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Original Article

Wound healing potentials of *Thevetia peruviana*: Antioxidants and inflammatory markers criteria

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ABSTRACT

Thevetia peruviana is a medicinal plant used in the treatment of external wounds, infected area, ring worms, tumours etc. in traditional system of medicine. The aim of the study was to evaluate the wound healing potentials of *T. peruviana* leaves hexane (LH) and fruit rind (FW) water extracts and to prove the folkloric claims. The antimicrobial, antioxidant and anti-inflammatory potentials could be important strategies in defining potent wound healing drug. Based on these approaches the current study was designed using incision, excision and dead space wound models with the biochemical, antioxidant enzymes and inflammatory marker analysis. The fruit rind water extract showed highest WBS of 1133 ± 111.4 g. The extracts in excision model retrieved the excised wound i.e. complete healing of wound at day 14. The hydroxyproline content of FW and LH treated dry granuloma tissue was increased to 65.73 ± 3.2 mg/g and 53.66 ± 0.38 mg/g, accompanied by elevations of hexosamine and hexauronic acid with upregulation of GSH, catalase, SOD, peroxidase and the down regulation of the inflammatory marker (NO) and oxidative stress marker (LPO) in wet granulation tissue was documented. Conclusively, both the extracts showed enhanced WBS, rate of wound contraction, skin collagen tissue development, and early epithelisation. Therapeutic wound healing effect was further proven by reduced free radicals and inflammatory makers associated with enhanced antioxidants and connective tissue with histological evidence of more collagen formation. The present research could establish *T. peruviana* as potential source of effective wound healing drugs.

1. Introduction

Healing of wound is the sign of growth and an important biological phenomena. Whether acute or chronic wounds can compromise an individual's wellbeing, self-image, workability and freedom.[17] The impact of wounds on physical, social and financial sectors of a person's life necessitates good wound management not only for the individual but also for the community.[12] The long history of wound care is practiced in, Tibb-e-Nabawi (SAW) in Arabic, use of smoke and hot meat burns in African, Medical Qi Gong and Feng Shui in Chinese and Varna Shodan and Varna Ropan in Indian systems of medicines.[3] The disruption of cellular and structural integrity of the skin tissue strata is called as wound and restoration of the same is healing. The variance in the number of phases and phase description diverges from author to author. In general four phases of wound healing are portrayed as hemostasis, inflammation, granulation and remodelling.

Plants have served as the healing agents for ages, like *Ficus bengalensis*, *Curcuma longa*, *Centella asiatica*, *Aloe vera*, *Cynodon dactylon*, *Rubis cordifolia*, *Ficus recemosa*, *Glycyrrhiza glabra*, *Berberis aristata*, are few medicinal plants, explored for their phyto-constituents and extensively been used in modern medicines too.[12] Beholding the present consequences the phyto-components could provide an excellent fountainhead to develop new wound healing drugs which will be more efficacious, safer and affordable for patients.

There are serious factors that postpones the process of wound healing by means of persuading tissue damage such as, repeated injury, infection, oxygenation, free radical generation. Hence, to tackle the above mentioned situations the use of a drug having

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antimicrobial, antioxidant and anti-inflammatory potentials could be an important tactic in healing of wounds. The ethno-medical uses of *Thevetia peruviana* is evident in treating the external wounds, infected area, ring worms, tumours etc., the use of grinded leaves of *T. peruviana* in ethno-veterinary medicine is the evidence for its plenteous use for healing of wounds.[22,25,44]

The plant has stolen the limelight with the existence of novel components having excellent therapeutic values such as cardiovascular effects, anticancer, antimicrobial, antioxidant, immunomodulatory and anti-inflammatory activities.[38] However, the lack of systematic information on wound healing potentials, despite the presence of voluminous reports regarding the pleotropic properties of the plant has called for the scientific validation of wound curing ethno medical claims of *T. peruviana*. To fulfil the lacuna between the folkloric use and scientific exploration, the wound healing efficacy of *T. peruviana* has been evaluated in the present study based on the above mentioned strategy.

