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EVALUATION OF ETHNO-MEDICINAL PLANT DRUGS FOR WOUND HEALING PRACTICED BY TRIBAL HEALERS OF BILIGIRIRANGANA HILLS (KARNATAKA), INDIA

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ABSTRACT

A tribal formulation comprises various combinations of different parts of ethno-medicinal plants and extracts of the identified candidate plants such as *Discorea hispida*, Dennst, (Dioscoreceae); *Glycosmis mauritiana* Tanaka, (Rutaceae); *Nothapodytes nimmoniana* Blume (Icacinaceae), *Andrographis serphyllifolia*, Vahl (Acanthaceae) *and Rauvolfia densiflora* (Wall.) Benth & Hook (Apocynaceae) are claimed to have a significant wound healing action followed by treating similar ailments owing to microbial infections (*both Gram negative and Gram positive pathogens*). The present study aimed to collect, analyze and evaluate the prosperous ethnopharmacologic knowledge on some less-known ethno-medicinal plants and their formulations practiced by Tribal healers in Biligirirangana Hills (BRT), Chamarajanagara district (Karnataka), India. The study initiated with identifying the important ethno-medicinal plants species used in traditional medicine for wound caused due to skin cuts, inflammation and skin infection by means of microorganisms. Supplementary analysis was also made by comparison of the traditional medicinal use of ethno-medicinal plants and their drug formulations with the available data of scientific literature. The Tribal medicinal formulation prepared with poly herbal mixtures was validated with the help of Ayurvedic Practitioner and

possible modifications was done in order to ascertain the efficacy of Tribal Medicine Formulation. The validated ethno-medicinal formulation (TMF) was then subjected for wound healing action in both water extract and ethanol extract formulation using exicision and incision models to uphold the gains in tensile strength of promoting epithelialization and wound contraction using excision wound models. Both Crude and Ethanol extracts of this TMF was studied for its remarkable effects on wound healing in rats, using excision, incision and dead-space wound models respectively at two different dose levels of 400 and 800 mg/kg. A significant acceleration of re-epithelialization was observed with crude and ethanol extracts of TMF compared to the controls after (16^{th} day) the Period of epithelialization. This property may be due to the effect of these formulations of ethno-medicinal herbs on migration and mitosis of epithelial cells and promotion of contraction of myo-fibroblasts which is responsible for the wound contraction. The TMF showed a definite, positive effect on wound healing, with a significant increase in the levels of the antioxidant enzymes, superoxide dismutase and catalase, in the granuloma tissue. The efficacy of this plant in wound healing may be due to its action on antioxidant enzymes, thereby justifying the claim by tribal/traditional healers. In addition, the tribal formulation used in the study is known to promote wound healing processes mainly due to their astringent and antimicrobial properties, which appears to be responsible for wound contraction and increased rate of epithelialization with reduced duration. After day 16, the animals were sacrificed and the histology of the wound area was examined. The best wound healing activity was observed with the crude extract of proposed TMF. Besides, Histopathology of Granulaoma tissue obtained from the group treated with both water and ethanol extracts of TMF showed significant increase in collagen deposition with more fibroblasts. The out-come of the study therefore attempts to bridge the lacunae in the existing literature and offers immense scope for researchers engaged in validation of the tribal/traditional claims and development of very safe, effective and globally accepted herbal drugs for cuts and wounds.

KEY WORDS: Ethno-medicinal plant drug, Tribal Medicine formulation (TMF), Tribal/Traditional Healers, Wound healing, Excision model, Aqueous/Water extract, Ethanolic extract.

INTRODUCTION

India has a rich tradition of plant-based knowledge of health-care (Anonymous, 1995 and 2002). A large number of plants/plant extracts/decoctions or pastes are equally used by tribal

medicine men and folklore-traditional healers in India for treatment of cuts, wounds and burns. Currently, several attempts have been made to analyse the ethno-pharmacological knowledge base and their practice for the treatment of cuts and wounds which includes a usage of plant drug, methods employed by tribal medicine men and folklore practices prevailing in India (Digraki *et al.*, 1999; Fatima *et al*., 2001; Kumara *et al.*, 2007; Amjad *et al.*, 2013). Pharmacological reports available on Indian ethno-medicinal plants employing various wound healing models and its underlying molecular mechanism, wherever available, has also been briefly reviewed (Begum and Nath, 2000; Babu *et al.*, 2002; Biswas *et al.*, 2004; Chaudhari and Mengi, 2006; Attama *et al.*, 2011 and Lingaraju *et al.*, 2013; De Wet *et al.*, 2013). The validation on herbal mixture of Indian medicinal plants is very inadequate and their pharmacological evaluation of also restricted. Besides, a large number of plant drugs used in both tribal and folklore with colossal prospective have not been validated, distinctively for their wound healing activity and related ailments (Ayyanar and Ignacimuthu, 2009; Wiart, 2001; Swati Rawat and Akhilesh Gupta, 2011; Alam *et al.*, 2011; Yogesh Sharma *et al*., 2013; Lingaraju *et al.*, 2013 and Sandra *et al.*, 2014).

'Wounds' are physical injuries that result in an opening or break of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin (Clark, 1996; Begum, 2000; Devi and Sampathkumar, 2011). Healing is a complex and intricate process initiated in response to an injury that restores the function and integrity of damaged tissues (Govindarajan *et al.*, 2007). Wound healing involves continuous cell–cell and cell–matrix interactions that allow the process to proceed in three overlapping phase's viz. inflammation (0–3 days), cellular proliferation (3–12 days) and re-modelling (3–6 months) (Glynn, 1981, Clark, 1996; Martin, 1996). Healing requires the collaborative efforts of many different tissues and cell lineages (Martin, 1997 and Fu Sc *et al*., 2005). It involves platelet aggregation and blood clotting, formation of fibrin, an inflammatory response to injury, alteration in the ground substances, angiogenesis and re-epithelialization. Healing is not complete until the disrupted surfaces are firmly knit by collagen (Buffoni *et al.*, 1993). The basic principle of optimal wound healing is to minimize tissue damage and provide adequate tissue perfusion and oxygenation, proper nutrition and moist wound healing environment to restore the anatomical continuity and function of the affected part (Gerald *et al.*, 1994 and Gall *et al.*, 2008). This study, therefore, attempts to bridge the lacunae in the existing literature and offers immense scope for researchers engaged

in validation of the traditional claims, development of safe, effective and globally accepted herbal drugs for cuts and wounds.

