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Antioxidant, anti-inflammatory and wound healing properties of ethanolic extracts of *Thevetia peruviana* (Pers.) K. Schum

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ABSTRACT: *Thevetia peruviana* (Pers.) K. Schum, well known as Karvira in the Ayurvedic text. The plant has been traditionally used for the treatment of wounds, inflammation and diverse types of skin disorders. The present study was designed to analyze the antioxidant and anti-inflammatory mediated wound healing potential of *T. peruviana* leaf (TPLE) and fruit rind (TPFE) ethanolic extracts. To establish the efficacy, the study was designed using *in vitro* antioxidant activities, carrageenan-induced inflammation model and incision, excision, dead space wound models associated with antioxidant enzymes, tissue biochemical and inflammatory marker analysis allied with acute toxicity studies in rats. The serum and tissue biochemical, histological and organ index studies of acute toxicity evaluation confirms the non-toxic nature of the extracts. The TPLE and TPFE treated groups represents complete restoration of normal skin structures after biopsy at day 14 comparable to the control group healed at day 24, the significant amount of hydroxyproline, hexosamine, hexuronic acid observed in dead space wound study, maximum wound breaking strength is intern related with the more collagen and fibroblast deposition can be correlated with the excellent wound healing efficiency of extracts. Consequently, the profound *in vitro* antioxidant efficacy, lowing of carrageenan induced inflammation, ESR, nitric oxide radicles and LPO (lipid peroxidation) content allied with an elevation of the GSH (reduced glutathione), SOD (superoxide dismutase), CAT (catalase), and GPx (glutathione peroxidase) in granuloma is a clear indication of good antioxidant and anti-inflammatory activity of TPLE and TPFE extracts. Our study provides evidence for the association of the excellent antioxidant, anti-inflammatory and wound healing capacity of *T. peruviana* present in a prudently balanced means which is required in a novel wound healing drug.

KEYWORDS: *Thevetia peruviana*; wound healing; antioxidant; lipid peroxidation; anti-inflammatory; nitric oxide; carrageenan.

1. INTRODUCTION

Healing of wound is the sign of development and essential biological phenomena. Acute or chronic wounds can affect an individual's persona, confidence, well-being, self-image and workability [1]. The influence of wounds on physical, social and financial areas of a person's life necessitates good wound treatment not only for the individual but also for the society [2]. Many factors play important role in the healing of wounds in prudently balanced means, while, the imbalance of the same factors are responsible for hindering the healing process. In a broader way, these factors are divided into three distinct fields such as, i) ROS (reactive oxygen species) and RNS (reactive nitrogen species) factor: Even though these molecules involved in the accretion of macrophages and settling of inflammatory cells to their respective positions, when surpassed may also be responsible for the delayed salvation of wounds by persuading tissue damage [3], ii) Inflammation factor: Even though the inflammatory cells are pivotal and are the very first response to the wound healing, while, increased scale and span of inflammation is responsible for delayed retrieval of wounds [4]. Similarly, iii) Infection factor: Microbial population at the wound site will increase the pro-inflammatory cytokines (interleukin-1 and TNF-α) and elongate the inflammatory phase responsible for the late recovery of wounds [5].

Hence, abolition of surpassed ROS and management of inflammation and infection could be an important stratagem in the restoration of chronic wounds. Because of the resilient stimulus of these hindering

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factors and lack of combinational effects of presently available wound management drugs the medical need of human population of the wound curing drugs is lagging behind, intern development of a wound healing drug with antioxidant, antimicrobial and anti-inflammatory potentials is a vital strategy.

In Ayurveda, many prominent scholars have advocated the use of leaves of plant *T. peruviana* (*Karvira*) for the treatment of external wounds, infected area, ringworms, inflammation, fever, tumors, skin disorders and in ethnoveterinary use [6, 7]. Considering the ethnomedicinal claims and the lack of systematic information on wound healing potentials of *T. peruviana* leaf and fruit rind, the current study has been called for a thorough exploration of wound curative capabilities, by evaluating the antioxidant, and anti-inflammatory means.

2. RESULTS

2.1. *In vitro* antioxidant activity

The *in vitro* antioxidant potentials of TPLE (*T. peruviana* leaf ethanolic extract) and TPFE (*T. peruviana* fruit rind ethanolic extract) has been displayed in Figure 1. In the current study the reduction potential of superoxide and nitric oxide radicles by TPLE and TPFE is excellent (IC_{50}: 240.7 ± 2.7 µg ml^{-1} and 230.6 ± 1.7 µg ml^{-1}) comparing to the standard BHT (Butylated hydroxytoluene) (IC_{50}: 787.3 ± 5.6 µg ml^{-1} and 279.1 ± 0.37 µg ml^{-1}) respectively. Moreover, TPFE showed the highest amount of iron-reducing capacity with IC_{50} of 130.7 ± 0.8 µg ml^{-1}. In the present investigation, TPLE and TPFE not only showed a good amount of antioxidant activity in terms of DPPH (2,2-diphenyl-1-picrylhydrazyl), hydroxyl radicle, FRAP (ferric reducing antioxidant power) and total antioxidant assays but also lessened the LPO (Lipid peroxidation) of egg yolk with IC_{50} 85.52 ± 0.71 and 95.97 ± 0.09 µg ml^{-1} compared with the standard BHT (IC_{50}: 87.55 ± 0.38 µg ml^{-1}).