2. Materials and methods

2.1. Plant material collection

Unripe fruits and leaves of *T. peruviana* (S) were collected from the surroundings of Kuvempu University, Shankaraghatta, Shimoga Dist., Karnataka, India. Plant identification was carried out by referring Flora of the Presidency of Madras (Gamble, 1915–1935).[8] The plant was further authenticated by Prof. V. Krishna, Taxonomist, Dept. of Biotechnology, Kuvempu University. The specimen was deposited at the Department of Biotechnology. As per the WHO published manual namely 'Quality control methods for herbal materials' the quality control of any herbal drug is based on three important criteria i.e. botanical identification, phytochemical screening, and content standardization.[34,36] In the present study all the above mentioned criteria are fulfilled to control the quality of the drugs.[36]

2.2. Chemicals

Hexane, chloroform, ethanol, sodium carbonate, NBT, EDTA, hydroxylamine hydrochloride, hydrogen peroxide, sulphosalicylic acid, DTNB, KCl, ferric chloride, HCl, TCA, TBA, BHT, naphthylethylene diamine dihydrochloride, sodium nitroprusside, sulphanilic acid, NaOH, H_2SO_4, para-dimethyl amino-benzaldehyde, n-Propanol, Na_2CO_3, Borax, carbazole etc. were purchased from Sigma–Aldrich, Merck and Himedia. All the chemicals and solvents used were of analytical grade.

2.3. Soxhlet extraction

Successive extraction was done using 500 g of powdered material of leaves and fruit rind material in soxhlet apparatus. The solvent hexane (2L, 50 °C˜15 cycles) was used for leaves extraction (LH), and for fruit rind material after successive extraction with hexane (2L, 50 °C˜15 cycles) chloroform (2 L, 45 °C˜15 cycles) and ethanol (2 L, 70 °C, ˜15–17 cycles) the cold water extraction (FW) was performed using 9:1 ratio of water and ethanol for 24 h. The LH and FW extracts were concentrated *in vacuo*. The yield of each dried extract was calculated. The available reports suggest that the hexane extract predominately contains most of the nonpolar components of plant metabolites like oils, fats, waxes. Currently, the study was designed to investigate the therapeutic potentials of extracts containing sole nonpolar and high polar phytochemicals. Therefore, the leaf hexane extract was selected for its nonpolar phytochemicals as the leaf material is evidenced with high content of latex, oils and fats.[36] The fruit rind of *T. peruviana* is evidenced with high carbohydrate content,[36] which are reported for wound healing[31] and hence it was selected for extraction with water: ethanol (9:1) as this combination of solvents dissolves most of the high polar components.[5]

2.4. Animals

Male Wister albino rats weighing 150–230 g were utilised, maintained under standard laboratory conditions (12 h light/darkness; at 25 ± 3 °C) with standard animal diet and water available ad libitum. The Institutional Animal Ethical Committee permitted the studies under the certification (Ref. No. NCP/IAEC/CL/247/2013-14).

2.4.1. Incision wound model

Incision wound model was taken up to evaluate the wound healing potentials of *T. peruviana* extracts in terms of tensile strength methods of Lee 1968 and Murthy *et al.*, 2013 were employed.[18,24] Twenty four (24) rats weighing 150–190 g were evenly distributed into four groups of six animals each grouping was done in following fashion: Control (ointment base), Positive control (povidone-iodine), LH and FW 5% w/w ointments [5 g of extract was mixed with 100 g of cream base (white petrolatum) until a uniform preparation is attained to prepare 5% w/w extract ointment] respectively.[11] Animals were anaesthetized with ketamine (30 mg/kg, i.p.), 6 cm paravertebral incisions were made through the full thickness of the skin. Wounds were sealed with interrupted sutures 1 cm apart, the extract ointment was topically applied up to 10 days. Sutures were removed on the 7th post wounding day. Wound breaking strength (WBS) was measured on the 10th post wounding day in anaesthetized rats. The wound breaking strength is expressed as the minimum weight (in grams) of water necessary to dragging apart the wound edges.

2.4.2. Excision wound model

To study the excision wound model twenty four (24) animals were evenly distributed into four groups of six animals each in the following order: Control (untreated), povidone-iodine, LH and FW extracts (5% w/w ointment). Rats were anesthetized with ketamine (30 mg/kg, i.p.) followed by a circular wound on the dorsal thoracic region of about 500 mm^2 was made. Wounds were traced on graph paper on the day of wounding and consequently on the alternate days until wound restoration was complete. The rate of wound contraction was calculated by the formula.[24]

$$\%\ \text{wound contraction} = \frac{\text{Healed area}}{\text{Initial wound area}} \times 100$$

Where, Healed area = Initial wound area − Remaining wound area.

