EVALUATION OF WOUND HEALING AND ANTIBACTERIAL ACTIVITY OF *LINDERNIA RUELLIOIDES* (COLSM.) PENNELL EXTRACTS IN WISTAR ALBINO RATS

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY OF MIZORAM UNIVERSITY, AIZAWL

CERTIFICATE

I certify that the thesis entitled "**Evaluation of wound healing and antibacterial activity of *Lindernia ruellioides* (Colsm.) Pennell extracts in Wistar albino rats**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Lalthansangi** is a record of the research work carried out during the period 2018 - 2025 under my supervision and guidance and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning.

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DECLARATION

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March, 2025

I, **Lalthansangi**, hereby declare that the subject matter of this thesis is the record of work done by me, that contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the **Degree of Doctor of Philosophy in Zoology.**

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ABBREVIATIONS

LRCE	*Lindernia ruellioides* chloroform extract
LREE	*Lindernia ruellioides* ethanol extract
LRAE	*Lindernia ruellioides* aqueous extract
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-HRMS	Liquid Chromatography High-Resolution Mass Spectrometry
N	North
E	East
No.	Number
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
GAE	Gallic acid equivalents
w/v	Weight per volume
µg	Microgram
mg	Milligram
g	Gram
kg	Kilogram
µl	Microliter
ml	Millilitre
s	Second
min	Minute
h	Hour
rpm	Rotation per minute
µmol	Micromole
nmol	Nanomole
mM	Millimole
M	Moles
nm	Nanometre
cm	Centimetre
mm^2	Millimetre squared
mm^3	Cubic millimetre

OD	Optical Density
ANOVA	One-way analysis of variance
SEM	Standard error of mean
DPPH	1,1-diphenyl-2-picrylhydrazyl
ABTS	2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
IC^{50}	50% Inhibitory concentration
PBS	Phosphate buffered saline
MDA	Malondialdehyde
GSH	Glutathione
GST	Glutathione-s-transferase
SOD	Superoxide dismutase
LPO	Lipid peroxidation
OECD	Organization for Economic Co-operation and Development
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
RNA	Ribonucleic acid
mRNA	Messenger RNA
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time-PCR
RT	Real time

GENERAL INTRODUCTION

Wounds can be defined as disruption of the normal anatomic framework and functioning of the skin, organ tissue or mucosal surface (Gupta, 2021). Skin wounds may occur from a variety of factors, including physical injuries that causes an opening and breaking of the skin (Gerald *et al.,* 1994) which could be accidental, incidental or due to a result from disease (Chhabra *et al.,* 2017; Young and McNaught, 2011; Teller and White, 2011). The most typical symptoms of wounds include bleeding, loss of feeling or function below the wound site, heat and redness adjacent to the wound, painful or throbbing sensation, swelling of tissue in the area and pus-like discharge (Rashed *et al.,* 2003). Healing is an intricate and sophisticated process that begins in reaction to an injury to restore the function and integrity of injured tissues (Govindarajan *et al.,* 2007). It is an extremely complex, multifactor chain of events involving various cellular and biochemical processes. (Yang *et al.*, 2021; Rodrigues *et al.*, 2019). The goal of these processes is to repair and reconstruct the disturbed anatomical coherence and functional condition of the skin. The healing process, a natural biological reaction to damage, begins immediately after wounding and proceeds in three stages, requiring the simultaneous efforts of different cells and tissues (Martin, 1997). In a normal condition, wounds heal by multiple processes, which are primarily a connective tissue response. The preliminary phase of this process involves an acute inflammatory phase, followed by the manufacture of collagen and extracellular macromolecules, and eventually leads to the formation of scar (Patil, 2009).

Animal models for wound healing are important biological tools to understand basic process of tissue repair and to validate strategies for treatment of wounds (Masson-Meyers *et al.,* 2020). Wound healing in human beings have many unique aspects that related to physiology, age and environmental factors, but the opportunities to carry clinical experiments to understand mechanism and to formulate therapy for wound healing are limited (Pandey *et al.,* 2012). Although animal wound healing models are imperfect reflection of wound healing processes

in humans and its clinical challenges, these models continue to be crucial tools for the establishment of novel concepts and approaches for therapy of wound healing (Davidson, 2001).

General process of wound healing

Wound healing is an action by which tissue regenerates. It is a simple yet dynamic process of rebuilding integrity and tissue layer, which entails an assortment of interrelated and concurrent processes (Marcandetti and Cohen, 2004). In general, the wound healing process is not affected by the type of injury and varies very slightly from one type to another. The process of wound healing consists of three phases: inflammatory phase, proliferative phase and remodelling phase (Garg and Paliwal, 2011). There is considerable temporal overlap of these phases of healing and the entire process lasts several months.

Inflammatory phase

The inflammatory response is initiated at the moment of injury. The goal of the inflammatory phase is to fight potential bacterial contamination of the wound and to activate cytokine secretion (Velnar *et al.,* 2009; Braiman-Wiksman *et al.,* 2007). This phase is characterized by hemostasis, chemotaxis and increased vascular permeability, limiting further damage, closing the wound, removing cellular debris and bacteria, and fostering cellular migration (Coger *et al.,* 2019). The initial step assists in the protection of the vascular system to maintain the functionality of the organ. The clot formed as a result of coagulation provides a matrix for the cells involved in subsequent steps of hemostasis and inflammation (Velnar *et al.,* 2009). Various pro-inflammatory cytokines and growth factors are released by the clot and wound tissue. Inflammatory cells then relocate to the wound site by the process of chemotaxis and promote the inflammatory phase (Broughton *et al.,* 2006; Campos *et al.,* 2008).

Within moments as an injury occurs, neutrophils are drawn to the wound from hindered arteries by chemoattractant, including interleukin 1 (IL-1), tumor necrosis factor-alpha (*Tnf-α*) and bacterial endotoxins, such as lipopolysaccharide (LPS) (Kolaczkowska and Kubes, 2013). Neutrophils generate their own cytokines

in reaction to pro-inflammatory cues and the stimulation of inflammatory signalling pathway. Through the mechanism of phagocytosis and the production of reactive oxygen species (ROS), antimicrobial peptides and proteolytic enzymes, neutrophils eliminate necrotic tissue and pathogens (Segel *et al.,* 2011). Additionally, they make use of what are referred as extracellular traps, an extruded DNA web covered with cytotoxic histones and antimicrobial peptides to capture and eliminate infections (Brinkmann *et al.,* 2004). Wound neutrophils decrease within a few days after the initiation of injury, unless there is infection (Kim *et al.,* 2008). While some neutrophils are eliminated by innate clearance processes such as macrophage efferocytosis, the majority of them adhere to the fibrin scab and emanate from the wound site (Martin and Leibovich, 2005). The few remaining neutrophils are eliminated by phagocytosis, necrosis and apoptosis or they can also depart from inflammatory tissue and re-enter the bloodstream by reverse trans endothelial migration (Buckley *et al.,* 2006).

An early wave of monocytes was observed entering the wound concurrently with neutrophils, despite the widespread belief that macrophages are recruited after neutrophils (Rodero *et al.,* 2014). Macrophages exhibit great plasticity and versatility, making them master effector cells in tissue repair (Das *et al.,* 2015). In mice, they peak at 72 hours after damage, whereas in humans, they do so 7 days after injury (Baum *et al.,* 2005). Although macrophages use anatomically safeguarded receptors to absorb necrotic cellular detritus and infectious debris, they differ from neutrophils in their activities and alterations in morphology brought on by cytokines (Mantovani *et al.,* 2005).

There are two types of wound macrophages: alternatively activated (anti-inflammatory) and classically activated (pro-inflammatory). In response to pro-inflammatory stimuli like lipopolysaccharide (LPS) and interferon-gamma (Ifn-γ), classically activated macrophages emit inflammatory cytokines and growth factors such as vascular endothelial growth factor (*Vegf*) and platelet-derived growth factor (*Pdgf*) and take the place of apoptotic neutrophils as the primary mediator of inflammation by phagocytosing them (Delavary *et al.,* 2011). The late phases of

inflammation are marked by a transition to alternative activation, which occurs by means of neo-differentiation of freshly recruited monocytes or deliberately by the transformation of circulating macrophages in situ to an anti-inflammatory phenotype. Environmental alterations in cytokines and efferocytosis can trigger this phenotypic transformation, despite the fact that it is not widely known (Das *et al.*, 2014). It could be further influenced by transcription factors (Nelson *et al.*, 2011), miRNAs (Das *et al.*, 2015) and regulating of both pro-inflammatory and anti-inflammatory receptors (Lin *et al.*, 2016).

Alternatively activated macrophages emit pro resolutory cytokines (IL-4, IL-10, IL-13) (Barrientos *et al.*, 2008; Eming *et al.*, 2007) and arginase, which is a prerequisite for efficient wound repair (Campbell *et al.*, 2013). A diverse set of growth factors are also discharged by anti-inflammatory macrophages that foster angiogenesis, fibroplasia and re-epithelialization (Jetten *et al.*, 2014). Recently, macrophages have been established to have an indispensable function in blood vessel stability and remodelling (Gurevich *et al.*, 2018). These macrophages are long-lived and reside in the wound until it recuperates completely. The healing process can be halted if inflammation fails to be controlled since it can negate the early migratory effect (Diegelmann and Evans, 2004).

Proliferative phase

The proliferation phase overlaps with the preceding inflammatory phase. This phase is characterized by vascular development, accumulation of collagen, granulation tissue development, epithelialization and contraction of wound and represents a proliferation of both epithelial and dermal elements which results in re-epithelialization of the wound and laying down of the primary extracellular matrix (ECM) (Guo and Dipietro, 2010). Alterations in mechanical tension and electrochemical gradients, together with prolonged contact with hydrogen peroxide, pathogens, growth factors, and cytokines, are capable of stimulating keratinocytes as promptly as 12 hours following injury (Shaw and Martin, 2016). Keratinocytes alongside the wound edge encounter an interim epithelial-mesenchymal switch as consequence of this stimulation, implementing more invasive and migrating

appearance (Li *et al.,* 2007). The leading-edge keratinocytes may propagate laterally throughout the wound to regenerate the epidermal layer, an event known as re-epithelialization, when front to rear polarity supplants top to bottom polarity (Wager and Leavesley, 2015). In an attempt to cater for the cellular demand necessary to resurface the wound, hair follicle stem cells are stimulated to multiply, and progeny epidermal cells ooze out of the follicle (Ito *et al.,* 2007). In superficial wounds, these cells proliferate from injured appendages while in full-thickness wounds, they originate from the epidermal margin. The re-epithelialization mechanism only recruits or triggers particular stem cell components (Ito *et al.,* 2005).

Fibroblasts are the primary cell type that replaces the intermittent fibrin-rich matrix with a more significant granulation tissue. They react to an array of signalling molecules from macrophages, endothelial cells and platelets, including transforming growth factor (*Tgf-β*) and *Pdgf* (Wilkinson and Hardman, 2020). Such signals cause fibroblasts to either differentiate into myofibroblasts, which cause wound contraction, or pro-fibrotic cells, which deposit extracellular matrix (ECM) proteins (Li *et al.,* 2007). In order to satisfy the metabolic requirements of the highly proliferative repairing tissue, angiogenesis creates new blood vessels. Hypoxia causes angiogenesis, which in turn causes cyclooxygenase 2 and hypoxia-inducible factors (HIFs) to be expressed, resulting to the discharge of *Vegf* and various factors (Huang *et al.,* 2005). As they proliferate, microvascular endothelial cells migrate into the wounded area in reaction to these alterations, creating new vessels that join with existing ones to form robust, tubular networks (Honnegowda *et al.,* 2015). While the fibrin matrix stimulates angiogenesis by causing endothelial cell phenotypic alterations to encourage their migration, *Vegf* suppresses the disintegration of endothelial cells by upregulating anti-apoptotic proteins (Kalebic *et al.,* 1983; Cai *et al.,* 2003).

By enhancing the function of microvascular endothelial cells, macrophages also contribute significantly to angiogenesis. They generate chemotactic factors like *Vegf* and *Tgf-β* to promote endothelial migration and proteases like matrix metalloproteinases (MMPs) to break down the thick fibrin network (Du *et al.,*

2019). Additionally, by drawing vessel tips in proximity, macrophages contribute to the development of new vasculature (Fantin *et al.,* 2010), phagocytosing extraneous vessels (Gurevich *et al.,* 2018; Poché *et al.,* 2015) and suppressing the angiogenic response avert extensive vascularization (Stefater *et al.,* 2011). The restoration of epithelial integrity occurs at the wound surface as these procedures continue to take place deeply within the wound. In case of an approximated incised wound, re-epithelization wraps up in less than 48 hours, but it may take considerably longer in the case of larger wounds with a significant tissue defect (Marcandetti and Cohen, 2004).

