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## Comparative evaluation of the wound healing potential of *Tinospora cordifolia* and its combination with local Insulin therapy in diabetic rabbits

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**Abstract**

Wound healing is impaired in diabetes mellitus. Therefore, the present study was carried out for the evaluation of healing potential of *Tinospora cordifolia* and its combination with local Insulin therapy in diabetic wounds. Diabetes was induced in the animals of group I and II. The wounds were created on the thoraco-lumbar area in each animal under general anesthesia. The animals of group I were treated with oral suspension prepared from methanolic extract of *T. cordifolia* stem and the animals of group II were treated with combination of oral *T. cordifolia* and local insulin therapy. The soframycin ointment was applied over wounds in both the groups. The rate of wound healing was assessed on the basis of colour digital imaging, wound morphometry, histopathological and Scanning Electron Microscopic (SEM) studies. On the basis of observations, the rate of wound healing recorded in group II animals was significantly higher and better than group I.

**Keywords:** healing potential, *Tinospora cordifolia*, Insulin therapy

**Introduction**

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and alteration in carbohydrate, fat and protein metabolism associated with absolute or relative deficiency of insulin secretion and/or insulin action (Kameswara *et al.*, 2003) [14]. It is one of the major contributors to delayed wound healing. Wound healing is the restoration of tissue architecture and function following an injury (Kumar *et al.*, 2007) [17]. Normal wound healing is a complex phenomenon covering four overlapping phases i.e. inflammation, proliferation, remodeling and maturation (Sumitra *et al.*, 2005) [5]. In diabetes mellitus, all these four phases are hampered and the wound remains in a chronic inflammatory state and fails to heal in a timely and orderly manner. Hyperglycemia and infection are the major causes for the delayed wound healing (Sandhya *et al.*, 2011) [30]. Bacteria thrive on the increased glucose available in the bloodstream, and that at the same time the high glucose concentration inhibits neutrophil action, leading to a proliferation of bacteria and infection. Infection and inflammation lead to imbalance of protease and reactive oxygen species, essential growth factors are degraded, the ability of angiogenesis is impaired and cell recruitment to the wound sites is inhibited (Blakytyn and Jude 2006) [4]. All these factors hampering the process of healing make the management of diabetic wounds difficult.

It has been proposed that absolute or relative lack of insulin contributes to delayed wound healing in diabetes (McIntosh and Pederson 1999) [24]. Clinical and animal experiments have indicated that local treatment with insulin may improve wound healing in diabetes (Madibally *et al.*, 2003) [22]. The role of topical insulin in the promotion of wound healing in several animal models has been reported by previous workers (Liu *et al.*, 2005; Zhang *et al.*, 2007) [20, 40]. In addition, insulin injected diffusely into the wound can accelerate wound re-epithelialization (Zhang *et al.*, 2007) [40]. In modern medicine, no satisfactory effective therapy is still available to cure the diabetic wounds and associated complications. Though insulin and other modern therapies are being used for the management of diabetic wounds, but they also have drawbacks like resistance (Piedrola *et al.*, 2001) [25]. Moreover, the modern therapeutic treatments are very expensive. Thus, all these factors compel for the search of safe and affordable alternatives with minimal adverse effects, which can be taken for long durations. Recently, there has been increasing interest in the use of medicinal plants. *Tinospora cordifolia* is one of them. It is a deciduous climbing shrub of the family *Menispermaceae*. It is distributed throughout the Indian subcontinent and China (Singh *et al.*, 2003) [33].

