Gupta et al., 2010; eCAM

In-vivo studies

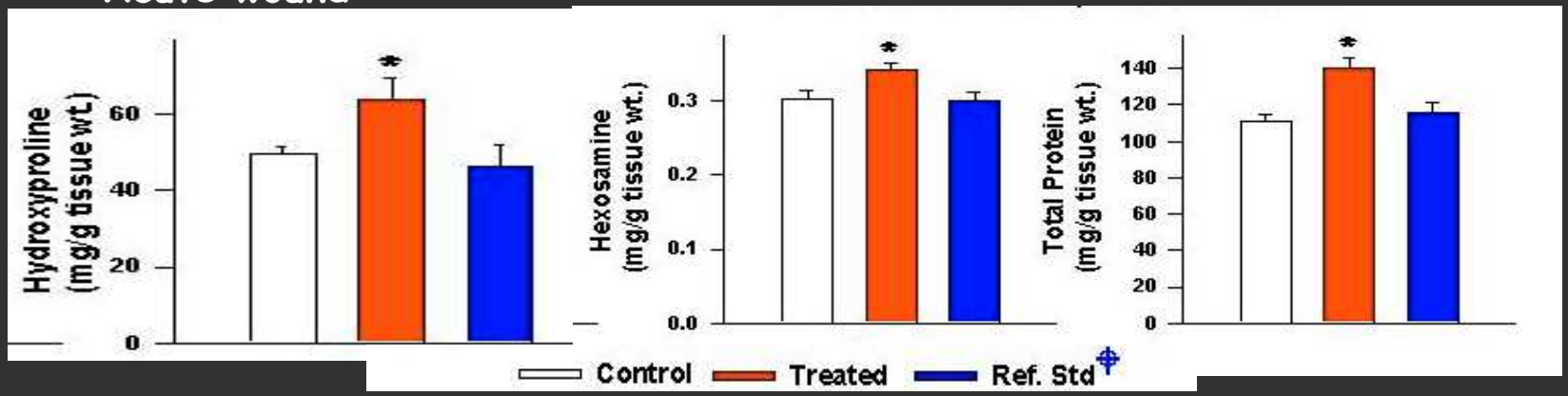
Wound area contraction



Visual observations



Acute wound



Gupta et al., 2005; Int J Lower Ext Wounds
Gupta et al., 2006; Mol Cellular Biochem

Effect of SBT Extract on Pro-healing Markers

? Collagen ? Hexosamine ? Protein ? DNA

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

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

Augments endogenous antioxidants
Reduces LPO levels

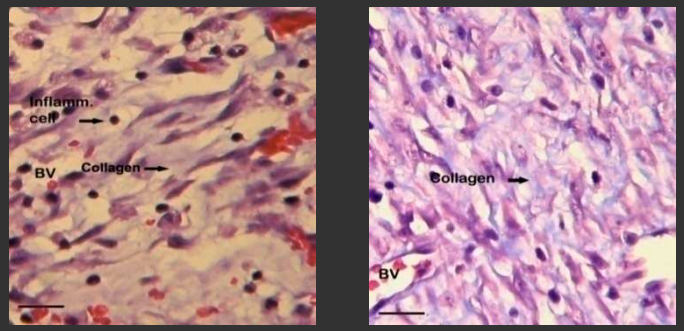
Parameters	Control	SBT
GSH (µg/mg protein)	1.68 ± 0.33	2.16 ± 0.18*
GST (U/mg protein)	2.04 ± 0.11	2.45 ± 0.20*
Vitamin C (µg/mg protein)	2.46 ± 0.37	3.60 ± 0.27*
CAT (U/mg protein)	8.18 ± 0.65	10.03 ± 0.68*
SOD (U/mg protein)	1.25 ± 0.20	1.61 ± 0.11*
MDA (n mol/mg protein)	2.41 ± 0.12	1.79 ± 0.21*

