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Original Article

A Prospective Experimental Study to Evaluate the Wound-Healing Potential of Citrus limon in Diabetic Ulcers in Sprague Dawley Rats

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ABSTRACT

Introduction: Diabetic wounds, particularly lower-limb ulcers, are a major complication of diabetes mellitus and are often associated with delayed healing. Despite multiple treatment options, no standardized therapy ensures optimal outcomes. *Citrus limon* peel extract has shown potential wound-healing properties in experimental studies.

Materials and Methods: Diabetes was induced in Sprague Dawley rats using streptozotocin (55 mg/kg, intraperitoneal), followed by excision wound creation. Animals (n = 30) were divided into five groups: normal control, diseased control, standard (silver nitrate), *C. limon* low dose (4%), and high dose (8%). Treatments were applied topically for 21 days. Parameters assessed included wound diameter, body weight, hematological and biochemical indices, antioxidant enzyme levels, and histopathology.

Results: Topical *C. limon* extract significantly enhanced wound contraction, improved antioxidant status (SOD, catalase, glutathione), and reduced lipid peroxidation compared to the diseased group. Treated groups also showed improved hematological and biochemical parameters, along with enhanced collagen deposition and tissue regeneration on histological evaluation. The low-dose formulation demonstrated effects comparable to the standard treatment.

Conclusion: *Citrus limon* peel extract promotes wound healing in diabetic rats through antioxidant and tissue-regenerative mechanisms. The low-dose formulation showed optimal efficacy, suggesting its potential as a safe and cost-effective therapeutic option for diabetic wound management.

Keywords*: Diabetic wound healing; Citrus limon; Streptozotocin-induced diabetes; Antioxidant activity; Excision wound model; Phytochemicals; Collagen synthesis; Sprague Dawley rats.*

INTRODUCTION

Diabetes mellitus (DM) is a chronic endocrine disorder characterized by persistently elevated blood glucose levels. Common clinical manifestations include polyuria, polydipsia, and polyphagia. When inadequately managed, diabetes can lead to acute complications such as diabetic ketoacidosis and hyperosmolar hyperglycemic state, as well as long-term complications including cardiovascular disease, stroke, nephropathy, foot ulcers, and retinopathy[1].

A wound is defined as a disruption in the normal anatomical structure and function of tissue. Clinically, wounds are classified as acute or chronic based on their duration and healing characteristics[2]. Acute wounds typically follow a predictable and orderly healing process, resulting in complete restoration of structure and function. In contrast, chronic wounds fail to progress through the normal stages of healing in a timely manner[3]. Disruption in any phase—haemostasis, inflammation, proliferation, or remodelling—can impair healing. In diabetic patients, defective glucose metabolism leads

to persistent hyperglycaemia, which significantly compromises wound repair and often results in chronic non-healing wounds. The global incidence of delayed wound healing among diabetic individuals continues to increase due to inadequate preventive and control strategies[4].

Diabetic wounds (DWs), particularly lower-limb and foot ulcers, are among the most serious complications of diabetes. The condition adversely affects all phases of wound healing—haemostasis, inflammation, proliferation, and remodelling—leading to delayed or incomplete repair. These wounds significantly reduce quality of life and contribute to increased morbidity and mortality[1]. Multiple metabolic and vascular abnormalities contribute to impaired wound healing in diabetes. Sorbitol, a toxic by-product of glucose metabolism, accumulates in tissues and contributes to renal, ocular, and vascular complications. Additionally, increased dermal vascular permeability leads to pericapillary albumin deposition, which impairs oxygen and nutrient diffusion to the wound site[5].

Treatment primarily focuses on symptomatic management, including protection of the exposed viable dermis, reduction of infection risk, minimization of pigmentation changes and scarring, and promotion of re-epithelialization—these constitute the fundamental principles of wound care.[6] Several clinical trials have evaluated therapies for diabetic wounds, including topical betulin gel[7], negative pressure wound therapy[8], honey-based dressings, royal jelly[9], Manuka honey-impregnated dressings[10], Dragon's blood cream[11], extracorporeal shock wave therapy, and autologous platelet-rich plasma gel. Despite these advances, a universally accepted standardized treatment protocol for diabetic wound healing is still lacking.

Therefore, the present study was designed to evaluate the wound-healing efficacy of *Citrus limon* in a diabetic wound model using Sprague Dawley rats.

