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Chemical composition and wound healing potential of *Lindernia ruelliioides* (Colson) Pennell from Mizoram, IndiaC Lalthansangi, RK Lalremtluangi, Esther Lalhmingliani[✉]

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ABSTRACT

Objective: To explore the wound healing potential and chemical components of *Lindernia ruelliioides* from Mizoram, India.

Methods: Plant extraction was conducted by cold maceration using chloroform, ethanol, and distilled water as solvents. To guarantee the safety of the plant extract, an acute dermal toxicity test was conducted before the experiment. Antioxidant assays were performed. Excision and incision wound models were used to assess the wound healing activities, including wound closure rate, epithelialization period, and tensile strength. A histopathological examination was carried out.

Results: *Lindernia ruelliioides* contained bioactive compounds, such as mycolactone F, 1-*O*-octadec-9-enyl glycerol, reserpine, tetracosanoic acid, 2-*O*-caffeoylglucuronate and several others which are found to possess various pharmacological activities. Acute dermal toxicity evaluation showed that the doses were deemed safe. Ethanol extract of *Lindernia ruelliioides* (LREE) treatment significantly elevated glutathione, glutathione-S-transferase, and superoxide dismutase, and decreased malondialdehyde in a dose-dependent manner. It showed increased wound contraction rate, shorter epithelialization time, and elevated wound breaking strength in the LREE-treated group when compared with the control. In addition, the histological examination showed enhanced neovascularization, fibroblasts, and collagen in LREE-treated animals.

Conclusions: *Lindernia ruelliioides* exhibits remarkable effects on wound healing. The study validates the traditional use of this plant in Mizoram region as a wound-healing agent.

KEYWORDS: *Lindernia ruelliioides*; Ethanolic extract; LC-MS; Antioxidant; Wound healing

1. Introduction

A wound may be referred to as the loss or disruption of cellular

and anatomical or functional continuity of living tissue[1]. Although wound healing occurs naturally, an infection may defer the healing process[2]. A multitude of processes are involved in the complicated (yet ordered) phenomena of wound healing. The process includes parenchymal cell regeneration, connective tissue and parenchymal cell migration and proliferation, extracellular matrix protein synthesis, connective tissue remodeling, and wound strength acquisition[3]. Alterations to any of these phases may result in a delay in healing or perhaps the incapacity to recover fully[4]. Despite their imprecise representation of human wound healing processes and associated clinical difficulties, animal wound healing models remain vital resources for the achievement of novel tactics and methods for therapy of wound healing[5]. In light of their antioxidant

Summary

Question: Is *Lindernia ruelliioides* a potential wound-healing candidate?

Findings: The present study involved excision and incision wound models. The wound closure rate was accelerated and tensile strength was increased, while epithelialization period was reduced in the extract-treated group in comparison with the control group.

Meaning: *Lindernia ruelliioides* could be used as a wound healing agent.

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properties and ability to stimulate fibroblast growth, plants have long been used to treat wounds and recent years have acquainted to uncover therapies that are less detrimental to the human body with ample evidence indicating the enormous efficacy of medicinal plants in several traditional systems[6].

Given this context, our attention turns to *Lindernia ruelliioides* (*L. ruelliioides*), belonging to the Linderniaceae family. It is an erect annual herb frequently seen in damp roadside and semi-shaded areas[7]. Traditionally, it was used for detoxification, treating injuries, cuts, wounds, bruises, and so on[8,9]. Popularly known as “Thasuih” among the natives of Mizoram, *L. ruelliioides* holds a rich traditional history in soothing sciatica, rheumatism, cuts, and wounds[10]. Regardless of the extensive use and compelling evidence of traditional medicine in Mizoram, there is currently insufficient scientific evidence to substantiate its pharmacological qualities. Our expected outcome will validate not just the traditional assertions but also enlighten the mechanisms that accelerate wound healing, which might be a stepping stone in the identification of novel therapeutic drugs in the future.