2.4.3. Dead space wound model

In dead space wound model study the animals were divided into four groups of six animals each in the following fashion: Control (1 ml/kg of 1% gum acacia p.o.), vitamin E (VTE 200 mg/kg b. w. p.o.), LH and FW extracts (200 mg/kg b. w. p.o.) respectively up to 10 days. Rats were anesthetized with ketamine (30 mg/kg, i.p.) incision of about 1 cm was made on both the dorsal paravertebral sides followed by grafting of sterilized cylindrical grass piths (2.5 cm × 0.3 cm) wounds were closed with sutures.[11] After 6 h of interval of the last dose on the 10th post wounding day, the animals were sacrificed and granulation tissue formed around the implanted piths were carefully removed out, weighed, and processed for the estimation of free radicals, antioxidants, and collagen tissue parameters.[24]

2.4.3.1. Estimation of antioxidant enzymes and inflammatory marker of granulation tissue. Antioxidants enzymes of granulation tissue were analysed, such as superoxide dismutase (SOD), catalase and reduced glutathione (GSH), peroxidase, oxidative stress marker like lipid peroxidation (LPO) and the percent nitric oxide (NO) inhibition were estimated in wet granulation tissue homogenate.

a) Superoxide dismutase (SOD)

The reaction mixture was prepared by mixing 0.5 ml of granulation tissue homogenate, 1 ml 50 mM sodium carbonate, 400 μl of 25 μM NBT (Nitro Blue Tetrazolium) and 200 μl 0.1 mM EDTA (Ethylene diamine tetraacetic acid), simultaneously control was prepared without tissue homogenate. The reaction was initiated by the addition of 400 μl of 1 mM hydroxylamine hydrochloride solution. The change in absorbance was recorded at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to prevent the reduction of NBT by 50%.

b) Catalase

The assay mixture contained 1.95 ml of phosphate buffer (0.005 M, pH7.0), 1.0 ml (0.019 M) H_2O_2 and 5 μl of granulation tissue homogenate. The mixture was incubated at room temperature for 2 min. The catalase activity was calculated by the change of the absorbance at 240 nm.

c) Glutathione peroxidase

Briefly, to the 0.5 ml of tissue homogenate 1 ml of 10 mM KI and 40 mM sodium acetate solutions were added and the absorbance was read at 353 nm. To this mixture 20 μl of 15 mM H_2O_2 was added, vortexed and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the optical density by 1 unit/min. The specific activity was expressed in terms of units per milligram of protein.

d) Reduced Glutathione (GSH)

An aliquot of 1 ml of granulation tissue homogenate (10%, w/v) was precipitated with 1 ml of sulphosalicylic acid (4% w/v). The samples were kept at 4 °C for 1 h and then centrifuged at 3500 rpm for 15 min at 4 °C. The reaction mixture in a total volume of 3 ml consisted of 100 μl supernatant, 2.7 ml 0.1 M phosphate buffer (pH 7.4) and 200 μl of DTNB (40 mg dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.4) The optical density of the yellow colour developed was measured at 412 nm.

e) Estimation of lipid peroxidation (LPO)

To the mixture of 500 μl 20% granulation tissue homogenate in 0.15 M KCl, 100 μl of 0.2 mM ferric chloride solution was added to initiate the peroxidation at 37 °C for 30 min. The reaction was stopped by adding 2 ml of ice cold mixture of 0.25 N HCl containing 15% TCA (Trichloroacetic acid), 0.30% TBA (Thiobarbituric acid) and 0.05% BHT (Butylated hydroxytoluene). The reaction mixture was heated at 80 °C for 60 min, the samples were cooled and centrifuged at 3500 rpm for 15 min and the optical density (O.D.) of the supernatant was measured at 532 nm. Lipid peroxidation was expressed as malondialdehyde (MDA) equivalents in nanomoles per milligram of protein.

f) Estimation of NO inhibition

At a physiological pH sodium spontaneously produces nitric oxide ions, which reacts with oxygen to produce nitrite ions.[9] The total reaction mixture of 3 ml contains 2 ml of 10 mM sodium nitroprusside, 0.5 ml of phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 ml of granulation tissue homogenate, the mixture was stirred well and incubated for 150 min at 25 °C. 0.5 ml of reaction mixture was mixed with 1 ml of sulphanilic acid reagent (0.33 % in 20 % glacial acetic acid) and incubated for 5 min. To the resultant mixture 1 ml of 0.1% naphthylethylene diamine dihydrochloride was added and allowed to stand for 30 min in diffused light. The O.D. was measured at 540 nm against the corresponding blank solution. Scavenging capacity of the extract was compared with standard drug Vitamin E.

