Evaluation of wound healing activity of hydroalcoholic extract of leaves of *Stachytarpheta jamaicensis* in streptozotocin induced diabetic rats

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ABSTRACT

The aim of the current study was to evaluate the wound healing effect of hydroalcoholic leaf extract of Stachytarpheta jamaicensis (HLS) on Streptozotocin-induced diabetic rats. The wound healing activity was evaluated by two methods: Excision and Dead space models. Diabetes was induced in rats by a single i.p. Streptozotocin (70 mg/kg). In Excision model, wound of circular area approx. 500 mm^2 was created on the back of each animal. Wound healing parameters like percentage wound contraction, period of epithelialization were observed by administering standard Mupirocin ointment (2% w/w) and HLS (2% and 5% w/w) topically. In Dead space wound model, polypropylene tubes were implanted subcutaneously and HLS extract was administered in two doses (200mg/kg and 400mg/kg). Granulation tissue was excised from implants and parameters like Tensile strength, Hydroxyproline, Hexosamine, DNA and Total protein content were measured. Animals treated with HLS had shown a significant increase in percentage wound closure, tensile strength, hydroxyproline, Hexosamine, DNA and Total protein content with a concomitant significant decrease in period of epithelialization and blood glucose levels when compared to untreated animals in a dose dependent manner. The HLS afforded significant wound healing potential in diabetic rats which may be due to the presence of phytochemical constituents such as flavanoids, triterpenes, sterols.

Key Words: Wound healing, *Stachytarpheta jamaicensis,* Streptozotocin, Excision, Dead space wound model.
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INTRODUCTION

Wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue and is a natural process. Wound healing process proceeds in three overlapping phases viz. inflammation cellular proliferation and remodeling [1]. Phase 1 is a coagulation and inflammatory phase (0–3 days) and this involves neutrophil migration towards fibrin clot. Phase 2 is a proliferative phase (3–12 days) in which the granulation tissue and collagen fibrils dominate the space. Phase 3 is a remodeling phase (3–6 months), involving synthesis of collagen fibers, leading to increase in tensile strength of the skin[2]. Healing is not complete until the disrupted surfaces are firmly knit by collagen[3].

Wound, though appearing minor are a major part of injuries occurring to the body. It can happen in everyday life and untreated uncared wounds can lead to dangerous consequences like microbial infection etc. Treating wounds at the earliest and carefully is important especially in cases of patients with diabetes with conditions like pressure ulcers which if left untreated requires amputation of the affected extremities[4].

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Diabetes mellitus is a condition known to be associated with abnormalities like reduced biosynthesis and/or accelerated degradation of synthesized which contribute to the impaired wound healing observed in diabetes [5]. *Stachytapheta jamaicensis* Vahl, family Verbenaceae (synonym *Stachytapheta indica* C.B. Clarke) is an invasive plant species found in many continents. Known by many names such Brazilian tea, bastard vervain, Blue Porterweed, it is a native to Mexico, USA, and naturalized in Tropical Africas, China, Indian Subcontinent, and the Pacific[6]. It was reported to have various activities as a febrifuge, anti-inflammatory, ulcers and rheumatic fever and also against diarrhoea and dysentery [7]. The leaves of *Stachytarpheta jamaicensis* have been traditionally claimed to possess wound healing activity[8] but has never been researched upon, therefore the present study was designed to evaluate the wound healing effect of the leaf extract on Streptozotocin-induced Diabetic rats.

MATERIALS AND METHODS

Plant material
The leaves of *Stachytarpheta jamaicensis* used in the present study were collected from the surroundings of Chittor district (Andhra Pradesh) and authenticated by Dr.P.Jayaraman Ph.D., Plant Anatomy Research Centre (PARC), Tambaram. Voucher specimen (PARC/2010/637) was submitted to Department of Pharmacology, SRM College of Pharmacy, Tamil Nadu, India.

Preparation of Extraction
The collected leaves were dried under shade and coarsely ground. 250g of the coarse powder was defatted, air dried and refluxed with ethanol (70% v/v) using Soxhlet extractor at 50°C for 72 h. Filtration using Whatman filter paper (no.1) and concentration under vacuum at 40°C using a rotavap was performed. The extract thus obtained was stored for further phytochemical and pharmacological evaluation. Portion of the extract was formulated with simple ointment base B.P. for the purpose of Excision model.