2. Materials and methods

2.1. Chemicals and reagents

Nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide, glutathione (GSH), phenazine methosulfate, phosphate buffered saline, and *n*-butanol reagent were obtained from HiMedia Laboratories Pvt., Ltd. (Mumbai, India). Hydrochloric acid, povidine iodine (PVI), chloroform and ethanol were procured from the local store/pharmacy. Thiobarbituric acid, 1-chloro-2,4 dinitrobenzene, 5, 5' dithio 2-nitrobenzoic acid, and trichloroacetic acid were obtained from Sigma Aldrich Inc (Louis, Germany).

2.2. Collection of plant material and preparation of extracts

L. ruelliioides was garnered from Reiek, Aizawl district, Mizoram, India (23° 41' 26" N; 92° 36' 19" E) during the period of June-August. Authentication of the plant was done at Botanical Survey of India, Eastern Circle, Shillong and herbarium sheet was submitted to Natural History Museum, Mizoram University with accession number NHMM-P/000160. After a thorough cleaning, it was carefully washed and preserved at room temperature to dry in the shade and subsequently pulverized. Next, it underwent a series of cold maceration in continuous agitation for 72 h using a variety of solvents, including chloroform, ethanol, and distilled water. After that, the extracts in liquid form underwent filtration, dried in an oven, and kept at 4 °C. Hereafter, the extracts using chloroform, ethanol, and distilled water as a solvent were referred to as LRCE,

LREE, and LRAE respectively.

2.3. Liquid chromatography–mass spectrometry (LC–MS) analysis

To identify the bioactive secondary metabolites in the ethanolic, chloroform, and aqueous extracts of *L. ruelliioides*, liquid chromatography combined with high-resolution mass spectrometry was employed. For the analysis, a triple quadrupole tandem mass spectrometer (ACQ-TQD-QBB1152, Waters acuity PDA detector, Waters Corporation, Milford, MA, USA) with an orthogonal ESI source was connected to an ACCUCORE HPLC (C18, 150 µm× 2.1 µm×1.7 µm) system. Mass acquisition spectra of every single extract have been recorded at wavelengths ranging from 150 to 2000 *m/z*. For both ES⁺ and ES[−], the source temperature was set at 120 °C and the desolvation temperature at 350 °C. The eluents in a linear gradient flow were mobile phases A (water and 0.1% formic acid) and B (mixed acetonitrile and 0.1% formic acid). Throughout the gradient, the amount to be injected was established at 5 µL, and the flow rate was maintained at 0.3 mL/min. OpenLynx™ and MassLynx™ (Agilent Technologies, India Pvt. Ltd.) were used to analyze the data.

2.4. Experimental rats

Healthy inbred Wistar albino rats (male) having a body weight between 180–200 g were employed. Each one was housed in a propylene cage at (23±2) °C and a cycle of twelve hours of light and darkness. They were well nourished and provided with water *ad libitum*. Ketamine hydrochloride (10 mg/kg) and xylazine (7:1 ratio) were used to anesthetize the animals before and during the surgical procedures, which were performed in sterile environments. Animals were continuously monitored for infections, and those that displayed symptoms were replaced and removed from the trial.

2.5. Ethical approval

The study was approved by the ethics committee for animal experimentation with clearance No: MZU/ZOOL/IAEC/2019-20/01. The study adhered to OECD guidelines No. 402[11].

2.6. Acute dermal toxicity test

Rats were divided into four groups (*n*=6). From the LC-MS study and from our preliminary work, LREE was found to be the most potent among the three extracts and therefore selected for the *in vivo* studies[12]. For acute dermal toxicity test, 5% LREE, 10% LREE, and 15% LREE ointments were applied on the freshly shaved back of the rats. Simple ointment was applied to the control group.

Animals were observed frequently for signs of acute toxicity during the first day and then once daily for 14 d.

2.7. Antioxidant assays

Animals were divided into four groups containing six animals each, where Group 1 was considered as negative control and received simple ointment, Group 2 was considered as positive control and received 5% PVI, Group 3 and 4 were the experimental groups and received 5% LREE and 10% LREE, respectively.

