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Research Article

WOUND HEALING POTENTIAL OF TRIDAX PROCUMBENS L. IN ALLOXAN-INDUCED IMMUNE COMPROMISED MICE

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A B S T R A C T

The therapeutic properties of hydro-methanolic extract of *Tridex procumbens* (*METP*) was screened for healing potential of dermal wounds of Alloxan-induced diabetic mice. The preliminary studies used hydro-methanolic plant extract (aerial parts) for evaluating antimicrobial properties against common pathogens. Lowering of fasting blood glucose (FBG) was monitored along with reduction of wound area in excision wound models. Dosing of 5mg/kg body weight and 2mg/kg body weight by topical application for 12 consecutive days showed a dose dependent reduction in elevated blood glucose and significant wound contraction as compared to diabetic control. *METP* also seemed to have an effect in regulating the body weights of the treated mice. Based on these results, it can be concluded that *METP* can be useful for immune suppressed wound healing and as skin repair agent in conditions similar to those applied by traditional medicine for hyperglycemia.

INTRODUCTION

The primary function of the skin is to serve as a vindicatory barrier against any harmful external attack that may result in the loss of the integrity of the skin such as wound and ultimately lead to disease or even death (Guo S *et al*. 2010), (J. Chen *et al*. 2005).Wound healing is a series if intercalating phases that restores the tissue integrity by replacing the lost tissue and repair the damaged (Hermes *et al*., 2013). There are a number of factors that adversely affect the healing process of wounds and bring unwanted delays, among them Diabetes mellitus (DM) is one of the most complicated impediment. Development of chronic, slow or non healing foot ulcer is seen as one of the most serious and debilitating complications in a majority of DM patients (Liane I.F. Moura *et. al*. 2013). Usually, the common complications associated with diabetic wound healing are: reduction in phagocytic and chemotactic activities of neutrophils (Agyare. C *et al*.,2011), (Liane I.F. Moura *et. al*. 2013), decreased collagen formation, decreased angiogenesis (Jaiswal S *et al*.2004), decreased vasculogenesis due to decreased endothelial progenitor cells (EPC), decreased endothelial nitric oxide synthetase (e-NOS) activity (Rashed AN *et al*., 2003), decreased number of growth factors like Vascular Endothelial Growth Factor (VEGF) (Sadaf *et al*., 2006) , (S. M. Ayuk *et al*., 2012.), Platelet Derived Growth Factor (PDGF) (Sadaf *et al*., 2006), (Liane I.F. Moura *et. al*. 2013), slower rate of transformation of fibroblasts to myofibroblasts (N. N. Houreld *et al*., 2012) etc. Similar findings have been reported in wound healing of experimental diabetic mice (Guo S *et al*. 2010), (N. N. Houreld *et al*., 2012), Cutaneous wounds in diabetes, not only cause pain and discomfort but also makes the patient vulnerable to chronic infections. Besides, the treatment also drains the patient financially due to the significant monetary costs associated with the treatment. Although several substances/ drugs/herbal products have been tried to enhance the wound healing process, no satisfactory therapy has been developed so far.

Tridax procumbens Linn. (Compositae family) is a native of tropical America and naturalized in tropical Africa, Asia, Australia and India. The plant is a small herb having short, hairy blade like leaves and is popularly called 'coat buttons' in English because of the appearance of its flowers (yellow corolla). It has been extensively used as a folkloric medicine in Ayurvedic system of treatment in India for various ailments (Saraf S *et al*., 1991), (Anjana, Sharma *et al*., 2009). Different pharmacological activities of *Tridax procumbens* have been reported in the last few years such as radical scavenging (Agrawal S *et al*., 2009), blood pressure lowering effect, wound healing activity (G. Babu *et al*., 2003), (Bhagwat D A, *et al*.,2008) and anti-diabetic activity ((Bhagwat D A *et al*., 2008).