Remodelling phase

This phase marks the final step in tissue maturation and differentiation leading to recovery of the skin and its aesthetic restoration (Diegelmann and Evans, 2004; Hackam and Ford, 2002). Reconstruction of the dermis occurs by reorganization of the matrix collagen (Braiman-Wiksman *et al.,* 2007). The primary cell type in charge of modifying the ECM of wounds are fibroblasts. They replenish the initial fibrin clot with fibronectin, hyaluronan, and proteoglycans, and subsequently in the healing process, they generate mature collagen fibrils (Darby *et al.,* 2014). Proteoglycans facilitate cell motility and assist in constructing mature, cross-linked collagen fibrils (Schultz and Wysocki, 2009). Adult skin that has not been impaired has about 80% collagen type I and 10% collagen type III. Granulation tissue, on the other hand, consists overwhelmingly of 30% embryo-associated collagen type III and just 10% collagen type I (Witte and Barbul, 1997). Collagen type I supersedes collagen type III throughout the healing process, which directly raises the growing scar's tensile strength (Diegelmann and Evans, 2004). The framework and integrity of scar ECM never quite revert to those of unwounded skin. In undamaged skin, collagen fibrils take on a basket weave orientation, whereas in the scar dermis, they form enormous parallel bundles. Therefore, only 80% of the pre-wounding tensile strength is conferred by wound scar tissue (Young and McNaught, 2011).

Temporal modulation of MMPs allows for the delicate equilibrium between the production of collagen and disintegration, which is mandatory for these successive alterations in the ECM. Collagenases, which are produced by fibroblasts, keratinocytes and anti-inflammatory macrophages, split the natural helical collagens across the entire healing process (Darby *et al.,* 2014). To maintain skin elasticity, elastin, another essential component of the dermal extracellular matrix, must reconstruct elastic fibers. It's interesting to note that elastin fragments or elastokines, which function as signalling molecules, are released when the regular dermal matrix breaks down (Duca *et al.,* 2004). Tropoelastin, the precursor of elastin, exhibits an abnormal configuration early in the healing process. Actually, it frequently takes months after damage for mature elastin fibers to become visible in scar tissue (Almine *et al.,* 2012; Amadeu *et al.,* 2004). While active scar remodelling lasts up to a year following injury, the high rate of collagen production within the wound returns to normal tissue levels by six to twelve months (Marcandetti and Cohen, 2004). As fibroblasts, endothelial cells and macrophages die or leave the wound site, the wound healing response diminishes, and a scar remains (Larouche *et al.,* 2018).

Investigating conventional methods for wound healing falls into utilizing herbal remedies in addition to of living organisms such as animal/insect products (Pereira and Bártolo, 2016). Wounds along with dermatological problems are among the top five reasons people sought medical attention in remote regions of developing nations (Ryan, 1992). Despite providing a cost-effective and safe method of treating wounds, conventional medicines have not gotten enough consideration (Monika *et al.,* 2022). The fact that this region is not part of WHO's priority disease zones is one of the explanations for why it gets ignored. A further explanation is the widespread belief that traditional health care systems are better suited for treating low-level, chronic diseases than for treating acute ones. The fact that wounds and injuries are typically treated locally rather than being brought to clinics at the most advanced stage of pathology could be an additional factor (Bodeker, 1995; Bodeker and Hughes, 1998).

Plants have been employed widely since the beginning of mankind to cure a variety of ailments but the people in ancient time keep no records and the information is mainly passed on verbally from generation to generation (Puspangadan and Atal, 1984). With the advancement in technology and increasing knowledge, several studies were accomplished by several researchers which demonstrates that traditional medicinal herbs have been acknowledged for an assortment of biological activities, including antimicrobial, analgesics, anticancer, anti-pyrexial, and antihypertensive activity and an important source of many biological active compounds (Inatani *et al.,* 1996; Alma *et al.,* 2003; Andrade *et al.,* 2007; Webster *et al.,* 2008), and these data have revealed a high degree of correlation between traditional medicinal plants uses and laboratory analysis (Singh *et al.,* 2008). For countless years, nature has been offering therapeutic remedies, and in 80% of the world's developing and impoverished nations, plant-based systems remain crucial to primary healthcare (Kalpana and Kodukkur, 2011). This can be evident from various studies of medicinal plants at several global locations to treat human diseases and infections (Caceres *et al.,* 1991; Nweze *et al.,* 2004; Vineela *et al.,* 2005).

Notably, several plants have been validated by scientific studies to actually exert biological action for having wound healing potential or its complications (Atangwho, 2009; Dash and Murthy, 2011; Shivananda *et al.,* 2009). Interestingly, herbal remedies are extremely valuable in wound care given that they hasten the healing process and leave the patients with fewer scars (Kumar *et al.,* 2007). The wound healing capability of hydro-methanolic *Dioscorea bulbifera* extract in experimental rats utilizing excision wound model was precisely documented, where animals treated with 200 mg/kg of the extract demonstrated full closure of the wound within 17 days and all the animals in the experimental groups had significantly elevated percentage of wound contraction compared to negative control ($P<0.05$) (Chinko BC and Precious-Abraham AD, 2024). *Simmondsia chinensis,* popularly known as jojoba was also evident to have a healing property in experimental rats. The ability of jojoba methanolic extract to heal wounds was studied by excision wound model where the extract treated group exhibited higher

percentages of wound contraction and shorter epithelialization periods. Additionally, the angiogenesis elements such as *Vegf, Tgf-β1* showed elevated level in comparison with the control which indicates an augmented re-epithelialization and angiogenesis process (El Sherif *et al.,* 2023). A multitude of other plants, which comprises of *Azadirachta indica, Emblica officinalis, Terminalia chebula, Curcuma longa, Cleome gynandra, Triticum aestivum, Desmodium gangeticum, Ficus religiosa, Punica granatum* and *Aloe vera* were also well documented for advocating wound healing in experimental animals (Narendhirakannan *et al.,* 2012; Jain *et al.,* 2006; Kalyon *et al.,* 2009; Lukiswanto *et al.,* 2019; Movaffagh *et al.,* 2022).

A comprehensive overview on the ethnopharmacological properties of the plant genus *Lindernia*

Lindernia species are an important source of herbal concoctions used in many traditional remedies used in India and other countries. (Umakrithika, 2021). The family Linderniaceae, which has about 13 genera and 195 species worldwide, primarily in the neotropics, incorporates plant species beneath the genus *Lindernia,* (Albach *et al.,* 2005) among which 22 species have been observed in India (Nayar *et al.,* 2006). Previously, in other classifications, it was a member of the Scrophulariaceae sensu lato family, and more recently, the Plantaginaceae sensu lato family. However, multiple researchers have established that this taxon ought to be distinguished from those families since Linderniaceae is a distinct family. (Oxelman *et al.,* 2005; Rahmanzadeh *et al.,* 2005). Among all the genera of Linderniaceae family, *Lindernia* scores a great importance owing to its reported ethnomedicinal uses, reported pharmacological activities and easy availability (Umakrithika, 2021).

Lindernia anagallis was reported to have antimicrobial activity as well as antioxidant activity. The essential oils of *L. anagallis* when analysed by GC-MS, demonstrated antimicrobial activity against a broad range of microorganisms (Tsai *et al.,* 2011). It also demonstrated antioxidant activities on 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay, β-carotene/linoleic acid assay, and nitric oxide radical scavenging assay. A study conducted by Shyura *et al.,* 2005 also

demonstrated that *L. anagallis* demonstrated potent antioxidant properties with IC_{50} values for DPPH radicals at 36 g/ml.

The hepatoprotective attributes of *Lindernia ciliata* methanol extract was assessed in rats administered ethanol, paracetamol and D-galactosamine along with its antioxidative potential (Praneetha *et al.,* 2014). Among the tested doses i.e., 100 mg/kg, 200 mg/kg and 400 mg/kg, 200 mg/kg was discovered to be the most efficient dosage which was indicated by the decreased level of ALT, AST, ALP. Concentration-dependent DPPH, nitric oxide, hydroxyl and superoxide radical scavenging properties have been demonstrated by the methanolic extract of *L. ciliata*. The hepatoprotective activity of *L. ciliata* was also reported by Devi *et al.*, 2013 along with Gupta and Pandey, 2014.

Lindersin B isolated from *Lindernia crustaceae* illustrated neuritogenic activity in PC12 cells that were generated from rat pheochromocytoma cells. Among two distinct compounds isolated from ethyl acetate extract of *L. crustacea* namely lindersin A and lindersin B, lindersin B exhibited significant neuritogenic activity. The results indicated that the activation of tyrosine kinase A/phosphatidylinositol 3 kinase/extracellular signal-regulated kinase signalling pathways is involved in neuritogenic action brought on by lindersin B (Lihong *et al.,* 2017). The methanolic extract of the leaves of *L. crustacea* was also documented to stimulate the activity of pancreatic lipases with percent activation recorded at 105.2 (Ado *et al.,* 2013). In this study 98 different parts (seeds, fruits, leaves, stems, flowers, roots) of some medicinal, herbal and aquatic plants was studied for their anti-lipase and pro-lipase activity in which *L. crustacea* was one of the plants studied.

In 2017, the antioxidative potential of *Lindernia madayiparense* was scientifically validated for the first time in the study conducted by Umakrithika *et al.,* 2017 in which four extracts such as petroleum ether, ethyl acetate, ethanol and aqueous extract of *L. madayiparense* were assessed for the *in vitro* antioxidant potential by DPPH free radical scavenging assay and reducing power assay. The results revealed that in both assays, The greatest amount of antioxidative action was

noted in the ethanolic extract of *L. Madayiparense*. The cytotoxic effect of petroleum ether, ethyl acetate and ethanol extracts of *L. Madayiparense* was also validated against Human hepatocellular carcinoma cell lines (HepG-2 cell line) in an *in vitro* study by MTT assay (Chiranjeevi *et al.*, 2017) where pet ether extract exhibited the maximum cytotoxic effect (92.47%) at a dose of 1000 µg/ml. In the following year, 2018, the anthelmintic activity of *L. madayiparense* was investigated against Indian earthworms, *Pheretima posthuma* and the outcome was that *L. madayiparense* exhibited a dose dependent paralytic effect and the time of death. It was determined that the observed anthelmintic activity might be possibly the existence of secondary metabolites which include alkaloids, flavonoids, terpenoids, tannins and saponins (Chiranjeevi *et al.*, 2018).

Lindernia ruellioides (Colsmann) Pennell, commonly called as 'Duckbill Pimpernel', locally called as 'Thasuih' in Mizo (Rai and Lalramnghinglova, 2010) belongs to the family Linderniaceae. This plant is considered helophyte when it comes to the growth form and flowers during the months of October-February (Hazarika and Borthakur, 2014). It is an erect annual herb with an average height of 10-15 cm. The leaves are elliptic or orbicular-elliptic, obtuse at apex, usually 5 x 1.8 cm. The flowers are pale purple in color and the seeds, which are trigonous, 0.5 mm long are brown in color (Umakrithika, 2021). *L. ruellioides* is usually found in semi-shaded areas including rice fields, riversides, stream sides and swampy grasslands. It is extensively dispersed throughout tropical and subtropical Asia such as Cambodia, India, Indonesia, Japan (Ryukyu Islands), Malaysia, Myanmar, Philippines, Singapore, Vietnam (Cook, 1996).

Traditionally, the whole part of the plant can be used medicinally for treating detoxification, injuries, menoxenia, dysmenorrhea. Additionally, it is frequently used to treat cuts, wounds, bruises, boils, jaundice, snakebite, diarrhoea, urinary issues, and wounds that heal quickly when applied externally and the leave's juices are often used to massage strains (Sikdar and Dutta, 2008). Among the natives of Mizoram, *L. ruellioides* holds a rich traditional history in soothing sciatica, rheumatism, skin worms, cuts, wounds as well as internally for issues with the eyes (Rai and Lalramnghinglova, 2010).