In addition, the pharmacological validation on Indian medicinal plants is considerably limited and a large number of plants used in tribal and folklore with enormous potential have not been validated for their wound healing activity. Hence, the present study is initiated to analyze the ethno-medicinal practices for the treatment of cuts and wounds which includes the usage of plants, formulation, methods employed by tribals and folklore practices prevailing in India. The investigation was also focused on the wound healing activity of a tribal medicine formulation comprising of *Dioscorea hispida,* Dennst, *Glycosmis mauritiana* Tanaka, *Nothapodytes nimmoniana* Blume, *Andrographis serphyllifolia,* Vahl *and Rauwolfia densiflora,* Benth & Hook. and were evaluated for their efficacy and mechanism of action in wound healing, using biophysical techniques.

MATERIALS AND METHODS

The field survey was carried out during the period, 2012-2013 at Biligirirangana Hill Tracts (BRT) of Chamarajanagara district, Karnataka. Interaction was held with available Tribal Medicine Men (TMM) at different podus of BRT area through open-ended and semi structured questionnaire to collect the data on ethnomedicinal plants such as *Dioscorea hispida*, Dennst, *Glycosmis mauritiana* Tanaka, *Nothapodytes nimmoniana* Blume, *Andrographis serphyllifolia,* Vahl *and Rauwolfia densiflora,* Benth & Hook. Along with formulation. The Tribal medicine formulation (TMF) for Wound healing and related ailments were assessed explicitly and then, the collected TMF data were analyzed both qualitatively and quantitatively. This ethnomedicinal knowledge was compared against the available scientific literature for reports of related uses and studies of phytochemical active compounds responsible for respective ailments (Ravishankar and Murthy, 2009).

Plant materials and Tribal Medicine Formulation

Ethno-medicinal plant materials and Tribal Medicine Formulations (TMF) were obtained from the Traditional Healers during interactions and then the samples were scientifically validated based on its physical characteristics in association with an authorized Ayurvedic practitioner, Nisarga Ayurveda Research Foundation, Sakaleshpur, Hassan district (India). The TMF constituent was subjected for devastating to small pieces using pestle and mortar, then powdered in an electric grinder for further analysis (Chaithra, 2013).

Preparation of Ethanol Extract

The shade-dried Ethno-medicinal plants samples were powdered to obtain the Formulation medicine (1kg) and was subjected for extraction exhaustively using 95% ethanol on a Soxhlet apparatus. The total ethanol extract was concentrated in vacuum container to a syrupy consistency (yield 270 g). The mixture was filtered, the filtrate placed in a hot air oven and maintained at 40^0C. After evaporation of the solvent, the resulting extract was placed in a sealed bottle until for further use (Wiart, 2001 and Khandelwal, 2005).

Phyto-chemical Screening

The powder of the Tribal Medicine Formulation (TMF) was subjected (50g) to successive extraction with different solvents in increasing order of polarity from petroleum ether to benzene, chloroform, acetone, and alcohol, finally to crude extract with water. The organic solvent was specified based on the dissolving efficiency and recovery of the TMF amongst the organic solvents used in the study, Meanwhile, the extracts were kept for evaporation to dryness and the dried extracts were subjected to various chemical tests in order to detect the presence of different phyto-constituents (Anonymous, 2002).

Ointment preparation for topical application

An alcohol free extract of Tribal Medicine Formulation (TMF) was used for the preparation of the ointment for topical application. The extract ointment of 10% and 15% (w/w) was formulated using soft white paraffin base, as per the standard procedures.

Experimental Animals

In the study, albino rats (*Rattus novergicus*) of either sex, weighing about 400–500mg/kg each, were used for the study (Reg No. *CSRF/IAEC/2013/022*). They were fed with standard chow and water *ad libitum* and they were housed in polypropylene cages maintained under standard conditions (12/12 hour light - dark cycle at 25 ± 3 °C; 35–60% RH). The experimental protocol was finalized as per the ethical standards of animal handling and also approved by Institutional Animal Ethics Committee.

Acute Toxicity Studies

Healthy adult albino rats of either sex were subjected for fasting for overnight. The animals were divided into 6 groups (n = 6 per cage) and were fed with increasing doses (1, 2, 4, and 8 g/kg body wt.) of Tribal Medicine Formulation (TMF), the total TMF crude and ethanol

extracts were administered orally in doses of up to 8 g/kg body wt., did not produce any evident sign of toxicity or mortality in rats up to 14 days after administration.

Wound models

The studies were carried out using ether anesthetized rats and their back was shaved, in three different wound models, at two different dose levels of 400 and 800 mg/kg body weight, respectively.

Excision wound model

The back of each rat was shaved under Pentobarbitone (4 and 8g/kg) anesthesia and prepared for operation. Thereafter, a circular skin piece of full thickness (approximately 500 mm^2) was removed by excising the skin from a predetermined dorsal area. For this purpose a marker was used to mark the area to be excised. The wounded animals were kept separately and rats wound were left undressed to the open environment, this model was used to monitor wound contraction and epithelialization time. The standard drug (0.2% w/w nitrofurazone ointment), simple ointment; ethanolic extract herbal ointment 10 and 15% w/w of Tribal drug (with water) formulation were applied everyday till the wound was completely healed. After the application of the drug, Tribal Medicine Formulation (TMF); changes in the wound area were calculated, giving an indication of the rate of wound contraction. The number of days required for falling of the eschar without any residual raw wound was determined as the period of epithelialisation (Sailesh, 2011).