Animals
The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of the SRM University, Kattankulathur, Tamil Nadu, India (Approval No.:662/02/C/CPCSEA) under CPCSEA guidelines. The animals selected were Albino Wistar rats of either sex weighing between 150-200g. They were housed and maintained in clean polypropylene cages and fed with commercially pelleted rat chow (M/s Hindustan Lever Ltd. Mumbai) and water *ad libitum.*

Induction of Diabetes
Diabetes was induced in rats by a single i.p. injection of Streptozotocin (70 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 [9].

Excision Model
Animals were divided into 4 groups of 5 animals each as follows:
Group I (control) untreated
Group II (standard) treated with Mupirocin ointment (2% W/W), topically for 21 days
Group III (test animals) treated with HLS extract (2%W/W), topically for 21 days
Group IV (test animals) treated with HLS extract (5%W/W), topically for 21 days

Post anaesthetization of the animals 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[10] [Fig 1]. Afterwards, wound was cleaned and haemostasized with normal saline [11] and left open to allow the regeneration of tissue[12,13]. Wound area measurement was recorded with suitable graph paper.

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Fig 1: Excision wound on day 0

Dead Space Wound (DSW) Model
Animals were divided into 3 groups of 5 animals each as follows:
Group I (control) untreated
Group II (test animals) treated with HLS extract (200 mg/kg), *p.o.* for 10 days
Group III (test animals) treated with HLS extract (& 400 mg/kg), *p.o.* for 10 days
Animals were anaesthetized and propylene tubes were subcutaneously implanted. Granulation tissue was dissected and tested according to method by Agarwal PK[14].

Percentage wound closure
The areas of wounds were measured on days 4, 8, 12, 16 post-wounding days and the mean percentage wound closure was calculated. The period of epithelization was calculated as the number of days required for falling of the dead tissue remnants without any residual raw wound.

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

where n = number of days[3].

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 a continuous constant water flow technique [15].

Blood Glucose Level
In DSW model, the fasting blood glucose levels in four hour-fasted rats were measured directly from Glucometer (ONE TOUCH^{TM}) with the help of strips. The glucose levels recorded for days 0, 5, 10.

Biochemical Estimations
To estimate collagen and hexosamine, the granulation tissue samples were defatted in chloroform : methanol (2:1) and dried, neutralized with sodium hydroxide before use. Collagen was estimated by the method of Woessner [16], whereas hexosamine was estimated by the method of Elson-Morgan [17].

Protein and DNA of wet granulation tissues were extracted in 5% trichloroacetic acid (TCA) as per Schneider [18]. 10 ml of 5% TCA was added to the tissue (100 mg wet weight) kept at 90°C for 30 min in a water bath to extract protein and DNA. The solution was then centrifuged and the supernatant was used to estimate DNA by the method of Burton [19] and protein by the method of Lowry *et al.* [20]

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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 5. $P < 0.05$ will be considered as significant.

<div align="center">

RESULTS

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Phytochemical Screening
The hydroalcoholic leaf extract of *Stachytarpheta jamaicensis* showed a positive result for Flavonoids, Glycosides, Saponins, Proteins, Steroids, and Terpenes.

Percentage wound contraction
The open area of the incised wound had decreased significantly when observed on day 8 [Fig 2] and even more on day 16 [Fig 3]. Animals of Group III and IV showed increased percentage of wound contraction when compared to control group in a dose dependent manner. (Table 1)

Figure 2: Excision wound on day 8

Figure 3: Excision wound on day 16

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Table 1: Percentage Wound Contraction (Original wound size = 500 mm^2)

Treatment	% Wound contraction				Epithelialization Period (days)
	Day 4	Day 8	Day 12	Day 16	
Group I	24.36± 0.54^a	53.17±0.89^b	71.99±0.36^c	91.71±0.26^d	21.83±0.30^e
Group II	60.97± 0.35***	75.93±0.84***	88.07±0.38***	99.73±0.14***	16.17±0.30***
Group III	31.54± 0.60***	60.67±0.27*	78.27±0.29***	93.19±0.24**	19±0.36***
Group IV	41.54±0.46***	72.18±3.59***	81.72±0.42***	96.75±0.35***	17.67±0.33***