The plant has been reported as anti-diabetic and anti-hyperglycemic (Raghuathan and Sharma 1969; Kar *et al.*, 2003) [26, 3], anti-inflammatory (Upadhyay *et al.*, 2010) [37], antioxidant (Chopra *et al.*, 1992) [8], anti-stress (Sharma *et al.*, 1995) [31]. The wound healing properties of *T. cordifolia* has also been reported (Nema *et al.*, 2012). Review of literature revealed the anti-diabetic and wound healing properties of *T. cordifolia* and Insulin. To the best of our knowledge, none of the studies reported the potential of *T. cordifolia* alone or in combination with insulin in diabetic wound healing. Hence, the present study was planned to evaluate the wound healing potential of *T. cordifolia* and its combination with local Insulin therapy in diabetic animals.

## 2. Materials and methods

### 2.1 Preparation of methanolic extract of *Tinospora cordifolia*

The stems of matured *T. cordifolia* (Fig-1) were collected, chopped, shade dried and finely powdered. The powder was then extracted with methanol using soxhlet extractor. The extract was dried and kept in refrigerator for further use. The methanolic extract of *T. cordifolia* was dissolved in distilled water 300 mg/ml. This prepared solution was given to the rabbits @ 2ml/ kg b.wt. orally.

### 2.2 Animals

Twelve clinically healthy New Zealand white rabbits were used in this study. All the animals were kept in individual cages. Prior to the start of the experiment, these animals were dewormed and kept under observation for one week to acclimatize the new environment and were randomly divided into two groups having six animals each. The animals were fed green fodder, wheat grains, maize and grams, ad libitum. Fresh and wholesome water was also provided ad libitum. Similar conditions of management and feeding were continued to all the animals during the entire period of study.

### 2.3 Induction of diabetes

The 12 animals of treatment groups were made diabetic by administering the sterile solution of 5% alloxan monohydrate (Alloxan monohydrate –Titan Biotech Ltd. Bhiwadi, Rajasthan, India) in normal saline at the dose rate of 100 mg/kg body weight, intra-peritoneally (Wang *et al.*, 2010) [38]. To avoid the mortality due to hypoglycemia alloxan was administered in well fed rabbits after estimation of the normal serum glucose levels. The animals were given 2 grams of glucose/kg b.wt. dissolved in 10 ml of distilled water to counteract the anticipated alloxan induced hypoglycemia. The blood glucose levels were estimated on day zero, seven, fourteen, and twenty eight after alloxan administration by using blood glucose determination kit and rabbits having 300 mg/dl or more were considered as diabetic (Dubey *et al.*, 1994 and Chang *et al.*, 2003) [10, 7].

### 2.4 Wound creation and design of study

Thoracolumbar area of the animals was shaved properly and prepared for aseptic surgery. The animals were anesthetized by administration of xylazine @ 10 mg/kg b.wt. and ketamine @ 50 mg/kg b.wt. intramuscularly (Jaiswal, 2012) [13]. After achieving surgical anesthesia, the animals were retained in ventral recumbency. The skin of thoraco-lumbar area was marked by sterile disk of 2 cm diameter for creation of wounds of equal sizes in all animals. The skin was excised in full thickness. The wounds were treated as follows-

**Group I:** Oral administration of methanolic extract of *T. cordifolia* @ 2 ml/kg and antiseptic dressing of the wounds with soframycin ointment.

**Group II:** Administration of methanolic extract of *T. cordifolia* orally, local insulin therapy @ 80 I.U. at the periphery of the wounds on alternative days and antiseptic dressing of wounds with soframycin ointment.

## 2.5 Assessment of wound healing

The rate of wound healing was assessed on the basis of color digital imaging, wound morphometry, histopathological and Scanning Electron Microscopic (SEM) studies.

### 2.5.1 Color digital imaging

Digital color photographs were taken on days 7, 14 and 28 with the help of digital camera at a fixed distance. Analysis of shape, size and irregularity of the lesion was determined.

### 2.5.2 Wound morphometry

To calculate the percent total wound healing (percent contraction), the wound area of both the groups was measured as per method given by Bohling *et al.* (2004) [5] on postoperative days 7, 14 and 28.