Measurement of wound area

The progressive changes in wound area were measured planimetrically by tracing the wound margin on a graph paper every alternate day. The changes in healing of wound i.e the measurement of wound on graph paper was expressed as unit (mm^2). The wound contraction was expressed as percentage reduction of original wound size as per the formula enuntiated hereunder (Esimone *et al*., 2005).

$$\% \text{ Wound Contraction} = \frac{\text{Healed Area}}{\text{Total Area}} \times 100$$

Method adopted

The albino rats were used for excision and incision wound models, the ointment is applied topically and animal were divided into following groups:

Group- I : Served as control without local application of any ointment.

Group- II : Nitrofurazone ointment (0.2% w/w) was applied, once daily

Group- III : Tribal Medicine formulation (TMF) 400mg/kg body weight.
Group- IV : Tribal Medicine formulation (TMF) 800mg/kg body weight.
Group- V : Ethanol extract of Formulation 400mg/kg body weight
Group-VI : Ethanol extract of Formulation 800mg/kg body weight

In the study, six animals were taken in each groups. All the above mentioned treatments were started from the day of operation and continued till the 20th day of healing. On 2^{nd}, 4^{th}, 8^{th} , 10^{th} , 12^{th} , 14^{th} , 16^{th} and 18^{th} days the wound area of each rat was traced on a graph paper and measured with the help of Planimeter.

Incision wounds

Two, 6-cm long para-vertebral incisions were made in shaved area of anaesthetized rat. Wounds were closed with interrupted sutures, 1 cm apart and the sutures were removed on the seventh day. Thereafter they were kept individually in different cages. Wound breaking strength was measured in anesthetized rats on the tenth day after wounding.

Six animals in each group were taken for the experiment, on the 10th day the animals were sacrificed and their tensile strength was measured. After sacrificing the animals subsequent to anaesthesia, sutures were gently pulled out. Both wound areas from each animal were removed carefully. Wound stripes of equal size (width) were then cut using a knife in which two blades were set at a fixed distance. Both ends of each strip were fixed with the help of a pair of steel clips. One clip allowed hanging on a stand and a polyethylene bottle was then allowed to fill with water gradually till the wound strip was broken at the site of wound. The amount of water required to break the wound was noted and expressed as tensile strength of wound (gm).

Dead-space wounds

In this experiment, the wounds were created by implanting two polypropylene tubes (0.5 × 2.5 cm each), one on either side, in the lumbar region on the dorsal surface of each rat. On the tenth post-wounding day, the granuloma tissue formed on the implanted tubes was dissected out carefully. Granuloma tissue from one tube was maintained (at –64 °C) for the estimation of antioxidant enzyme levels. The other was used for the determination of tensile strength, after which it was dried in an hot air oven at 60°C for 24 h and the dry weight was recorded (Aebi, 1973; Devi and Sampath Kumar, 2011). The acid hydrolysate of the dry tissue was used for the estimation of hydroxyproline content in the tissue.

Biochemical Attributes: The granuloma tissue from the dead-space model was homogenized in phosphate buffered saline (pH 7.0) and centrifuged under cold conditions. The clear supernatant was assayed spectrophotometrically to determine the levels of the antioxidant enzymes explicitly; superoxide dismutase and the catalase (Andrea *et al.*, 1998; Beauchamp and Fridovich, 1971 and Bekerecioglu *et al*., 1998).

Histopathological studies

A section of the granuloma tissue was subjected to histopathological examination to determine the pattern of lay-down for collagen using two particular stains *i.e*. hematoxylin and eosin (Neuman and Logan, 1950 and Ozturk *et al.*, 2006).

Statistical Analysis

All the generated data of results were expressed as mean ± SE and the same were evaluated using one-way ANOVA with posthoc-Scheffe's *post hoc* test. The values obtained were considered statistically significant ($p < 0.05$).

RESULTS

In the present study, the data obtained from the tribal healers of the study area were analyzed for ethnomedicinal knowledge qualitatively and quantitatively. This was compared against the available scientific literature for reports of related uses and studies of phytochemical compounds responsible for respective ailments. The obtained plant materials and Tribal Medicine Formulation (TMF) from the Traditional Healers and was subjected for scientific validation with authorized Ayurvedic practitioners (Table-1 & 3).

Further, preliminary phyto-chemical screening of the TMF revealed that the presence of tannins, phenolic compounds. The acute toxicity studies showed that the drug was safe up to a maximum dose of 8 g/kg body wt. of the animal.

The effect of Tribal Medicine Formulation (TMF) was screened on both Excision and Incision wound models with the control (simple ointment base B.P) and reference standard (framycetin sulfate cream 1% w/w) .The measurements of the progress of the wound healing induced by the control (simple ointment base B.P), reference standard (Nitrofurazone ointment -0.2%w/w) and test formulations in the excision wound model are shown in Table-2 and 4 and Graph-1. It is observed that, the wound contraction ability of the test formulation was significantly greater than that of the control and reference standard ($p < 0.005$).

For excision wound model, the effect of topical treatment of extract at 10 and 15% w/w ointment showed, the significant ($P<0.05$) increase in the contraction rate of animals treated as compared with control on all days of the treatment (Table-4 and Graph-1). Whereas, the tensile strength of incised wound after 10th day of wounding, were treated with extract (10 and 15% w/w) was considerabley significant at ($P<0.005$) as compared with control (Table-5 and Graph-2).

In the incision wound model, increase in tensile strength was found to be noteworthy and of the 10 days of wound due to treatment with test formulation (515 ± 2.42). The measurements of the tensile strength are shown in Table-5 and Graph-2. It is evident from the results obtained in the study that, the traditional medicine formulation has significant wound healing activity in both models of wound healing and hence, justifying its use in tribal practice at BRT.