MATERIALS AND METHODS

Study Design and Experimental Animals

The present study evaluated the wound-healing efficacy of a topical *Citrus limon* formulation in experimentally induced diabetic ulcers in adult Sprague Dawley (SD) rats. A total of 30 healthy male SD rats (weighing 180–250 g) were included and randomly divided into five groups (n = 6 per group): Normal Control, Diseased Control, Standard (Silver Nitrate), *C. limon* Low Dose (CL-LD), and *C. limon* High Dose (CL-HD). The experimental protocol was approved by the Institutional Animal Ethics Committee (Reg. No. 1201/PO/Re/S/08/CPCSEA). Animals were procured from the National Institute of Pharmaceutical Education and Research (NIPER), Mohali, following protocol approval (Reg. No. CCP/IAEC/Feb2022/8). All animals were housed under standard laboratory conditions at the animal facility of Chandigarh College of Pharmacy, Landran, Mohali, with free access to food and water.

Plant Material Collection and Authentication

Fresh fruits of *Citrus limon* were procured from Adda Market, Nangal Township, Punjab, India. The peels were washed thoroughly, separated, cut into small pieces, shade-dried, and pulverized into coarse powder using an electric blender. Botanical authentication of the plant material was performed at NIPER, Mohali (Authentication No. NIP-M-525).

Pharmacognostic Standardization

Standardization of the plant material was carried out according to Indian Pharmacopoeia (1996) guidelines. Parameters evaluated included moisture content, total ash, acid-insoluble ash, water-soluble ash, extractive values, foaming index, and swelling index (WHO, 2011). The extract was concentrated under reduced pressure at 40–50°C, and the percentage yield was calculated.

Phytochemical Screening and TLC Analysis

Preliminary phytochemical screening was performed to qualitatively identify bioactive constituents such as alkaloids[12], glycosides, saponins[2], flavonoids[13], tannins, steroids, proteins, amino acids, phenols, and reducing sugars using standard chemical tests. Thin Layer Chromatography (TLC) was carried out for component separation using the following solvent systems:

- Chloroform: Ethyl acetate (6:4)
- Petroleum ether: Ethyl acetate (6:4)

Rf values were recorded for characterization of phytoconstituents[4].

Induction of Diabetes

Diabetes was induced in overnight-fasted rats by intraperitoneal administration of streptozotocin (STZ) at a dose of 55 mg/kg, dissolved in citrate buffer (pH 4.5). After 72 hours, fasting blood glucose levels were measured from the tail vein. Rats with blood glucose levels ≥250 mg/dL were considered diabetic and included in the study.[14]

Excision Wound Model
Using a sterile biopsy punch, a circular full-thickness excision wound of approximately 6 mm² diameter and 0.2 mm depth was created on the dorsal surface of 25 diabetic rats under aseptic conditions. These animals were subsequently used to evaluate wound-healing activity using different topical formulations.

Preparation and Application of Formulations
Topical formulations of *C. limon* peel extract were prepared in two concentrations:

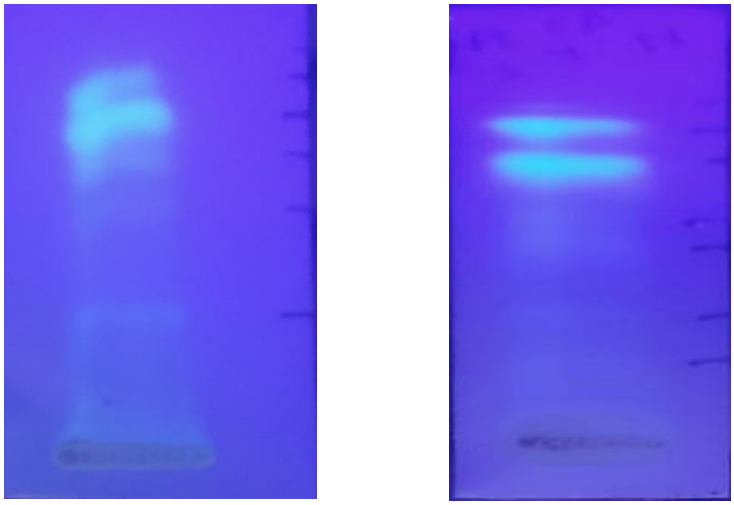
- CL-LD (4%)
- CL-HD (8%)

Silver nitrate was used as the standard treatment. All formulations were applied topically once daily for a period of 21 days.