Although different extracts of *Tridax procumbens* have been shown to have an array of reported uses as folklore medicine but the plant has not been established as a healer in immune compromised wound models through any scientific report.

Hence, based on its ethno pharmacological profile and reputed use in traditional therapeutic practice, the present study is an attempt to- (i) evaluate the antimicrobial potential (against various wound pathogens) of hydro-methanolic extract of aerial parts of *T.procumbens (METP*) (ii) evaluate systematically the possible *in vivo* diabetic wound healing potential of *METP* in excision wound models.

MATERIALS AND METHODS

Chemicals: Alloxan was purchased from Fluka BioChemika, India, , India, Sodium dihydrogen orthophosphate, disodium hydrogen phosphate (Himedia, India),. Metformin was purchased from Sun Pharmaceuticals Ltd, India were purchased from Sigma-Aldrich, USA. All the other chemicals used in experiments were of analytical grade.

Plant material and Preparation of Extracts

Aerial parts of *Tridex procumbens (Tp)* were collected from the premises of Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, Madhya Pradesh, India during the months of December 2015 to February 2016. They were authenticated by Dr. Madhuri Modak, Professor, Department of Botany, Shaheed Bhagat Singh Govt. Degree College, Ashta, Sehore, Madhya Pradesh, India, with voucher number 1212-88.01-408. They were allowed to shade dry for 2-3 weeks, pulverized, weighed and macerated in a separating funnel with 50% methanol. The mixture was vigorously shaken intermittently for 72 hours. The extract was collected in beaker and concentrated in water bath at 45°C. This process was repeated 3 times at least till colorless marc was obtained. It was defatted, dried at 45°C in oven, powder of crude extract collected and weighed (yield 21% $\pm$ 0.67) and was used for in vitro studies and formulation of extract-based-gel (100mg/ml stock solution) using Polyethylene Glycol-400 as vehicle.

Antimicrobial Activity

Microbial strains used: The extract was tested against a group of 8 wound pathogens including 5 bacteria: *Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae;* one yeast*: Candida albicans;* and two fungi*: Aspergillus flavus* and *Aspergillus niger*. The microbial work was carried out in aseptic conditions. The additions of the extracts, agar medium and microbial cultures were done as per standard procedure.
Procedure

The disc-diffusion assay was used to perform the antibacterial assay (Sankaranarayanan S *et al.*, 2012) using nutrient agar medium (Hi Media (P) Ltd Mumbai) which was prepared by dissolving 2.8 g of nutrient agar in 100 ml of double distilled water. The solution was autoclaved at a temperature of 121°C for 15 min for sterilization. It was cooled and the solution poured into sterile Petri dishes to solidify. The depth of the agar medium was measured (4 cm).

Clinical isolates of the selected microbes were grown overnight in aseptic tubes. The tubes were incubated at temp 37°C for 24 hrs and observed for the turbidity produced. Aseptic swab was used to inoculate the dried surface of agar plate by streaking few times over the surface, rotating the plate approximately by 90° to ensure uniform distribution of the inoculums. The medium was allowed to dry for few minutes before adding a sterile paper disc of 6 mm diameter. A dilution series of the extract in 10 % Methanol was prepared over the range of 0.5-2mg/ml. 10µl of the solutions were introduced on each disc and 10 % methanol alone served as a negative control. Meropenem (M)-10 mcg/disc was used as positive control for bacterial strains. For fungal cultures, Sabouraud Dextrose Broth (SBD) was used and Penincillin (P)—30mcg/disc was used as positive control. The plates were left undisturbed at a temperature of 37 °C for 12 hours; inhibition zones were measured and recorded.