A significant increase in wound contraction at both excision and incision wound models, were observed in the skin tensile strength of the Tribal Medicine Formulation (TMF) followed by Ethanol extract of formulation-treated group on the tenth post-wounding day at both dose levels (Table-4 and 5 and Graph-1 and 2). The TMF drug-treated animals of the dead-space wound model showed a significant increase in dry granuloma weight, granuloma breaking strength and the level of hydroxyproline content at both dose levels as compared to Ethanolic extract of TMF. The histological assessment revealed increased collagen deposition in the TMF drug, treated group as compared to control (Fig.- 1 and 2, Table-5 and 6 and Graph-1 and 2). Further, studies relating to the activity of antioxidant enzymes reveal that the TMF extract significantly increased the levels of superoxide dismutase and catalase, the two powerful antioxidant enzymes of the body that are known to quench superoxide radicals (Table-6). The animals treated with the ethanol extract of formulation medicine also showed significant decrease in the epithelialization period whereas, the treatment with TMF drug was found to be significantly superior over all other treatments including control. It is evidenced by the shorter period for the fall of wound closure process as compared to control. The extract of TMF followed by Ethanol also facilitated the rate of wound contraction significantly at both dose levels (Table-4 and Graph-1). The results in this study are in support that the wound healing and repair is accelerated by applying Tribal Formulation Medicine which was highlighted by the full thickness coverage of the wound area by an organized epidermis in the presence of mature scar tissue in the dermis (Fig.-1 and 2).

Table-1: List of Ethno-medicinal plant drugs practiced by Tribal healers for wound related problems at Biligirirangana Hill Tracts, Karnataka

Sl. No.	Ethno-medicinal plant with Botanical name and Vernacular Name in Kannada	Family	Plant parts used	Ethno-medicinal value	Formulation & mode of treatment against ailments/diseases	Dosage and Duration of the treatment
1.	Dioscorea hispida, Dennst. Vr. Name: *Noolana hambu*	Dioscoreaceae	Tuber, leaf, seeds	Wound healing, excess bleeding, Pharynx inflammation, disturbances in Gastro-intestinal tract, Anti-allergic and inflammatory bowel disease etc.	Decoction of tuber, stem & leaf Tonic form Oral administration & Paste with lime juice for external applications for wounds and infected area	Decoction/ Tonic 1tsp three times a day for a week Paste application four times/week
2.	Glycosmis mauritiana (Lam), Tanaka. Vr. Name: *Orrange berry*	Rutaceae	Root, stem, leaf	Wound healing, Healing of Cancer tumour, Antimicrobial and Antigangrene etc	Paste with water And apply externally Crushed with warm water and swallowed	Apply paste at wound area & cover with a thin cloth 3times/week Tonic One tsp three times a day for a week
3.	Nothapodytes nimoniana, Blume. Vr. Name: *Durvasane mara*	Icacinaceae	Leaf stem	Wound healing, Anticancer activity, Microbial infection etc	Paste with warm water Decoction with cold /warm water	Tablets/ Decoction One tab three times a day for a week. Decoction-two times/week
4.	Andrographis serphyllifolia, Vahl. Vr. Name: *Kasinasara*	Acanthaceae	Stem, leaf	Gangrene, skin infection by microbes, wound treatment etc	Decoction with warm water. Paste with Honey for external	Tonic One tsp three times a day for a week. Paste application three times/week
5.	Rauwolfia densiflora Benth & Hook. Vr. Name: *Snake root*	Apocynaceae	Leaf, stem & root	Decoction for reduce Blood pressure, Snake bite, Skin infection, treating insomia etc.	Ground & juice boiled with warm water & swallowed. Paste with warm water and apply	Tonic One tsp two times a day for 8 days Paste for external wound application

Source: Ethno-medicinal wealth of B.R.Hills, Karnataka, India (Data base: Ravishankar and Murthy, 2009)

Further, it was found that, a significant acceleration of re-epithelialization with TMF followed by Ethanol extract of Formulation compared to the controls after 48 h (Fig.-1 and 2). Additionally, it was also analyzed whether the use of TMF extracted in water and ethanol resulted in a beneficial effect on wound healing in both animal models used in the study. This approach allowed us to have a rational basis for our further experiments on the elucidation of the underlying molecular mechanisms of the wound healing properties of TMF, because these studies were performed with primary human keratinocytes in culture where, the oleogels or the oils can not be used. Indeed, we could show significantly accelerated wound healing with 10 µg/ml TMF of water and ethanol extracts compared to other treatments alone 48 h after wounding (Table-4 and 5 and Graph-1 and 2). Therefore, it can be assumed that, the action and active constituent present in the TMF (poly herbal formulation) of both water and ethanol is exclusively responsible for the effect observed (Fig.-1 and 2).

Table-2: Efficacy of crude extracts of Ethno-medicinal plants (individual drug) on wound healing/ closure through excision wounds

Sl.No	Treatment	% Wound healing/closure				Period of epithelization in days*
		4th day Mean±SE	8th Day Mean±SE	12th day Mean±SE	16th day Mean±SE	
1.	Control Ointment base	20.00±1.20	24.90±1.06	64.00±2.40	85.80±0.68	26
2.	Nitrofurazone 0.2% (Ref std)	23.70±0.44	36.00±0.16	69.00±0.40	96.00±0.46	20
3.	*Dioscorea hispida* (2% w/w) *Crude extract*	15.90±0.28	19.80±1.60	55.00±0.25	90.00±1.80	22
4.	*Glycosmis maruitiana* (2% w/w) *Crude extract*	9.80±1.02	14.60±2.20	48.00±1.52	76.00±1.14	29
5.	*Nothapodytes nimmoniana* (2% w/w) *Crude extract*	14.00±0.68	17.00±0.86	54.00±2.12	82.00±2.23	25
6.	*Andrographis serphyllifolia* (2% w/w) *Crude extract*	18.80±0.39	28.00±0.42	75.00±0.38	92.00±1.90	21
7.	*Rauwolfia densiflora* (2% w/w) *Crude extract*	20.70±1.36	32.00±1.22	78.00±0.45	96.70±0.84	18