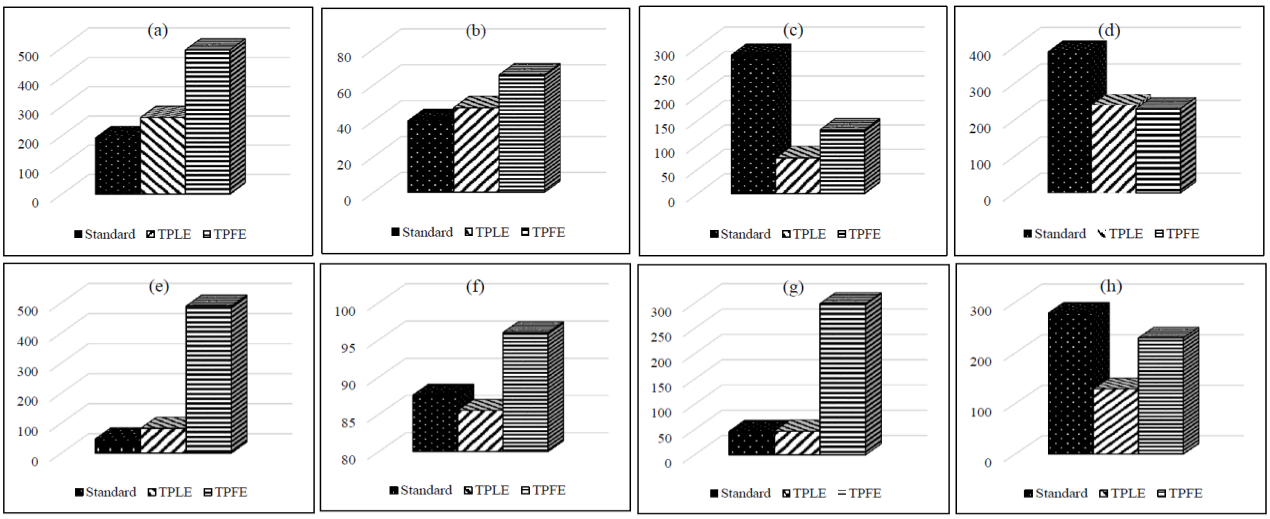

Figure 1. IC_{50} values of *in vitro* antioxidant activity assays. (a) total antioxidant assay, (b) DPPH scavenging assay, (c) ferric reducing power assay, (d) superoxide anion scavenging assay, (e) TPTZ scavenging assay, (f) hydroxyl radicle scavenging, (g) nitric oxide radicle scavenging assay, (h) lipid peroxidation assay.

2.2. Acute toxicity

The relative liver weight of TPLE (3.63 ± 0.044%) ($P < 0.0001$) and TPFE (3.76 ± 0.13%) ($P < 0.0001$) was slightly decreased (Table 1). The serum biochemicals too displayed non-significant alteration in the levels of serum albumin, bilirubin, γ-GT (gamma-glutamyl transferase), creatinine, and protein levels. Whereas, the levels of ALT (alanine aminotransferase) and ALP (alkaline phosphatase) plummeted considerably as tabulated in the Table 2. Upon examining the liver tissue-specific antioxidant makers like GSH (reduced glutathione), SOD (superoxide dismutase), CAT (catalase), and GPx (glutathione peroxidase) were found to be normalized, associated with the fall of LPO levels in treated rat and liver histopathological normal architecture instincts towards the intact integrity of the tissues, confirms the non-toxic nature of the extract at the treated dose. Results are tabulated in Table 3, Figure 2A.

Table 1. Percent body weight, absolute and relative organ weights of acute toxicity analysis.

Group	Treatment	Increase in Body weight	% Increase in Body weight	Absolute organ weight (g)			Relative organ weight (%)		
				Liver	Kidney	Lungs	Liver	Kidney	Lungs
1.	**Control**	24.5 ± 1.5	15.16 ± 0.6	10.11 ± 1.18	1.67 ± 0.5	1.66 ± 0.47	4.93 ± 0.05	0.6 ± 0.05	0.6 ± 0.06
2.	**TPLE**	20 ± 0	7.3 ± 1.4 ***	8.57 ± 0.11	1.765 ± 0.13	1.62 ± 0.05	3.63 ± 0.044 ****	0.68 ± 0.027	0.68 ± 0
3.	**TPFE**	25.5 ± 0.5	10 ± 0 **	8.995 ± 0.09	1.815 ± 0.08	1.66 ± 0.0	3.76 ± 0.13 ****	0.67 ± 0.02	0.68 ± 0.02

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). *T. peruviana* leaf (TPLE) and fruit rind (TPFE) ethanolic extracts.

Table 2. Serum biochemical markers of acute toxicity analysis.

Group	Treatment	Biochemical parameters							
		Albumin (g/dl)	Bilirubin (mg/dl)	ALT (U/l)	ALP (U/l)	γ-GT (U/l)	Urea mg/dl	Creatinine mg/dl	Protein (g/dl)
1.	**Control**	2.88 ± 0.22	0.21 ± 0.04	316.7 ± 6.3	574.4 ± 12.9	8.97 ± 1.9	38.59 ± 1.4	0.6 ± 0.19	1.6 ± 0.07
2.	**TPLE**	2.9 ± 0.23	0.12 ± 0.005	155.3 ± 3 ****	249.1 ± 5.05 ****	6.3 ± 0.2	52.25 ± 0.2 ****	0.43 ± 0.008	1.8 ± 0.02
3.	**TPFE**	3.2 ± 0.02	0.18 ± 0.01	130.7 ± 1.8 ****	249.5 ± 3.8 ****	2.04 ± 0.5	52.2 ± 0.2 ****	0.47 ± 0.003	1.7 ± 0.02

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). *T. peruviana* leaf (TPLE) and fruit rind (TPFE) ethanolic extracts.