2.4.3.2. Estimation of connective tissue parameters. Granulation tissue was dried at 50 °C for 24 h. To each tube containing 40 mg of the dried granulation tissue, 1 ml of 6 N HCl was added and incubated on boiling water bath for 24 h (12 h each day for two days) for hydrolysis. Unnecessary acid was neutralized by 10 N NaOH phenolphthalein as indicator. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/mL with distilled water. The final hydrolysate was used for the assessment of hydroxyproline, hexosamine, and hexuronic acid.[24]

a) Hydroxyproline (HPR)

Hydroxyproline content was estimated by adding 0.2 ml each of hydrolysate, 2.5 N NaOH, 0.01 M $CuSO_4$, and 6% H_2O_2 mixed and placed on water bath at 80 °C for 15 min. After incubation, the tubes were removed and cooled for 5 min in cold water 4 ml of 3N H_2SO_4 was added with continuous agitation followed by addition of 2 ml of freshly prepared 5% solution of para-dimethyl amino-benzaldehyde in n-Propanol. The test tubes were once again incubated at 75 °C for 15 min and cooled for 5 min. Colour intensity was measured at 540 nm against the blank. Standard curve was prepare by using L-Hydroxyproline 50–1000 μg/0.3 ml.[29]

b) Hexosamine (HXA)

Hexosamine concentration was evaluated in the dried granulation tissue by adding 0.2 ml of hydrolysed fraction to 1 ml of acetyl acetone reagent (1 ml of acetyl acetone in 50 ml of 0.5 N sodium carbonate solution) incubated on water bath at 96 °C for 40 min and cooled. To the reaction mixture 1 ml of 95 % alcohol was added, followed by addition of 1 ml of *p*-dimethylaminobenzaldehyde solution (0.8 g *p*-dimethylaminobenzaldehyde dissolved in 30 ml alcohol + 30 ml conc. HCl) thoroughly mixed and left at room temperature for 1 h. O.D. was red at 530 nm against blank. Standard curve was prepared from D (+) glucosamine hydrochloride 5–50 μg/0.5 ml.[7]

c) Hexuronic acid (HUA)

The hexuronic acid content was analysed by adding 2.5 ml of 0.025 M Borax in concentrated sulphuric acid in tubes and cooled to 4 °C. To this mixture 0.5 ml of hydrolysate was carefully added and incubated at 4 °C. The content was mixed by vigorously shaking, with constant cooling. The content was heated for 10 min in boiling water bath and cooled to room temperature. 0.1 ml of 0.125% carbazole reagent in absolute alcohol was added, vortexed and heated on the boiling water bath for 15 min, and finally cooled to room temperature. O.D. was measured at 530 nm against the blank. Standard curve was prepared from the D (+) Glucurono-6, 3 lactone 5–40 μg/0.5 ml.[24]

2.4.3.3. Histopathological observations. The hematoxylin and eosin stained cross-sections of deep granulation tissues from the

implanted piths were collected on the 10th day of the experiment for the histopathological alterations like mononuclear inflammatory cells, scattered fibroblasts (minimal fibrosis), proliferating vasculature in granulation tissue, abundance of eosinophilic collagen tissue and neovascularisation.

2.5. Statistical analysis

The parametric data were expressed as the mean ± SEM. To determine the differences between groups one-way analysis of variance (ANOVA) was carried out by using the Graph Pad Prism 6 for windows software (version 6.07). Fisher's LSD test was performed for intergroup comparisons using the least significant difference (LSD) at 0.05, 0.001 and 0.0001 was used to determine the level of significance among the various treatments.

3. Results and discussion

3.1. Incision wound model

The wound healing potential in leaves hexane and fruit rind water extracts of *T. peruviana* has been evaluated in the present study based on the earlier findings of antioxidant[20–22,24,31,35] and antimicrobial[10,16,20,26–28,36,37] abilities which come under the most influential paradigms for the plants having wound healing ability.[6,19,21] The parametric data were expressed as the mean ± SEM. To determine the differences between groups one-way analysis of variance (ANOVA) by Fisher's LSD test was carried out using the Graph Pad Prism 6 for windows software (version 6.07). The incision wound model resides on the principle of granuloma tissue development, i.e. increase in collagen production leads to the stabilization of the fibres formation which intern give rise to stable intra and intermolecular crosslinks.[45] The studies of incision wound model of control rats showed wound breaking strength (WBS) as 455.2 ± 25.49 g on 10th post wound day, while LH and FW extracts treated rats showed significant ($P < 0.05$ to $P < 0.01$) WBS as 850 ± 10 g, and 1133 ± 11.4 g respectively. The animals treated with vitamin E at 200 mg/kg body weight showed WBS as 539.6 ± 8.74 g ($P < 0.01$). The excellent healing abilities of *T. peruviana* extracts are proved by means of two folds more granulation development by FW extract, followed by the activity of LH extract.