**** Values based on the average of Mean ± SE (n=4)***

Table-3: Validated Tribal Medicine formulation (TMF) practiced for wound healing and related ailments by Tribal Medicine Men at Biligirirangana Hill Tracts, Karnataka

Sl. No.	Ethno-medicinal plant with Vernacular Name.	Family	Plant parts used	Quantity (powder) (g/kg)	Dosage & Duration of TMF (asper TMM)	Validated Quantity of TMF (g)*
1.	Dioscorea hispida Dennst. **(A)** Vr. Name: *Noolana hambu*	Dioscoreaceae	Tubers	15	Decoction with warm water/goat milk/honey for oral administration *(3 times/week)*	(A) 20+ (B) 25+ (C) 15+ (D) 25+ (E) 15 = 100
2.	Glycosmis mauritiana (Lam) Tanaka. **(B)** Vr. Name: *Orrange berry*	Rutaceae	leaves	10		
3.	Nothapodytes nimoniana, Blume. **(C)** Vr. Name: *Durvasane mara*	Icacinaceae	Leaves	10	Paste with warm water/lime juice and apply externally to wound region *(4 times/week)*	
4.	Andrographis serphyllifolia Vahl. **(D)** Vr. Name: *Kasinasara*	Acanthaceae	Whole plant	15		
5.	Rauwolfia densiflora Benth & Hook. **(E)** Vr. Name: *Snake root*	Apocynaceae	Leaves	15		

***TMF obtained from TMM and was Validated by Authorized Ayurvedic Practitioner*

Table-4:Effect of the Tribal Medicine Formulation (TMF) Water extract and Ethanolic extract of Medicine formulation (EMF) on Wound healing using Excision Wound Model

Sl.No	Treatment	4th day	8th Day	12th day	16th day	Epithelization Period in days
1.	Control Ointment base	20.00±1.20	24.90±1.06	64.00±2.40	85.80±0.68	26
2.	Nitrofurazone 0.2% (Ref std)	23.70±0.44	36.00±0.16	69.00±0.40	96.60±0.46	20
3.	Tribal Medicine formulation (TMF-w/w) 400mg/kg body weight	22.90±0.28	49.70±1.60	55.00±0.25	90.00±1.80	16
4.	Tribal Medicine formulation (TMF-w/w) 800mg/kg body weight	26.00±1.44	58.00±2.26	69.80±1.4	98.00±0.21	16
5.	Ethanol extract of Formulation-w/w 400mg/kg body weight	28.60±1.46	52.00±1.34	65.00±2.42	94.00±0.22	18
6.	Ethanol extract of Formulation-w/w 800mg/kg body weight	34.60±0.84	54.00±0.88	68.00±2.87	95.60±0.84	18

P<0.05 compared with control . P<0.05 compared with 800mg, Values are mean ± SEM (n=4)

Table-5: Effect of the TMF Water extract and Ethanol extract of Meidicine formulation (EMF) on wound healing parameters using animal models*

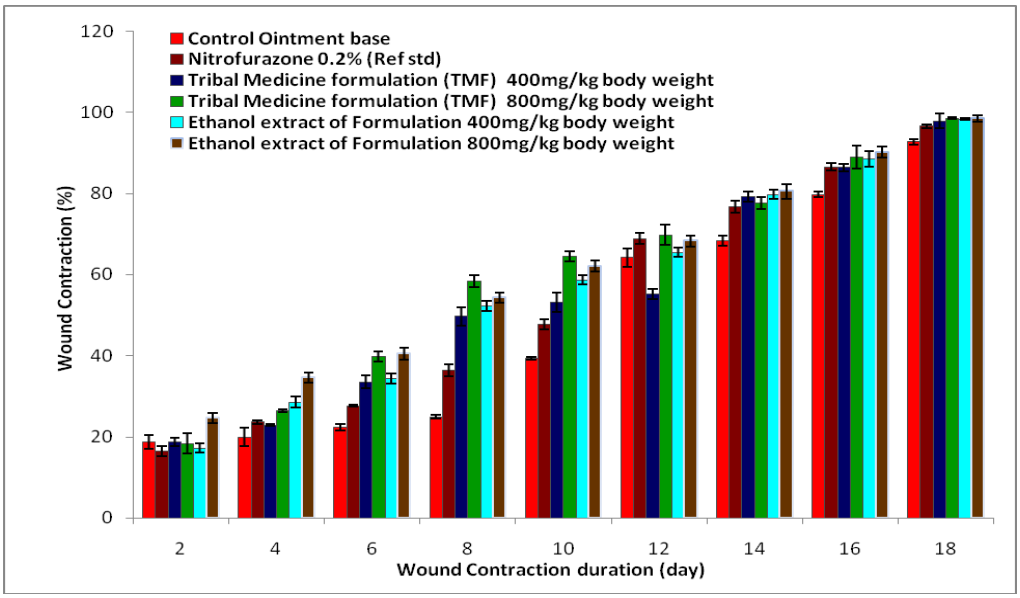
Sl. No.	Wound parameter studied	Excision Model Breaking strength (g)	Incision Model Breaking strength (g)	Granuloma Weight (g/100g)	Dead Space Model Breaking strength (g)	Hydroxyproline (mg/100g)
01	Control	415.10±6.44	340.20±8.26	19.20±2.04	165.50±15.44	985.40±154.28
02	Nitrofurazone 0.2% (Ref std)	540±8.42	465±2.62	40.45±4.02	459±6.42	1659±144.22
03	Tribal Medicine formulation (TMF-w/w) 400mg/kg body weight	546±4.42	475.60±4.42	44.80±2.22	455.20±14.06	1686.20±145.26
04	Tribal Medicine formulation (TMF-w/w) 800mg/kg body weight	725±6.20	515.70±2.42	52.80±4.04	565.20±12.08	1896.20±164.66
05	Ethanol extract of Formulation-w/w 400mg/kg body weight	525±2.22	465.50±2.46	45.50±4.24	456.20±10.2	1606.30±164.62
06	Ethanol extract of Formulation-w/w 800mg/kg body weight	740±6.46	496.20±2.22	54.20±6.44	575.60±14.46	1888.04±176.46