### 2.5.3 Histopathological study

For the histopathological study, wounds were first cleaned with savlon. Thereafter, samples from the healing sites were collected on seventh, fourteenth and twenty eighth day and preserved in 10% formal saline solution. After fixation of samples they were washed and dehydrated with a sequence of ethanol-xylene series of solutions (Kanai *et al.* 2000) [15]. The tissues were processed by routine paraffin (54 °C- 56 °C melting point) embedding technique and sections of 4-5 μ thickness were cut. The tissues were processed in a routine manner (Lillie and Fullmar, 1976) [18] and 4μ thick longitudinal sections were cut and stained with Hematoxylin and Eosin (H&E) as per the standard procedure (Luna, 1992) [21].

### 2.5.4 Scanning Electron Microscopic (SEM) Study

The wound specimens for this study were collected from the test wounds on days fourteen and twenty eight, postoperatively. Each specimen consisted of both wound healing and adjacent soft tissue. The samples were fixed in 2% solution of glutaraldehyde in PBS (Cornovsky's fluid) for 24 hours. Further tissue processing was done as described by Gangwar (2012) [11].

## 3.6 Statistical Studies

The statistical analysis of the collected data was performed by using SPSS 20 software. The data obtained was subjected to analysis of variance (ANOVA) and means were compared using Duncan's Multiple Range Test.

## 4. Results and Discussion

The colour digital photographs of diabetic wounds treated with different therapies at different time intervals are presented in figure 2. On days 7, 14 and 28, the wounds of the animals of groups II showed better healing tendency than the group I wounds to which only single treatment was given. Wound contraction was also evident and better in group II when compared to group I. The percent of wound contraction was calculated by measuring the changes in wound size (Table- 1). On seventh day, the maximum wound contraction

was recorded in animals of group II followed by group I. But there was no significant difference between both the groups at this stage. On fourteenth day, at this duration wound contraction was still higher in group II followed by group I. The difference in total wound contraction in group II was significantly higher from group I. On, twenty eighth day, the wounds were nearly healed in groups II at this stage except in group I where only 79.71% wound contraction was recorded upto this stage as compared to 97.12% in group II. There was significant difference in wound contraction between group II and group I. The group I showed significant difference in wound contraction at different time intervals but in group II there was no significant difference from day fourteen to day twenty eight.

Tissue biopsy samples stained with H&E stain were evaluated histopathologically on different days (Fig. 3). On seventh day, in group I, there was very thin and incomplete layer of epithelium along with highly immature capillaries and granulation tissue, tissue debris was having polymorphonuclear cells. In group II, there was relatively more epithelization than group I along with numerous budding capillaries, abundant fibroblasts and granular tissue. On fourteenth Day, in group I, superficial epithelium was not formed completely. Epidermal layer was not properly formed. Presence of newly formed capillaries (angiogenesis) and fibrosis in dermal tissue was evident at this time. In group II, thin continuous layer of epithelium along with epidermis was formed. Abundant newly formed blood vessels along with fibrous tissue and collagen were present at this stage. On twenty eighth day, In group I, epidermis was not properly formed, mild tissue reactions with few fibrous and collagen tissue were evident. In group II, epidermal healing was complete but in dermis, proliferation of fibrous connective tissue was present.

Scanning Electron Microscopic Study of the samples taken from wounds was also done (Fig. 4). On fourteenth day, irregularly arranged collagenous tissue along with numerous newly formed blood vessels was seen in group I. Epithelialization was in initial phase in group II, regularly arranged dense collagenous tissue was seen with few blood vessels and epithelialization had started. On twenty eighth day, in group I, the collagenous tissue became denser and arranged in a little bit regular pattern. The epithelial layer formed but was still incomplete and angiogenesis was reduced but still present. In group II, regularly arranged dense collagenous tissue was evident and epithelialization was nearly completed.