Statistical Analysis
All data were expressed as Mean ± Standard Error of Mean (SEM) (n = 6). Statistical significance was determined using appropriate analytical methods, and *p* values < 0.05 were considered statistically significant.

RESULTS
The percentage yield of *C. limon* peel extract was found to be 3.96%, obtained from 120 g of semi-solid extract prepared from 3027 g of plant material. Air-dried peel material was used for pharmacognostic evaluation. The moisture content was 12.05 ± 0.1686, total ash 15.53 ±0.0352, acid-insoluble ash 5.29 ± 0.0491, water-soluble ash 4.72 ± 0.1568, extractive value 10.49±0.3334, swelling index 13.33 ±0.8819, and foaming index 250.

Qualitative phytochemical identification was performed using Thin Layer Chromatography (TLC) based on characteristic color development and retention factor (Rf) values. The Rf values for C limon peel extract using chloroform:ethyl acetate (6:4) were 0.909, 0.818, 0.590, and 0.363, while petroleum ether ethyl acetate (6:4) yielded Rf values of 0.923, 0.846, 0.666, 0.589, 0.384, and 0.256 as depicted in **Figure 1**.



Solvent System 1 Chloroform: EA (6:4) Solvent System 2 PE: EA (6:4)
Figure 1: TLC of hydroalcoholic peel extract of *C. limon*

Herbal creams formulated using *C. limon* peel extract exhibited smooth semisolid consistency, good homogeneity, and were easily washable with water. The formulations demonstrated satisfactory spreadability (0.223 for low dose and 0.120 for high dose), viscosity (11,600 cP) for hydroalcoholic peel extract of *C. limon*.

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In-Vivo Diabetic Wound Healing Activity
The wound-healing study was conducted using five groups (Normal Control, Diseased, Silver Nitrate (Standard), CL-LD, and CL-HD), with six rats per group. Initial and final body weights were recorded, and organ weights (brain, heart, liver, and kidneys) were measured at sacrifice.

STZ administration caused a significant reduction in body weight in the diseased group (31.67±3.073 g), whereas treatment with *C. limon* formulations effectively prevented weight loss, comparable to the standard silver nitrate group

Table 1.

Table 1: Change in Body Weights

Group	Initial body weight (g)	Final body weight (g)	Change in body weight (g)
Normal Control	193.3±6.667	215.0±6.708	$21.67±3.073^{ns}$
Diseased	201.7±3.073	170.0±5.774	$31.67±3.073^{ns}$
Standard(Std)	195.0±6.455	217.0±6.292	$22.00±4.082^{ns}$
CL LD	204.0±5.099	228.8±5.099	$24.80±3.162^{ns}$
CL HD	198.3±3.073	226.5±5.164	$28.21±3.073^{ns}$

Legend: Values are expressed as Mean ± SEM (Standard Error of Mean), n = 6.
CL-LD: *Citrus limon* Low Dose (4%); CL-HD: *Citrus limon* High Dose (8%);
ns: non-significant.

On Day 21, wound diameter in the diseased group remained significantly larger (2.533 ±0.166 mm) compared to the standard group (1.200 ± 0.289 mm). Both *C. limon*-treated groups showed enhanced wound contraction, with CL-LD showing results comparable to the standard treatment. Delayed wound closure in the diseased group was evident throughout the study period **Table 2**.

Table 2 Wound diameter of different plant extracts

Day	Diseased	Standard	CL LD	CL HD
3	$5.000±0.000^{ns}$	$5.000±0.000^{ns}$	$5.000±0.000^{ns}$	$5.000±0.000^{ns}$
6	$4.000±0.000^{ns}$	$4.000±0.289^{ns}$	$4.600±0.244^{ns}$	$4.333±0.210^{ns}$
9	$3.500±0.223^{ns}$	$3.500±0.289^{ns}$	$4.000±0.000^{ns}$	$4.000±0.258^{ns}$
12	$3.000±0.000^{ns}$	$2.500±0.289^{ns}$	$3.000±0.000^{ns}$	$3.000±0.258^{ns}$
15	$3.000±0.000^{ns}$	$2.000±0.000^{ns}$	$2.000±0.000^{ns}$	$2.500±0.223^{ns}$
18	$2.833±0.210^{ns}$	$2.000±0.289^{ns}$	$1.900±0.210^{ns}$	$2.333±0.166^{ns}$
21	$2.533±0.166^{b}$	$1.200±0.289^{\beta}$	$1.780±0.244^{ns}$	$1.900±0.258^{ns}$

Legend: Values are expressed as Mean ± SEM, n = 6.
CL-LD: *Citrus limon* Low Dose; CL-HD: *Citrus limon* High Dose.