Regardless of the extensive use as a traditional medicine, only a countable number of pharmaceutical applications of *L. ruellioides* have been accounted. Wei *et al.,* 2017 reported the anti-HBV effect *L. ruellioides.* In this study, five caffeoyl phenylethanoid glycosides isolated from the stems and leaves of *L. ruellioides* such as linderruelliosides A, linderruelliosides B, desrhamnosylverbascoside, plantainoside A and acteoside were tested for their anti-HBV activity. The *in vitro* anti-HBV assay showed that each of those compounds possessed inhibitory activities on HBsAg and HBeAg. The most active compound, desrhamnosylverbascoside could inhibit the secretions of HBsAg and HBeAg with IC_{50} values of 30.74 and 5.17 μM, respectively.

In vitro antioxidant activity of the methanolic extract of *L. ruellioides* was studied by Imtilemla *et al.,* 2020. In their study, *in vitro* antioxidant activity such as DPPH scavenging activity, reducing power assay and nitric oxide scavenging activity of methanolic extract of *L. ruellioides* was determined and the result highlighted that the methanolic extract revealed potential antioxidant activity in terms of DPPH scavenging activity, reducing power assay and nitric oxide scavenging activity. The preliminary phytochemical analysis was additionally determined which demonstrated the existence of amino acid, flavonoids, tannins, steroids and triterpenoids.

The present thesis

The present thesis was designed to study the wound healing potential of *Lindernia ruellioides* (Colsm.) Pennell by using excision and incision wound models. The study begins with the analysis of its phytochemical components. Three extracts such as chloroform, ethanol and aqueous extracts were obtained by cold maceration and they have been investigated for their phytochemical components, free radical scavenging activities and antibacterial activities. From the three extracts, ethanolic extract was chosen for further use in the *in vivo* experiment. Wistar albino rats was employed as an animal model for conducting the wound healing potential of *L. ruellioides.* The studies conducted in this thesis was classified into four chapters as follows.

Chapter I: Preparation of Lindernia ruellioides extracts, phytochemical analysis, and identification of bioactive compounds using LC-MS.

In accordance with their increasing polarity, *Lindernia ruellioides* was cold macerated using chloroform, ethanol and distilled water. Qualitatively and quantitatively phytochemical assessment of the extracts was performed along with the Liquid Chromatography-Mass Spectrometry (LC-MS) analysis.

Chapter II: Free radical scavenging activities and antibacterial activities of various extracts of Lindernia ruellioides.

The ability of *L. ruellioides* extracts to efficiently scavenge DPPH, ABTS and superoxide radicals was evaluated in this chapter. The antibacterial activity of *L. ruellioides* extracts along with the minimum inhibitory concentration (MIC) were further determined.

Chapter III: Wound healing activity of Lindernia ruellioides using excision and incision wound models in Wistar albino rats.

In this chapter, the wound healing potential of *L. ruellioides* was determined by assessing its antioxidant level as well as its oxidant status, wound closure rate, epithelialization period, skin tensile strength and histopathological examination by employing excision and incision wound models in male Wistar albino rats.

Chapter IV: Study of the expression pattern of Vegf and Tgf-β1 using quantitative RT-PCR.

Here, the expression pattern of two growth factors *Vegf* and *Tgf-β1* were evaluated by employing quantitative RT-PCR in an excision inflicted granulation tissue.

CHAPTER-I

Preparation of *Lindernia ruellioides* extracts, phytochemical analysis and identification of bioactive compounds using LC-MS.

INTRODUCTION

Nature has blessed us with countless number of plants that contains health benefits for all living creatures. The plant kingdom is a treasure house of potential drugs and in the past few years there has been an increasing awareness about the importance of medicinal plants (Shabi Ruskin and Ajina, 2017). Herbal remedies become increasingly more popular as an outcome of the desire to harness the knowledge of traditional treatment methods (Tyler, 2000). It would seem realistic to speculate that man's experience with illness ultimately contributed to the practice of eating or rubbing plant parts, such as seeds and leaves, for curing illness (Hassan, 2015). Plants have long been used in pharmacological treatment of illnesses worldwide (Schulz *et al.,* 2001). A dozen formulas for the medication preparation of more than 200 different plants were inscribed on Sumerian clay slabs approximately 5000 years ago, making it one of the oldest records and thought to be the first documented proof of the use of medicinal herbs (Sumner, 2000). In India, Ayurveda, a medical system which is based on herbal remedies has been recognized for almost 5000 years (Morgan, 2002) which serves an important sector in the field of medicine till date. Plant-based therapies are still crucial to healthcare, and there is a wealth of evidence supporting their usage across cultural boundaries (Moerman, 1986; Johnson, 1999).

The naturally occurring bioactive substances found within plants are recognized as phyto-constituents. Every portion of the plant body, including the bark, leaves, stem, root, flower, fruits and seeds, naturally synthesizes them (Gordon and David, 2001; Tiwari *et al.,* 2011). Phytochemicals are essentially separated into fundamental constituents based on their roles in plant metabolism. These primary

constituents include common sugars, amino acids, proteins, lipids and organic acids that are prevalent in all plants and carry out metabolic tasks that are essential to the plant's survival (Croteau *et al.,* 2000), and secondary constituents, which are the organic substances that accumulate unintentionally and are not particularly crucial to plant life given that they don't explicitly contribute to the growth, development, and reproduction of plants. Nonetheless, they are recognized to be the primary source of a plant's distinct flavor, color and odor. They also significantly contribute to the defence systems of plants (Bennet and Wallsgrove, 1994). The most important and commonly encountered secondary metabolites within plants are saponins, tannins, alkaloids, phenolic compounds, glycosides, terpenoids and flavonoids (Edeoga *et al.,* 2005). The majority of the secondary metabolites found in plants are recognized to be physiologically active substances that exhibit a variety of pharmacological activities, which likely explains why they are used in traditional medicine. (Egwaikhide *et al.,* 2007; Okeniyi *et al.,* 2007). Moreover, the qualitative and quantitative analysis of the phytochemical constituents of a medicinal plant is regarded as a significant step in medicinal plant research (Kokate, 1994).

Due to the fact that secondary metabolites are produced in certain cell types and at different phases of development, it is challenging to extract and purify them (Kamala *et al.,* 2014). Consequently, secondary metabolites that are economically utilized as biologically active substances are typically low volume, high value products and their biological functions were widely unrecognized due to the frequently low concentrations in plants (Twaij and Hasan, 2022). Nevertheless, knowledge about secondary metabolites for their ecological importance and numerous other benefits, has emerged over the past few decades (Weinberg, 1962; Pawlikowski, 2010). According to several studies, it has been demonstrated that the bioactive secondary metabolites slow the onset and spread of illnesses like cancer, cardiovascular, neurodegenerative diseases and possess antioxidant, antibacterial, anti-inflammatory activities, strengthening the immune system and shielding the skin from ultraviolet radiation (Kumar and Pandey, 2013; Chen *et al.*, 2015; Dziato *et al.*, 2016; Andreu *et al.,* 2018; Meng *et al.*, 2018) by employing a variety of biological processes to scavenge free radicals. To date, plant secondary metabolites

have played a major role, which is demonstrated by the fact that more than 30% of medicinal products derive directly or indirectly from natural products (Cragg and Newman, 2013; Newman and Cragg, 2004; Cragg *et al.,* 2009). Traditional herbal medicine practitioners have described the healing properties of various wild plants (Kumar *et al.,* 2007; Jari´c *et al.,* 2007). Various healing constituents in these plants have prompted researchers to examine them with a view to determine their healing potential.

A variety of plants used in the traditional medicine have now become a part of the modern world health care system (Srivastava *et al.*, 1996). Among those traditional herbal medicines, *Lindernia ruellioides* has been employed for its healing properties in different regions of the world (Wei *et al.,* 2017; Sikdar and Dutta, 2008; Rai and Lalramnghinglova, 2010). Hence, effort has been undertaken to investigate the specific phytochemical components of *L. ruellioides.*

MATERIALS AND METHODS

Collection of plant material and preparation of extracts

L. ruellioides was collected from Reiek, Aizawl district, Mizoram, India (23° 41' 26" N; 92° 36' 19" E) during the months of June-August. Identification and authentication of the plant was done at Natural History Museum Mizoram, Mizoram University with accession no. NHMM-P/000160. The plant was thoroughly cleaned with fresh water to remove all dirt and unwanted materials. It was allowed to thinly spread on a cleaned surface for drying at room temperature. In the course of the drying process, a hygienic and clean environment was maintained, and the plants were periodically checked and cared for in order to impede the development of fungus and other unnecessary materials. The complete drying of the plant requires about a month if the weather is favourable. After that, the dehydrated plant material was grounded to powder using an electrical grinder.

A known amount of the powdered plant (150 g) was then subjected to cold maceration. The process of maceration was done in a beaker which is completely sealed using aluminium foil to avoid evaporation. Firstly, petroleum ether was used to defatting the powdered plant and then sequentially macerated using chloroform, ethanol, and distilled water for approximately 72 hours each with continuous agitation. The liquid extracts were later filtered through Whatman No.1 filter paper and evaporated to dryness in an oven and was stored at 4℃ for further use. Hereafter, *L. ruellioides* chloroform extract, *L. ruellioides* ethanolic extract and *L. ruellioides* aqueous extract will be referred to as LRCE, LREE and LRAE respectively.

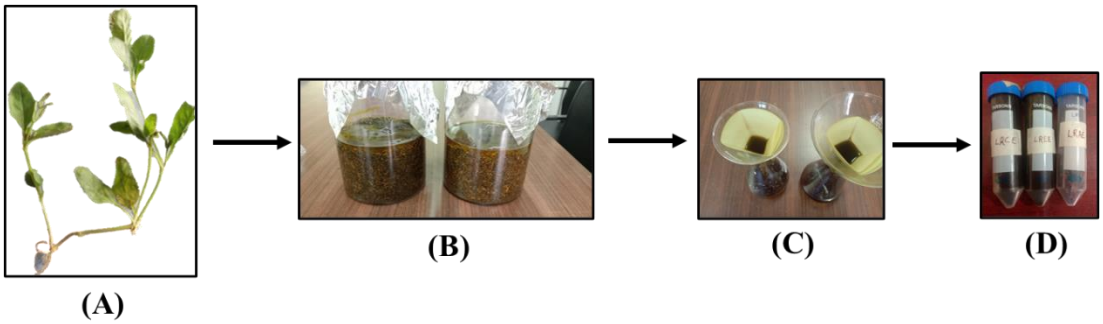


Figure 1.1: (A) *Lindernia ruellioides*; (B) Extraction by cold maceration; (C) Extract filtration; (D) Extracts obtained (LRCE, LREE and LRAE).

Estimation of percent yield of various *L. ruellioides* extracts

By measuring the weight of the powdered plant prior to extraction and the residue that was ultimately retrieved upon termination of extraction, the yield percentage of all the extracts produced was determined.

Yield (%) = Weight of the resulting dry extract /Weight of the powdered plant prior to extraction × 100

Qualitative phytochemical analysis

Qualitative phytochemical analysis of various extracts of *L. ruellioides* was done following standard protocols described by Trease and Evans, 2002 and Harborne, 1998.

Alkaloids

The Dragendorff's test was implemented to confirm that *L. ruellioides* extracts contained alkaloids. In short, 0.5 ml of Dragendorff's reagent was combined with 0.1 g of several *L. ruellioides* extracts. Assuming alkaloids are present, a reddish-brown precipitate will appear.

Saponins

Three drops of olive oil were combined with 0.1 g of *L. ruellioides* extract, and the mixture was shaken violently for a few minutes. Saponins were present because a reasonably stable emulsion formed.

Flavonoids

2 ml of extracts of the crude powdered plant sample were incorporated with a couple of droplets of sodium hydroxide (NaOH) solution. The presence of flavonoids was revealed by the formation of a bright yellow color that vanished when copious hydrochloric acid (HCl) was added.

Tannins

The ferric chloride test was used to detect the presence of tannins. In short, a few drops of 0.1% ferric chloride were added after 0.1 g of *L. ruellioides* extract had been dissolved in the corresponding solvents. The presence of tannins was indicated by the creation of a blue-black or brownish green tint.

Steroids

A few drops of strong sulfuric acid (H_2SO_4) were blended with 0.1 g of extract that had been dissolved in its corresponding solvent. The development of red color indicates the presence of steroids (at lower level).

Glycosides

2 ml of extracts of the crude powdered plant sample were treated with 3 ml chloroform and 10% ammonia solution. The sighting of a pink color indicated positive result.

Phlobatannins

When 0.1 g of several *L. ruellioides* extracts were heated in 1% aqueous hydrochloric acid, a reddish precipitate formed, signifying the presence of phlobatannins.