Wet granulation tissue homogenates were used to estimate GSH, glutathione-S-transferase (GST), superoxide dismutase (SOD), and malondialdehyde (MDA). A glass homogenizer (10% *w/v*) (ABGIL, India) in phosphate buffered saline (phosphate buffered saline, pH 7) at 4 °C was used to homogenize the granulation tissues. After centrifuging the homogenates for half an hour at 12000 rpm and 4 °C, the supernatants were used to test for antioxidant activity[13].

2.7.1. GSH

A total of 900 µL of sodium phosphate buffer at 0.02 mol/L, 20 µL of 10 mmol/L 5,5'-dithiobis(2-nitrobenzoic acid), and 80 µL of the cell homogenate were mixed, and the resulting product was allowed to sit at room temperature for 2 min. A UV-visible spectrophotometer (Thermo Fisher Scientific, India) was used to measure the sample's absorbance at 412 nm in relation to a blank. Using a standard graph, the GSH concentration was expressed in µmol/mg protein[14].

2.7.2. GST

The standard methods were used to assay GST[15]. A total of 50 µL of 1-chloro-2,4-dinitrobenzene at 20 mmol/L was mixed with 850 µL of 0.1 mol/L phosphate buffer (pH 6.5). The mixture was incubated at 37 °C for 10 min, and then supplemented with 50 µL of cell homogenate and 20 mmol/L GSH. The absorbance of the sample was assessed at 340 nm and the activity was expressed in unit/mg of total protein.

2.7.3. SOD

A standard approach was used to determine the SOD activity[16]. In brief, 300 µL of 3.0 mmol/L NBT and 200 µL of 780 µmol/L nicotinamide adenine dinucleotide were mixed with 100 µL of cell homogenate and 186 µmol/L phenazine methosulfate. To halt the reaction, after 90 seconds of incubation at 30 °C, 1 mL of acetic acid and 4 mL of *n*-butanol were added. The impact of the enzyme was quantified in units (1U = 50% inhibition of NBT reduction)/mg protein, and the absorbance of the sample and blank was measured at 560 nm.

Inhibition % = [(OD of blank – OD of test sample)/OD of blank] × 100

SOD unit = 1/50 × % inhibition.

2.7.4. MDA

MDA was assessed by following the standard method[17]. A 1:2 mixture of 10% trichloroacetic acid, 0.8% thiobarbituric acid, and 0.02 mol/L hydrochloric acid was mixed with cell homogenate. After 10 min of boiling, the liquid was promptly allowed to cool at ambient temperature and then centrifuged for 10 min at 1000 rpm. The absorbance at 535 nm was measured in contrast to a blank. The quantity of MDA in the specimen was determined using the extinction coefficient of 1.56×10^{-6} mol/L/cm.

2.8. Wound healing activity

Excision and incision wound models were used to assess the ability of LREE to promote healing of wounds. Animals were divided into four groups of six animals: Group 1 (negative control) animals received applications using a simple ointment base from polyethylene glycol. Animals of Group 2 (positive control) were topically treated with 5% PVI. Groups 3 and 4 were treated with 5% *w/w* and 10% *w/w* LREE in ointment, respectively. The wound closure rate, the duration of epithelialization, and the strength of the wound breaking were used to evaluate the impact of LREE on wound healing.

2.8.1. Excision wound model

After the animals were anesthetized, excision wounds were induced by the protocol given by Morton and Malone[18] and recommended by Kamath *et al.*[19]. The dorsal fur of the animal was meticulously shaved using a medical blade and electric clipper. The intended wound area was marked on the animal's back. Together with the markings, a surgical blade was used to make a full-thickness excision wound that was 300 mm² in diameter and 2 mm deep.

2.8.2. Measurement of wound area

Using translucent paper and graph paper, the wound region was measured on days 3, 6, 9, 12, 15, 18, and 21 post-wounding to ascertain the degree of contraction in the wound. The wound area was then estimated. Day 0 was considered the wounding day. The percentage of the decrease in wound area was calculated as follows[20].