Superscripts (^{b}, $^{\beta}$) indicate statistical significance compared to diseased and standard groups, respectively; ns: non-significant.

Hematological and Biochemical Parameters
STZ-induced diabetes resulted in a marked reduction in hemoglobin and RBC count in the diseased group (Hb: 9,467 ± 0.095 g/dL; RBC: 3.185 ± 0.384 × 10°/µL) compared to normal controls. Treatment with CL-HD restored hematological parameters, closely matching the silver nitrate group. Elevated WBC and platelet counts observed in the diseased group were normalized following treatment, particularly in the CL-HD group, indicating reduced inflammation as depicted in **Table 3**. Similar results were identified in liver function tests and glycemic control as shown in **Table 4 and 5.**

Table3 Hematological Parameters among Different Groups in Diabetic Wound Healing Model

Parameter	Normal	Diseased	Standard	CL LD	CL HD
Haemoglobin (g/dl)	$13.48± 0.2810^{\alpha}$	$9.467± 0.09545^{m}$	$13.58± 0.3172^{ns}$	$12.38± 0.04773^{ns}$	$12.87± 0.2376^{ns}$
RBC Count $\times10^{6}$/µl	$6.065± 0.0866^{ns}$	$3.185± 0.3840^{ns}$	$6.095± 1.117^{ns}$	$5.004± 0.5467^{ns}$	$5.167± 0.1941^{ns}$
WBC Count $\times10^{3}$/µl	$4.717± 0.1833^{\alpha}$	$9.183± 0.1327^{m}$	$5.175± 0.8014^{ns}$	$8.040± 1.083^{ns}$	$6.717± 0.4833^{ns}$
MCV (fL)	$76.08± 3.752^{\delta c}$	$56.55± 0.3585^{pd}$	$69.25± 4.776^{o\delta}$	$66.82± 2.236^{p}$	$58.22± 1.026^{p\delta d}$
MCH (pg)	$21.48± 0.5885^{ns}$	$19.03± 0.1838^{ns}$	$20.28± 0.4328^{ns}$	$19.58± 0.7102^{ns}$	$19.60± 0.3435^{ns}$
MCHC (%)	$33.80± 0.3386^{\delta}$	$25.33± 0.3528^{pd}$	$33.48± 0.5865^{\delta}$	$30.70± 1.179^{\beta}$	$31.67± 0.6059^{\gamma}$
Platelet Count (In lacs)	$5.620± 0.1206^{ns}$	$6.277± 0.1198^{ns}$	$5.700± 0.7521^{ns}$	$3.882± 0.4444^{ns}$	$4.820± 0.7565^{ns}$

Legend: Values expressed as Mean ± SEM, n = 6.
RBC: Red Blood Cells; WBC: White Blood Cells;
MCV: Mean Corpuscular Volume;
MCH: Mean Corpuscular Hemoglobin;
MCHC: Mean Corpuscular Hemoglobin Concentration.

Table 4- LFT and Lipid Profile Parameters among Different Groups in Diabetic Wound Healing Model

Parameter	Normal	Diseased	Standard	CL LD	CL HD
Total bilirubin (mg/dL)	0.533 ± 0.0143^{ns}	1.293 ± 0.0217^{ns}	0.655 ± 0.0272^{ns}	0.858 ± 0.0274^{ns}	0.948 ± 0.011^{ns}
AST (U/L)	$53.35\pm1.252^{\delta d}$	271.9 ± 0.662^{pd}	$81.54\pm0.851^{p\delta}$	$97.69\pm1.174^{p\delta d}$	$98.18\pm0.704^{p\delta d}$
ALT (U/L)	$63.40\pm1.367^{\delta d}$	226.3 ± 0.956^{pd}	$83.23\pm1.831^{p\delta}$	$95.34\pm2.549^{p\delta d}$	$103.9\pm1.199^{p\delta d}$
Total cholesterol (mg/dL)	$91.45\pm0.2513^{\delta a}$	169.7 ± 0.5618^{pd}	$83.14\pm4.814^{m\delta}$	$58.24\pm0.5921^{p\delta d}$	$72.86\pm1.153^{p\delta c}$

Legend: Values expressed as Mean ± SEM, n = 6.
AST: Aspartate Aminotransferase;
ALT: Alanine Aminotransferase.