Terpenoids

Salkowski's test was implemented to determine whether terpenoids were present in different *L. ruellioides* extracts. To enable the creation of a layer, 5 ml of each extract (0.1 g/ml) was merged with 2 ml of chloroform and then carefully added 3 ml of saturated H_2SO_4. The presence of terpenoids was established by the development of a reddish-brown color near the interface.

Quantitative phytochemical analysis

Determination of the total phenolic content

The method outlined by Singleton et al. (1999) was used to determine the total phenolic content of several *L. ruellioides* extracts. In general, 1 ml of *L. ruellioides* extract, dissolved in its corresponding solvent, was mixed with 5 ml of ten-fold diluted Folin-Ciocalteau's reagent at concentrations ranging from 0.25 to 8.0 mg/ml. Following five minutes of incubation at room temperature, sodium carbonate (4 ml, 0.115 mg/ml) was incorporated to the mixture. A UV-visible spectrophotometer was then used to measure the absorbance at 765 nm after the combination had been incubated for two hours at room temperature. The reagents mentioned above were combined with a 1 ml methanolic solution of gallic acid to create the calibration curve, and the absorbance was measured at 765 nm. Each determinant was performed in triplicate. The total phenolic content in all the extracts was expressed as gallic acid equivalents (GAE) mg/ml of the dry extract.

Determination of the total flavonoids

Total flavonoid content was determined using the standard method given by Barreira *et al.*, 2008. Briefly, 0.25 ml of extract (0.25-8.0 mg/ml; dissolved in the appropriate solvent) 75 µl of a 5% (w/v) sodium nitrite solution was added after 1.25 ml of distilled water had been incorporated with the quercetin standard solution. 150 µl of a 10% (w/v) aluminium chloride solution was added after a few minutes, and it was let to stand for an additional five minutes before 0.5 ml of 1M sodium hydroxide was poured. The mixture was then diluted with distilled water to a volume of 2.5 ml. At 510 nm, absorbance was immediately measured. Quercetin equivalents were used to represent the result (mg/g extract). Every estimate was made in triplicate.

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

Chloroform, ethanolic and aqueous extracts of *L. ruellioides* were tested for the presence of bioactive secondary metabolites using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS). In order to perform 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 X 2.1, 1.7 um) system. Each extract's mass acquisition spectra were captured between 150 and 2000 m/z. For both ES+ and ES-, the source temperature was set to 120°C and the desolvation temperature to 350°C. In a linear gradient flow, the eluents were mobile phases A (water and 0.1% formic acid) and B (mixed acetonitrile and 0.1% formic acid). Throughout the gradient, the flow rate was kept at 0.3 ml/min and the injection volume was 5 µl. MassLynx™ and OpenLynx™ software was used to examine the data.

Statistical analysis

The mean ± standard error of mean is used to express the data. Tukey multiple comparison of means was used after one-way analysis of variance (ANOVA) was used to test for significant differences. *P* value of less than 0.05 was considered statistically significant. SPSS version 27 and Graph pad prism version 7 were used for statistical and graphical evaluations.

RESULTS

Extract yield

Ethanolic extract was recorded to possess the highest yield (13.83%) among the three extracts in which the aqueous extract (10.23%) comes next and later chloroform extract (2.8%). Table 1.1 displays the amount of extract yield using different solvents.

Table 1.1. Yield of different extracts of *L. ruellioides* in percentage.

Solvents	Powdered sample (g)	Extract obtained (g)	Yield (%)
Chloroform	150	4.2	2.8
Ethanol	150	20.75	13.83
Aqueous	150	15.35	10.23

Qualitative phytochemical analysis

Qualitative phytochemical investigation revealed the existence of several naturally existing chemical compounds, such as alkaloids, saponins, flavonoids, tannins, phlobatannins and terpenoids in ethanolic extract. Alkaloids, flavonoids, tannins and steroids were present in aqueous extract. The chloroform extract contains alkaloids, flavonoids, saponins and tannins. Among the three extracts, the ethanolic extract encompassed the vast majority of the phytochemicals that were examined, after which were the aqueous extract and the chloroform extract.

Table 1.2. Qualitative phytochemical evaluation of different *L. ruellioides* extracts. ('+' sign denotes the existence of phytochemicals while '-' sign denotes the absence of phytochemicals. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanolic extract; LRAE- *L. ruellioides* aqueous extract).

Phytochemicals	Reagent	Color indication	LRCE	LREE	LRAE
Alkaloids	Dragendroff's reagent	Reddish brown precipitate	+	+	+
Saponins	Olive oil	Whitish emulsion	+	+	-
Flavonoids	Sulphuric acid, Magnesium turnings	Pink red color	+	+	+
Tannins	Ferric chloride	Brownish green or blue-black color	+	+	+
Steroids	Sulphuric acid	Red color	-	-	+
Glycosides	Glacial acetic acid, Ferric chloride, Sulphuric acid	Brown ring	-	-	-
Phlobatannins	Hydrochloric acid	Red precipitate	-	+	-
Terpenoids	Sulphuric acid	Reddish brown	-	+	-

Quantitative phytochemicals analysis

Total phenolic content

The extracts of *L. ruellioides* showed an increase in total phenolic content in a dose dependent manner (Figure 1.1). At 8 mg/ml, LREE has considerably higher (*P* 0.001) total phenolic content (327.97 ± 1.77 mg GAE /g of dry extract) compared to LRAE (209.63 ± 0.69 mg GAE /g of dry extract) and LRCE (196.51 ± 1.71 mg GAE /g of dry extract).

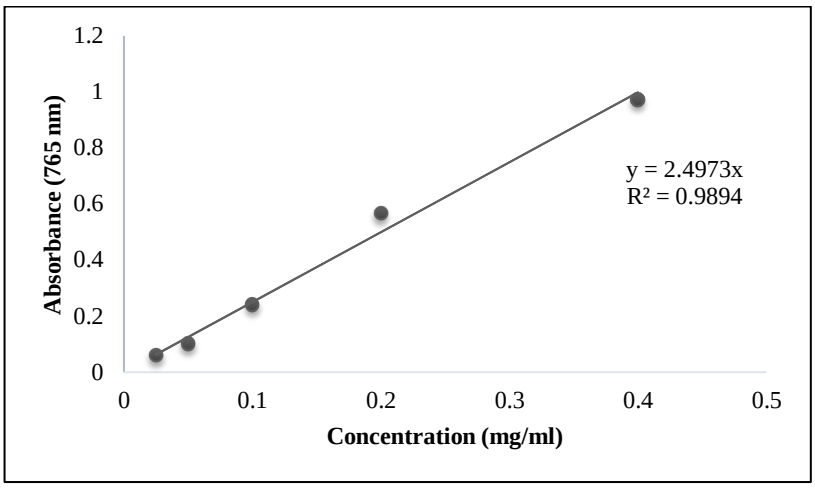


Figure 1.2: Standard gallic acid graph.

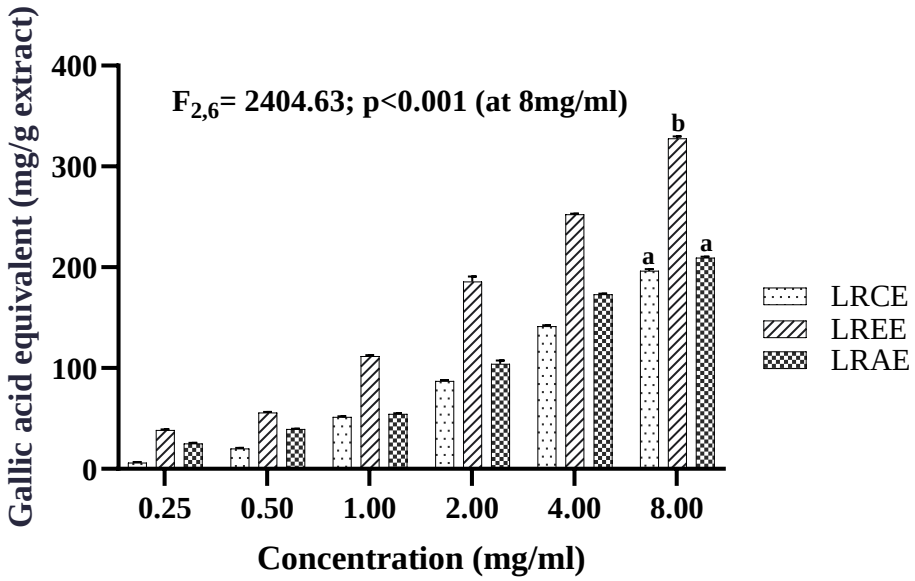


Figure 1.3: Phenolic content of *L. ruellioides* extracts determined as Gallic acid equivalent. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

Total flavonoid content

The total flavonoid content of *L. ruellioides* extracts also increased in a dose dependent manner (Figure 1.2). At 8 mg/ml, the ethanolic extract has significantly the highest (*P* 0.001) content (264.95 ± 0.71 mg quercetin equivalent/g of dry extract) compared to aqueous extract (217.44 ± 0.15 mg quercetin equivalent/g of dry extract) and chloroform extract (210.59 ± 0.41 mg quercetin equivalent/g of dry extract).

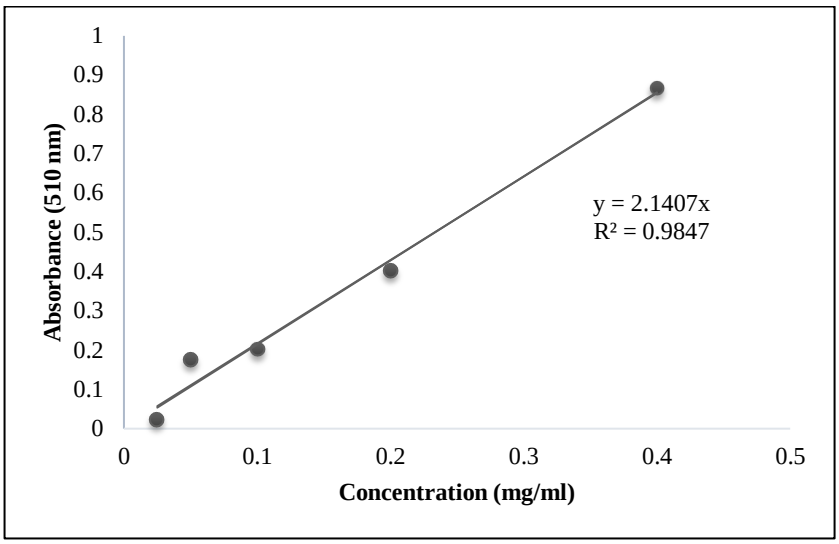


Figure 1.4: Standard quercetin graph.

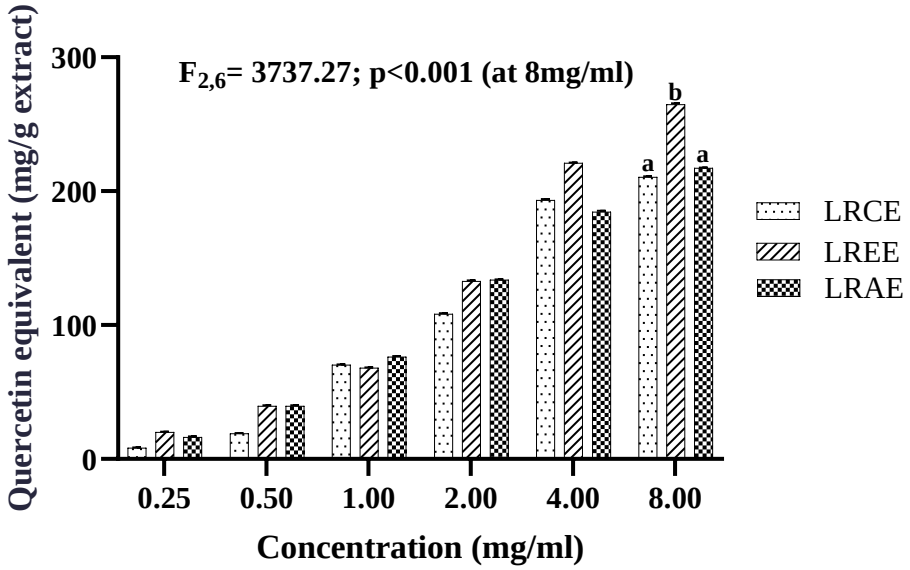


Figure 1.5: Flavonoid content of various extracts of *L. ruellioides* determined as Quercetin equivalent. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

Liquid Chromatography-Mass Spectrometry (LC-MS)

The chloroform, ethanolic and aqueous extracts of *L. ruellioides* were analysed using LC-HRMS. The results of the LC-MS analysis for LRCE, LREE, and LRAE confirmed that a number of crucial bioactive components having various pharmacological activities were present. Among several compounds mycolactone F was identified to exist in each of the three extracts and compounds such as L-cysteine, 4-hydroxybenzoate and tetracosanoic acid was discovered to have existed in both LREE and LRAE. The discovered compounds were highlighted in Table 1.3, 1.4 and 1.5, along with their related biological activities, peak area and retention time (RT). Remarkably, it has already been discovered that several of these compounds have therapeutic effects, highlighting the latent healing qualities that are inherent to *L. ruellioides.*

Table 1.3. Phytochemical constituents identified from LRCE using Liquid Chromatography-Mass Spectrometry.