Animal Study and In Vivo Experimental Protocol

The use of animals was as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) norms (CPCSEA registration no. 500/1A/2002/CPCSEA). Approval for experimental work was as per ethical committee of Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, M.P., India. Swiss Albino mice of either sex, mean weight 25-30 gms, and 6-7 weeks old were obtained from the animal house of the institute. They were housed, fed and treated in accordance with in-house guidelines for animal protection. They were given standard mouse pellet diet and water *ad libitum.* The mice were kept at controlled light and temperature conditions (light: dark, 12:12 hrs) according to CPCSEA norms. For the experimental purpose, the animals were kept fasting overnight but were allowed free access to water.

Induction of Experimental Diabetes

A freshly prepared solution of Alloxan Monohydrate (150 mg/kg b.w) in double distilled water was injected intraperitoneally to overnight fasted mice. After 6 days, blood sample was taken by retro-orbital puncture and the blood glucose level was assessed by glucometer (AACU-CHK Sensor comfort, Roche diagnosic, GmbH, D-68298, Manheim, Germany). For animals with blood glucose level $\geq$ 250mg/dl, a standardized wound area (6mm X 6mm skin in full thickness removed) was induced on the shaved, dorsal surface of excision area under anaesthetization of with 2% lidocaine hydrate. The animals were left undisturbed overnight.

Grouping and Treatment

After wounding, the animals were divided into the following groups (n=6):
Group I: Normal Control mice: (non-diabetic) left untreated.
Group II: Diabetic Control mice- wound left untreated;
Group III: Placebo group-wounds treated topically with PEG-400
Group IV: Standard group-wounds treated topically with Betadine-Povidone ointment;
Group V: Test group 1 (DT1)- wounds treated topically with 5mg/kg b.wt *METP* based gel;
Group VI: Test group 2 (DT2)- wounds treated topically with 2mg/kg b.wt *METP* based gel;
The herbal gel along with standard and vehicle were applied once daily for 12 consecutive days.

Determination of wound contraction

For the determination of wound contraction, digital vernier caliper was used. The wound size was measured and photographs were taken on 0^{th}, 3^{rd}, 6^{th} 9^{th} and 12^{th} days. Changes in wound size were measured to calculate the

percentage of wound healing based on the equation by Baie and Sheikh, 2000:

% wound healing = [A_0-A_t]/A_0 X 100
Where A_0 is the initial wound area and A_t is the size of wound on t^{th} day.

Monitoring Blood Glucose: On days 0,3, 6, 9 and 12 blood samples were drawn retro-orbital puncture from overnight fasted mice of each group and the blood glucose level was assessed by glucometer (AACU-CHK Sensor comfort, Roche diagnosic, GmbH, D-68298, Manheim, Germany). The body weights of the animals were also recorded.

RESULTS AND DISCUSSION

The phytochemical screening of *Tridex procumbens* extracted in different solvents is shown in Table 1. Aqueous and Methanolic extracts seem to possess a variety of active components that include alkaloids, flavonoids, glycosides, saponins, polyphenols, tannins etc which may relate to its therapeutic properties.

Table 1 Phytochemical Screening of extracts of *T.procumbens* in different solvents

S.No.	Phytochemicals	Aqueous Extract	Methanolic Extract	Petroleum Ether Extract
1.	Flavonoids	+	+	+
2.	Tannins	+	+	+
3.	Alkaloids	+	+	–
4.	Glycosides	–	+	–
5.	Saponins	-	+	–
6.	Phenols	+	+	–
7.	Tepenoids	+	+	+
8.	Steroids	-	-	–
9.	Carbohydrates	+	+	+
10.	Proteins	+	+	+

+ Tests positive; -- Tests negative

Antimicrobial activity of the methanolic extract: The inhibitory effects of crude methanolic extracts from aerial parts of *T.procumbens* against 8 wound pathogens are given in Table 2.