Table 3. Liver antioxidant markers of acute toxicity analysis.

Group	Treatment	Liver Antioxidants Parameters				
		GSH (µmol/mg)	Catalase (mmol/mg)	LPO (nmol/mg)	SOD (U/mg)	GPx (U/mg)
1.	**Control**	39.39 ± 1.2	13.59 ± 0.87	0.25 ± 0.1	12.84 ± 2.4	3.72 ± 0.19
2.	**TPLE**	24.14 ± 0.4 ***	17.03 ± 0.46	0.097 ± 0.02	6.89 ± 0.5 *	2.93 ± 0.07 **
3.	**TPFE**	21.84 ± 1.4 ***	6.98 ± 1.6 *	0.072 ± 0.02	6.23 ± 0.35 *	3.52 ± 0.04

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). *T. peruviana* leaf (TPLE) and fruit rind (TPFE) ethanolic extracts.

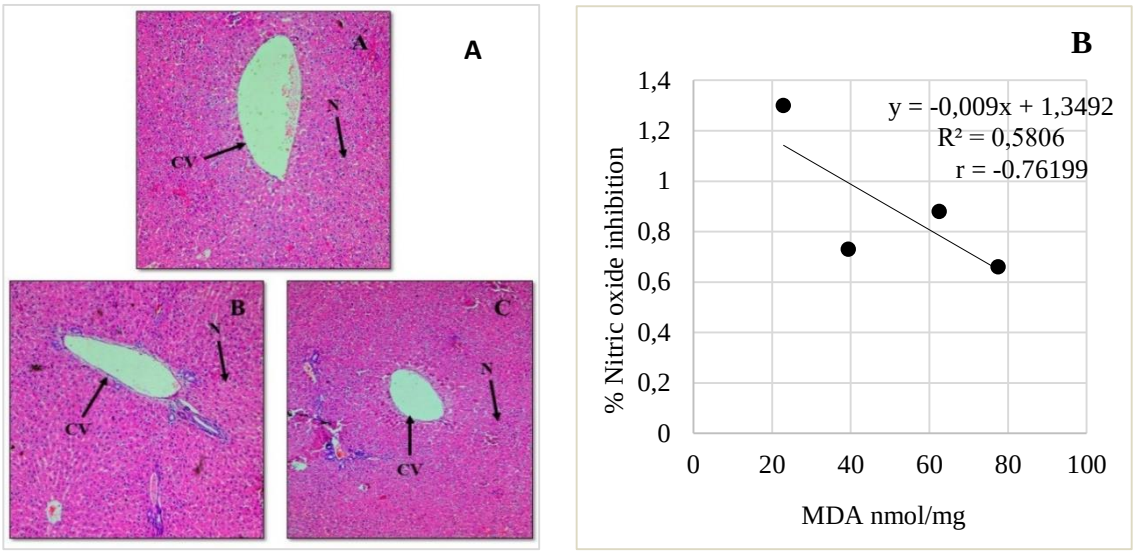

Figure 2. A: Histopathology of Liver in Acute toxicity studies: A) Control, B) TPLE and C) TPFE. Hepatocyte with their normal nuclei (N), central vein (CV). Scale bars: 50µm, B: Correlational studies between LPO and % NO inhibition content of granulation tissue.

2.3. Anti-inflammatory activity

The anti-inflammatory effect of TPLE and TPFE in carrageenan-induced paw oedema is represented in Table 4 a marked inhibition of inflammation was seen in TPFE treated rats 70.28 ± 18% ($P < 0.001$) and TPLE 66.38 ± 10.1% ($P < 0.001$), compared with the animal groups treated with standard drug indomethacin displayed 89.53 ± 0.46% ($P < 0.0001$) activity. Results were confirmed by ESR (erythrocyte sedimentation rate) evaluation which evidenced ESR level fall between normal ranges in the extract treated groups.

Table 4. Study of anti-inflammatory effect in carrageenan-induced paw edema in rats.

Group	Oral treatment	Oedema induced by carrageenan (mm)						Inflammation parameters		
		Dosage (mg/kg)	0 min	30 min	60 min	120 min	240 min	% IR	% II	ESR (mm)
1.	**Control**	10	2.2 ± 0.04	2.4 ± 0.07	2.77 ± 0.08	2.685 ± 0.1	2.43 ± 0.03	25.81 ± 0.58		4 ± 0
2.	**Positive control**	10	2.2 ± 0.005	2.5 ± 0.01	1.95 ± 0.02	1.2 ± 0.005	0.25 ± 0.01	6.15 ± 0.38	89.53 ± 0.46	2 ± 0
3.	**TPLE**	200	1.7 ± 0.2	1.64 ± 0.2	1.94 ± 0.13	1.42 ± 0.77 *	0.81 ± 0.23 ***	18.13 ± 4.2	66.38 ± 10.1	1 ± 0 *
4.	**TPFE**	200	1.5 ± 0.18	1.64 ± 0.7	1.54 ± 0.81 *	1.0 ± 0.01 **	0.73 ± 0.45 ***	14.35 ± 7.9	70.28 ± 18	1.5 ± 0.5 **

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). %IR: % inflammation remained, % II: % inflammation inhibition. *T. peruviana* leaf (TPLE) and fruit rind (TPFE) ethanolic extracts.