% Wound contraction = [(Area of initial wound – Area of wound for a specific day) / Area of initial wound] × 100

2.8.3. Period of epithelialization

The duration of epithelialization was defined as the amount of time (in days) needed for the eschar to snap off leaving no raw wounds behind[21].

2.8.4. Incision wound model

An incision wound was inflicted as described by Ehrlich and

Hunt[22]. Using a medical blade and an electric clipper, the anesthetized animal's dorsal hair was shaven. A 6-cm longitudinal paravertebral incision was created along the cutaneous muscle and skin of the back. Using a curved needle and a surgical thread, surgical sutures were firmly and constantly placed on the separated skin at 1 cm intervals. The wounds were not dressed. Eight days after the injury, the sutures were taken out, and rats were treated as above mentioned.

2.8.5. Determination of tensile strength

On the 10th day, the tensile strength of the injured skin was assessed following the methods given by Lee[23]. On either side of the wound, a line extending approximately 3 mm from the suture line was drawn, and the sedated animal was fastened to the table. A pair of forceps were used to grasp the line from both sides of the suture. While the other end of the forceps was attached to a lightweight measuring jar that was free to hang, the other end was securely held in place. Water was gradually and steadily introduced until the wound started to open up. The water addition was halted as soon as the wound gaped. As a measurement of breaking strength in grams, the weight of water was recorded. The mean of three readings for a particular incision wound was used to calculate each breaking strength[23].

2.9. Histopathological examination

Histopathological examination was done following Bancroft and Gamble[24]. Cross-section full-thickness skin tissues were taken from each group to assess histological changes. Samples were fixed/preserved in buffered formalin (10%). Following a range of ethanol solutions with different grades (70%, 90%, and 100%) for dehydration, the tissues were cleared with xylene before being encased with paraffin wax and then divided into 5 µm thick portions. Poly-L-lysine was applied to ensure adequate adhesion

and tissue sections were gently spread in a water bath at 43 °C. The slides were deparaffinized using xylene and then rehydrated using a gradual sequence of ethanol concentrations for 10 min at each level. Hematoxylin and eosin were used to stain. To enhance clarity, the slides were treated with xylene in the final steps before being mounted with dibutylphthalate polystyrene xylene. The slides were observed under a Nikon E200 microscope (JJM Instruments, New Delhi, India).

2.10. Statistical analysis

SPSS software version 27 and Graph Pad Prism version 7 were utilized. The data were expressed as mean ± standard error mean. One-way analysis of variance (ANOVA) followed by Tukey multiple comparisons of means was performed to test significant variations. The *P*-value was deemed statistically significant if it was less than 0.05.

3. Results

3.1. LC-MS analysis of *L. ruelliioides*

LC-MS chromatogram of LRCE, LREE, and LRAE revealed several bioactive compounds having various pharmacological activities. Mycolactone F was found in all three extracts. *L*-cysteine, 4-hydroxybenzoate and tetracosanoic acid were found to be present in LREE and LRAE (Supplementary Tables 1-3).

3.2. Dermal acute toxicity

No animal died during the study period. No changes were observed in the eyes, fur, respiratory, circulatory, autonomic, or central nervous systems, or in somatomotor action or trends of behavior.