In group I, swelling and exudation (inflammatory changes) reduced slowly by fourteenth day after treatment. This might be due to the anti-inflammatory property of *T. cordifolia*. Rawal *et al.* (2004) [29] reported that *T. cordifolia* could significantly enhance VEGF and markedly decreases the expression of pro-inflammatory mediators such as COX-2 and cell adhesion molecules such as VCAM (vascular cell adhesion molecule). In group II, the reduction in pain and exudation may be due to the combined anti-inflammatory

effect of *T. cordifolia* (Upadhyay *et al.*, 2010) [37] and insulin (Dandona *et al.*, 2007) [9]. On day twenty eight, the mean wound contraction was in group I (79.71%). These results were in accordance with the findings of Barua *et al.* (2010) [2]. The early reepithelialization and faster wound closure in *T. cordifolia* treated wounds might be associated with the increased keratinocytes proliferation and migration on the wound surface (Blakytyn *et al.*, 2006) [4]. Wound contraction was higher in group II when compared to group I. This may be attributed to combined wound healing potency of *T. cordifolia* and insulin. The increased angiogenesis in *T. cordifolia* treated group indicated early and better wound healing in non diabetic rats (Barua *et al.*, 2010) [2]. It indicated that wound healing in *T. cordifolia* treated diabetic rabbits was slightly less as compared to combined therapy. Local insulin therapy enhanced the wound healing by shortening the time needed for complete epithelialization in diabetic rats (Apikoglu-Rabus *et al.*, 2010) [1]. Lima *et al.* (2012) [19] observed that the IRS1 (*Insulin receptor substrate 1*), IRS 2 (*Insulin receptor substrate 2*), SHC (adaptor protein), ERK (extracellular-signal-regulated kinases), and AKT (Protein Kinase B) are increased in the wounded skin of diabetic rats. They also reported that topical application of insulin normalizes the wound healing time in diabetic animals.

The healing response as observed in group I animals treated with oral suspension prepared from alcoholic extract of stem powder of *Tinospora cordifolia* may be attributed to its antimicrobial (Thatte *et al.*, 1992) [36], antioxidant (Ramya and Lakshmidevi 2010) [28], anti-inflammatory (Sharma and Singh 1980; Mary *et al.*, 2003) [32, 23], immunobiological (Biswadev *et al.*, 2002) [3], antidiabetic (Rajalakshmi *et al.*, 2012) [27] activities. Singh *et al.* (2010) [34] reported that the anti-inflammatory activity of *T. cordifolia* was due to berberine, cordifolioside A, cordioside, ecdysterone, isocolumbin, jatrorrhizin, magnoflorine, palmatine and syringin. The wound healing activity of insulin is due to its attribute of antidiabetic function and to stimulate the growth and development of different cell types and effects proliferation, migration, secretion by keratinocytes, endothelial cells and fibroblasts (Brem and Tomic 2007; Gurtner *et al.*, 2008) [6, 4]. Topical or local application of insulin promotes the synthesis of wound collagen, accelerates the wound angiogenesis and speed up wound healing in diabetic rats (Wu *et al.*, 2009) [39].

Group II animals, treated with oral *T. cordifolia* and local insulin therapy showed better wound healing when compared to group I. This better healing might be due to additive or synergistic effects of *T. cordifolia* and insulin. On account of their properties, oral use of methanolic extract of *T. cordifolia* and local insulin therapy prevented the wound from infections and led to reduction of swelling, inflammation, formation of scab and hence early setup of fibroplasia, fibroblast maturation and differentiation, collagen fiber formation, maturation and organization, angiogenesis, epithelialization and keratinization which resulted in better wound healing in group II as compared to group I.

**Table 1:** Mean Wound Contraction (%) in Different Groups at Different Time Intervals

S.N.	Groups	Days		
		7	14	28
1	G-2	12.946 <sup>Aa</sup> ±3.07	27.593 <sup>Ba</sup> ±4.96	79.716 <sup>Ca</sup> ±2.58
2	G-4	24.833 <sup>Aa</sup> ±5.73	70.166 <sup>Bb</sup> ±13.05	97.126 <sup>Bb</sup> ±1.59

**Note:** Mean bearing different superscript in small alphabets show significant difference between groups at the same day interval, and superscript in capital alphabets show significant difference within group at different day intervals ( $p \leq 0.05$ ).