2.4. Wound healing activity

2.4.1. Incision wound model

Incision wound model was evaluated by wound breaking strength (WBS), in the present study TPLE and TPFE extract treated rats showed significant WBS of 949.3 ± 52.67g ($P < 0.01$) and 771.5 ± 190.4g respectively on 10th post-wound day, which is considerably higher expanse of WBS development than the animals treated with povidone-iodine (539.6 ± 8.74 g) ($P < 0.01$), whereas, rats of control group showed WBS of 455.2 ± 25.49 g.

2.4.2. Excision wound model

The excision wound model studies are accessible in Table 5, accordingly the control rats showed 11.8% to 80.6% of wound contraction at day 4 to day 12 and 83.0% to 95.4% from day 14 to day 20, while complete epithelization and healing was observed on day 24. Interestingly, the rats treated with TPLE showed highest rate of wound contraction ie 13.4% at day 4 and 92.7% at 12th day and 100% ($P < 0.0001$)on day 14, whereas TPFE displayed 8.87% to 86.4% ($P < 0.0001$) of wound contraction with complete healing at day 14. The povidone iodide treated rats showed an increase in wound contraction from 50.7% on day 4 to 98.3% on day 12 and 100% at day 14 as well.

Table 5. Effect of TPLE and TPFE on excision wound healed area.

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 W C	509.7 ± 3.0 (0.0 ± 0.0)	449.3 ± 9.96 (11.8 ± 2.2)	265.7 ± 3.4 (47.8 ± 0.6)	155.3 ± 6.1 (69.5 ± 1.2)	98.83 ± 2.6 (80.6 ± 0.5)	86.5 ± 3.3 (83.0 ± 0.6)	80.2 ± 2.8 (83.8 ± 0.6)	56.5 ± 4.46 (88.9 ± 0.8)	28.8 ± 1.5 (95.4 ± 0.2)	1.97 ± 0.2 (99.6± 0.1)	24
2.	H A W C	509.7 ± 3.1 0.0 ± 0.0	251.3 ± 4.12 **** (50.7 ± 0.6)	155.3 ± 6.1 **** (69.51 ± 1.2)	60.17 ± 1.6 **** (88.2 ± 0.3)	8.5 ± 0.88 **** (98.3 ± 0.2)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	14
3.	H A W C	515.7 ± 5.8 (0.0 ± 0.0)	368.3 ± 8.9 **** ++++ 13.4 ± 2.2	283.5 ± 5.3 ++++ 22.6 ± 3.8	73.5 ± 1.3 **** ++ 79.97± 1.2	25.3 ± 2.5 **** 92.7 ± 2.5	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	14
4.	H A W C	505 ± 3.6 (0.0 ± 0.0)	33 ± 9.2 **** ++++ 8.87 ± 3.3	224.2 ± 12.7 *** ++++ 22.2 ± 9.8	54.1 ± 3.1 **** 74 ± 0.62	16.1 ± 0.4 **** 86.4 ± 3.2	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	0.0 ± 0.0 (100 ± 0.0)	14

Group 1: control 1% gum acacia, Group 2: Povidone-Iodide 5%v/v, Group 3 TPLE 5%v/v, Group 4: TPFE 5%v/v, HA: healed area (mm), 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. + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ and ++++ $P < 0.0001$ compared to the positive control (Fisher's LSD test). *T. peruviana* leaf (TPLE) and fruit rind (TPFE) ethanolic extracts.

2.4.3. Dead space wound model

Dead space wound model studies revealed that the TPLE and TPFE extract treatment caused an increase in wet weight of granulation tissue per 100g body weight of rats. Where TPFE showed 206.5 ± 0.73 mg/100g and TPFE showed 142.4 ± 50.02 mg/100g, compared with control group 187.5 ± 1.9 mg/100g (Table 6), and interestingly the protein content was increased in extract treated granulation tissues in comparison with the control group. The efficacy of extracts was comparable with that of VTE (Vitamin E).

The analysis of antioxidants enzymes in wet granulation tissues of extract treated animals showed a significant increase in the level of GSH with 37.28 ± 0.08 µmol/mg and 37.72 ± 0.14 µmol/mg ($P < 0.0001$) in TPLE and TPFE treated animals. In the same way, SOD was also significantly increased to 7.5 ± 0.02 U/mg and 7.45 ± 0.03 U/mg ($P < 0.01$) in either of the extract treated rat's granuloma. Catalase and peroxidase contents were also amplified in both extracts treated rats when compared with control group. Oxidative stress marker LPO in TPLE and TPFE extracts treatment was reduced to 0.66 ± 0.1 nmol/mg and 0.73 ± 0.007 nmol/mg ($P < 0.0001$) respectively.

Table 6. Granulation tissue and tissue specific antioxidant markers studies.

		Wet tissue parameters		Antioxidants markers and Free radicals					
Group	Animal B.Wt.	Total wet wt. (g)	Wet wt. mg/100 g	Protein (g/dl)	GSH (µmol/ mg)	SOD (U/mg)	GPx (U/mg)	CAT (mmol/ mg)	MDA (nmol/ mg)	% NO Inhibition
Control	175 ± 5.00	0.27 ± 0.02	187.5 ± 1.9	1.31 ± 0.05	21.14 ± 0.43	3.48 ± 0.06	0.16 ± 0.01	42.2 ± 0.66	1.3 ± 0.01	22.92 ± 2.55
Vitamin E	185 ± 5.00	0.52 ± 0.01	305.7 ± 1.9	1.74 ± 0.08 *	25.14 ± 0.64 **	7.84 ± 0.05 ****	1.56 ± 0.01 ****	103 ± 2.58 ****	0.66 ± 0.1 **** ++++	77.49 ± 3.16 ****
TPLE	215.5 ± 6.5 **	0.445 ± 0.015	206.5 ± 0.73	1.62 ± 0.13 *	37.28 ± 0.08 **** ++++	7.5 ± 0.02 **	1.20 ± 0.04 ** ++	103 ± 2.58 **** ++++	0.88 ± 0.04 **** +	62.5 ± 1.6 **** ++++
TPFE	297 ± 6 ****	0.42 ± 0.14	142.4 ± 50.02	1.81 ± 0.6 *	37.72 ± 0.14 **** ++++	7.45 ± 0.03 **	0.87 ± 0.07 ** +++	84.9 ± 2.44 **** ++++	0.73 ± 0.007 **** +	39.4 ± 0.67 **** ++++