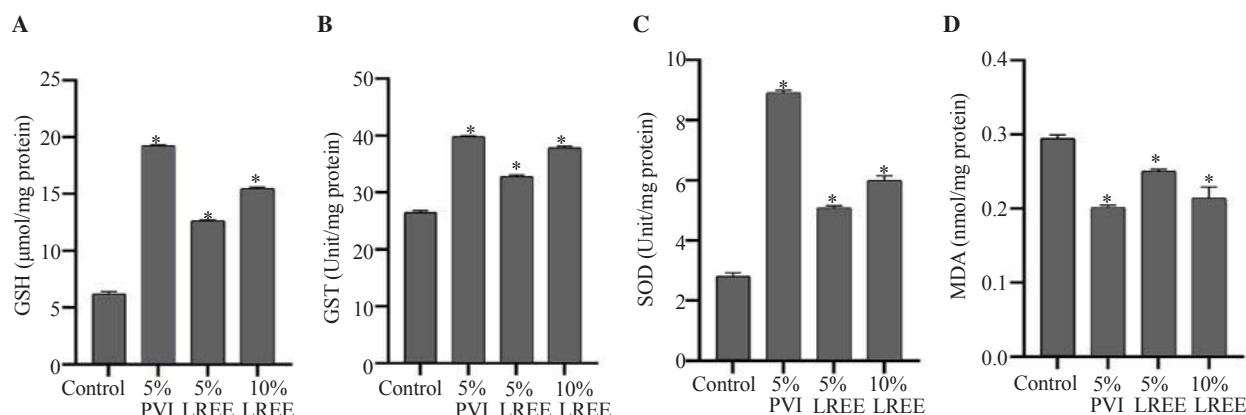


Figure 1. Impacts of *Lindernia ruelliioides* (*L. ruelliioides*) ethanolic extract (LREE) on (A) glutathione level (GSH); (B) glutathione-S-transferase activity (GST); (C) superoxide dismutase (SOD); (D) malondialdehyde (MDA). **P*<0.05 compared with the control group. PVI: povidine iodine.

Considering there was no discernible harm at the levels that were stipulated, the doses were deemed safe.

3.3. Antioxidant activity

LREE treatment significantly elevated GSH, GST, and SOD, and decreased MDA in a dose-dependent manner (Figure 1) in contrast to the control group.

3.4. Wound area change

Figures 2-3 and Supplementary Table 4 display the wound area on days 3, 6, 9, 12, 15, 18, and 21. A significant rise in % wound contraction was observed from the 6th day in *L. ruelliioides* extracts-treated animals in comparison with the control group ($P<0.05$). On day 21, the reduction of wound area in *L. ruelliioides* administered animals was 98.91% and 97.57% for 10% and 5% LREE,

respectively, and the differences were significant when compared with the control group (95.98%, $P<0.05$).

3.5. Period of epithelialization

The period of epithelialization was significantly ($P<0.001$) shorter for 10% LREE (16.6 ± 0.2) and 5% LREE (18.1 ± 0.5) as compared to control (23.4 ± 0.6). The effect of 10% LREE was comparable to 5% PVI (Figure 4).

3.6. Wound breaking strength

Both 10% LREE and 5% LREE showed higher wound breaking strength [(478.72 ± 2.36) g and (412.00 ± 0.96) g, respectively] compared with control group [(290.44 ± 3.38) g] ($P<0.001$). The effect of 10% LREE was comparable to 5% PVI (Figure 5).

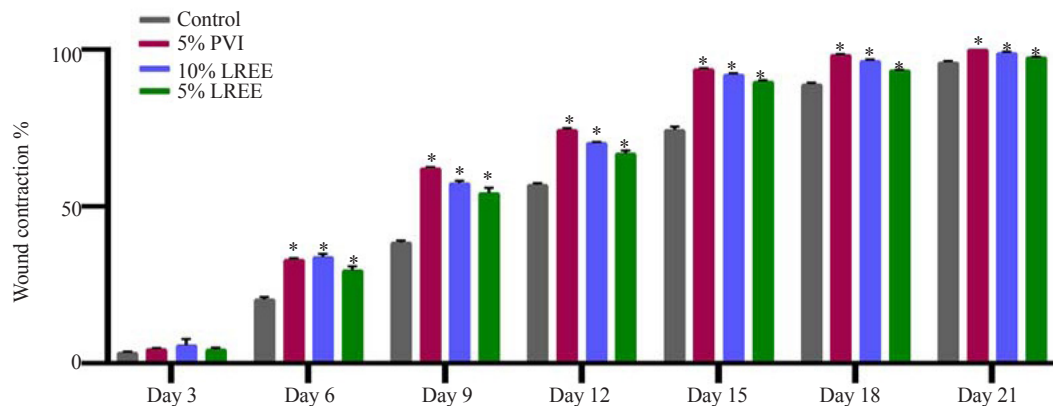


Figure 2. Effect of LREE on wound contraction in percentage. * $P<0.05$ compared with the control group.