Table 5 HBA1C Parameter among Different Groups in Diabetic Wound Healing Model

Groups	HBA1C(%)
Normal	$4.630\pm0.137^{\delta}$
Diseased	8.017 ± 0.079^{pd}
Standard	$4.923\pm0.306^{\delta}$
CL LD	$5.440\pm0.492^{\delta}$
CL HD	$5.800\pm0.258^{n\delta}$

Legend: Values expressed as Mean ± SEM, n = 6.
HbA1c: Glycated Hemoglobin.

Antioxidant Enzyme and Oxidative Stress Markers
STZ-induced diabetes significantly reduced superoxide dismutase (SOD), catalase (CAT), and glutathione (GR) levels, while increasing lipid peroxidation (LPO) in all organs studied. CL-LD treatment significantly enhanced antioxidant enzyme activity and reduced lipid peroxidation levels, comparable to silver nitrate ($p < 0.0001$) as shown in **Tables 7**. These findings indicate strong antioxidant and free-radical scavenging effects of *C. limon* peel extract.

Table 7: Effect of Citrus limon Peel Extract on Antioxidant Enzymes and Lipid Peroxidation in Different Organs

Group	Brain				Heart				Kidney				Liver			
	SOD	CAT	GR	LPO	SOD	CAT	GR	LPO	SOD	CAT	GR	LPO	SOD	CAT	GR	LPO
NC	42.74	4.36	51.18	12.67	43.18	4.30	51.47	13.03	44.09	4.46	52.88	13.02	47.01	4.44	55.40	14.03
DC	19.58	2.40	20.61	22.36	20.36	2.09	20.16	25.98	20.16	2.09	22.79	25.98	21.33	2.31	23.61	23.42
STD	44.09	3.48	50.45	13.80	44.77	3.46	51.53	14.80	43.64	3.53	50.96	15.03	45.33	3.50	53.88	13.87
CL LD	34.44	3.28	43.70	16.60	35.18	3.18	43.60	16.34	34.92	3.16	44.05	17.33	35.03	3.31	45.08	19.28
CL HD	29.55	2.77	26.79	20.04	27.81	2.82	27.27	20.85	28.03	2.87	28.78	21.96	29.14	2.95	29.52	24.00

Legend:
SOD: Superoxide Dismutase;
CAT: Catalase;
GR/GSH: Reduced Glutathione;
LPO: Lipid Peroxidation;
NC: Normal Control; DC: Diseased Control; STD: Standard;
CL-LD: *Citrus limon* Low Dose; CL-HD: *Citrus limon* High Dose.

Histopathological Evaluation

Histological examination on Day 21 revealed excessive inflammatory cells, poor epithelial regeneration, and delayed healing in the diseased group. In contrast, *C. limon*-treated groups demonstrated enhanced re-epithelialization, increased granulation tissue formation, dense neovascularization, and reduced inflammation. CL-LD showed superior histological improvement compared to CL-HD and was comparable to the standard group, likely due to bioactive phytoconstituents promoting epithelial and endothelial cell proliferation as shown in **Figures 2-6**.