H & E Staining

Control SBT Silver-sulfadiazine

(Scale bar, 100 μm)

MT staining -Collagen



Control SBT

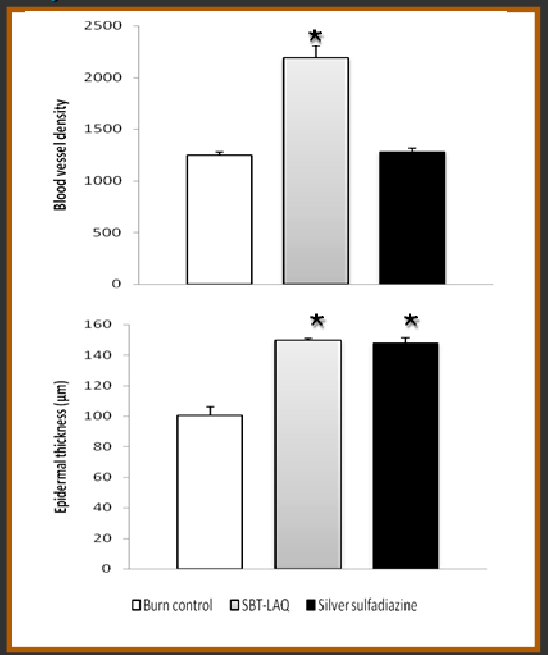
(Scale bar, 20 μm)