*Values are expressed in mean± SEM (n=6).Value comparisons were made between Negative control Vs standard, group III and group IV (^{a}P< 0.0001, ^{b}P< 0.0001,^{c}P<0.0001, ^{d}P<0.0001, ^{e}P<0.0001). Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001. ns-not significant.*

Tensile Strength

Extract-treated groups (Group II and Group III) showed a significant increase in tensile strength of granulation tissue of dead space wounds in a dose dependent manner when compared to control group. (Table 2)

Table 2: Tensile strength of Granulation Tissue of *Stachytarpheta jamaicensis* treated and untreated wounds on diabetic rats

Treatment	Tensile strength (g)
Group I	37.87±2.62
Group II (200 mg/kg of HLS)	70.85±2.94***
Group III (400mg/kg of HLS)	94.22±3.20***

*Values (g) are expressed am mean± SEM (n=6). Inter group comparisons were made between Negative control Vs reference, group II and group III using one way ANOVA followed by Dunnett test. P<0.0001. Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001*

Weight of Granulation Tissue

A significant increase in both wet granulation weight and dry granulation weight was shown by extract-treated groups in a dose dependent manner when compared to control animals. (Table 3)

Table 3: Wet and Dry Granulation Tissue Weight

Treatment	Wet granulation weight	Dry granulation weight
Negative control	79.54±2.66^a	23.82±1.43^b
Group II (200 mg/kg of HLS)	98.62±2.36**	32.41±1.52**
Group III(400 mg/kg of HLS)	121.9±4.18***	38.56±1.61***

*Values (g) are expressed am mean± SEM (n=6). Inter group comparisons were made between Negative control Vs group II and group III using one way ANOVA followed by Dunnett test. ^{a}P<0.0001, ^{b}P<0.0001 Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001*

Biochemical Parameters
Blood Glucose Levels

The blood glucose levels in extract-treated groups were significantly decreased in a dose dependent manner. (Table 4)

Table 4: Blood Glucose Levels

Treatment	Blood glucose levels		
	Day 0	Day 5	Day 10
Negative control	216.1±4.2^a	218.1±4.2^b	221.5±3.83^c
Group II (200 mg/kg of HLS)	211.5±1.72*	195.3±1.42^{ns}	144.6±1.17**
Group III(400 mg/kg of HLS)	221.1±3.83**	144.6±1.42***	108.5±1.76*

*Values (g) are expressed am mean± SEM (n=6). Inter group comparisons were made between Negative control Vs reference, group II and group III using one way ANOVA followed by Dunnet test. ^{a}P<0.01, ^{b}P<0.05, ^{c}P<0.01 Symbols represent *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001,*

Table 5: Hydroxyproline Content

Treatment	Hydroxyproline content
Negative control	97±3.45
Group II (200 mg/kg of HLS)	109.5±2.11*
Group III (400mg/kg of HLS)	117.5±2.41***

*Values (g) are expressed am mean± SEM (n=6). Inter group comparisons were made between Negative control Vs reference, group II and group III using one way ANOVA followed by Dunnett test. P<0.0003. Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001*

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Hydroxyproline (Collagen) Content
Extract-treated groups showed a significant dose-dependent increase in hydroxyproline content when compared to control animals. (Table 5)

Hexosamine Content
Significant increase in hexosamine content was shown by extract-treated groups in a dose dependent manner when compared to control animals. (Table 6)

Table 6: Hexosamine Content

Treatment	Hexosamine content
Negative control	21.83±1.10
Group II (200 mg/kg of HLS)	30.08±0.75***
Group III (400mg/kg of HLS)	38.33±0.99 ***

Values (g) are expressed am mean± SEM (n=6). Inter group comparisons were made between Negative control Vs group II and group III using one way ANOVA followed by Dunnett test. P<0.0001. Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001