Table 2 Antimicrobial potency of *METP* and Standards

S.N	Name of Organism	Zone of Inhibition (mm)*	
		METP	Meropenem/ **Penicillin
1.	*Staphylococcus aureus*	13.1 ± 0.53	13.86±0.16
2.	*Streptococcus pneumoniae*	13.1 ± 0.45	12.75± 0.21
3.	*Escherichia coli*	12.0 ± 0.61	13.10±0.06
4.	*Klebsiella pneumoniae*	11.2 ± 0.34	11.91±0.04
5.	*Pseudomonas aeruginosa*	11.3 ± 0.83	13.34±0.67
6.	**Candida albicans*	10.0 ± 0.22	12.87±0.22
7.	**Aspergillus flavus*	10.4 ± 0.46	12.24±0.31
8.	**Aspergillus niger*	10.84± 0.45	12.88 ±0.66

*Data expressed are as mean± S.D. of triplicate experiments. **Zones of Inhibition of fungi are compared against Penicillin as standard. The microbes were clinical isolates provided by the research lab of Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, Madhya Pradesh, India.

The activity of the extract at a concentration of 2mg/ml was more pronounced for gram positive bacteria than gram negative bacteria, although it showed good inhibition against *E.coli* at 1.5mg/ml. In comparison to bacterial strains, the fungal strains were found to be less sensitive towards *METP* though its activity was significant. Similar findings have demonstrated the efficacy of *T. procumbens* extracts against *Staphylococcus aureus*, *E.coli* and *Klebsiella pneumoniae* (Sharma and Kumar, 2009; Das *et al*., 2009; Yoga Lathe *et al*., 2010). Since MIC values of *METP* were substantially low against common human pathogens and taking into account the presence of flavonoids, alkaloids and tannins that work synergistically in inhibition of bacterial DNA gyrase and cytoplasmic membrane enzymes (Cushine and Lamb, 2005), it was proposed to undertake it's *in vitro* antidiabetic capacity.

Studies on animal models

In vivo diabetic wound healing study: The effect of *METP* on body weights, plasma glucose levels and wound size reduction in diabetic mice are shown in figures 3-5 at a clinically relevant doses of 5mg/kg b.wt (shown as T1) and 2mg/kg b.wt (shown as T2) . Post wound creation, all the groups showed reduction in body weights in the subsequent days. *METP* at lower dose (DT2) reverses weight lowering from 25.8gm on day 3^{rd} to 27.29 gm on the 12^{th} day while at higher dose (DT1) it shows significant effect in reversing weight loss from 26.5gms on 3^{rd} day to 29.95gms on day 12^{th}. Similar effect is seen in the group IV treated with standard Betadine-povidone ointment. In the groups I, II and III weight loss is observed till day 12.

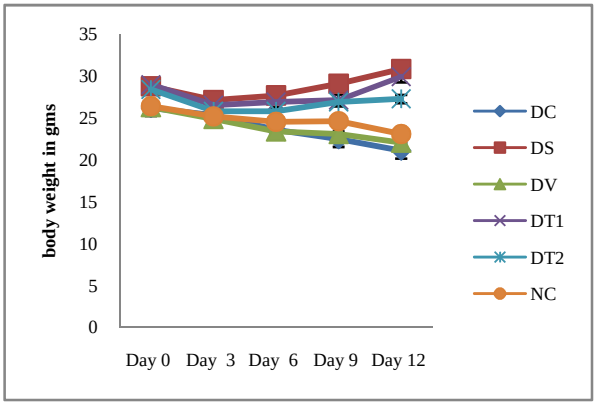

Fig 1 Result of Body Weight Studies for Diabetic wound Group

Data are expressed as mean± SD of four animals from each group. DC shows values for Diabetic Control group. DS shows values for Diabetic Standard (Betadine treated) group , DV shows values for Diabetic Vehicle group, DT1 shows data for Diabetic group treated with 50mg/gm *METP*, DT2 shows data for Diabetic group treated with 20mg/gm *METP* and NC shows data for Non-Diabetic Control group.