**P<0.05 compared to control. Values are mean ±SEM (n=4)*

Table-6: Effect of Ethanolic extract of Tribal Formulation on the status of antioxidant enzymes in granuloma tissue *

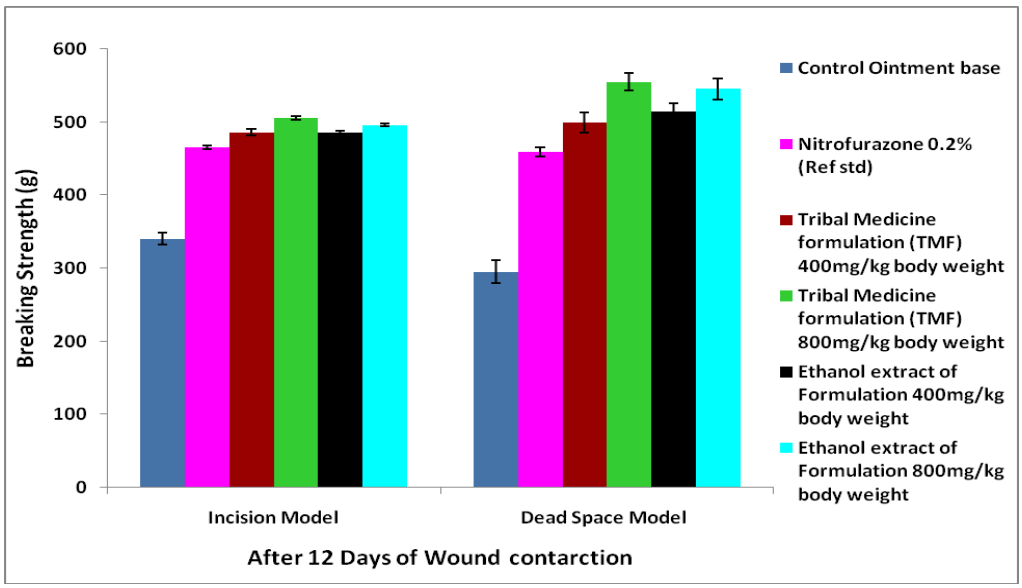
Sl.No	Enzymes	Superoxide Desmutase (1µg/mg)	Catalase (1µg/mg)
1.	Control	0.128±0.014	$3.28x\ 10^{-2}$ $\pm 2.8x10^{-3}$
2.	Tribal Medicine formulation (TMF-w/w) 400mg/kg body weight	0.166±0.024	$5.12x\ 10^{-2}$ $\pm 6.7x10^{-3a}$
3.	Tribal Medicine formulation (TMF-w/w) 800mg/kg body weight	0.182±0.044	$8.28x\ 10^{-2}$ $\pm 6.4x10^{-3}$
4.	Ethanol extract of Formulation-w/w 400mg/kg body weight	0.187±0.026	$5.82x\ 10^{-2}$ $\pm 4.7x10^{-3a}$
5.	Ethanol extract of Formulation-w/w 800mg/kg body weight	0.266±0.042	$7.68x\ 10^{-2}$ $\pm 6.8x10^{-3a}$

**P<0.05 v/s. control. Values are mean ±SEM (n=4)*



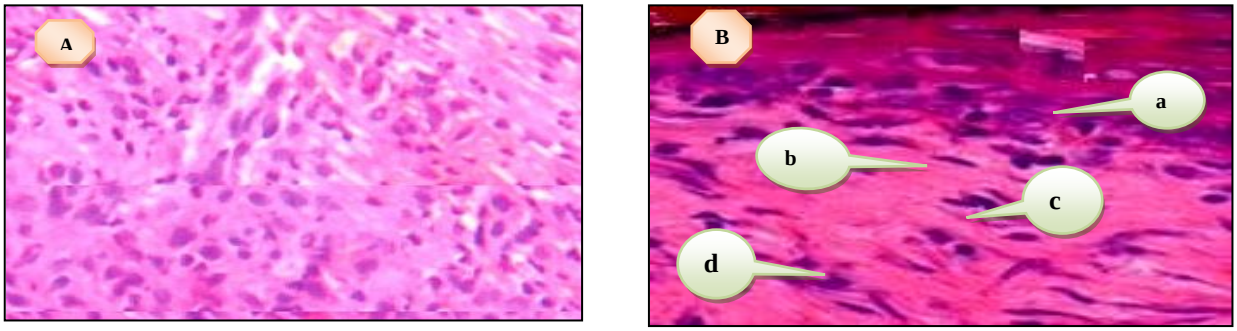
Graph-1: Effect of TMF and Ethanol extract formulation on Wound contraction by Excision model compared with Control and Standard Reference.