DNA content
Extract-treated groups showed significant increase in DNA content in a dose dependent manner when compared to control animals. (Table 7)

Table 7: DNA Content

Treatment	DNA content
Negative control	7.3±0.34
Group II (200 mg/kg of HLS)	9.41±0.33***
Group III (400mg/kg of HLS)	12.73±0.23***

Values (g) are expressed am mean± SEM (n=6). Inter group comparisons were made between Negative control Vs group II and group III using one way ANOVA followed by Dunnett test. P<0.0001. Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001

Total Protein Content
Extract-treated groups showed significant increase in Total protein content in a dose dependent manner when compared to control animals. (Table 8)

Table 8: Total protein Content

Treatment	Total protein content
Negative control	106.8±3.32
Group II (200 mg/kg of HLS)	119±2.63**
Group III (400mg/kg of HLS)	128±1.69***

Values (g) are expressed am mean± SEM (n=6). Inter group comparisons were made between Negative control Vs group II and group III using one way ANOVA followed by Dunnett test. P<0.0002. Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001

DISCUSSION

Diabetes mellitus is known to be associated with a variety of alterations in connective tissue metabolism, as a result of which diabetics face the problem of poor wound healing. Loss of collagen observed in diabetes may be due to decreased levels of synthesis or enhanced catabolism of newly synthesized collagen, or both [21]. As *Stachytarpheta jamaicensis* was reported to cause hypoglycemic effects [22], it was felt that it would be interesting to study its influence on the healing of wounds in diabetic conditions. Results obtained in the present study suggested that treatment of diabetic rats with hydroalcoholic extract of leaves of *Stachytarpheta jamaicensis* may have a beneficial influence on wound healing [22].

Collagen is the predominant extracellular protein in the granulation tissue of a healing wound and there is a rapid increase in the synthesis of this protein in the wound area soon after an injury. In addition to providing strength and integrity to a tissue matrix, collagen also plays an important role in homeostasis. Subsequent epithelialization also requires collagen. In the present study, we examined the influence of hydroalcoholic extract of leaves of *Stachytarpheta jamaicensis* on the collagen content in granulation tissues. Treatment of wounds with *Stachytarpheta jamaicensis* increased the maximum levels of collagen in the granulation tissue, as compared to the untreated diabetic control as supported by a previous research report [14].

Glycosaminoglycans and proteoglycans are synthesized by fibroblasts in the wound area. These substances form a highly hydrated gel-like ground substance, a provisional matrix on which collagen fibers are embedded. Treatment with hydroalcoholic extract of leaves of *Stachytarpheta jamaicensis* increased the content of ground substance in the granulation tissues. As collagen accumulated, hexosamine levels were increased [23]. It may be seen that the increase in hexosamine content was associated with a concomitant increase in collagen content.

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 [14]. Since *Stachytarpheta jamaicensis* has been reported to have anti-inflammatory effect [24] and also higher protein and DNA contents (compared to the untreated controls) have been observed, it is possible that *Stachytarpheta jamaicensis* might contribute to wound healing through the mechanism of cellular proliferation.

The collagen molecules synthesized were laid down at the wound site and become cross linked to form fibers. Wound strength was acquired from both, remodeling of collagen, and the formation of stable intra- and inter-molecular cross links [25]. Since granulation tissue from dead space wounds treated with the hydroalcoholic extract of leaves of *Stachytarpheta jamaicensis* 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. *Stachytarpheta jamaicensis* 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 [26], any drug that antagonizes lipid peroxidation helps in increased circulation therefore increased collagen viability, thus increasing DNA synthesis and reducing cell damage [27]. Phytochemical screening of *Stachytarpheta jamaicensis* which showed presence of flavanoids and also previous research on its antimicrobial activity [28, 29] also attributed to its wound healing capability.

CONCLUSION

The results suggested that hydroalcoholic extract of leaves of *Stachytarpheta jamaicensis* 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 hydroalcoholic extract of leaves of *Stachytarpheta jamaicensis* was a result of its phytochemical constituents such as flavonoids, triterpenes and isosterols, which are known to produce wound healing and hypoglycemic activity and also antiinflammatory activity of *Stachytarpheta jamaicensis*.

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