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Research Article

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Evaluation of wound healing properties of *Salvia splendens* leaves in streptozotocin induced diabetic rats

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ABSTRACT

The ethyl acetate fraction of methanolic extract of Salvia splendens leaves was evaluated for its wound healing effect in Streptozotocin induced diabetic rats. The wound healing activity was evaluated by both Excision and Dead space wound model. A single i.p. dose Streptozotocin (60 mg/kg) was used to induce Diabetes in rats. In the Excision model, wound of circular area (approx. 500 mm$^{2)}$ was created on the back of each animal. Wound healing parameters like percentage of wound contraction, period of epithelialization were observed by topically administering Mupirocin ointment (2% w/w) as standard and methanolic extract (5% and 10% w/w) of S. splendens . Dead space wounds were inflicted by implanting sterile cotton pellets (10mg each) subcutaneously. 5 mg/kg of Glibenclamide standard and methanolic extract of S. splendens were administered in doses of two strengths (200mg/kg and 400mg/kg). The excision of granulation tissue formed on the implanted cotton pellets was done and parameters like Tensile strength, Hydroxyproline content, and Total protein content were measured. Animals treated with extract showed a significant increase in percentage wound closure, tensile strength, hydroxyproline, and Total protein content with a significant decrease in period of epithelialization and blood glucose levels when compared to untreated animals in a dose dependent manner. A significant wound healing potential was seen in the Ethyl acetate fraction of methanolic extract in diabetic rats which may have been due to the presence of phytochemical constituents such as flavonoids, triterpenes, sterols.

Key words: *Salvia splendens*, wound healing, Excision, Dead space wound model.

INTRODUCTION

Wound is a loss or breaking of cellular and anatomical or functional continuity of living tissues. The physical injuries results in an opening or breaking of the skin and appropriate method for healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin [1].

Healing of wounds, a fundamental response to tissue injury occurs by a process of connective tissue repair. The end product of this process is a fibrous scar . The highly vascular granulation tissue synthesize Collagen (the pre-dominant constituent of a fibrous scar) and other components of the ground substance that is formed within the wound space. The strength and integrity to the dermis is provided by Collagen [2]. Diabetes mellitus is a condition which is known to be associated with a variety of connective tissue abnormalities. The collagen content of the skin is decreased as a result of reduced biosynthesis and/or accelerated degradation of newly synthesized collagen. The impaired wound healing observed in diabetes is contributed by these abnormalities [3].

The name *Salvia* (sage) is derived from salvare which means healer in Latin. Besides their antioxidant, antiseptic and antibacterial properties they also possess antifungal, antiviral, cytotoxic, carminative, diuretic, hypoglycemic, hemostatic,& wound healing properties, Many compounds isolated from *Salvia* extracts are associated with antiseptic, antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, hypoglycemic, cytotoxic, and antitumor activities.. The aerial parts of these plants contain flavonoids, triterpenoids and monoterpenes, particularly

in the flowers and leaves, while diterpenoids are found mostly in the roots. *Salvia splendens*, is known as scarlet sage mainly consists of anthocyanins and terpenoids. [4,5].

EXPERIMENTAL SECTION

Plant material

Leaves of *Salvia Splendens* were collected from the campus of Birla Institute of Technology, Mesra, Ranchi. The plant was identified and authenticated by taxonomy department of Botanical Survey of India (BSI), Kolkata. The voucher specimen (CNH/76/2012/Tech.II/898) was retained in the Department of Pharm. Science & Technology, BIT-Mesra, Ranchi, Jharkhand (India) for future reference.

Preparation of plant extracts

The Salvia leaves collected were washed to remove the adhered debris. It was dried in shade at room temperature, kept away from sunlight. The dried leaves were coarsely powdered using tissue blender. The air dried powder was successively extracted by hot extraction process using Soxhlet apparatus with solvents of increasing polarity index viz., petroleum ether (40-60 grade), chloroform and methanol for 72 hours. After filtering the extracts , the filtrates were dried using rotary evaporator to get dried crude fractional extracts[6]. A portion of the extract was formulated with a simple ointment base B.P. for Excision model.