3.2. Excision wound model

The excision wound model studies have indicated that the rate of wound contraction in control rats as 11.8%–80.6% at day 4 to day 12 and 83.0%–95.4% from day 14 to day 20, while complete epithelization and healing observed on day 24. The Povidone-Iodine 5% treated rats showed increase in wound contraction from 50.7% on day 4–98.3% on day 12 and 100% at day 14 as shown in Table 1 and Fig. 2. The percent rate of wound contraction in rats, treated topically with FW, was from 13.7% ($P < 0.0001$) to 92.1% ($P < 0.0001$) on day 4 to day 12 and 100% wound contraction on day 14. Similarly, the LH treated animals group had wound contraction of 50.7% ($P < 0.0001$) at 4th day and 98.1% ($P < 0.0001$) at 12th day and complete epithelisation was observed at 14 day. No any signs of inflammation or toxicity were observed from the extracts of *T. peruviana* and further, it did not show any cytotoxicity on skin cells. Studies of excision wound models reveal that in both the groups treated by the respective extracts there is an enhancement in the restoration of normal skin structures, where complete epithelisation was at day 14 and the same results were retrieved from standard povidone-iodine representing the plant extract's effective wound healing efficacy. An explanation for the excellent wound healing activity of *T. peruviana* could be the linkage between the fast recovery of epidermis with the augmentation of proper inflammatory response at the wound site.[13] The process of wound healing in rats is much faster when compared with the humans and accordingly rat wound heals days ahead of human wounds. Due to the difference in the skin morphologies of rats and humans, rat skin wound restoration does not entirely mimic human skin.[32]

3.3. Dead space wound model

The same effect of enhanced collagen and granulation tissue development is evidenced in dead space wound model studies which revealed LH and FW extract treatment can cause significant increase in wet weight in mg per 100 g body weight by 94.24 ± 0.69 mg and 197.1 ± 58.03 mg respectively compared with control showing 87.5 ± 1.9 mg/g (Table 3). The biochemical data of the hydrolysate of dry dead space tissue was tested for the levels of hydroxyproline, hexosamine and hexauronic acid to validate the effective repair of normal skin structures (Table 3). The increased hydroxyproline content in the dead space wounds treated by FW and LH directly associates with the collagen turn over, associating to rapid healing with concurrent increase in the breaking strength of the treated wounds. Hexauronic acid and hexosamine act as basis for the production of novel extracellular medium. Their ability to bind and alter protein-protein interactions has branded them as vital factors of cellular approachability in homeostasis, growth and repair.[43] Interestingly, LH extract treated animals didn't display much granulation tissue deposition as compared to standard VTE treated animals, yet the concentrations of all the biochemical markers of dry granulation tissue are shown to be upregulated and this signifies the potent healing capability by means of stabilization of collagen fibres and instincts towards the action of the active principles present in LH extract.

A thorough review of reactive oxygen species (ROS) role in wound healing process[6] states that ROS at wound area plays a

Table 1
Effect of *T. peruviana* LH and FW extracts in excision wound healing studies[*,**,***,****].

Group		1 Day	4 Day	6 Day	8 Day	12 Day	14 Day	16 Day	18 Day	20 Day	22 Day	EIN
1	H A	509.7 ± 3.0	449.3 ± 9.96	265.7 ± 3.4	155.3 ± 6.1	98.83 ± 2.6	86.5 ± 3.3	80.2 ± 2.8	56.5 ± 4.46	28.8 ± 1.5	1.97 ± 0.2	24
	W C	(0.0 ± 0.0)	(11.8 ± 2.2)	(47.8 ± 0.6)	(69.5 ± 1.2)	(80.6 ± 0.5)	(83.0 ± 0.6)	(83.8 ± 0.6)	(88.9 ± 0.8)	(95.4 ± 0.2)	(99.6 ± 0.1)	
2	H A	509.7 ± 3.1	251.3 ± 4.12****	155.3 ± 6.1****	60.17 ± 1.6****	8.5 ± 0.88****	0.0 ± 0.0****	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	14
	W C	(0.0 ± 0.0)	(50.7 ± 0.6)	(69.51 ± 1.2)	(88.2 ± 0.3)	(98.3 ± 0.2)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	
3	H A	513.2 ± 5	269.3 ± 4.5****	251.2 ± 4.1 ns	62.5 ± 1.3****	24.1 ± 0.6****	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	14
	W C	(0.0 ± 0.0)	(13.7 ± 2.8)	(17.8 ± 2.1)	(79 ± 0.26)	(92.1 ± 0.3)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	
4	H A	516.3 ± 3.1	213 ± 2.3****	116 ± 3.5****	40.17 ± 0.5****	8.1 ± 0.19****	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	14
	W C	(0.0 ± 0.0)	(31.6 ± 4.1)	(61.3 ± 4.8)	(86.5 ± 1)	(98.1 ± 0.4)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	(100 ± 0.0)	

Group 1: Control, Group 2: Povidone-Iodine 5% w/w, Group 3: LH 5% w/w, Group 4: FW 5% w/w, HA: healed area, WC: % of wound contraction, EIN: Epithelialization in days. Data indicate mean ± SE, n = 6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ compared to the control group (Fisher's LSD test).