Data indicate mean ± SE, n = 6. * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 compared to the control group and + P < 0.05, ++ P < 0.01, +++ P < 0.001 and ++++ P < 0.0001 compared to the positive control (VTE) Group (Fisher's LSD test). *T. peruviana* leaf (TPLE) and fruit rind (TPFE) ethanolic extracts.

Additionally, upon estimation of dried granulation tissue for connective tissue parameters, TPLE treated rat's granuloma shown substantially increased hydroxyproline (55.39 ± 7.1 mg/g), hexosamine (41.68 ± 0.67 mg/g) and hexuronic acid (49.63 ± 6.06 mg/g) concentration when compared to control group (Table-8). Surprisingly, TPFE was found to be the best extract in enhancing the hydroxyproline (72.2 ± 7.54 mg/g), hexosamine (49.25 ± 2.3 mg/g) and hexuronic acid (62.74 ± 4.11 mg/g) content. The evaluation of %NO (nitric oxide) inhibition was performed to know the probable effect of extracts in the *in vivo* system in terms of inflammation reduction. TPLE and TPFE extract treatment inhibited NO radicles to 62.5 ± 1.6% and 39.4 ± 0.67% ($P < 0.0001$) respectively.

2.4.4. Histopathological observations

Histoarchitecture of control granulation tissue exhibited inadequate healing evidenced by dispersed fibroblasts, mononuclear inflammatory cells, some proliferating vasculature, while the TPLE and TPFE extract treated rats granulation tissue showed profound healing by fibrosis with a profusion of eosinophils, intensified collagen tissue, neovascularization and curtailed inflammatory cells (Figure 3).

3. DISCUSSION

Healing process takes place by cellular activities of victim itself, but various risk factors such as excessive inflammation, oxidative stress, and infection delay it. Multiple lines of evidence shown that the antioxidant, anti-inflammatory, and antimicrobial potentials are the three affecting domains of a potent wound healing drug [3, 4, 5], but only few animal studies and even less clinical studies are obtainable [8]. The phytomedicinal therapy for wound healing is promising as the bioactive compounds are postulated to control wound oxidative, inflammatory and infective stress and thereby accelerating wound healing [7]. The phytochemical abundance of *T. peruviana* has been documented [9], similarly, the available advanced analytical technoques reported the existence of therapeutically importent phytocomponents as tabulated in Table 7 [10-18]. Moreover, the presence of excellent antimicrobial and antioxidant potentials in *Thevetia peruvina* has been extensively reported [19-21].