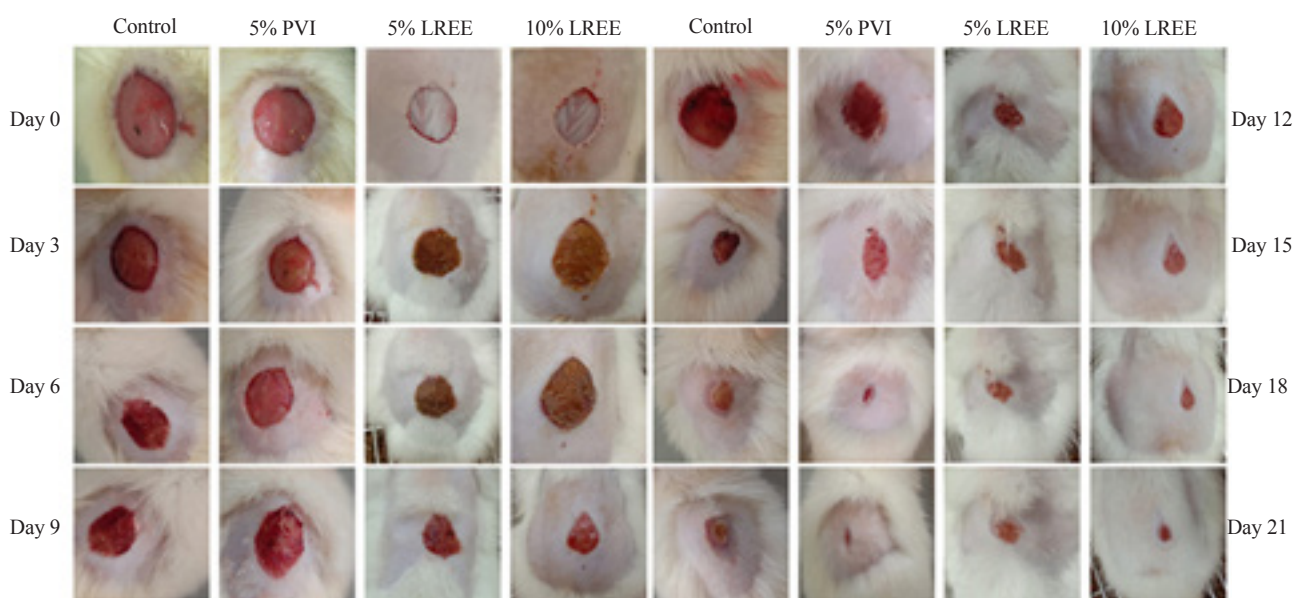


Figure 3. Effect of LREE on wound area. Day 0 was considered the day of wound infliction.

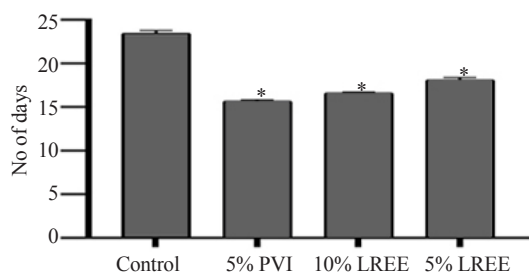


Figure 4. Effect of LREE on epithelialization. * $P < 0.05$ compared with the control group.

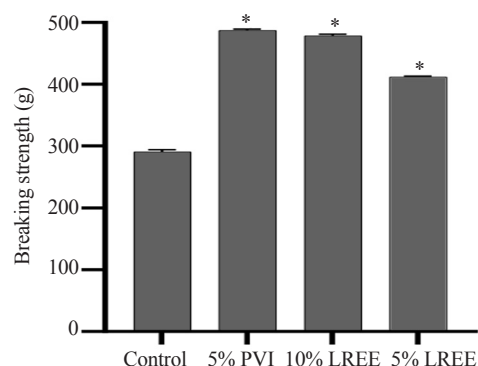


Figure 5. Effect of LREE on wound breaking strength. * $P < 0.05$ compared with the control group.