Peak	Name	RT	Peak area	Activity	References
1	1-O-Octadec-9-enyl glycerol	26.07916667	4826126.841	Antimicrobial, tuberculostatic, anti-inflammatory, wound healing	Berger *et al.*, 1953; Emmerie *et al.*, 1952; Burford and Gowdey, 1968; Bodman and Maisin, 1958.
2	2-O-Caffeoylglucarate	24.84455	2151399.835	Anticancer	Hanausek *et al.*, 2003
3	Aspidodasycarpine	28.16468333	1098621.071	No activity reported	
4	Betulinic acid	26.51295	540577.0336	Anticancer	Chichewicz and Kouzi, 2004.
5	Delphinidin 3-O-sophoroside	28.18136667	372089.6208	No activity reported	
6	Justicidin B	16.75278333	8.18E+07	Antibacterial	Apers *et al.*, 2003.
7	(-)-Jamine	25.5286	1758218.704	No activity reported	
8	Mycolactone F	24.21055	1704390.589	Analgesic, anti-inflammatory	Guenin-Macé *et al.*, 2015; Isaac *et al.*, 2017
9	Ophiopogonin C	28.61515	380463.1333	No activity reported	

10	Reserpine	28.41493333	4173438.701	Treatment of hypertension, cardiovascular diseases, nervous disorders	Weiss and Fintelmann, 2000
11	Veatchine	26.31275	8765088.083	No activity reported	

Table 1.4. Phytochemical constituents identified from LREE using Liquid Chromatography-Mass Spectrometry.

Peak	Name	RT	Peak area	Activity	References
1	Tetracosanoic acid	29.06918333	1.23E+08	Anticancer, antioxidant, antibacterial	Millaty *et al.*, 2020; El-Agbar *et al.*, 2018; Desbois and Smith, 2010.
2	(S)-N-[3-(3,4-Methylenedioxyphenyl)-2-(mercaptomethyl)-1-oxoprolyl]-(S)-alanine	25.7157	5318579.237	No activity reported	
3	4-Hydroxybenzoate	28.4185	4333032.093	Antimicrobial, antioxidant, anti-inflammatory	Manuja *et al.*, 2013; Cho *et al.*, 1998
4	Limonin	27.91798333	3597461.164	Antioxidant, anti-inflammatory	Yu *et al.*, 2005; Li *et al.*, 2022
5	Mycolactone F	24.23081667	3266435.085	Analgesic, anti-inflammatory	Guenin-Macé *et al.*, 2015; Isaac *et al.*, 2017
6	Alangicine	23.78035	2842577.786	Anti-inflammatory	Shravya *et al.*, 2017
7	L-Cysteine	28.43518333	1961539.535	No activity reported	
8	Sterol 3-beta-D-glucoside	23.96386667	2615175.517	No activity reported	
9	Deserpidine	27.73446667	1563961.705	Used in the treatment of hypertension and psychosis	Kossover and Goldman, 1962; Leon *et al.*, 1962; Varchi *et al.*, 2005
10	Cucurbitacin A	28.28503333	752122.6767	Anti-inflammatory, antioxidant, antimalarial, antimicrobial	Varela *et al.*, 2022; Tannin-Spitz *et al.*, 2007.

Table 1.5. Phytochemical constituents identified from LRAE using Liquid Chromatography-Mass Spectrometry.

Peak	Name	RT	Peak area	Activity	References
1	4-Hydroxybenzoate	28.39465	3049230.906	Antimicrobial, antioxidant, anti-inflammatory	Manuja *et al.*, 2013; Cho *et al.*, 1998
2	Aurantio-obtusin beta-D-glucoside	27.31018	2926222.432	Hypotensive, hypolipidemic, anti-inflammatory	Xu *et al.*, 2021
3	Bixin	27.41028	6397321.722	Antioxidant, antimicrobial, anticancer, anti-inflammatory, nephroprotective	Moreira *et al.*, 2014; Handayani *et al.*, 2021; Dos Santos *et al.*, 2012; Pacheco *et al.*, 2019; Li *et al.*, 2020
4	Delta-Tocotrienol	27.62718	1324710.29	No activity reported	
5	L-Cysteine	28.27785	3366081.885	No activity reported	
6	Mycolactone F	24.24032	1164641.405	Analgesic, anti-inflammatory	Guenin-Macé *et al.*, 2015; Isaac *et al.*, 2017
7	Tetracosanoic acid	25.67515	1.31E+08	Anticancer, antioxidant, antibacterial	Millaty *et al.*, 2020; El-Agbar *et al.*, 2018. Desbois & Smith, 2010.

DISCUSSION

Phytochemicals are non-nutritive substances that are present in plants and cooperate with dietary fiber and minerals to avert illness (Johanna and Jyh-Lurn, 2007; Agbafor and Nwachukwu, 2011). In accordance with studies, numerous phytochemicals have been found in therapeutic plants, which are considered beneficial health effects (Scalbert *et al.,* 2005). The current investigation discovered several phytochemicals such as alkaloids, tannins, saponins, flavonoids, steroids, terpenoids and phlobatannins along with a significant amount of phenolic and flavonoid compounds in *L. ruellioides* extracts. These secondary metabolites, referred to as phytochemicals contribute to flavor and color (Molefe-Khamanga *et al.,* 2012) and are documented to possess several pharmacological potentials which includes antioxidants, antimalarial, antimicrobial activities, promote the function of the immune system and minimize the chance of numerous illnesses (Dolara *et al.,* 2005; Wink *et al.,* 1998; Dudareva *et al.,* 2004; Agbafor and Nwachukwu 2011; James *et al.,* 2007; Cai *et al.,* 2003).

Polyphenols are remarkably prevalent antioxidants in the plant kingdom, and they were being claimed to protect our body against the detrimental consequences of free radicals, strengthen immunity and stimulate natural defences by exhibiting various healing properties and preventing chronic diseases (Masiala *et al.,* 2024; Lamoral-Theys *et al.,* 2010). It is speculated that the polyphenolic compound's redox characteristics, which are crucial for adsorbing and abolishing free radicals, suppressing singlet and triplet oxygen, or breaking down peroxides, are primarily responsible for their antioxidant activity (Zheng and Wang, 2001).

Phenolic compounds, probably the largest group of secondary metabolites present in plants range from simple compounds with a single aromatic ring to complex compounds which are polymers (Teoh, 2015). They are potent antioxidants that are capable of eliminating free radicals, eradicate a-tocopherol radicals, triggers antioxidant enzymes, chelate metal catalysts and suppress oxidases. Their strong scavenging ability on free radicals maybe because of their hydroxyl groups and intertwined ring structures (Amic *et al.,* 2003). It has been shown that the

antioxidant effects of phenolic acids are mediated by their redox characteristics, ability to chelate metals and quenching of singlet oxygen (Rice-Evans *et al.*, 1996; Chandra *et al.*, 2014). Notably, the phenolic content of plants may contribute directly to their antioxidant action (Tosun *et al.*, 2009). Furthermore, phenolic compounds are being reported to exhibit antiallergenic, antimicrobial, anti-inflammatory, hepatoprotective and cardioprotective effects (Alpinar *et al.*, 2009; Kumar and Pandey, 2013; Sulaiman *et al.*, 2011; Noreen *et al.*, 2017).

Flavonoids are the most ubiquitous groups of plant secondary metabolites and additionally alleged to possess antioxidative action and reduce their formation by chelating the metals (Treutter, 2006). Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups (Sharififar *et al.*, 2008; Aryal *et al.*, 2019). There have been reports of flavonoids to serve as health-promoting compound and protect against several diseases (Tiwari, 2001). Some flavonoids are antibacterial, antiviral (against the common cold sore virus), anti-allergic, antiplatelet and antineoplastic (Liu, 2011). Furthermore, flavonoids have a broad spectrum of pharmacological properties encompassing anti-inflammatory, antioxidant, antimicrobial, antimutagenic, antitumor, antidiabetic, vasorelaxant, immunomodulatory and both oestrogenic and anti-oestrogenic activities (Lin, 2014; Kamtekar *et al.*, 2014; Ren *et al.*, 2003). Flavonoids, a large group of phytochemicals, possess various known health beneficial effects linked to health including antioxidant and free radical scavenging activity (Bouaziz-Ketata *et al.*, 2015; Durkar *et al.*, 2014; Bhatt and Patel, 2015). Flavonoids are known to possess neuro-protective, anti-inflammatory effects, anticancer, anti-genotoxic and antiglycative activities, which are basically related to their antioxidant properties (Xiao and Kai, 2012).

In addition, there are a number of secondary metabolites uncovered in plants that are known for their pharmacological properties. Among them, tannins are regarded to be among the most guaranteeing polyphenolic substances (Tomczyk *et al.*, 2010). Tannin is found to present in all the three extracts of *L. ruellioides*. It has also been demonstrated that tannin molecules inhibit the mutagenic effects of

several mutagens. In order to interact with biological macromolecules, a variety of carcinogens and mutagens generate oxygen free radicals. The antioxidative qualities of tannins, which are crucial for preventing cellular oxidative damage, including lipid peroxidation, may be coupled to their antimutagenic and anticarcinogenic potential. (Chung *et al.*, 1998). Alkaloids are also confirmed to be inherent in every extract of *L. ruellioides* which contains numerous pharmacological effects, including its antibacterial capabilities among certain ethnomedicinal plants (Singh and Bhat, 2003). Several other compounds having a wide range of pharmacological activities were also revealed by LC-MS analysis highlighting the potential healing activities of *L. ruellioides.* From our study, we can conclude that *L. ruellioides* extracts contain a number of phytochemicals along with a significant amount of phenolic and flavonoid compounds showcasing its possible pharmacological potential as a wound healing agent.

CHAPTER-II

Free radical scavenging activities and antibacterial activities of various extracts of *Lindernia ruellioides*

INTRODUCTION

Free radicals are produced naturally in our bodies as part of the regular metabolic process. Free radical can be referred to as an atom or molecule containing one or more unpaired electrons, and thus unstable. When electrons from nearby molecules, like proteins, lipids and DNA couple with this unstable radical, it tends to become stable (Liu *et al.,* 2020; Gilgun-Sherki *et al.,* 2002). It is increasingly evident that as transitional metabolites with tremendous biological value, free radicals are formed or converted in the body through various endogenously and exogenously enzymatic or non-enzymatic reactions, which maintains the metabolic balance of the human body (Zhu *et al.,* 2023). Free radicals can broadly be classified into three major types such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and other radicals without O or N elements. Typically, ROS mainly consists of singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), and lipid peroxide (LPO) such as alkoxyl radical ($RO^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$). RNS comprises nitric oxide (NO•) and nitrogen dioxide (NO_2•) (Zhao *et al.,* 2021).

ROS as well as RNS play a dual role in our body as both detrimental and beneficial effects (Sharma and Kumar, 2011). At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they are highly reactive and toxic and cause damage to cell membrane, proteins, lipids and deoxyribonucleic acid (DNA) which induces oxidative stress. This oxidative stress cause tissue damage resulting in several diseases such as neurodegenerative disease, cardiovascular disease, diabetes, cancer, aging, rheumatoid arthritis and cataract (Pham-Huy *et al.,* 2008; Bandyopadhyay *et al.,* 1999; Sung *et al.,* 2013). Antioxidant play a vital role in protecting our body towards free radical damage. They balance free radical production and detoxify them when in excess (Palaniswamy and Padma, 2011; Valko *et al.,* 2007; Lobo *et*

al., 2010). The toxic effects of ROS and RNS opposed by enzymatic as well as non-enzymatic antioxidants system such as catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), ascorbic acid, tocopherol, flavonoids, glutathione and carotenoids mitigate the consequences of reactive oxygen species and thus help in preventing a number of diseases (Ahmad *et al.*, 2010).