Fig. 1: *Tinospora cordifolia*

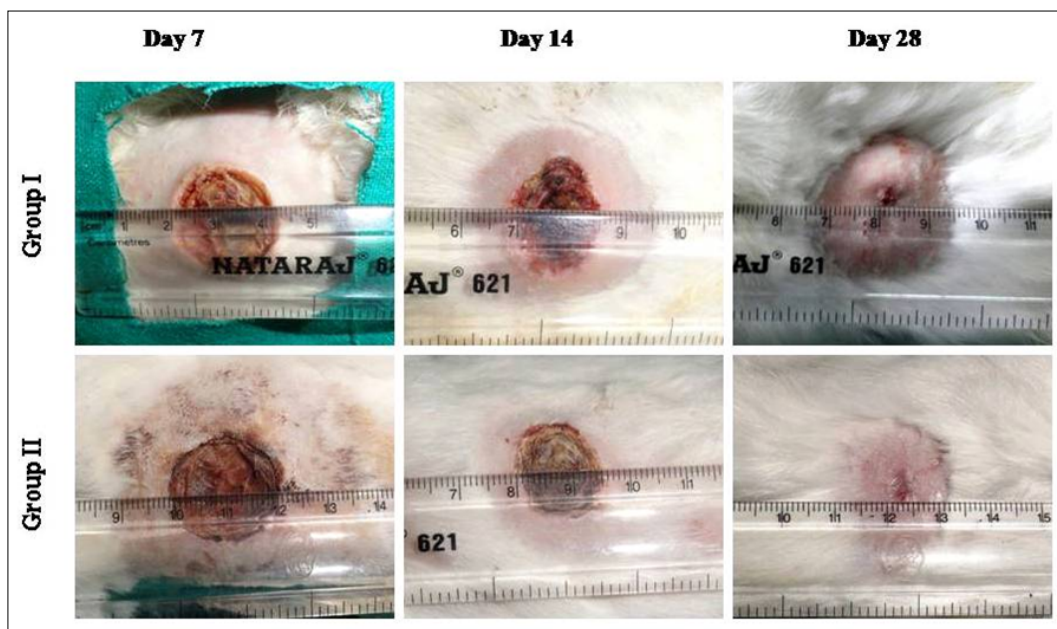


Fig 2: The colour digital photographs of the wounds of group I and II at different time intervals.