Animals

The experimental protocol was approved by Institutional Animal Ethics committee, Department of Pharmaceutical Sciences & Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand (India). Approval No: BIT/PH/IAEC/23/2013 in September 2013 under CPCSEA guidelines.

The animals selected were Wistar strain Albino rats of either sex weighing between 150-200 gms, procured from the animal house of Department of Pharmaceutical Sciences & Technology, BIT, Mesra, Ranchi, Jharkhand (India).They were housed and maintained in clean polypropylene cages and allowed to acclimatize for 1 week before the commencement of the experimental study.They were fed with commercially pelleted rat chow and water *ad libitum.*

Housing conditions were maintained at 22 ± 2ºC at 12 h day/ night cycles.

Drugs and Chemicals

All the chemicals and solvents used in this study were of analytical grade.(Merck Millipore)

The marketed formulation viz; Mupirocin 2% ointment (Glaxo Smith Kline) and Glibenclamide (US vitamins, India) were used in the study.

The other Chemicals were procured from Central Drug House, Delhi.

Induction of Diabetes

The rats were induced diabetes by a single i.p. injection of Streptozotocin (60 mg/kg) in 0.1 M citrate buffer, pH 4.0. Animals with fasting blood glucose levels greater than 160 mg/dl, seven days after induction were used for further studies. [5].

Excision Model

Animals were divided into 4 groups of 6 animals each:
Group I (control) untreated
Group II (standard) treated with Mupirocin ointment (2% W/W), topically for 21 days
Group III (test -1) treated with EA-MESS ointment (5%W/W), topically for 21 days
Group IV (test -2) treated with EA-MESS ointment (10%W/W), topically for 21 days.

The animals were anaesthesized with 10mg/kg ketamine,.The dorsal fur area of each animal was shaved with electric clippers and a circular wound of 500mm^2 was created by surgical blade according to Morton and Malone[7] [Fig 1]. Afterwards, wound was cleaned, haemostasized with normal saline [8] and left open to allow the regeneration of tissue [9]. The measurement of wound areas was done on days 4,8,12 and 16 for all groups using a transparent sheet and a marker and recorded on a suitable graph paper.

Dead Space Wound (DSW) Model

Animals were divided into 4 groups of 6 animals each as follows:

Group I (control) untreated
Group II(standard) treated with Glibenclamide (5 mg/kg), *p.o.* for 10 days
Group III (test -1) treated with EA-MESS extract (200 mg/kg), *p.o.* for 10 days
Group IV (test -2) treated with EA-MESS extract (400 mg/kg), *p.o.*for 10 days
Animals were anaesthetized and sterile cotton pellets(10mg each) were subcutaneously implanted. Granulation tissue was dissected and tested according to the method described by Agarwal P[11].

Percentage wound closure
The areas of wounds were measured on 4^{th}, 8^{th}, 12^{th}, 16^{th} post-wounding days and the mean percentage wound closure was calculated.[12] .

$$\% \text{ wound closure} = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{wound area on day 0}} \times 100$$

where *n* =number of days.

The period of epithelization
When no raw wound was left behind, it was taken as end point of complete epithelization. The period of epithelization was the number of days required for this. [13]

Tensile strength
In dead space model, the excisions of granulomas from subcutaneous implants were performed on the 10th post wounding day. The breaking strength of the piece measuring about 15mm length and 8 mm in width was determined by continuous constant water flow technique [14,15]

Blood Glucose Level
In DSW model, the fasting blood glucose levels in four hour-fasted rats were measured directly using calibrated Glucometer (ONE TOUCHTM) with the help of strips. The glucose levels were recorded at days 0, 5 & 10.

Biochemical Estimations
Protein concentration was estimated according to the method of Lowry et al using BSA

(bovine serum albumin) as standard[16].

Wound tissues were analysed for hydroxyproline content, which is the basic constituent of collagen. [17].

Statistical analysis
The mean value ± SEM was calculated for each parameter. Results were statistically analyzed by one way analysis of variance (ANOVA) followed by Dunnet's *t*-test using software Graph pad prism 6. $P < 0.05$ was considered as significant.