Under certain circumstances, oxygen can have a significant adverse effect on our health by producing ROS, which are both free radicals and non-free radicals. These ROS can have detrimental effects like atherosclerosis, ischemic heart disease, aging, inflammation, diabetes, immunosuppression, neurodegenerative diseases, cancer and other illnesses (Jadhav and Bhutani, 2002). Consequently, both the avoidance and treatment of diseases driven by free radicals may greatly benefit from the use of antioxidants having free radical scavenging properties (Hamid *et al.*, 2010). Indeed, topical application of compounds having antioxidant status was previously demonstrated to be important in healing of cutaneous wound (Martin, 1996). Moreover, malfunctioning at the cellular level may be prevented by a couple antioxidants which reduce damage caused by free radicals (Kalyon, 2009).

Antioxidants are compounds that provide electrons to damaged cells, preventing and stabilizing the damage caused by free radicals. Additionally, free radicals are converted by antioxidants into waste products that the body can discard (Rahman *et al.*, 2015). It is well known that intake of foods that are packed with antioxidants minimizes the incidence of an assortment of diseases spurred on by free radicals (Hamid *et al*., 2010). The natural abundance of phytochemicals including polyphenols, carotenoids, and vitamins E and C is primarily responsible for these positive health impacts (Steinmetz and Potter, 1996). Even though the available synthetic antioxidants can effectively inhibit oxidation (Brewer, 2011), they are suspected to bring about a multitude of negative health consequences in humans (Lu *et al.*, 2013) as well as in experimental animals (Anagnostopoulou *et al.*, 2006). As a result, extensive research is being done on naturally occurring antioxidants with fewer adverse consequences to prevent diseases relating to free radicals (Brewer, 2011; Soobrattee *et al.*, 2005).

The human body and its surrounding environment are home to a diverse array of microorganisms in a biological balance, yet unbridled rapid microbial development may trigger some serious issues (Scheepmaker *et al.,* 2019; Parham *et al.,* 2016). Although antimicrobial compounds are used as antibiotic medications to treat infections in the human body, they can have a number of negative effects, most notably an increase in ROS (Shaikh *et al.,* 2019) that may pose a serious threat to human health and raise possible health hazards (Panieri *et al.,* 2016; Parham *et al.,* 2019). Antibiotic resistance in bacteria evolves through intrinsic or acquired resistance, by chromosomal mutation, or by horizontal gene transfer (HGT) (Sundin and Wang, 2018; Breijyeh *et al.,* 2020). Several mechanisms can lead to the development of antibiotic resistance such as alteration in cell membrane permeability either by reducing antibiotic penetration or increasing its elimination by efflux pumps; moreover, bacteria can deactivate the antibiotic itself or modify the antibiotic targets, in addition to other alternative pathways (Verhaegen *et al.,* 2023; Blair *et al.,* 2015). Several investigations have disclosed that various chemicals components of herbal plants possess antibacterial properties that can protect the human body against diseases without being damaging to cells (Parham *et al.,* 2020). Herbal or synthetic antimicrobial agents are both possible. The main negative effect of synthetic substances including antibiotics, metals, and metal oxide nanoparticles is the development of ROS, which can be exceptionally hazardous and can lead to cancer (Yu *et al.,* 2020).

In several impoverished nations, herbal remedies are the mainstay of treatment for infections. To date, there are over 11,000 species of herbal plants that are in use medicinally and, of these, roughly 500 species are commonly used in Asian and other countries (Saklani and Kutty, 2008; Zhou *et al.,* 2007). Herbal plants that are traditionally used as medicine are considered to be harmless which is consumed or taken by many people without any prescription (Ranjith, 2018). Due to the potential toxic or detrimental effects of many chemical antimicrobial agents, employing natural materials has recently become excellent for treating microbial diseases (Bereksi *et al.,* 2018) Incorporating herbal ingredients could serve as a perfect substitute (Torkan *et al.,* 2015; Tribess *et al.,* 2015) and may also present a

fresh opportunity to create antiviral, anticancer, and antibacterial medications with fewer adverse effects. Aromatic ingredients represent ongoing efforts to look into novel substances that may have antibacterial properties. Numerous studies have demonstrated that various herbal remedies include a variety of compounds, many of which have antibacterial and radical scavenger qualities that can protect the body from infections and cellular oxidation reactions. Consequently, owing to their antibacterial, antiviral, and antioxidant properties, these components are important for the synthesis of several herbal medicines (Saratale *et al.,* 2018; Miraj *et al.,* 2017; Salazar-Aranda *et al.,* 2011). Such varying substances are regarded as potential materials for future research in antimicrobial therapy since they have the ability to inhibit and control infections with minimal cellular harm. Many studies have concentrated on investigating antibacterial and antiviral medications with fewer side effects in light of the aforementioned control measures for the catastrophic effects of the fast growth of bacteria and viruses (Sharma *et al.,* 2017; Waris *et al.,* 2017; Pourghanbari *et al.,* 2016).

MATERIALS AND METHODS

Preparation of plant extract

The extraction process of different extracts of *L. ruellioides* was previously discussed in chapter 1. The chloroform extract (LRCE), ethanolic extract (LREEE), and aqueous extracts (LRAE) were employed to evaluate the scavenging of free radicals and antibacterial activity.

In vitro antioxidant assays

DPPH radical scavenging activity

The radical scavenging activity of DPPH was conducted in accordance with the method described by Leong and Shui, 2002. At several quantities of *L. ruellioides* extracts (0.5 ml, 20-1000 µg/ml), 1 ml of a 0.1 M DPPH methanolic solution was added. After 30 minutes of the whole thing being exposed in the dark, the absorbance at 523 nm was taken into account. The baseline correction used was methanol. The outcomes were contrasted with the previously generated control without sample. IC_{50}, or the concentration of extract (µg/ml) that obstructed 50% of DPPH radicals, was used to express the extract's antioxidant activity. Every trial was conducted in triplicate, with ascorbic acid serving as the standard. The calculation was then used to determine the amount of scavenging:

$$\% \text{ scavenging} = [(A_{blank} - A_{sample})/A_{blank}] \text{ X } 100$$

where A_{blank} the is absorbance of the control and A_{sample} is the absorbance of the solution containing the plant extract.

ABTS radical scavenging activity

The technique outlined by Re *et al*. (1999) was used for assessing the ABTS radical scavenging activity of *L. ruellioides* extracts. Equal volume of 2.45 mM potassium persulfate solution and a 7 mM ABTS solution were combined to create a stock solution. A dark-colored solution containing $ABTS^{\cdot+}$ radicals were obtained by incubating the solution for 12 hours at room temperature in the dark. Before every test, a working solution was made by diluting the stock solution with 50%

methanol to achieve an initial absorbance of roughly 0.700 (± 0.02) at 745 nm. The scavenging activity was later evaluated by combining 150 µl of different extract fractions (20-1000 µg/ml dispersed in their individual solvents) with a working standard of 1.5 ml of ABTS. At 745 nm, the drop in absorbance was measured. Ascorbic acid was employed as positive control. Following that, the scavenging activity was calculated using the formula:

$$\% \text{ scavenging} = [(A_{blank} - A_{sample})/A_{blank}] \text{ X } 100$$

where A_{blank} is the absorbance of the control A_{sample} is the absorbance of the solution containing the plant extract.

Superoxide ($O_2^{\bullet -}$) radical scavenging activity

Considering minor adjustments, the nitroblue-tetrazolium (NBT) reduction technique (Hyland *et al*., 1983) was used to measure superoxide scavenging activity. To the reaction combinations that includes 0.2 ml of nitroblue tetrazolium (1 mg/ml in dimethyl sulfoxide) and 0.6 ml of extract (20-1000 µg/ml), 2 ml of alkaline dimethyl sulfoxide (DMSO) (1 ml DMSO in 5 mM sodium hydroxide) was included to provide a complete volume of 2.8 ml. The absorbance measurement was made at 560 nm. Rather than alkaline DMSO, the blank was made of pure DMSO. The formula given below was used to determine the extracts of *L. ruellioides'* capacity to scavenge the superoxide radical, with ascorbic acid serving as the standard:

$$\% \text{ scavenging} = (A_e - A_o/ A_e) \times 100$$

where A_o is absorbance in the absence of a sample and A_e is absorbance of the solution containing the plant extract.

Antibacterial activity

Microorganisms

The antibacterial activity was assessed against three bacteria species: *Escherichia coli* (MTCC-40), *Bacillus subtilis* (MTCC-121) and *Klebsiella pneumoniae* (MTCC-39). Ahead of the experiment, the bacteria were sub-cultured in nutrient broth and incubated for 24 hours at 37˚C.

Disc diffusion method (Bauer *et al.*, 1966)

Preparation of test microorganisms

Sterile distilled water was utilized to regulate the turbidity of the sub-cultured microorganisms adopting 0.5 Mc Farland as a standard (~1.5 X 108 microorganisms/ml).

Preparation of plant extract disc

Whatman filter no. 3 was used to create a paper disc with a diameter of 5 mm. Before the extracts were applied, the paper discs were sterilized. The sterilized paper discs were then coated with 10 µL of the plant extracts in varying concentrations, ending up with discs bearing 20 mg and 10 mg of the extract, respectively. The discs were subsequently allowed to air dry.

Preparation of media

Nutrition agar powder was dissolved in distilled water to create 200 ml of nutrition Agar (HiMedia). The agar that had been dissolved was then sterilized.

Evaluation of antimicrobial activity of plant extract

The disc diffusion method was used to evaluate the plant extract's antimicrobial properties. Using the pour plate method, agar plates were made, inoculated with the test microorganisms and left to dry at room temperature. The paper disc with two distinct concentrations (20 mg and 10 mg) of plant extract, 25 µg of streptomycin disc (standard) were cautiously maintained on the agar plate's surface. Furthermore, paper disc that contain 5% DMSO for LRCE and distilled water for LREE and LRAE were additionally retained as a negative control to ensure that the extract's dissolving solution lacked antibacterial properties. Afterward, the plates were incubated for 24 hours at 37°C in inverted position. After incubation, the plates were monitored for antimicrobial activity. The extract possessing that activity was taken for measuring the zone of inhibition.

Minimum inhibitory concentration (MIC)

Using the disc diffusion susceptibility test method in accordance with Mendoza (1998), the lowest inhibitory concentration of the crude extract of *L.*

ruellioides was established on the test organisms where the extract was confirmed to be active.

Preparation of test microorganisms

Sterile distilled water was utilized to regulate the turbidity of the sub-cultured microorganisms adopting 0.5 Mc Farland as a standard (~1.5 X 108 microorganisms/mL).

Preparation of plant extract disc

Using Whatman filter paper no. 3, a paper disc with a diameter of 5 mm was created. The extracts were applied to the paper discs after they had been sterilized. The sterilized paper discs were then coated with 10 µL of the plant extracts in varying quantities, ensuring that the discs contained 10 mg, 5 mg, 2.5 mg, 1.25 mg, 0.625 mg and 0.3125 mg of the extract respectively, and the discs were then allowed to air dry.

Preparation of media

Mueller Hinton Agar (HiMedia) is made in 100 ml by immersing premade powder in distilled water. Sterilization was then performed on the dissolved Mueller Hinton Agar.

Evaluation of antimicrobial activity of plant extract

The disc diffusion method was used to determine the plant extract's MIC. Using the pour plate method, agar plates were made, inoculated with the test microorganisms, and left to dried out at the ambient temperature. Following that, the paper discs with six different concentrations (10 mg, 5 mg, 2.5 mg, 1.25 mg, 0.625 mg and 0.3125 mg) of plant extract and 25 µg streptomycin disc were carefully placed on the agar plate's surface. Negative control was not kept as 5% DMSO and distilled water exhibits no antimicrobial activity in the previous disc diffusion experiment. The plates were then incubated for 24 hours inverted at 37°C. Following the completion of the incubation period, the antibacterial activity of the plates was assessed, and the MIC for each test microorganism was identified.

Statistical Analysis

The mean ± standard error of mean is used to express the data. With the use of Graph Pad Prism version 7, the IC_{50} values were determined. Tukey multiple comparison of means was used after one-way analysis of variance (ANOVA) was used to evaluate for significant differences in the phytochemical contents, free radical scavenging activities and antibacterial activities of different extracts. The statistical and graphical analyses were conducted using SPSS version 27 and Graph Pad Prism version 7. *P* values below 0.05 were regarded as statistically significant.