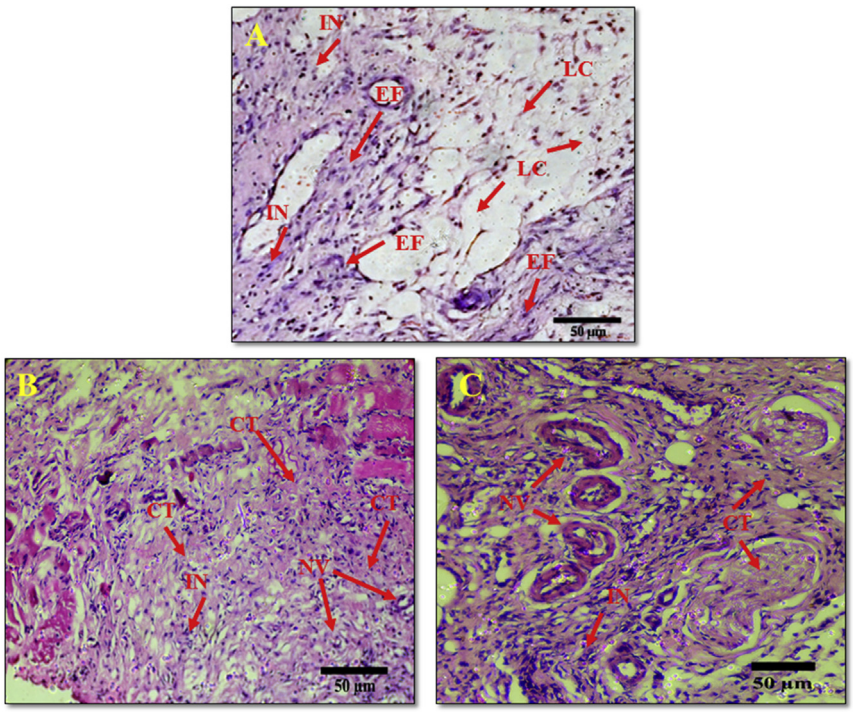

Fig. 1. Histopathology of granulation tissue of *T. peruviana* LH and FW extracts treated rats at day 10 stained with H&E. (A) Granulation tissue of control rat showing mononuclear inflammatory cells (IN), scattered abundance of eosinophilic fibroblasts indicated (EF), Lose Collagen (CT). (B–C) Granulation tissue of rat treated LH and FW showing large number of collagen tissue (CT) (fibrosis) and neovascularisation (NV) with minimal inflammatory cells.