RESULTS

1. Percentage wound contraction
There was significant decrease in the open area of the incised wound on Day 4, Day 8 and more on day 16 (Fig 1) .Animals of standard, test1 & test2 groups showed increased percentage of wound contraction when compared to control group, in a dose dependant manner. (Table 1**).**

Table 1: Effect of EA-MESS ointment on % wound contraction in STZ induced diabetic rats

Post-wounding days	Control (Untreated)	Standard (Mupirocin 2% ointment)	Test 1 (5%Ointment of EA-MESS)	Test 2 (10% Ointment of EA-MESS)
Day 4	6.667±0.42	26.83±0.47****	13.83±0.79***	19.5±0.99***
Day 8	11.33±0.33	45±1.07****	24.33±0.42****	36.83±0.31****
Day 12	23.67±0.56	74.33±0.33****	34.67±0.42****	67.±0.58****
Day 16	56.33±0.61	93.5±0.89****	68.8±0.7****	84.5±0.43****

*The results are expressed as mean ±SEM (n=6). Inter group comparisons were made using one way ANOVA followed by Dunnett test. Probability values of ****p< 0.0001, *** p<0.001, ** p<0.01 and * p<0.05 when compared to control.*

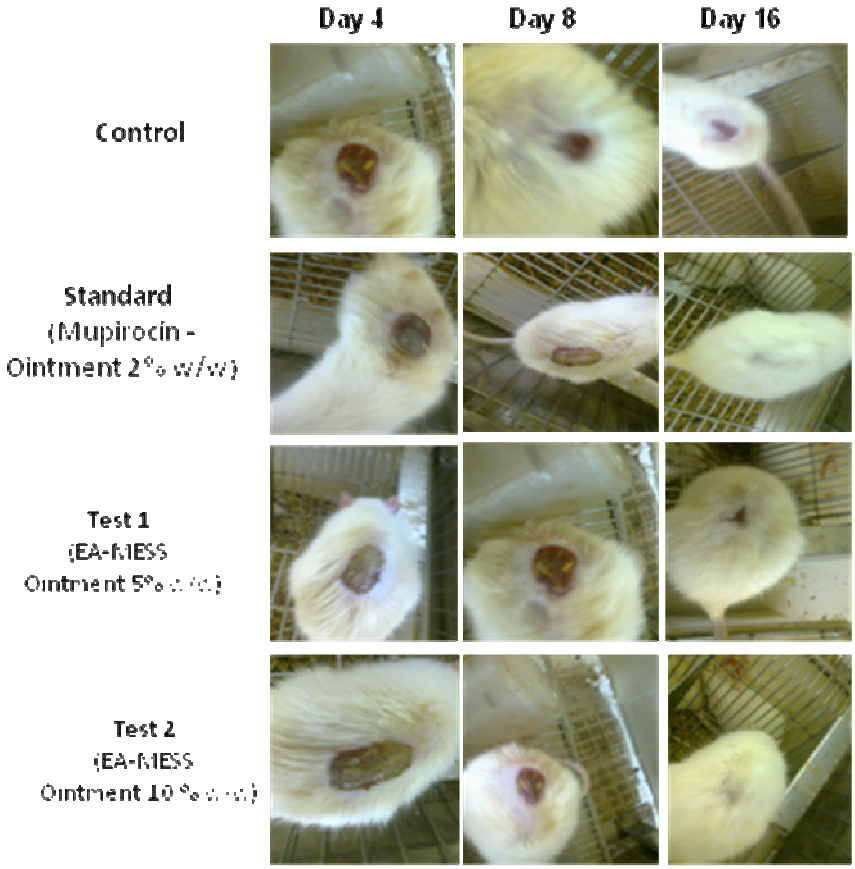

Fig 1: Excision wound on 4th, 8th & 16th day

2. Period of epithelization of wound

When there was no raw wound left behind, it was taken as end point of complete epithelization. The days required for this was taken as the period of epithelization. (Table 2**).**

Table 2: Period of epithelization of wound with EA-MESS extract in STZ induced diabetic rats