RESULTS

Free radical scavenging activity

DPPH radical scavenging activity

L. ruellioides scavenged DPPH radicals in a dose dependent fashion as indicated by the discolouration of DPPH. The maximum activity of LREE, LRCE and LRAE to scavenged DPPH was noted at 500 µg/ml, 600 µg/ml and 900 µg/ml respectively. LREE was most potent as it effectively inhibited DPPH radical formation and showed the highest scavenging activity (IC_{50} 158.0 ± 4.82 µg/ml) followed by LRAE (IC_{50} 276.4 ± 6.32 µg/ml) and the lowest scavenger was LRCE (IC_{50} 330.43 ± 5.34 µg/ml). IC_{50} of all the extracts are statistically significant in contrast to the standard ascorbic acid (IC_{50} 12.47 ± 0.13 µg/ml).

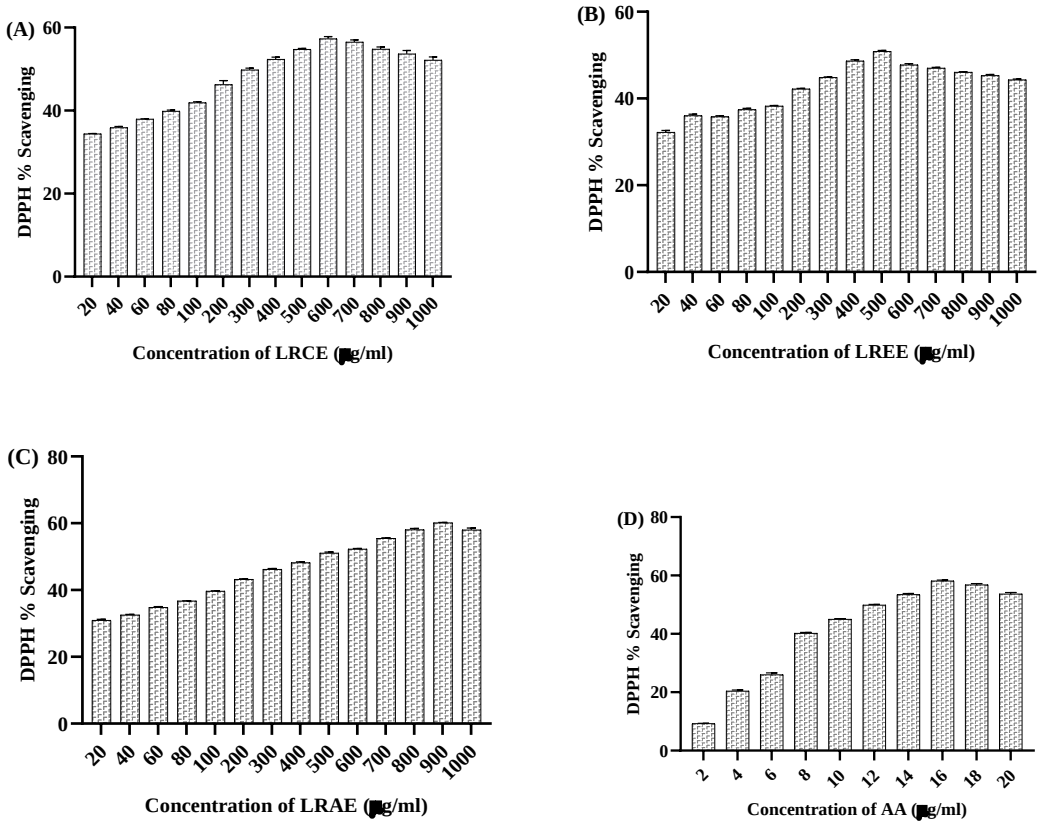


Figure 2.1: DPPH radical scavenging activity of several extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA- Ascorbic acid. Values are expressed as mean ± SEM, n=3.

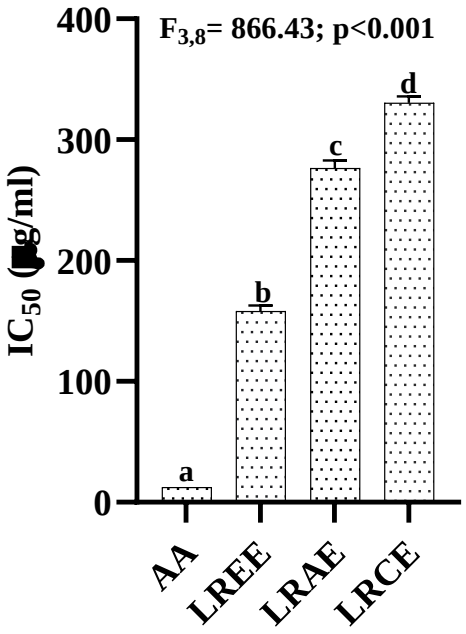


Figure 2.2: IC_{50} (µg/ml) for DPPH. AA- Ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

ABTS radical scavenging activity

L. ruellioides also showed a dose dependent increase in the scavenging of ABTS radical. The highest scavenging action was noted at 200 µg/ml for both LREE and LRAE and 500 µg/ml for LRCE. LREE scavenged the ABTS radicals more efficiently as it showed the highest scavenging activity (IC_{50} 112.9 ± 6.47 µg/ml), followed by LRAE (IC_{50} 222.33 ± 3.18 µg/ml). LRCE was least effective in neutralizing the ABTS (IC_{50} 318 ± 12.19 µg/ml). IC_{50} of all the extracts were statistically significant in contrast to the standard ascorbic acid (IC_{50} 13.52 ± 0.25 µg/ml).

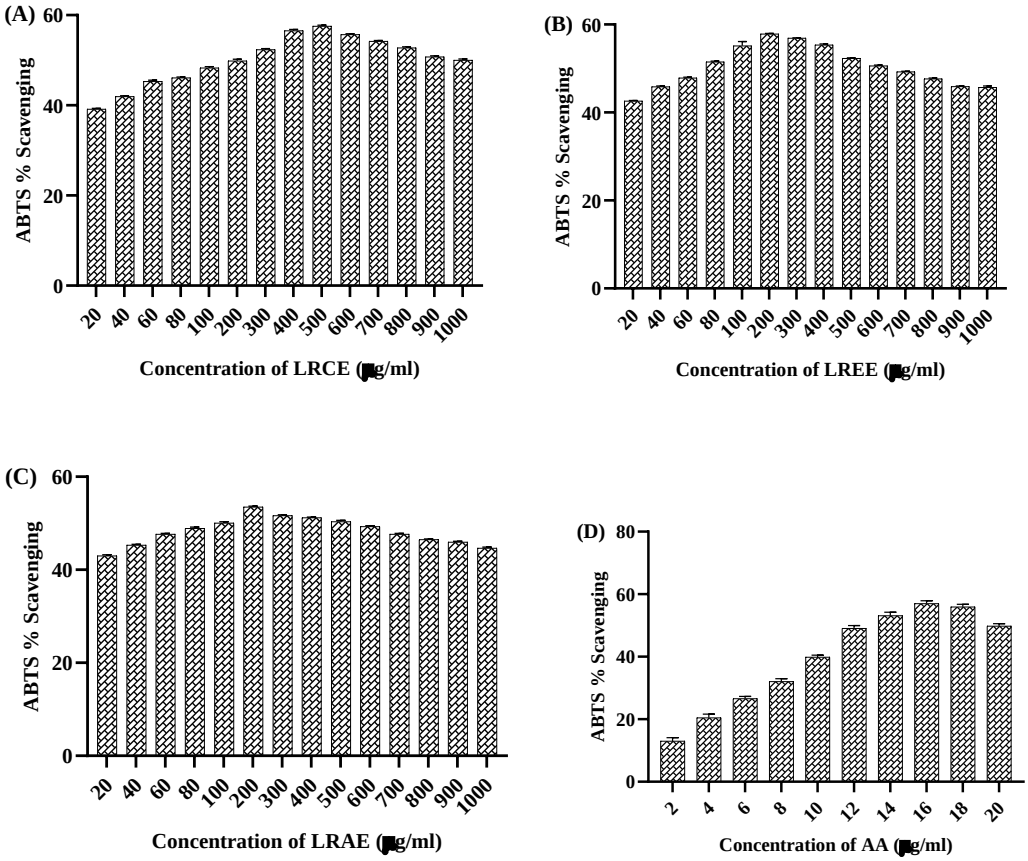


Figure 2.3: ABTS radical scavenging activity of various extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA-Ascorbic acid. Values are expressed as mean ± SEM, n=3.

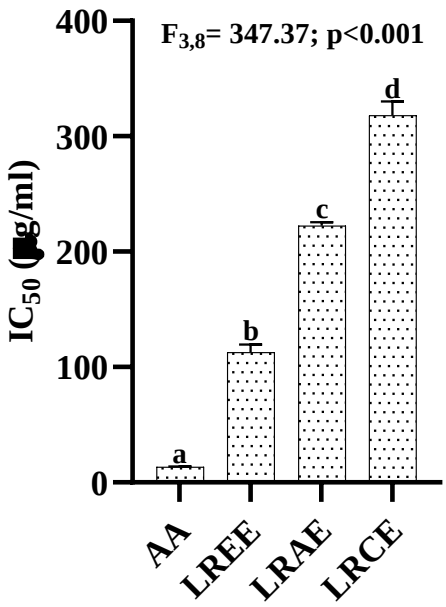


Figure 2.4: IC_{50} (µg/ml) for ABTS. AA- Ascorbic acid; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

Superoxide radical scavenging activity

Various extract of *L. ruellioides* inhibit the formation of superoxide radicals in a dose dependent manner. LRAE scavenged the $O_2^{\cdot -}$ most effectively with a peak scavenging activity at 400 µg/ml (IC_{50} 118.1 ± 7.88 µg/ml). For LREE and LRCE peak scavenging activity occurred at 600 µg/ml (IC_{50} 235.6 ± 10.9 µg/ml) and 800 µg/ml (IC_{50} 381.8 ± 5.6 µg/ml) respectively. The standard ascorbic acid had an IC_{50} 14.13 ± 0.13 µg/ml for $O_2^{\cdot -}$.

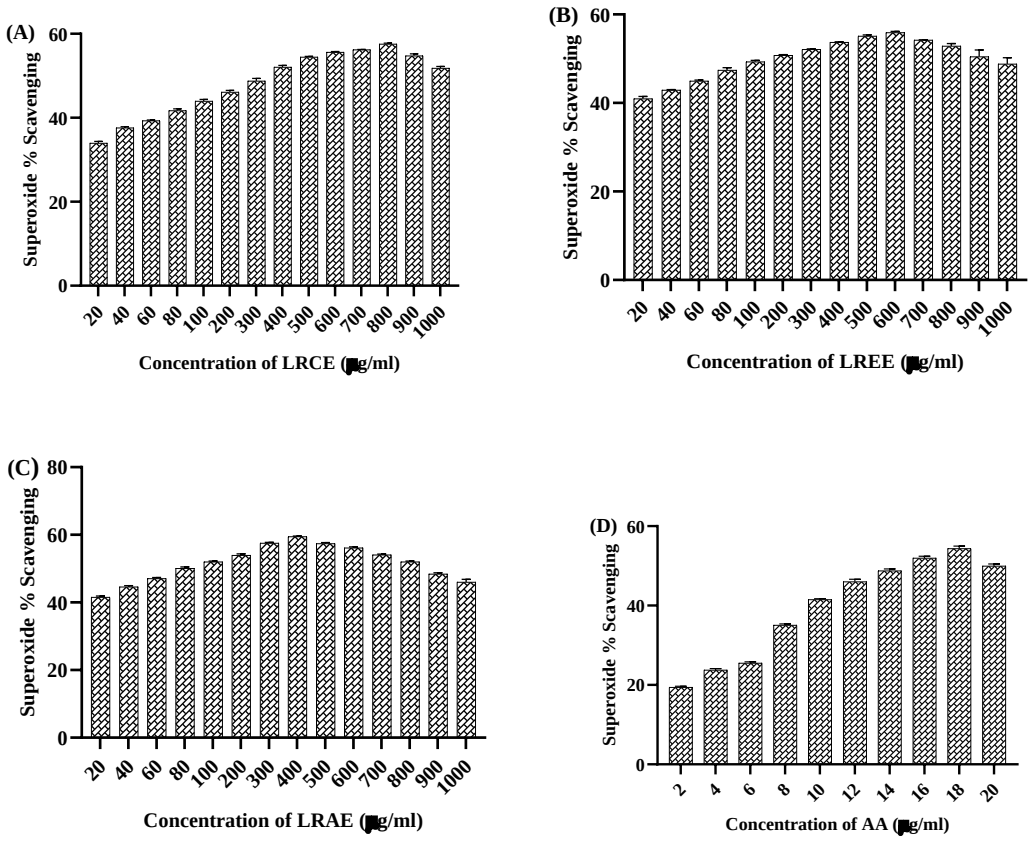


Figure 2.5: Superoxide radical scavenging activity of various extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA- Ascorbic acid. Values are expressed as Mean ± SEM, n=3.