'SBT' treatment showing compact and well-aligned collagen fibers

Morphometric Analysis

? Blood vessel density

? Epidermal thickness



Upadhyay & Gupta et al., 2010; eCAM

a- SM ACTIN

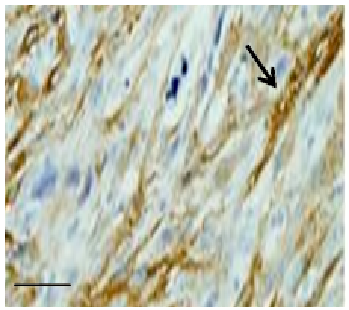
Burn Wounds

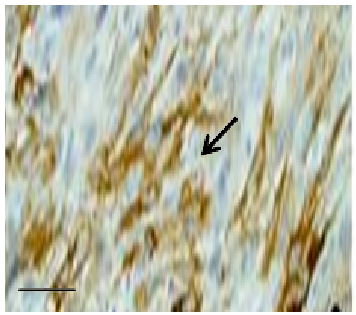
Immunohistochemical Analysis

TGF-ß1

Up-regulates the expression of

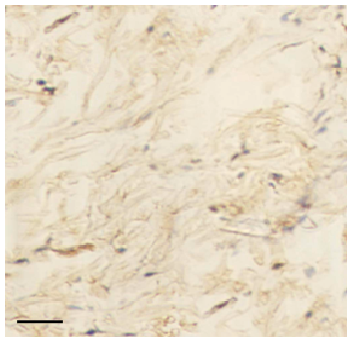
a-SM actin & TGF-ß1

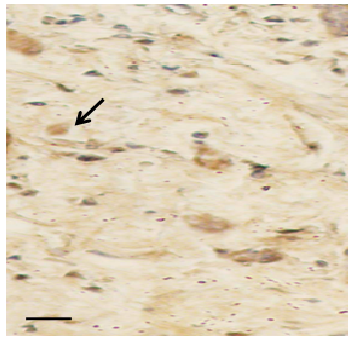




Control

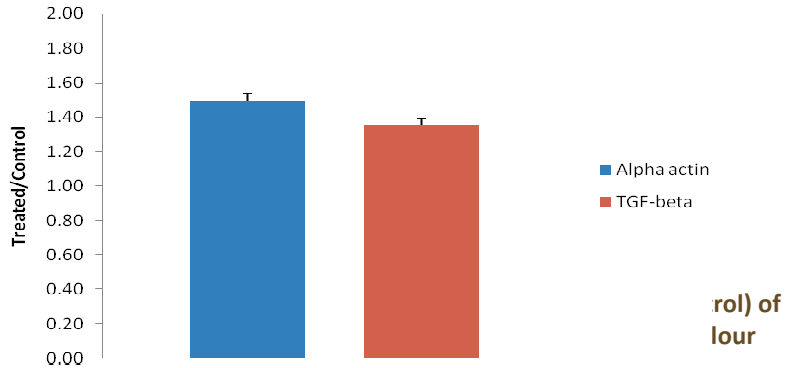
SBT





Control

SBT



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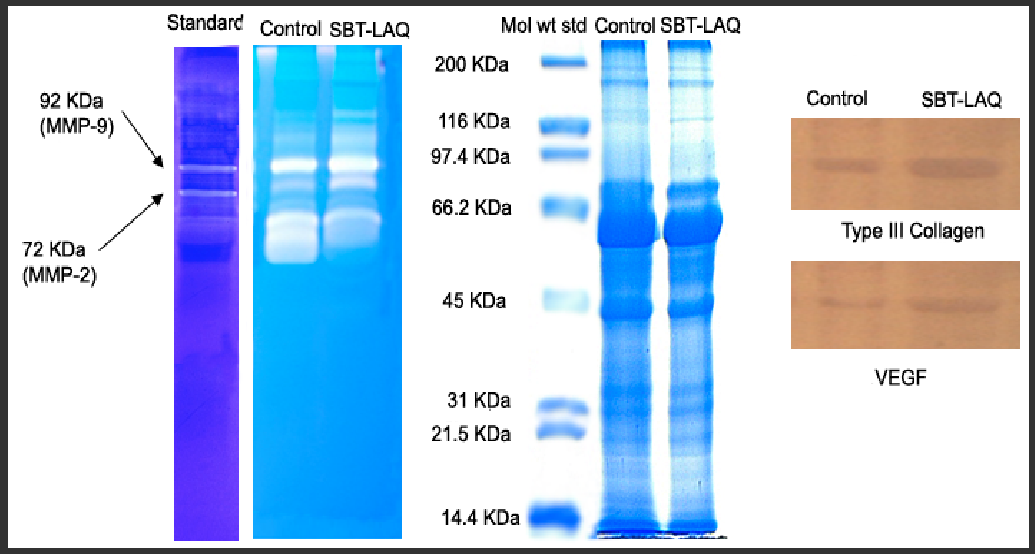
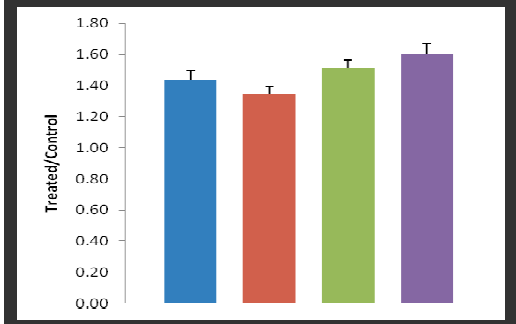
Densitometric Analysis

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

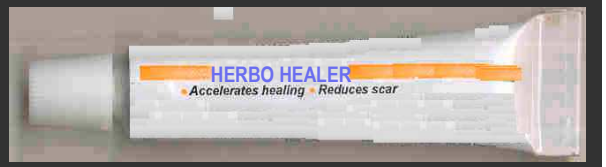
Upadhyay & Gupta et al., 2010; eCAM

‘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)
*Patent file number – 837/DEL/2009



SBT-encapsulated cryogel dressing advantages:

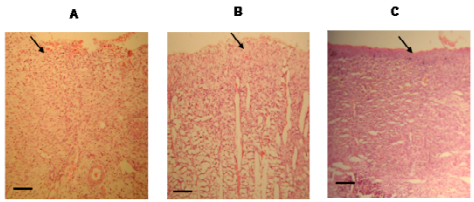
- Maintain moist wound micro-environment
- Controlled and sustained drug release
- Barrier against bacteria
- Oxygen permeability and good handling