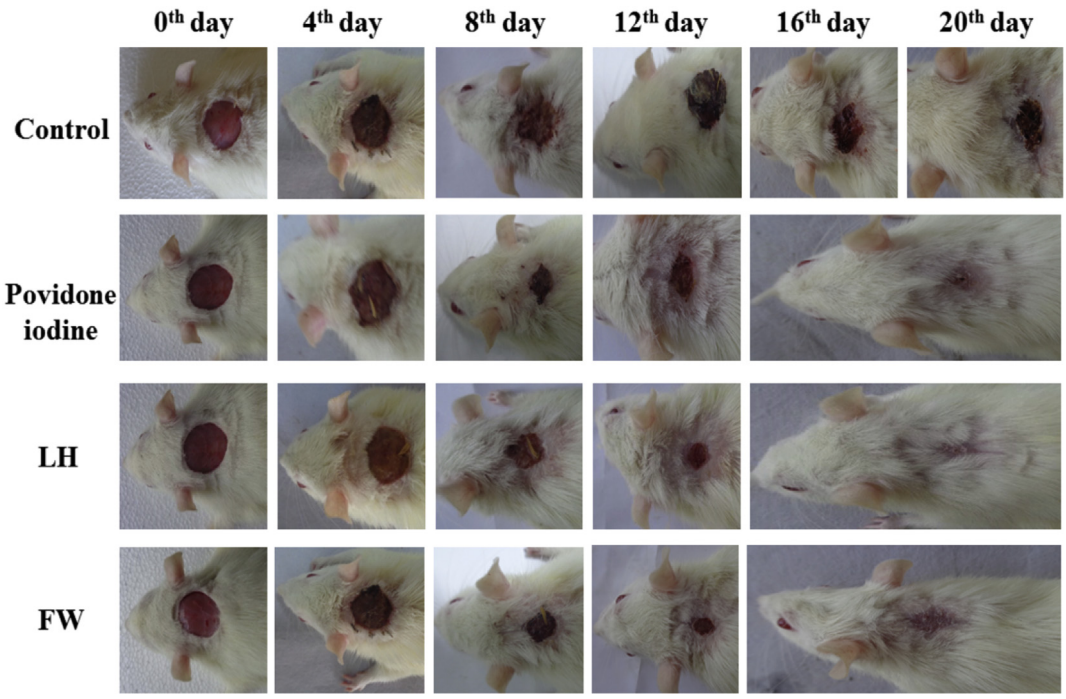

Fig. 2. Wound contraction area on post-excision days of, control, povidone-iodine, LH and FW (5% w/w) extracts treated rats.

crucial role in organisation of normal process. Among ROS especially NO regulate and recruit the role of immunocytes and helps in generation of blood vessels,[39] the excess production of them at the place of wound itself is the biggest barrier of the healing process and leads to oxidative stress thereby causing cytotoxicity and delayed wound healing. Hence the elimination of excessive ROS could be an important strategy in healing of chronic wounds,[21] therefore, the wound healing drug having antioxidant potentials would surely be beneficial. In the view of above facts, the analysis of antioxidants molecules content in wet granulation tissue of extract treated groups was evaluated which showed significant upregulation in the levels of antioxidant enzymes, where GSH level is

Table 2
Granulation tissue and tissue specific antioxidant markers studies of *T. peruviana* LH and FW extract treatment*.

Group	Oral treatment	Animal body weight	Total wet weight (g)	Wet weight mg/100 g	GSH (μmol/mg)	CAT (mmol/mg)	SOD (U/mg)	Peroxidase (U/mg)	LPO (nmol/mg)	% NO inhibition
1	Control	175 ± 5	0.275 ± 0.02	187.5 ± 1.9	21.1 ± 0.4	42.2 ± 0.6	3.47 ± 0.06	0.16 ± 0.01	6.34 ± 0.012	22.92 ± 2.5
2	Vitamin E 200 mg/kg	185 ± 5 ns	0.525 ± 0.01	305.7 ± 1.9 ns	25.1 ± 0.6***	103 ± 2.5****	7.84 ± 0.05***	1.56 ± 0.01	1.66 ± 0.12****	77.49 ± 3.16****
3	LH 200 mg/kg	313 ± 3****	0.29 ± 0.005	94.24 ± 0.69 ns	30.5 ± 0.3****	73.06 ± 0.2****	14.5 ± 1.2****	0.44 ± 0 ns	3.95 ± 0.4****	56 ± 1****
4	FW 200 mg/kg	191.5 ± 4.5 ns	0.38 ± 0.12	197.1 ± 58.03 ns	37.02 ± 0.37****	82.05 ± 0.5****	7.47 ± 0.05**	0.27 ± 0.09***	0.57 ± 0.094****	69.38 ± 1.2****

Control: 1 ml/kg of 1% gum acacia, LH: leaves hexane, FW: Fruit water, Data indicate mean ± SE, n = 6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$, ns-non significant compared to the control group (Fisher's LSD test).

Table 3
Effect of *T. peruviana* LH and FW extract on granulation tissue of dead space wound model*.

Group	Oral treatment	Animal body weight	Dry tissue parameters		Connective tissue parameters		
			Total dry weight	Dry weight mg/100 g	Hexosamine mg/g	Hydroxyproline mg/g	Hexuronic acid mg/g
1	Control	175 ± 5	0.0762 ± 0.0008	42.89 ± 1.65	19.11 ± 0.76	16.74 ± 0.5	12.09 ± 0.5
2	Vitamin E 200 mg/kg	185 ± 5 ns	0.105 ± 0.005**	57.02 ± 1.22**	28.07 ± 0.43**	31.02 ± 0.7 ns	29.67 ± 0.8**
3	LH 200 mg/kg	313 ± 3****	0.145 ± 0.005****	46.31 ± 1.15****	48.6 ± 1.05****	53.66 ± 0.38**	49.91 ± 0.7***
4	FW 200 mg/kg	191.5 ± 4.5 ns	0.1205 ± 0.0005***	62.97 ± 1.741***	53.48 ± 2.42****	65.73 ± 3.2***	60.95 ± 2.31****

LH: leaves hexane, FW: Fruit water, Data indicate mean ± SE, n = 6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.000$, ns-non significant compared to the control group (Fisher's LSD test).