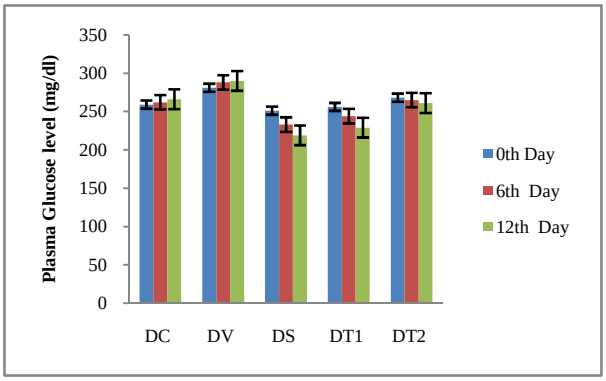

Fig 2 Effect of *METP*, standard and vehicle on plasma glucose level of Diabetic mice. Data are expressed as mean± SD of four animals from each group

The Standard group IV showed 12.7% reduction in blood glucose, group V showed 10.5% , group VI showed 2.61% reduction in blood glucose from day 0 to day 12 (Fig 2).

The restoration of a functional barrier during the healing process is dependent on the successful regeneration of new skin with architecture that closely resembles the inured tissues. Wound contraction in various groups is shown in Fig 3.

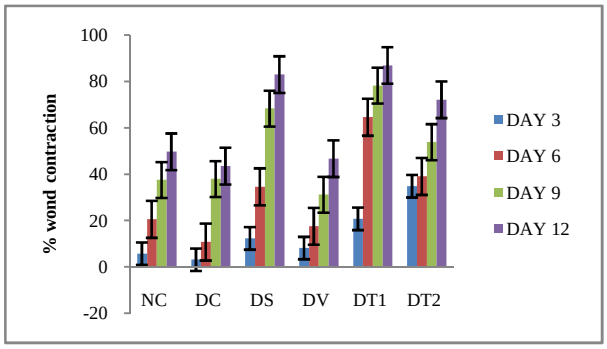

Fig 3 Effect of Vehicle, standard and *METP* on % wound contraction in Diabetic models. Data are expressed as mean+ SD of four animals from each group

It clearly appears from above data, that wound contraction started immediately from day 3, in all groups. However, the rate of wound closure is higher in later days in the standard and *METP* treated groups as compared to the control and vehicle groups. On day 6, animals of all the treated groups (IV, V and VI) exhibited significant increase in wound contraction as compared to control and vehicle groups. In group II (the diabetic control) the rate of contraction is the lowest with 10.79% on 6th day and 43.54% on the 12th day showing immune compromised wound healing as compared to untreated non-diabetic group I. On day 12 post wounding, it was observed that the standard group showed 82.97% healing whereas *METP* treated groups V and VI showed 86.95% and 72.15% healing respectively, when compared to the controls (49.73%, 43.54% and 46.72% for non-diabetic, diabetic and vehicle groups respectively). The wounds treated with *METP* were also characterized by appearance of fur from day 9.

CONCLUSION

Tridex procumbens, a wild herb, has been used since ages in various folkloric and traditional medicines in the past decades, there has been important focus on its bioactive moieties of its different parts which showed potential as a wide range of therapeutic activities like antioxidant, anti-inflammatory, antimicrobial, antihyperglycemic, hepatoprotective etc. These attributes are mainly attributed to the presence of Polyphenols, alkaloids and flavonoids in its different parts. Hence, the aerial parts were selected for the study.

The results of in vivo experiments clearly proved that the alcoholic extracts showed dose dependent weight stabilizing and glucose lowering effects. The extract also showed good wound healing potential in diabetic as well as non-diabetic mice which confirms its folkloric use to treat normal wounds. Thus, it can be suggested that this plant could be a potential diabetic wound healer especially at concentration of 5mg/kg body weight. However the exact healing mechanism of immune suppressed wound is not clearly understood. Therefore, further approaches are needed to clearly elucidate the full mechanism of action of such natural prohealers.

Declaration of Interests: We declare no conflicts of interests.
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