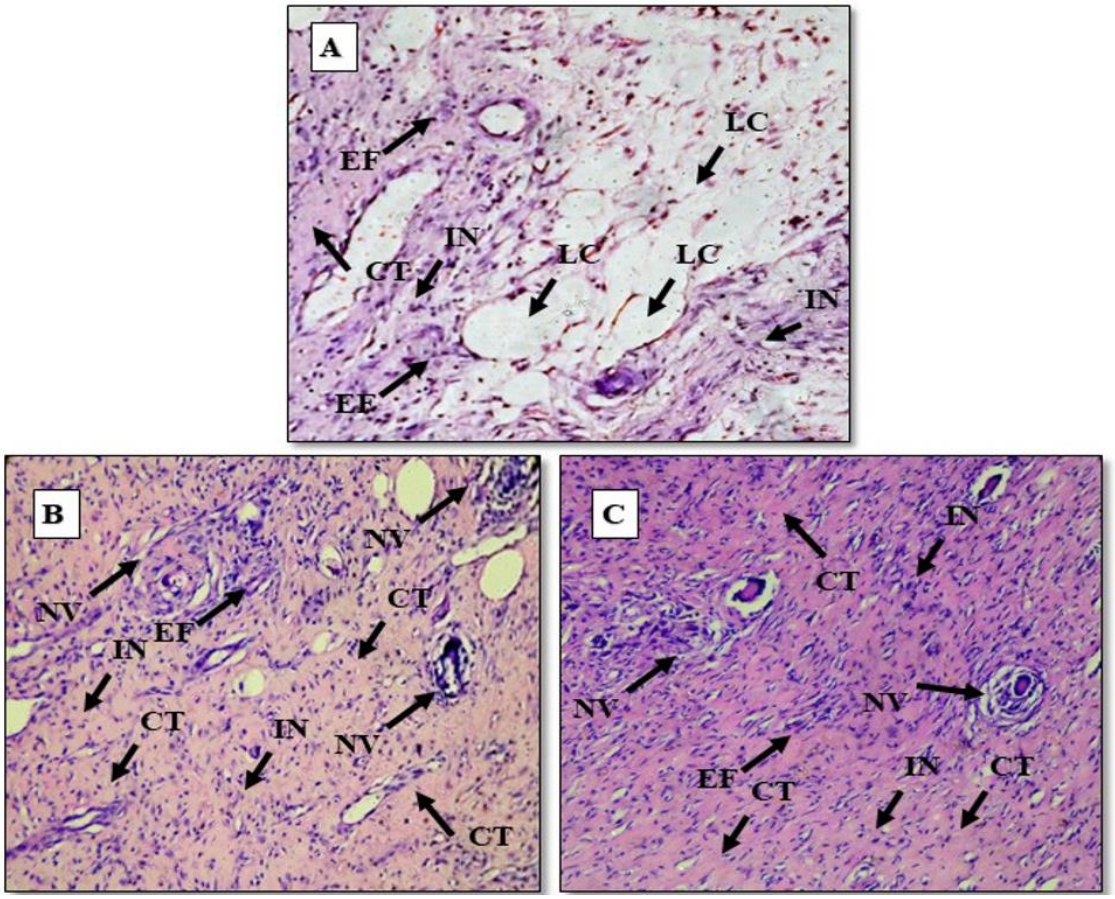

Figure 3. Histopathology of granulation tissue of TPLE extract treated rats at day 10 stained with H&E. Scale bars: 50µm (A) Granulation tissue of control rat showing mononuclear inflammatory cells (IN), scattered abundance of eosinophilic fibroblasts (EF) and large number of collagen tissue (CT) were indicated. (B) TPLE (C) TPFE treated granulation tissue of rat showing mononuclear inflammatory cells (IN), scattered abundance of eosinophilic fibroblasts (EF) (fibrosis) and neovascularisation (NV) with minimal inflammatory cells.

In the present study *T. peruviana* leaves and fruit rind ethanolic extracts demonstrated potential *in vitro* antioxident activity as compared to the earlier reports [19, 22]. The aute toxicity evalutions are in agreement with the previous report of *Plumeria alba* Linn (*Apocynaceae*) where the same non-significant changes were observed during the acute toxicity analysis [23] demonstaring nontoxic nature of extracts at the tested concentation. The complete restoration of normal skin structures after biopsy of excised skin at day 14, the significant amount of collagenation observed in dead space wound tissue and maximum wound breaking strength is intern related with the more collagen and fibroblast deposition in the TPLE and TPFE treated groups. The *in vivo* treatment indicates a significant escalation in the antioxidant profile of granulation tissue of dead space wound model in rats. SOD is a cardinal enzyme bioavailability of whom depends on O_2^- concentration. The amplified level of superoxide dismutase (SOD) leads to transformation of superoxide radicals into oxygen and H_2O_2 and prevent further generation of free radicals like peroxynitrite and hydroxyl radicals and decreases ROS generation and oxidative stress at the wound site [24]. The increased activity of SOD may designate the amount of enzyme produced against the amelioration of oxidative stress in granuloma of rats. The ability of TPLE and TPFE to enhance the SOD enzyme in the *in-vivo* system is directly correlated with the excellent superoxide radicle quenching capacity in the *in-vitro* antioxidant analysis. A significant escalation in the GSH, GPx and CAT activity observed in the extract treated animals suggest that H_2O_2 and lipid hydroperoxides collected due to increased activity of SOD enzyme was properly neutralized to O_2 and H_2O which is associated with a decreased level of MDA (malondialdehyde) in the granuloma.

Table 7. Reported phytochemical in *T. peruviana*.

Sl. No.	Components	Reference
1.	Neriifolin, thevetin A, thevetin B, oleandrin, acetyl neriifolin and peruvoside	[10]
2.	Kaempferol 3-O-fl-D-glucopyranosyl-β-D-galac-Topyranoside, Kaempferol 3-O-[6"-O-sinapoyl-β-D-glucopyranosyl]-β-D-galactopyranoside, Kaempferol 3-O-β-D-glucopyranosyl [6"-O-sina-poyl-β-D-glucopyranosyl], Kaempferol 3-O-[2""-O-sinapoyl-β-D-glucopyranosyl], [6"-O-sinapoyl-β-D-glucopyranosyl]-β-D-galactopyranoside, Quercetin 3-O-β-D-glucopyranosyl-β-D-galacto- pyranoside, Quercetin 3-O-[6"-O-sinapoyl-β-D-glucopyranosyl]-β-D-galactopyranoside.	[11]
3.	Favanone glucosides (2R)- and (2S)-5-Oββ-D-glucopyranosyl-7,49-dihydroxy-39,59-dimethoxyflavanone[pervianoside I (3), peruvianoside II (4)], and flavonol glycoside quercetin3-O-{ββ-D-glucopyranosyl-[ββ-L-rhamnopyranosyl], ββ-D-galactopyranoside} (peruvianoside III, 13).	[12]
4.	3-Methylcyclohexanone, 1-Nonene Isopulegol, 2-(2-Butoxyethoxy)ethanol, Benzoic acid, Caprylic acid, Pulegone, Tetrahydrogeraniol 4-Isopropyl-1,3-cyclohexanedione, 3-Butyl-hexa-3-ene-2-one, Carvacrol 2-Butoxyethyl acetate, 2-Nonenal 2-Propyl-1-heptanol 8-Methyl-1-undecene, Citronellol, cis-Pulegone oxide Spathulenol, Nerolidol, Methyl isosterate, Palmitic acid Methyl palmitate, Methyl elaidate, Linoleic acid Oleic acid 9-Octadecenal 9-Octadecenol 9-Octadecenamide b-Ergostenol, Terpenes, Fatty acids and derivatives.	[13]
5.	Neriifolin, thevefolin, peruvoside, and (20S)-18,20-epoxydigitoxigenin RL-thvetoside	[14]
6.	3-Dihydro-3,5-Dihydroxy-6-Methyl-4H Pyran-4-one, 3-methyl 1-hepatanol, 3,6-Dimethylundecane, Tetradecane, Lauric acid, Mome inositol, Palmitic acid, Ethyl palmitate, Phytol, Ethyl(9z,12z)-9,12-octadecadienoate, Ethyl linolenate.	[15]
7.	Thevetin C, acetylthevetin C, 18, 20-oxido 20, 22-dihydro, thevetin B, and acetylthevetin B	[16]
8.	8-Hydroxy-6, 7-epoxydendrolasin, Citronellyl tiglate Methyl 16-hydroxy-3-3dimethylhepatadecanoate 1-xAllyloxy-1-ethynyl-5-methylcyclohexane 2-(Methoxycarbonyl)-2-propargyl-1-cyclopentanol 1, 2-Benzenedicarboxylic acid,dicyclohexyl ester 1-(-R)-endo-Methylbornyl E-butenoate 1, 3, 4, 5-Tetramethylbicyclo [3.2.0] hex-3-ene-2-one	[17]
9.	Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	[18]