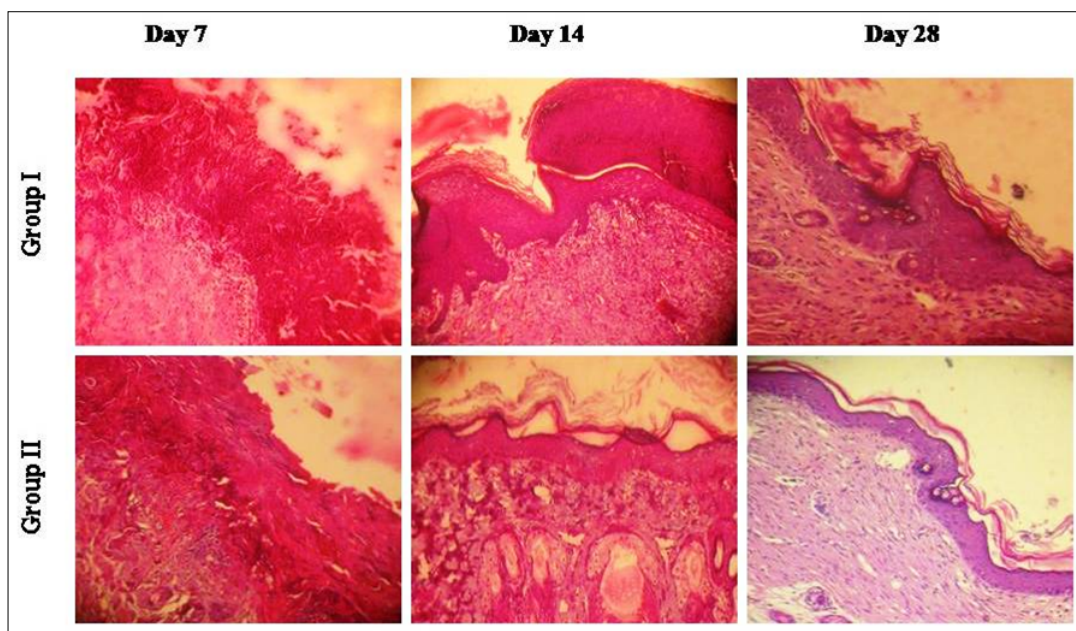
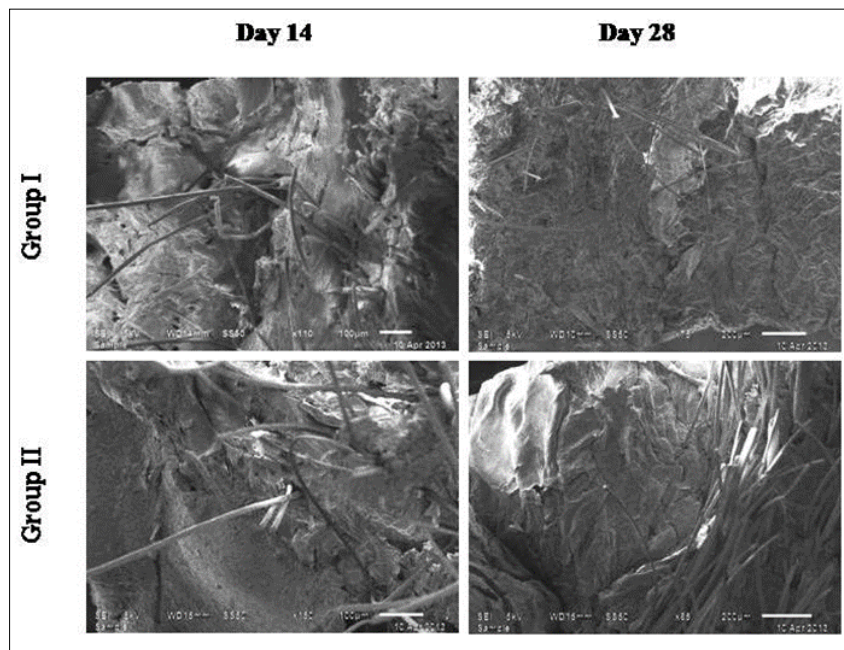


Fig 3: Histopathological observations of the wound biopsies collected from the animals of groups I and II at different time intervals (H&E, 40x).



**Fig 4:** Scanning Electron Microscopic (SEM) pictures of the healing sites in group I and II at different time intervals.

### Conclusion

In present study, *T. cordifolia* and insulin both have shown better potential for wound healing in diabetes when used orally and locally respectively, but the wound healing potential of *T. cordifolia* and insulin was more than the *Tinospora cordifolia* alone. For the clear understanding of mechanism of action and clinical applications of *Tinospora cordifolia* alone or in combination with insulin require further investigations.

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### Competing Interests

The authors declare that they have no competing interests.

### Authors' Contributions

All the authors participated in the study and collected data. Ajit Kumar Singh prepared manuscript. B. Om Preethi, H.N. Singh, A.K. Gangwar, D. Niyogi and Kh. Sangeeta Devi revised the manuscript. All authors read and approved the final manuscript.

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