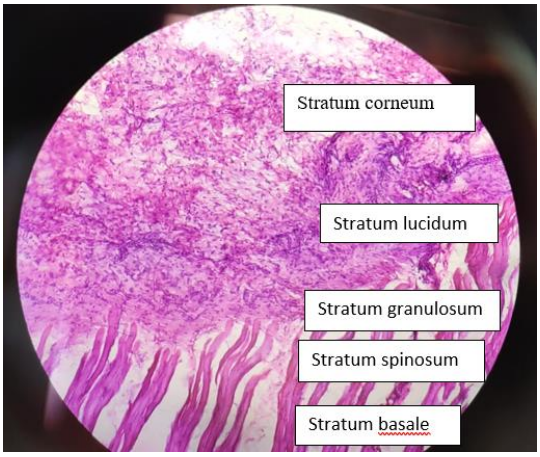

Fig 2 Normal Control

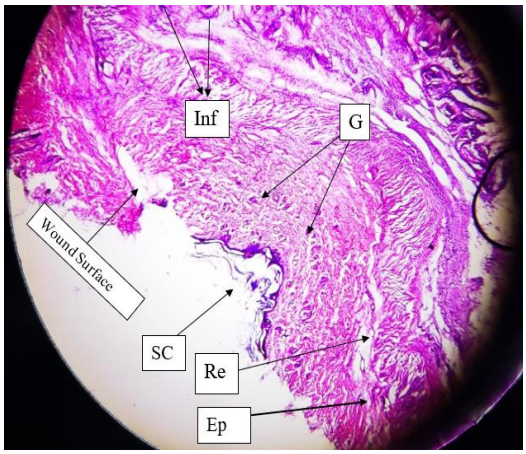

Fig 3 Diseased

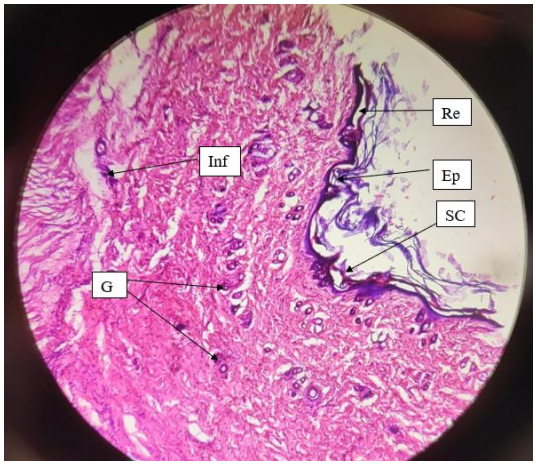

Fig 4 Standard Histology

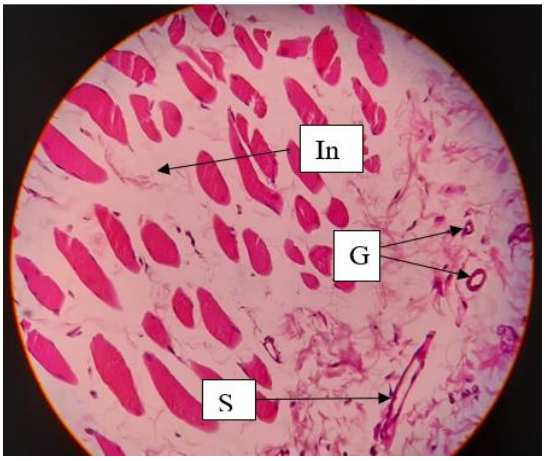

Fig 5CL LD (4%)

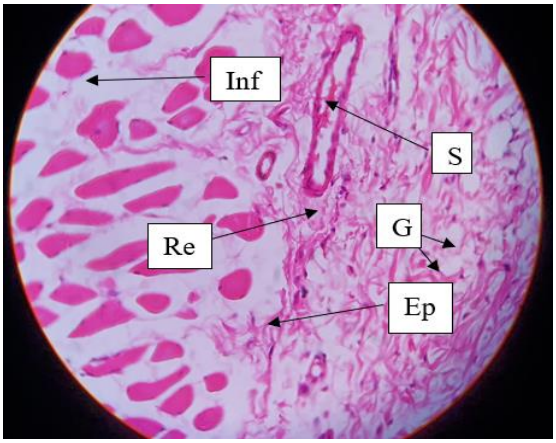

Fig 6 CL HD (8%)

Discussion & Conclusion

Diabetes is a well-established factor that adversely affects the normal programmed phases of wound healing through persistent hyperglycaemia, altered metabolism, and changes in body weight. Previous studies indicate that diabetic patients require special attention to wounds and ulcers due to impaired healing capacity. Delayed wound repair is a common complication of diabetes mellitus, as wounds are continuously exposed to the external environment and are

susceptible to microbial invasion, leading to excessive free radical generation. Alcoholic extracts of citrus peel have been reported to possess antimicrobial activity, which may contribute to accelerated wound healing by reducing microbial burden at the wound site.[15]

The findings of the present study demonstrate that *C. limon* peels contain a wide range of bioactive constituents, including flavonoids, alkaloids, tannins, triterpenoids, coumarins, carotenoids, saponins, and glycosides. Pharmacognostic standardization parameters such as ash values, extractive values, swelling index, and foaming index-confirmed the quality and purity of the plant material, and the extract was found to be non-toxic. In diabetic rats, delayed wound healing is associated with impaired fibroblast activity, resulting in reduced collagen(hydroxyproline) and protein synthesis within granulation tissue. Antioxidant compounds are known to activate fibroblasts, enhance collagen deposition, and promote extracellular matrix formation. Improved oxygen diffusion, increased lymphatic drainage, reduction in oxidative stress, and enhanced collagen synthesis collectively contribute to accelerated wound repair, consistent with the findings of the present study.[16]