The inflammatory response is considered as a warranted step of the wound healing process associated with the generation of NO radicals which plays important role in human physiology as a regulator of wound healing [25]. Accordingly, the current study exhibited potential inhibition of the nitric oxide production in granulation tissue of TPLE and TPFE treatments. The collective evidence can be hypothesized that the blockade of NO synthesis contributes to the regulation of inflammation response and may be favorable for the collagen formation [4]. It is interesting to note that in the current study a negative correlation has been seen between the LPO and % NO inhibition, with a Pearson correlation coefficient of -0.76199 (Figure 2B) exhibiting potential antioxident ability. Extending, the anti-inflammatory potential displayed as reduced paw edema and ESR level in carrageenan-induced model portrayed the ability of the extracts hindering excessive inflammatory stimuli, affirming the strong anti-inflammatory property which makes *T. peruviana* an ideal candidate for the management of inflammatory responces.

Similarly, our previous reports too suggest the strong antioxidant and anti-inflammatory mediated wound healing activity of leaf hexane and fruit rind water extracts of *T. peruviana* [7]. The commendable antioxidant and anti-inflammatory mediated wound healing activity of *T. peruviana* is attributed to the presence of phytochemicals like triterpenoids, tannins, flavonoids and phenolics, similarly, the presence of the highest amount of carbohydrates in TPLE and TPFE [18] is definitely a strong reason for the manifestation of curative property, as an immunomodulator, encouraging the propagation of skin fibroblasts and keratinocytes [26]. As wound healing course comprises of many circles of biochemical processes which overlaps on one another, eventually balancing of them by one single compound doesn't seems logical, while effective healing rather instincts towards a cumulative response of more than one compound as established by the extracts of *T. peruviana*.

4. CONCLUSION

In conclusion, this study demonstrated the *T. peruviana* extracts accelerates the wound healing in excision, incision and dead space wound models, via managing the inflammation, microbial load and oxidative stress at the wound site. These healing effect of *T. peruviana* leaves and fruit rind ethanolic extracts authenticates *T. peruviana* usage in ayurvedic and other folkloric medicines in the restoration of the wound and recommends a novel therapeutic stratagem.

5. MATERIALS AND METHODS

5.1. Plant material collection

Leaves of *T. peruviana* were collected from the surroundings of Kuvempu University, Shankaraghatta, Shimoga Dist (13.5994° N, 75.8153° E)., Karnataka, India. Plant identification was carried out by referring Flora of the Presidency of Madras [27]. The plant was further authenticated by Prof. V. Krishna, Taxonomist, Dept. of Biotechnology, Kuvempu University. The specimen was deposited at the Department of Biotechnology (BTRM 014). The name of the plant appears in www.theplantlist.org as *Thevetia peruviana* (Pers.) K.Schum.

5.2. Soxhlet extraction

The leaf and fruit rind of *T. peruviana* were shade dried and pulverized mechanically (sieve no. 10/44). Successive extraction was done using 500 g of powdered leaf and fruit rind material in soxhlet apparatus. The solvents hexane (TPLH and TPFH) (2L, 50 °C ~ 15 cycles), chloroform (TPLC and TPFC) (2 L, 45 °C ~15 cycles) and ethanol (TPLE and TPFE) (2 L, 70 °C, ~15–17 cycles) were used. All the extracts were concentrated *in vacuum*, the yield of dried extract was calculated. Based on the highest yield ethanol extracts of leaf and fruit rind were selected for the further estimations.

5.3. Animals

Male Wister albino rats weighing 150-230 g were utilized, 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).

5.4. *In vitro* antioxidant evaluation

The present study was designed to evaluate the *in vitro* antioxidant activity of ethanolic extracts of *T. peruviana* leaf and fruit rind at concentration gradient of 25, 50, 75, 100 and 125 µg/ml by following standerd method for the assays like nitric oxide radical (NO•) scavenging, hydroxyl radical (HO•) scavenging, superoxide anion scavenging [28], DPPH free radical scavenging potential [29], total antioxidant capacity [30], ferric reducing the ability of plasma (FRAP) [31] and lipid peroxide inhibiting assay [32].