Period of epithelization	Control (Untreated)	Standard (Mupirocin 2% ointment)	Test 1 (5%Ointment of EA-MESS)	Test 2 (10% Ointment of EA-MESS)
	23.5±0.62	16.33±0.33**	20.5±0.43*	19.5±1.43

*The results are expressed as mean ±SEM (n=6). Inter group comparisons were made using one way ANOVA followed by Dunnett test. Probability values of ****p< 0.0001, *** p<0.001, ** p<0.01 and * p<0.05 when compared to control.*

3. Tensile strength of granulation tissue

Standard and extract treated groups showed a significant increase in tensile strength of granulation tissue of dead space wounds in a dose dependant manner when compared to control group. (Table 3**).**

Table 3: Tensile strength of granulation tissue with EA-MESS in STZ induced diabetic rats

Treatment groups	Tensile strength (gms)
Control (Untreated)	53.33±1.08
Standard (5 mg/kg of Glibenclamide)	96.17±2****
Test 1 (200 mg/kg of EA-MESS)	68±1.12****
Test 2 (400 mg/kg of EA-MESS)	83.33±2.43***

*The results are expressed as mean ±SEM (n=6). Inter group comparisons were made using one way ANOVA followed by Dunnett test. Probability values of ****p< 0.0001, *** p<0.001, ** p<0.01 and * p<0.05 when compared to control.*

4. Weight of granulation tissue

A significant increase in both wet granulation weight and dry granulation weight was observed in standard and test treated groups in a dose dependant manner, when compared to control animals (Table 4).

Table 4: Wet and Dry granulation tissue with EA-MESS in STZ induced diabetic rats

Treatment groups	Wet granulation tissue weight(mg)	Dry granulation tissue weight(mg)
Control (Untreated)	75.5±1.48	21.67±1.05
Standard (5 mg/kg of Glibenclamide)	144.8±2.05****	41.67±1.05****
Test 1 (200 mg/kg of EA-MESS)	90.83±1.08**	28.33±1.05*
Test 2 (400 mg/kg of EA-MESS)	125.5±2.28****	38.83±1.54***

*The results are expressed as mean ±SEM (n=6). Inter group comparisons were made using one way ANOVA followed by Dunnett test. Probability values of ****p< 0.0001, *** p<0.001, ** p<0.01 and * p<0.05 when compared to control.*

Biochemical Parameters:

5. Blood glucose level

The blood glucose levels in standard and test treated groups were significantly decreased in a dose dependant manner when compared to control animals.(Table 5).

Table 5: Blood glucose level of EA-MESS in STZ induced diabetic rats

Treatment groups	Blood glucose levels (mg/dl)		
	Day 0	**Day 5**	**Day 10**
Control (Untreated)	333.5±1.28	336.88±1.28	341.2±1.17
Standard (5 mg/kg of Glibenclamide)	337.8±1.94	185.3±5.17****	119±1.48****
Test 1 (200 mg/kg of EA-MESS)	333.3±1.33	310.5±0.89****	234.3±1.08****
Test 2 (400 mg/kg of EA-MESS)	340±1.24	224.3±2.62****	168.3±2.17****

*The results are expressed as mean ±SEM (n=6). Inter group comparisons were made using one way ANOVA followed by Dunnett test. Probability values of ****p< 0.0001, *** p<0.001, ** p<0.01 and * p<0.05 when compared to control.*

6. Protein and Hydroxyproline content

Standard and Extract treated groups showed significant increase in total protein content and hydroxyproline content in a dose dependant manner when compared to control group animals. (Table 6**).**

Table 6: Protein and Hydroxyproline content of granulation tissue with EA-MESS extract in STZ induced diabetic rats

Treatment groups	Protein content ((mg/gm tissue))	Hydroxyproline content (mg/gm tissue)
Control (Untreated)	44±0.86	66.83±0.65
Standard (5 mg/kg of Glibenclamide)	162.5±1.02****	139.5±0.62****
Test 1 (200 mg/kg of EA-MESS)	106.7±.33****	108.3±0.8****
Test 2 (400 mg/kg of EA-MESS)	138.8±1.4****	127.8±0.7****