P<0.05 v/s. control. Values are mean ±SEM (n=4)

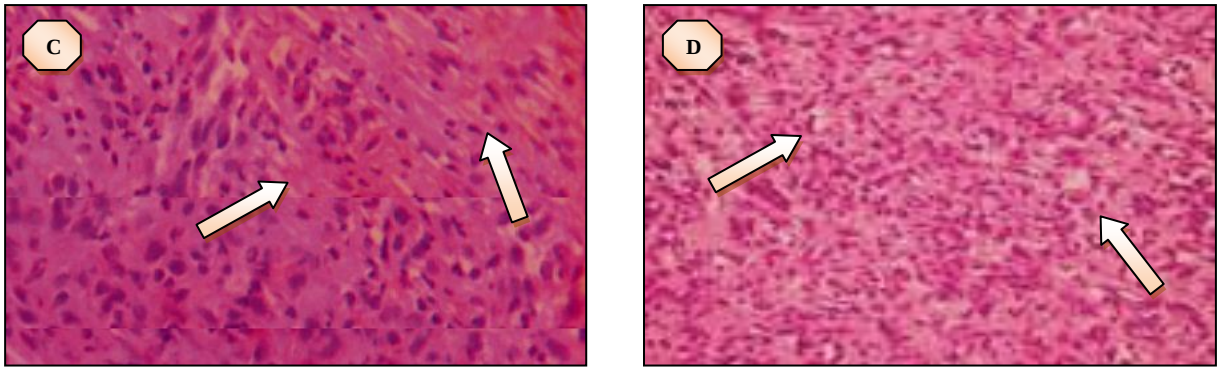


Graph-2: Effect of TMF and Ethanol extract formulation on Breaking Stength of Incision Model and Dead Space Model after 12 days of Wound Contraction

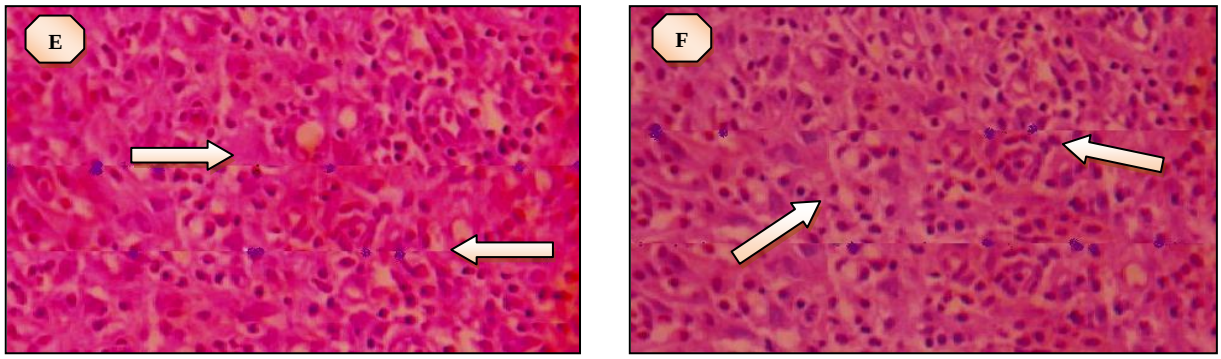
P<0.05 v/s. control. Values are mean ±SEM (n=4)



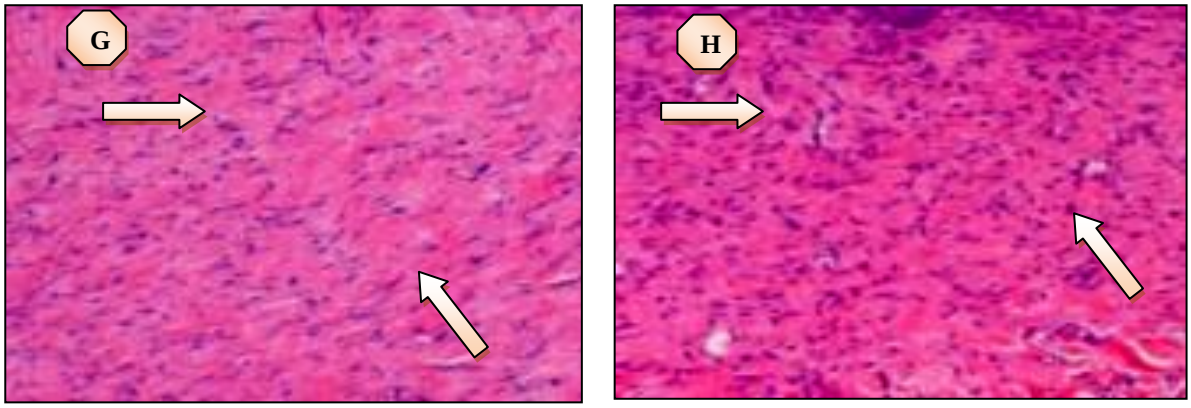
A: View of Histological section of Granuloma tissue B- a: Epidermis Layer, b: Dermis Layer, c: Collagen after treatment (magnification-40x). -layer and d: Fibroblast Cell (magnification-100x).



C: Granulation of the tissue treated with control showing incomplete healing of the wound with less epithelialization, macrophages and trivial collagen formation (magnification-40x).
D: Granulation of the tissue treated with Nitrofurazone showing significant healing with closure of the wound followed by considerable epithelialization, macrophages and prominent collagen formation (magnification-40x).