Pro-healing markers in SBT-encapsulated dressing treated wounds

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

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

H & E Staining



Control Cryogel SBT-Cryogel

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

Single dose dermal toxicity

(Organ/body weight ratio)

Organ	Control	2 gm/kg (bw)
Liver $\times 10^{-3}$	30.5±1.4	30.1±0.8
Heart $\times 10^{-3}$	3.5±0.1	3.74±0.07
Kidney $\times 10^{-3}$	3.7±0.08	3.6±0.09
Spleen $\times 10^{-3}$	1.89±0.07	1.93±0.13
Testis $\times 10^{-3}$	4.8±0.17	4.9±0.29
Adrenal $\times 10^{-3}$	9.5±0.3	8.9±0.4
Lung $\times 10^{-3}$	4.8±0.3	5.1±0.20

Biochemical and Hematological parameters

Parameters	Control	2 gm/kg (bw)
Cholesterol (mg/dl)	78.8±3.8	78.8±3.4
Triglyceride (mg/dl)	56.7±1.9	53.8±4.8
Creatinine (mg/dl)	0.67±0.05	0.68±0.04
Direct Bilirubin (mg/dl)	0.39±0.08	0.42±0.1
Alkaline Phosphatase (IU)	7.8±0.3	8.1±0.55
SGOT (IU)	28.7±2.8	30.6±3.8
SGPT (IU)	7.9±0.5	7.8±0.8
LDH (nmol/mg protein)	10.8±0.8	11.2±0.9
Blood glucose (mg%)	89.7±6.9	92.3±5.8
Protein (g/dl)	8.8±0.7	7.5±0.4
Sodium (meq/l)	140.6±2.7	138.8±4.6
Potassium (meq/l)	5.7±0.03	4.8±0.03
WBC ($\times 10^3$ µl)	7.8±0.4	8.3±0.6
RBC($\times 10^6$ µl)	6.6±0.3	6.8±0.4
Hemoglobin (g%)	14.1±0.4	14.8±0.7
Hematocrit (%)	47.2±0.8	48.6±1.8
MCV (fl)	58.9±1.0	56.4±1.8
Platelets (10^3 µ/l)	778.8±23.8	755.3±13.2

Repeated dose dermal toxicity study (28-Days)

(Organ/body weight ratio)

Organ	Control	1 gm/kg (bw)
Liver $\times 10^{-3}$	23.3±0.4	30.1±0.8
Heart $\times 10^{-3}$	3.2±0.08	3.1±0.3
Kidney $\times 10^{-3}$	3.1±0.06	3.3±0.11
Spleen $\times 10^{-3}$	1.9±0.06	1.8±0.03
Testis $\times 10^{-3}$	4.7±0.12	4.6±0.0.1
Adrenal $\times 10^{-3}$	8.4±0.6	8.0±0.5
Lung $\times 10^{-3}$	5.4±0.1	5.2±0.2