Flavonoids and their derivatives play a crucial role in inhibiting lipid peroxidation by improving vascularity and preventing or slowing cellular necrosis.[17] Furthermore, vitamin C exhibits enhanced biological activity in the presence of flavonoids, as these compounds spare intracellular vitamin C and augment overall antioxidant capacity.[18] Citrus peel extracts rich in flavonoids and vitamins have therefore been widely recognized for their antioxidant, anti-inflammatory, and protective effects, particularly in diabetes-associated wound conditions.[19]

The principal finding of this study is that topical administration of *C. limon* peel extract significantly accelerates wound healing in diabetic rats with full-thickness excision wounds. The extract exhibited strong lipid peroxidation inhibitory activity, as reflected by reduced LPO levels in treated groups.[20] Defective wound repair observed in untreated diabetic rats was associated with decreased collagen and protein content, while treatment with *C. limon* extract restored these parameters. These results are consistent with earlier reports demonstrating the wound-healing efficacy of citrus peel extracts and with previous findings where oral administration of *C. limon* ethanol extract reduced blood glucose levels, shortened wound healing time, and enhanced tissue growth and collagen svnthesis in diabetic rats.[21]

The increased deposition of granulation tissue, reflected by elevated hydroxyproline and total protein content, may be attributed to improved oxygen and nutrient delivery following treatment. Overall, this study concludes that a topical cream formulated with hydroalcoholic extract of *C. limon*, particularly at the high dose, possesses significant wound-healing activity and demonstrates therapeutic efficacy comparable to the standard silver nitrate treatment in diabetic ulcer healing.

Limitations

Despite demonstrating promising findings, the present study has certain limitations that should be acknowledged. First, the study was conducted on a relatively small sample size (n = 30), which may limit the generalizability of the results. Second, the experimental model utilized streptozotocin-induced diabetes in rats, which, although widely accepted, may not fully replicate the complexity of chronic diabetic wounds in humans. Additionally, the study primarily focused on biochemical, antioxidant, and histopathological parameters without exploring detailed molecular mechanisms underlying the observed effects. Key pathways involved in wound healing, such as growth factor signaling, cytokine modulation, and gene expression related to angiogenesis and collagen synthesis, were not evaluated.

Furthermore, only two concentrations of *Citrus limon* extract were tested. A broader dose–response analysis could provide a more comprehensive understanding of the optimal therapeutic range. Long-term safety, stability of the formulation, and potential dermal toxicity with prolonged use were also not assessed.

Future Directions

Future research should focus on elucidating the molecular mechanisms underlying the wound-healing effects of *Citrus limon*, particularly its role in modulating inflammatory cytokines, growth factors (such as VEGF and TGF-β), and signaling pathways involved in tissue regeneration. Dose optimization studies involving a wider range of concentrations are warranted to establish the most effective and safe therapeutic dose. Additionally, formulation refinement, including incorporation into advanced delivery systems such as hydrogels, nanoparticles, or bioengineered dressings, may further enhance therapeutic efficacy.

Preclinical studies should be extended to larger animal models to improve translational relevance. Ultimately, well-designed randomized controlled clinical trials are necessary to evaluate the safety, efficacy, and applicability of *C. limon* formulations in human diabetic wound management.

CONCLUSION

The findings of the present study demonstrate that topical application of *Citrus limon* peel extract significantly enhances wound healing in streptozotocin-induced diabetic rats. The observed effects are likely mediated through multiple mechanisms, including antioxidant activity, reduction of lipid peroxidation, enhancement of collagen synthesis, and promotion of tissue regeneration. Among the tested formulations, the low-dose preparation (CL-LD, 4%) exhibited optimal therapeutic efficacy, showing results comparable to the standard treatment. The extract effectively improved wound contraction, restored antioxidant enzyme levels, normalized biochemical parameters, and enhanced histopathological outcomes.

These results highlight the potential of *Citrus limon* peel extract as a safe, cost-effective, and promising topical therapeutic agent for diabetic wound management. Further studies are required to validate these findings and facilitate translation into clinical practice.

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