3.7. Histopathological evaluation

The control group demonstrated a delayed healing process and contained fewer collagen fibers, blood capillaries, fibroblasts, and more inflammatory cells. In comparison with the control, faster remodeling was noticed in extracts-treated groups. Histological photomicrographs displayed a significant increase in the deposition of collagen, neovascularization, and more fibroblasts, and comparatively few inflammatory cells in 5% PVI as well as *L. ruellioides* extracts-treated groups. There was full-thickness reepithelialization, in which the epidermis was well organized (Figure 6).

4. Discussion

Many studies have proved the wound-healing potential of plant extracts[25–28]. This study investigates the wound-healing abilities of *L. ruellioides*.

Reactive oxygen species can result in significant harm to the tissue and even neoplastic changes, which further hinder the healing process. Consequently, removing reactive oxygen species may be an essential approach in the treatment of chronic wounds[29]. Notably, compounds or plant extracts with antioxidant capacity could be potentially beneficial medicinal agents for hastening the healing of wounds. Our study shows that LREE treatment significantly elevated GSH, GST, and SOD, and decreased MDA, which is in line with the study of Lalthansangi *et al*[12]. Besides, the astringent and antibacterial activities of phytochemical elements of plants[30] have been proven to support the wound healing process. These attributes also seem to be accountable for the healing of the wound and enhanced rate of epithelialization[31].

This study investigated wound contraction, duration of epithelialization, and wound breaking strength using excision and incision wound models, respectively. 10% LREE applied topically increased the proportion of wound contraction, reduced epithelialization time, and elevated wound breaking strength. The rapid wound contraction may be driven by an upsurge of an inflammatory α -chemokine which influences the recruitment and function of different cells that cause inflammation, including keratinocytes as well as fibroblasts. Additionally, it could also enhance the gap junctional intracellular communication in fibroblast and stimulate increased granulation tissue maturation[32]. An increase in wound breaking strength indicated enhanced collagen maturation and stabilization of the fibers, which facilitate wound healing[33]. At the site of the injury, collagen molecules are deposited and cross-linked to create fibers. It can be inferred that the plant extract not only enhances the synthesis of collagen per cell but also facilitates protein crosslinking because of increased tensile strength. The extract could support tissue granulation and remodeling in addition to fastening wound closure.

The effect on healing of wounds is further supported by the histological analysis. According to histopathological observations,

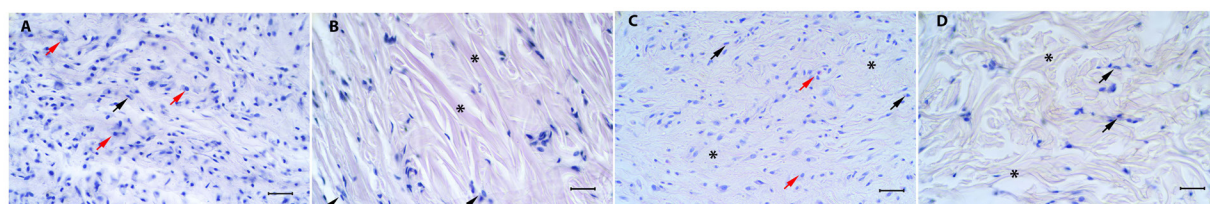


Figure 6. Histological results. A: the control, B: 5% PVI groups, C: 5% LREE-treated group; D: 10% LREE-treated group. The asterisk indicates collagen fibers, the black arrow indicates fibroblasts and red arrow indicates inflammatory cells (Scale bar: 50 μm ; magnification: 20 $\times$).

the extract could promote collagen creation during the proliferative stage, with an increase in the number of fibroblasts.

Furthermore, the results of LC-MS analysis revealed the presence of several bioactive compounds including mycolactone F, 1-*O*-octadec-9-enyl glycerol, reserpine, tetracosanoic acid, 2-*O*-caffeoylglycerate which have been reported to have pharmacological properties like antioxidant activity, anti-inflammatory activity, and antimicrobial activity^[34–38], which in turn may have a synergistic effect on the wound healing activity.