($P < 0.0001$) significantly elevated by LH 30.5 ± 0.3 μmol/mg and FW 37.02 ± 0.37 μmol/mg treatment compared to the control with 21.1 ± 0.4 μmol/mg. Similarly, the SOD levels of granulation tissue were also elevated to 14.5 ± 1.2 U/mg ($P < 0.0001$) and 7.47 ± 0.05 U/mg ($P < 0.01$) which is almost two to three fold increased than the control group with 3.47 ± 0.06 U/mg. Similarly, Catalase content of LH and FW treated rats 73.06 ± 0.2 mmol/mg and 82.05 ± 0.5 mmol/mg also increased to nearly two folds than standard 42.2 ± 0.6 mmol/mg as tabulated in Table 2.

The results of *T. peruviana* having antioxidant activity were retrieved by the analysis of percent nitric oxide inhibition where NO can also considered as inflammatory marker. The LH and FW treated rats granuloma exhibited significant increase in percent of nitric oxide inhibition to 56± 1% and 69.38± 1.2% ($P < 0.0001$) respectively. Conversely, the oxidative stress mediated tissue damage marker (LPO) levels were lowered to 3.95 ± 0.4 nmol/mg and 0.57 ± 0.094 nmol/mg. The obtained results are statistically significant and comparable with control group as tabulated in Table 2. However, the analysis of levels of LPO is directly related with the oxidative damage of the tissue which in the present study has been decreased in extract treated animals. Apparently, the lowering of the LPO and NO content is a clear indication of active role of *T. peruviana* extracts in preventing the oxidative damage and inflammation which indeed promotes the healing process. Thus, it can be postulated that that the *T. peruviana* has the wound healing potentials by means of antioxidant and anti-inflammation. The potent antioxidant mediated wound healing activity of *T. peruviana* leaf and fruit rind extracts are in agreement with previous reports.[26–28,30,41,46] This activity could be attributed to the presence of beneficial phytochemicals present in the plant parts.[1,20,35,40]

Medicinal plants are widely used for the treatment of several illnesses. However, toxicological and pharmacological studies are needed to assess the risks and true benefits of these plants. As many components are mixed in herbal treatment and may produce some level of toxicity which makes its toxicological analysis very crucial. Even though, there are some of the side effects observed in the use of herbal drugs such as allergic reaction, burning sensation, constipation, dizziness, gastrointestinal upset, menstrual disorders and muscle spasms etc. have been reported by many researchers,[2,4,14,15,33] but the benefits are definitely more. Herbs are natural and safe as the herbal medicine acts in a synergised way where the side effects of the main active compounds are reduced by secondary compounds. Hence, the basis of ethno pharmacology does not always be in a single active compound but rather is a result of the combination of more than one active compounds found in the extract.[42]

3.4. Histopathological observations

Histological studies of granulation tissue of control rat showed mononuclear inflammatory cells, dispersed fibroblasts, and some proliferating vasculature in granulation tissue indicating incomplete healing of wound in control animals, while the granulation tissue of rats treated with LH and FW extracts displayed abundance of eosinophils and increased collagen tissue and neovascularisation with few inflammatory cells indicating profound healing by fibrosis (Fig. 1). Where, the macrophages washes out the unwanted materials and orchestrate the multiplication of endothelial cells with the emerging of new blood vessels, replication of smooth muscles and the establishment of fibroblasts aims to upsurge the gap junctional intracellular communication and persuades speedy maturation of granulation tissue.[23]

The main limitation of the present work is reproducibility of the same results in humans due the morphological changes of skin. Surely, the current findings have drawn an outline of therapeutic potentials of *T. peruviana* in wound healing. Further, thorough metabolomics characterisation of active extracts is needed to identify the active constituents present and their therapeutic mechanism in appropriate models definitely be the future line of work.

4. Conclusion

The bioactive efficacy of *T. peruviana* extracts in incision, excision and dead space wound models stands for the remarkable

wound healing ability which intern substantiates the ethnopharmacological use of *T. peruviana* in traditional system of medicine. The present results clearly exhibiting the enhanced wound breaking strength, reepithelization with upregulated levels of tissue specific antioxidant enzymes with the down regulation of oxidative stress (LPO) and inflammatory marker (NO). It is established that *T. peruviana* leaves and fruit rind extracts possess the antioxidant and anti-inflammatory based wound healing properties, which signifies the importance of this plant for future use in pharmaceutical industry for the development of effective wound healing drugs.

Conflicts of interest

All authors declare no conflicts of interest.

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