*The results are expressed as mean ±SEM (n=6). Inter group comparisons were made using one way ANOVA followed by Dunnett test. Probability values of ****p< 0.0001, *** p<0.001, ** p<0.01 and * p<0.05 when compared to control.*

DISCUSSION

Wound healing process proceeds in three stages viz. Inflammation, cellular proliferation and remodeling [18]. The first stage is a coagulative and inflammatory phase and in this Neutrophils migrate towards fibrin clot. The second stage is a proliferative phase in which the space is dominated by granulation tissue and collagen fibrils. The third stage being remodeling phase which involves synthesis of collagen fibers, leading to increase in tensile strength of the skin[19]. Healing is completed when the disrupted surfaces are firmly knit by collagen[20].

In connective tissue metabolism a variety of alterations is known to be associated with Diabetes mellitus, which results in poor wound healing in diabetics. Loss of collagen in diabetes is either due to decreased levels of synthesis or enhanced catabolism of newly synthesized collagen or both [21]. As *S. splendens* was reported to cause hypoglycemic effects , it was perceived that it would be interesting to study its influence on the healing of wounds in diabetic conditions. The present study results suggest that diabetic rats treated with methanolic extract of leaves of *S. splendens* may have a beneficial influence on wound healing [22]

The granulation tissue of a healing wound contains collagen as the predominant extracellular protein and soon after an injury, there is a rapid increase in the synthesis of this protein in the wound area. Besides providing strength and integrity to a tissue matrix, collagen also plays an important role in homeostasis and subsequent epithelialization. The influence of methanolic extract of leaves of *S. splendens* on the collagen content in granulation tissues was studied in the present study, The maximum levels of collagen in the granulation tissue, was increased by treatment of wounds with *S. splendens* as compared to the untreated diabetic control [11]

Fibroblasts synthesize Glycosaminoglycans and proteoglycans in the wound area. These substances form a highly hydrated gel-like ground substance, a provisional matrix on which collagen fibers are embedded. The content of ground substance in the granulation tissues was increased by treatment with methanolic extract of *S. splendens*

leaves. The protein and DNA content of granulation tissues indicate the levels of protein synthesis and cellular proliferation. It has also been reported that inhibition of proinflammatory markers and stimulation of IL-8 and various growth factors may lead to increased rate of wound contraction [10]. Since animals treated with *S. splendens* have been reported to have higher protein and DNA contents (compared to the untreated controls), it is possible that *S.splendens* might contribute to wound healing through the mechanism of cellular proliferation.

The collagen molecules synthesized are laid down at the wound site and become cross linked to form fibers. Wound strength is acquired from both, remodeling of collagen, and the formation of stable intra- and intermolecular cross links [23]. Since granulation tissue from dead space wounds treated with methanolic extract of leaves of *S. splendens* showed greater tensile strength, it may be inferred that it not only increases collagen synthesis per cell, but also aids in cross linking of the protein. *S. splendens* treated wounds also showed an increase in rate of wound contraction which led to quicker healing as confirmed by decreased period of epithelialization when compared to untreated control wounds.

Flavonoids have been recognized as agents that can be used to antagonize lipid peroxidation that usually occurs in case of wound injury. Similar to antioxidants like vitamin C and vitamin E [24], any drug that antagonizes lipid peroxidation helps in increased blood circulation therefore increased collagen viability, thus increasing DNA synthesis and reducing cell damage [25]. Phytochemical screening of *S. splendens* which showed presence of flavanoids and its antimicrobial activity as shown in previous research [26, 27]. also attributed to its wound healing capability.

CONCLUSION

The results suggested that methanolic extract of the leaves of *S.splendens* treatment may have a beneficial influence on the various phases of wound healing like fibroplasia, collagen synthesis and contraction resulting in faster healing. It was quite possible that the enhanced healing of wounds in diabetic rats by methanolic extract of leaves of *S. splendens* was a result of its phytochemical constituents such as flavonoids, triterpenes and isosterols, which are known to produce wound healing and hypoglycemic activity.

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