E: Granuloma tissue trated with TMF (400mg/kg body weight) showing complete healing of the wound in with augmented epithelialization, macrophages and foremost collagen formation (magnification-40x).
F: Granuloma tissue with TMF (800mg/kg body weight) showing remarkable closure of the wound via complete healing treated representing with striking epithelialization, macrophages and outstanding collagen formation (magnification-40x).
Fig-1: Evaluation of Tribal Medicine Formulation (TMF), standard Nitrofurazone along with control on Hist-pathological appraisal of wound tissues in excision model



G: Histological section of granuloma tissue trated with extract of Ethanol Medicine Formulation (EMF-400mg/kg body weight) showing absolute healing of the wound in with increased epithelialization, macrophages and foremost collagen formation (magnification-40x).
H: Histological section of granuloma tissue trated with extract of Ethanol Medicine Formulation (EMF-800mg/kg body weight) showing complete healing of the wound in with amplified epithelialization, macrophages and the collagen formation is excellent (magnification-40x).
Fig-2: Evaluation of extract of Ethanol Medicine Formulation (EMF) on Hist-pathological appraisal of wound tissues in excision model

DISCUSSION

The progress of the wound healing induced by extract of TMF followed by Ethanol formulation, ointment (10% and 15% w/w) treated groups, simple ointment (control) treated group and Nitrofurazone (standard drug, 0.2%w/w) treated group of animals are represneted in the data. Wound healing involves various phases which include granulation, collagenation, collagen maturation and scar maturation (Esimone *et al.*, 2001; Jaggetia and Rajnikant, 2004; Kumara *et al.*, 2007; Nwala *et al.*, 2013). Many plant extracts and medicinal herbs have shown potent antioxidant activity. Tannins, the main components of many ethno-medicinal plant extracts, act as free radical scavengers (Lee, 1968; Kapoor *et al.*, 1989; Kokate, 1994; Marja *et al.*, 1999; Maquart *et al.*, 1999; Hernandez *et al.*, 2001; Kaur *et al.*, 2004 and Panduranga Murthy *et al*, 2011). Research into the role of antioxidants from plant extracts in wound healing has been published widely (Joharapurkar *et al.*, 2003). Wound healing process consists of different phases such as granulation, collagenation, collagen maturation and scar maturation which are concurrent, but independent to each other. Hence, in the present study three different wound models were used. In the incision wound model, a significant increase was observed in the skin tensile strength of TMF in both water and the ethanol treated groups at different dose levels. The drug-treated animals at both dose levels of the dead-space wound model showed a significant increase in dry granuloma weight, granuloma breaking strength and the level of hydroxyproline content, respectively. The histopathological study revealed

increased collagen deposition in the drug, treated group (Fig.-1 and 2) as compared to control followed by standard treatments (Leite *et al*, 2000; 2000; Mukarjee *et al.*, 2000; Letts *et al.*, 2006; Ozgen *et al.*, 2006; Shafiuddin *et al.*, 2009).

Studies on the estimation of antioxidant enzyme revealed that the extract significantly increased the levels of superoxide dismutase and catalase, the two powerful antioxidant enzymes of the body that are known to quench superoxide radicals (Table-6). The antioxidant enzymes (superoxide dismutase and catalase) are known to quench the superoxide radical and thus, prevent the damage of cells caused by free radicals. In studies using the excision wound model, animals treated with the ethanol extract of *Formulation Medicine* showed a significant decrease in the epithelialization period, as evidenced by the shorter period for the fall of eschar compared to control (Phillips *et al.*,1991; Pierce and Mustoe, 1995; Sabale *et al.*, Tran *et al*., 1996; Hussein *et al.*,2000; 2012; Rashed *et al.*, 2003; Rupesh *et al.*, 2011; Sailesh narayan *et al.*, 2011 and Sandra *et al.*, 2014).

The TMF drug extract significantly facilitated the rate of wound contraction. The Phyto-chemical parameters revealed that, the ethanolic extract of Formulation Medicine (EMF) showed high amount of tannins, presence of alkaloids and other active constituents which may be responsible for the antioxidant activity. So, in this study scavenging effect might be one of the most important components of wound healing which may be responsible to support wound healing property. Thus, the enhanced wound healing may be due to the free radical scavenging action of the Formulation Medicine as well as enhanced antioxidant enzyme level in granuloma tissues (Purna and Babu, 2000; Babu *et al*., 2002; Hernandez *et al*., 2001; Raquel *et al*., 2002). This finding also provides an insight into the usage of the galls of Formulation Medicine in traditional treatment of wounds or burns associated with bacterial infections (Samuelson, 1992; Shukla *et al.*, 1999; Saleem *et al.*, 2006).

It is observed that, the wound contraction ability of the extracts of TMF and Ethanol formulation were significantly greater than that of the control and other treatments (simple ointment treated group) respectively. The 15%w/w of TMF extract showed most significant wound healing from the sixth day onwards, which was comparable to that of the Nitrofurazone treated group of animals. The wound closure time was considerably lesser, as well as the percentage of wound contraction was much more with the 15%w/w of TMF extract treated groups (18th day for 100% contraction which was superior to that of the Nitrofurazone treated group) followed by Ethanol extract of Medicine formulation (EMF)

treatments (Suguna *et al.*, 1996; Tran *et al.*, 1996; Tripathi and Sharma, 1998; Hong *et al.*, 2005; Trombetta *et al.*, 2006 and Panduranga Murthy *et al.*, 2011b). The TMF extract treated group of animals showed most significant wound contraction from the 18th day onwards and achieved 100% with the wound closure time of 20th days.

CONCLUSION

There are a number of ethnomedicinal plants which are used specifically by the tribal medicine men of India are not been validated or such plant drugs not been evaluated keeping the traditional and conventional claim in mind. In the present study, the tribal medicine formulation prepared with both water and ethanolic extracts exhibited a excellent wound healing activity comparable to those of control and, Nitrofurazone, a standard antibiotic commonly used in wound healing. This finding therefore, justifies the practice of TMF in Tribal medicine system for wound healing. Hence, the pharmacologist can harness the potentials in the ethnomedicinal plants and in the TMF extracts of ointments for the treatment of contemporary diseases relating to wound, skin cut, skin infection etc. The ethnomedicinal plants and formulation certainly which are the real gift from the nature having traditional knowledge, provides excellent novel raw material for the treatment of wound related ailments. Besides, the findings of the study on ethnomedicinal plant/plant drugs (extracts) screens the soluble extracts in the development of safe, cost effective and an acceptable wound healing herbal formulation, which is validated appropriately and the efficiency has been proved scientifically. Further, this drug formulation can act as effective substitute or may even swap the modern wound healing agents.

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