However, this study solely relied on the whole ethanolic extract of *L. ruelliioides* for its wound healing activity instead of the isolated compounds, which makes it exceedingly uncertain which phytochemical is responsible for wound healing activity. This can be addressed in the future study.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

CL contributed to designing the experiment, data curation, investigation, writing the manuscript, and reviewing and editing the manuscript. RKL assisted with the experiment. EL was involved in supervision, and conceptualization as well as provided lab equipment and chemicals. The final manuscript was read and approved by all of the authors.

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Supplementary Table 1. Phytochemical constituents identified from LRCE.

Peak	Name	Retention time (min)	Peak area
1	1- <i>O</i> -Octadec-9-enyl glycerol	26.07916667	4826126.841
2	2- <i>O</i> -Caffeoylglucarate	24.84455	2151399.835
3	Aspidodasycarpine	28.16468333	1098621.071
4	Betulinic acid	26.51295	540577.0336
5	Delphinidin 3- <i>O</i> -sophoroside	28.18136667	372089.6208
6	Justicidin B	16.75278333	8.18E+07
7	(-)-Jamine	25.5286	1758218.704
8	Mycolactone F	24.21055	1704390.589
9	Ophiopogonin C	28.61515	380463.1333
10	Reserpine	28.41493333	4173438.701
11	Veatchine	26.31275	8765088.083

Supplementary Table 2. Phytochemical constituents identified from LREE.

Peak	Name	Retention time (min)	Peak area
1	Tetracosanoic acid	29.06918333	1.23E+08
2	(S)- <i>N</i> -[3-(3,4-Methylenedioxyphenyl)-2-(mercaptomethyl)-1-oxopropyl]- <i>(S)</i> -alanine	25.7157	5318579.237
3	4-Hydroxybenzoate	28.4185	4333032.093
4	Limonin	27.91798333	3597461.164
5	Mycolactone F	24.23081667	3266435.085
6	Alangicine	23.78035	2842577.786
7	<i>L</i> -Cysteine	28.43518333	1961539.535
8	Sterol 3- β - <i>D</i> -glucoside	23.96386667	2615175.517
9	Deserpidine	27.73446667	1563961.705
10	Cucurbitacin A	28.28503333	752122.6767

Supplementary Table 3. Phytochemical constituents identified from LRAE.

Peak	Name	Retention time (min)	Peak area
1	4-Hydroxybenzoate	28.39465	3049230.906
2	Aurantio-obtusin beta- <i>D</i> -glucoside	27.31018	2926222.432
3	Bixin	27.41028	6397321.722
4	Delta-Tocotrienol	27.62718	1324710.29
5	<i>L</i> -Cysteine	28.27785	3366081.885
6	Mycolactone F	24.24032	1164641.405
7	Tetracosanoic acid	25.67515	1.31E+08

Supplementary Table 4. Effect of ethanolic extract of *L. ruellioides* on reduction in wound area (mm²).

Groups	0 day	3 rd day	6 th day	9 th day	12 th day	15 th day	18 th day	21 st day
Group 1 (Control)	298.56±0.7 5	288.38±0.5 1	237.63±0.82	183.35±0.69	128.44±0.5 3	76.47±1.67	32.99±0.89	11.99±0.61
Group 2 (5% PVI)	301.39±1.7 3	287.70±1.0 9	201.80±0.33 *	114.13±1.10 *	76.97±0.78 *	18.74±0.52 *	5.00±0.37*	0*
Group 3 (5% LREE)	288.89±0.9 4	276.08±1.9 1	202.98±2.61 *	131.91±3.09 *	95.38±1.71 *	29.44±0.63 *	18.99±0.36 *	7.03±0.31*
Group 4 (10% LREE)	292.11±2.1 9	275.16±2.1 2	192.95±3.12 *	124.39±1.42 *	86.72±1.00 *	23.22±0.52 *	10.46±0.68 *	3.18±0.58*

Values are expressed as mean ± SEM (*n*=6); **P*<0.001 when compared to the control group.