5.5. Acute toxicity studies

The acute toxicity study was conducted according to the Organisation of Economic Co-operation and Development (OECD) guideline 420 (2001). The rats were fasted overnight but with free access to water. A single dose of 2000 mg/kg body weight of TPLE and TPFE was orally gavaged to three groups (n=6) of male Wistar rats. The rats in the control group (n = 6) were fed with 1% DMSO as a vehicle. All of the experimental animals were upheld under keen observation for any ciphers of toxicity and mortality for 14 days. After 14 days the animals were sacrificed blood and liver, kidney and lungs were collected for the further analysis.

5.5.1. Evaluation of serum and tissue-specific markers

Valuation of serum nonspecific marker enzymes ALP, γ-GT, ALT, serum albumin, bilirubin, urea, total creatinine and total protein were analysed in blood samples using automatic Robonic prietest Touch biochemistry analyser and standard kits, procured from Robonic (India) Pvt. Ltd., Mumbai, India.

5.6. Anti-inflammatory activity

Rats were divided into four groups of six animals each. The animals were fasted overnight prior to the start of the experiment with water ad libitum. The first control group received distilled water (10 ml/kg, p.o.), while the second group was treated with standard drug indomethacin (10 mg/kg, p.o.). Group 3 and 4 were administered with the extracts TPLE and TPFE (200 mg/kg body weight, p.o.). The animals were pre-treated

1 h before the injection of carrageenan for the proper absorption of the drug in the body. Acute inflammation was produced by the subplantar injection of 0.1 ml of 1% carrageenan in the right hind paw of the rats. The thickness (mm) of the paw was measured immediately at 30, 60, 120 and 240 min interval by using digital vernier caliper [33]. The percentage of inflammation remained was calculated by using the formula:

$$\% \text{ inflammation remained} = \frac{\text{Pt}A - \text{Pt}B}{\text{Pt}A} \times 100$$

The percentage inflammation inhibition was calculated by the formula:

$$\% \text{ inflammation inhibition} = \frac{\text{Pt}C - \text{Pt}A}{\text{Pt}C} \times 100$$

Where Pt*A* is the Paw thickness at 240 min, Pt*B* is normal paw thickness, PtC is paw thickness of control at 240 min of carrageenan injection.

5.6.1. Determination of erythrocyte sedimentation rate (ESR)

At the end of the carrageenan-induced paw edema experiment, the animals were sacrificed by cervical decapitation blood was collected and used for erythrocyte sedimentation rate analysis by Westergren's method [34].

5.7. Wound healing activity

5.7.1. Incision wound model

The wound healing potentials of *T. peruviana* extract was evaluated by ıncision wound model and wound breaking strength on the eleventh day [7]. Twenty four rats weighing 150-190 g were equally dispersed into four groups of six animals each group1 as control (ointment base), group 2 was positive control (povidone-iodine) and group 3 and 4 were treated with TPLE and TPFE (5% w/w ointments) respectively. 6 cm paravertebral incisions were made in anesthetized rats (ketamine, 30 mg/kg, i.p.). Sutures distancing 1 cm were made throughout the wound, to that topical application of extract ointment was carried out for 10 days. On the seventh post-wounding day, sutures were evacuated. The WBS is designated as the minimum weight (in grams) required to drag apart the wound edges.

5.7.2. Excision wound model

Twenty four animals were evenly distributed into four groups of six animals each to study the excision wound model, as group 1 named as control (untreated), group 2 was povidone–iodine and group 3 and 4 were TPLE and TPFE extracts (5% w/w ointment). Ketamine (30 mg/kg, i.p.) was used to anesthetize rats, followed by crafting about 500 mm^2 circular wound on the dorsal thoracic region. The rate of wound contraction was measured using graph paper on alternate days until complete wound healing [7]. The rate of contraction was calculated as : $\% \text{ wound contraction} = \frac{\text{Healed area}}{\text{Initial wound area}} \times 100$

Where Healed area = initial wound area - remaining wound area.

5.7.3. Dead space wound model

The animals were divided into four groups of six animals and the grouping for dead space wound model experiment was as follows: control (1 ml/kg of 1% gum acacia p.o.), positive control (vitamin E (VTE) 200 mg/kg b. w. p.o.), TPLE and TPFE extract treated groups (200 mg/kg b. w. p.o.). About 1 cm incision was made on both the dorsal paravertebral sides of ketamine (30 mg/kg, i.p.) anesthetized rats, followed by grafting of sterilized cylindrical grass piths (2.5 cm x 0.3 cm) wounds were sealed with sutures [28] and treated orally up to 10 days. The animals were euthanized on the tenth post wounding day after six hours of the interval of the last dose, granulation tissue around the implanted piths was carefully removed, and processed for the biochemical and histological examinations [7].

5.7.4. Estimation of antioxidant enzymes and inflammatory marker of granulation tissue

Wet granulation tissue homogenate was assessed for antioxidants enzymes such as SOD, catalase, GSH, peroxidase, LPO and the percent NO inhibition [7].

5.7.5. Estimation of connective tissue parameters

1 ml of 6 N HCl was added to hydrolyze the 40 mg of dried granulation tissue (50 °C for 24 h) and kept at bubbling water bath for 24 h (12 h/day for two days). 10 N NaOH phenolphthalein as an indicator was used to neutralize the excessive acid. The neutral hydrolysate was diluted to 20 mg/ml with distilled water and utilized for the hydroxyproline, hexosamine, and hexuronic acid estimation [7].

5.7.6. Histopathological observations

The histological changes in hematoxylin and eosin stained dead space tissues was examined for mononuclear inflammatory cells, multiplying vasculature, fibrosis characterized by scattered fibroblasts, neovascularization, and eosinophilic collagen tissue.

5.8. 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 were used to determine the level of significance among the various treatments.

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