Upadhyay et al., 2009; FCT

Biochemical and Hematological parameters

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

Salient findings

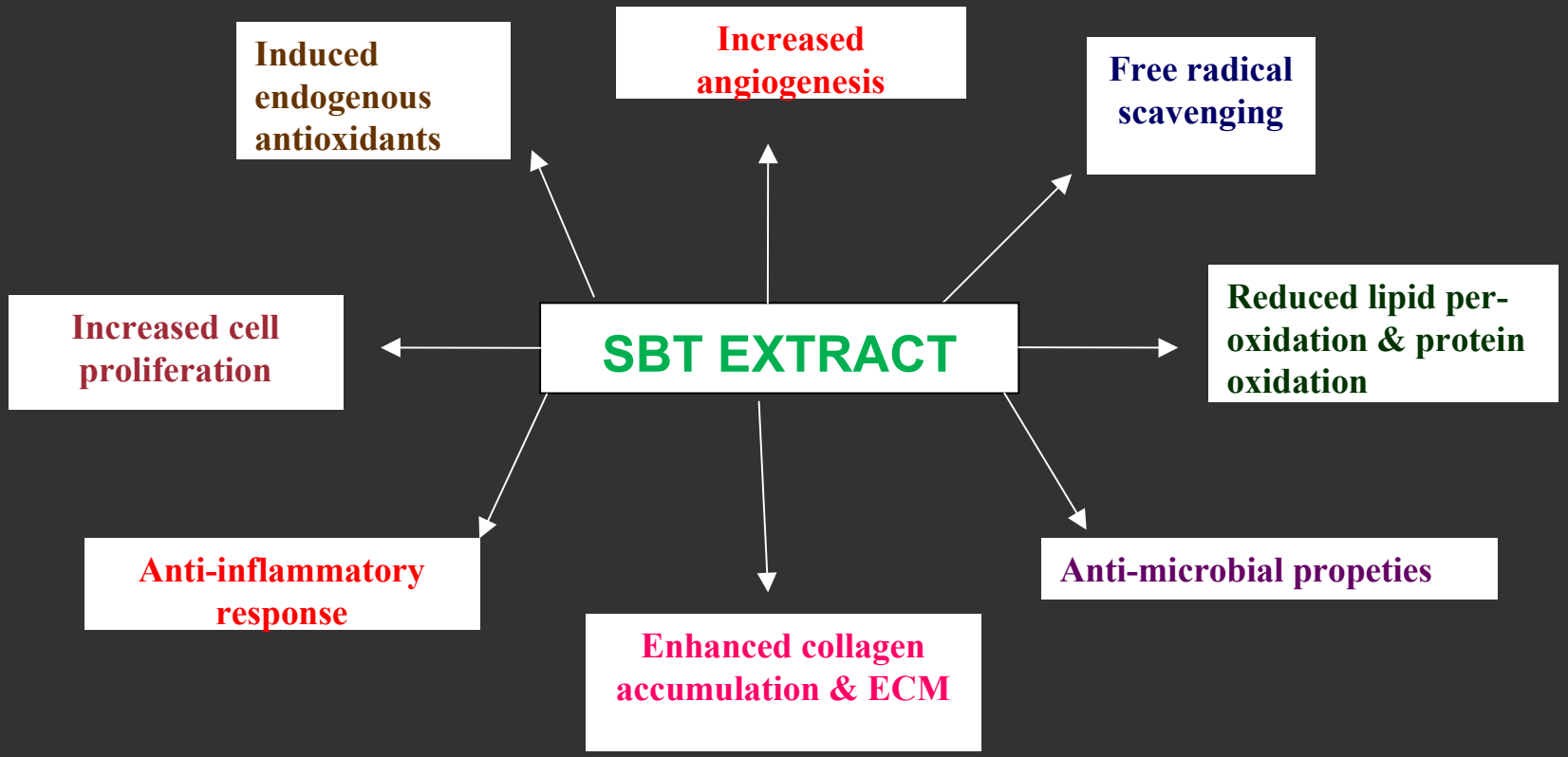
- Possesses significant healing potential for acute & chronic burns wounds

- 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-ß1
  - 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

Publications:

- SBT extract — *Int J Low Extrem Wounds 4: 88-92, 2005*
- SBT flavone (from fruit pulp) — *Mol Cellular Biochem 290: 193-98, 2006*
- Poly-herbal formulation (PHF) including SBT — *Wound Rep. Reg. 16: 784-90, 2008*
  - SBT supercritical CO2- extracted seed oil — *Food Chem. Toxicol., 47: 1146-53, 2009*
- SBT extract for burn wound treatment — *eCAM 73: 774-77, 2010*
  - Antioxidant, anti-bacterial activity & phytochemical, HPLC analysis of SBT extracts — *Food Chem. Toxicol., 47: 1146-53, 2009*

Patent:

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


THANKS