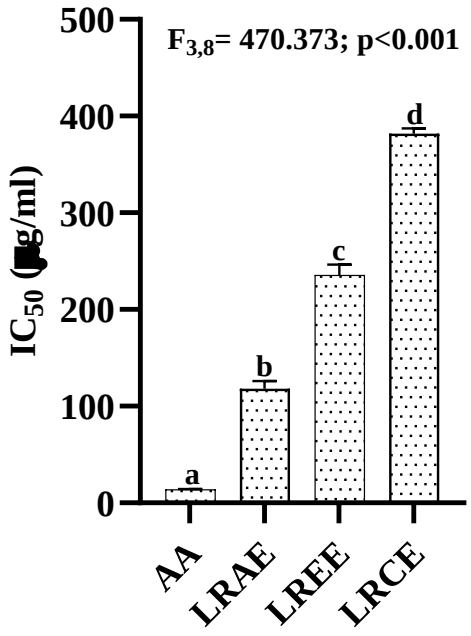


Figure 2.6: IC_{50} (µg/ml) for superoxide radical. AA- Ascorbic acid; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

Antibacterial activity

Disc diffusion method

The results of antibacterial activity showed that LRCE, LREE and LRAE were active against the test organisms as they show remarkable zone of inhibition.

LRCE inhibited the growth of *E. coli* with a moderate degree of inhibition (df= 2, 6; F= 264.33; $p<0.001$). At 20 mg/ml, LRCE showed an inhibition zone of 9.3 ± 0.16 mm while 10 mg/ml LRCE showed 7.1 ± 0.16 mm in diameter. The standard streptomycin exhibited a significantly high inhibition value of 17.5 ± 0.28 mm diameter of a clear inhibition zone.

The growth of *B. subtilis* is also inhibited by both 20 mg/ml and 10 mg/ml LRCE with significant variation (df= 2, 6; F= 441; $p<0.001$). 20 mg/ml and 10 mg/ml LRCE showed inhibition zone of 8.1 ± 0.16 and 6.1 ± 0.16 mm in diameter respectively in which a standard streptomycin showed 19.1 ± 0.16 mm of inhibition zone.

LRCE also revealed a significant variation of growth inhibition in *K. pneumoniae* (df= 2, 6; F= 296.33; $p<0.001$). 20 mg/ml and 10 mg/ml showed an inhibition zone of 9.1 ± 0.16 mm and 6.5 ± 0.28 mm, respectively. A standard streptomycin inhibited *K. pneumoniae* growth by 17.3 ± 0.16 mm diameter of a clear zone.

The graphical representation of inhibitory activity of LRCE against the three tested bacteria was represented in Figure 2.7.

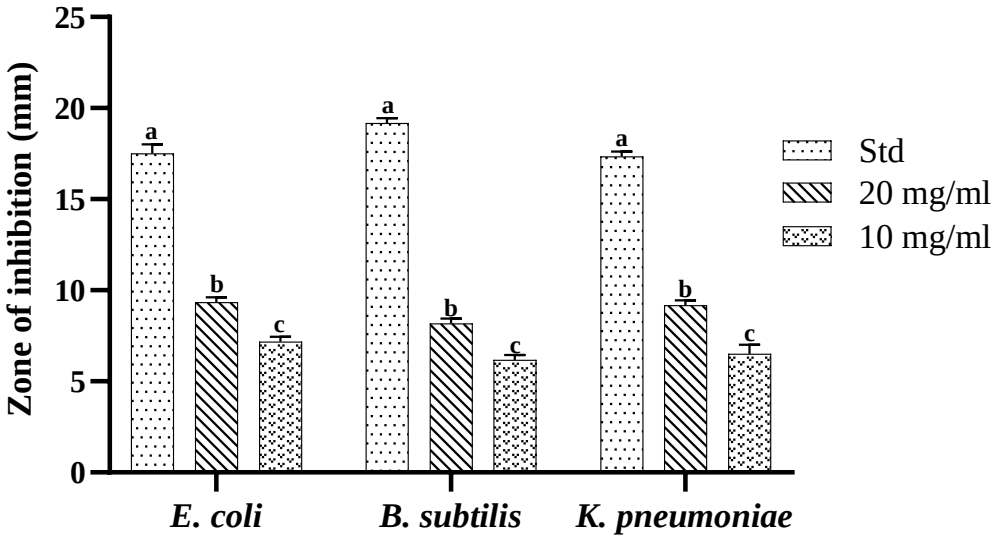


Figure 2.7: Graphical representation of antibacterial activity of LRCE against *E. coli*, *B. subtilis* and *K. pneumoniae*.

Considering LREE, a significant growth inhibition of *E. coli* was observed (df= 2, 6; F= 650.4; p<0.001). 20 mg/ml and 10 mg/ml LREE inhibited the growth of *E. coli* with a zone measuring 8.1 ± 0.16 mm and 7.6 ± 0.16 mm in diameter respectively, whereas the standard drug streptomycin indicates a clear zone measuring 17.6 ± 0.16 mm in diameter.

A significant inhibition of growth was also observed in *B. subtilis* (df= 2, 6; F= 661.78; p<0.001). 20 mg/ml exhibited an inhibition activity with a clear zone of 7.1 ± 0.16 mm in diameter and 10 mg/ml showed a zone of 6.1 ± 0.16 mm in diameter. The standard streptomycin showed 19.6 ± 0.16 mm clear zone.

LREE also demonstrated significant inhibition against *K. pneumoniae* (df= 2, 6; F= 327; p<0.001). LREE with 20 mg/ml and 10 mg/ml indicate a clear zone measuring 8.1 ± 0.16 mm and 7 ± 0.28 mm in diameter respectively. And the standard streptomycin showed a zone of 16.8 ± 0.16 mm. Graphical representation of inhibitory activity of LREE against the tested bacteria was represented in Figure 2.8.

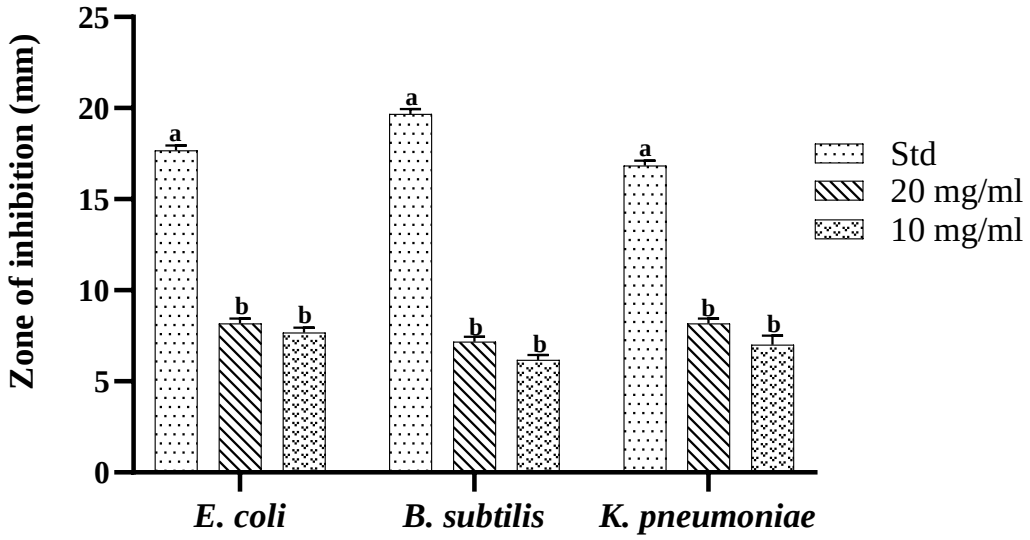


Figure 2.8: Graphical representation of antibacterial activity of LREE against *E. coli*, *B. subtilis* and *K. pneumoniae*.

For LRAE, there was a noticeable growth inhibition for *E. coli* (df= 2, 6; F= 339.11; $p<0.001$). 20 mg/ml and 10 mg/ml showed 7.6 ± 0.16 mm and 6.6 ± 0.33 mm in diameter respectively while the standard streptomycin showed a zone of 16.1 ± 0.16 mm.

There was a significant growth inhibition of *B. subtilis* (df= 2, 6; F= 675.17; $p<0.001$). Only a slight inhibition of growth was noted at 20 mg/ml along with 10 mg/ml LREE showing 6.1 ± 0.16 mm and 5.3 ± 0.16 mm in diameter of clear zone. However, the standard streptomycin showed an inhibition zone of 16.1 ± 0.16 mm diameter.

A significant inhibition of growth was observed in *K. pneumoniae* (df= 2, 6; F= 652.17; $p<0.001$). 20 mg/ml LRAE exhibited an inhibition activity with a clear zone of 7.1 ± 0.16 mm diameter while 10 mg/ml LRAE showed a zone measuring 6.6 ± 0.16 mm diameter. A standard streptomycin showed an inhibition zone of 17.1 ± 0.16 mm diameter. Graphical representation of inhibitory activity of LREE against the tested bacteria was represented in Figure 2.9.

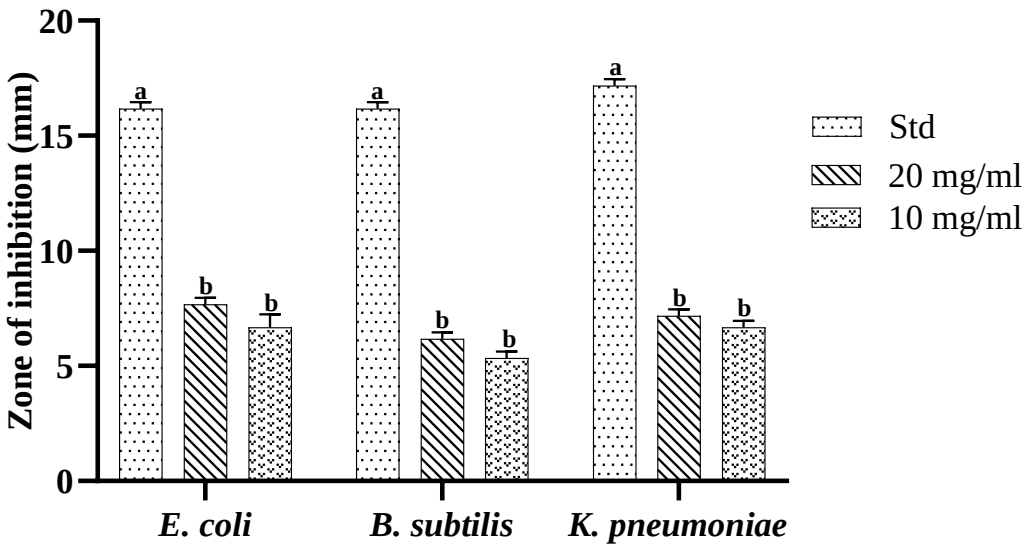


Figure 2.9: Graphical representation of antibacterial activity of LRAE against *E. coli*, *B. subtilis* and *K. pneumoniae*.

Comparing the three extracts, at 10 mg/ml, LREE was most active against the three microorganisms with zone of inhibition of 7.6 ± 0.16 mm, 6.1 ± 0.16 mm and 7 ± 0.28 mm for *E. coli*, *B. subtilis* and *K. pneumoniae* respectively. However, at 20 mg/ml, LRCE was most effective with zone of inhibition of 9.3 ± 0.16 mm, 8.1 ± 0.16 mm and 9.1 ± 0.16 mm for *E. coli*, *B. subtilis* and *K. pneumoniae* respectively. Moreover, from the study, we found that 5% DMSO and distilled water, the solvents used for dissolving the extract fails to exhibit any antibacterial activity. The inhibitory activity of LRCE, LREE and LRAE against *E. coli*, *B. subtilis* and *K. pneumoniae* was represented in Figure 2.10.

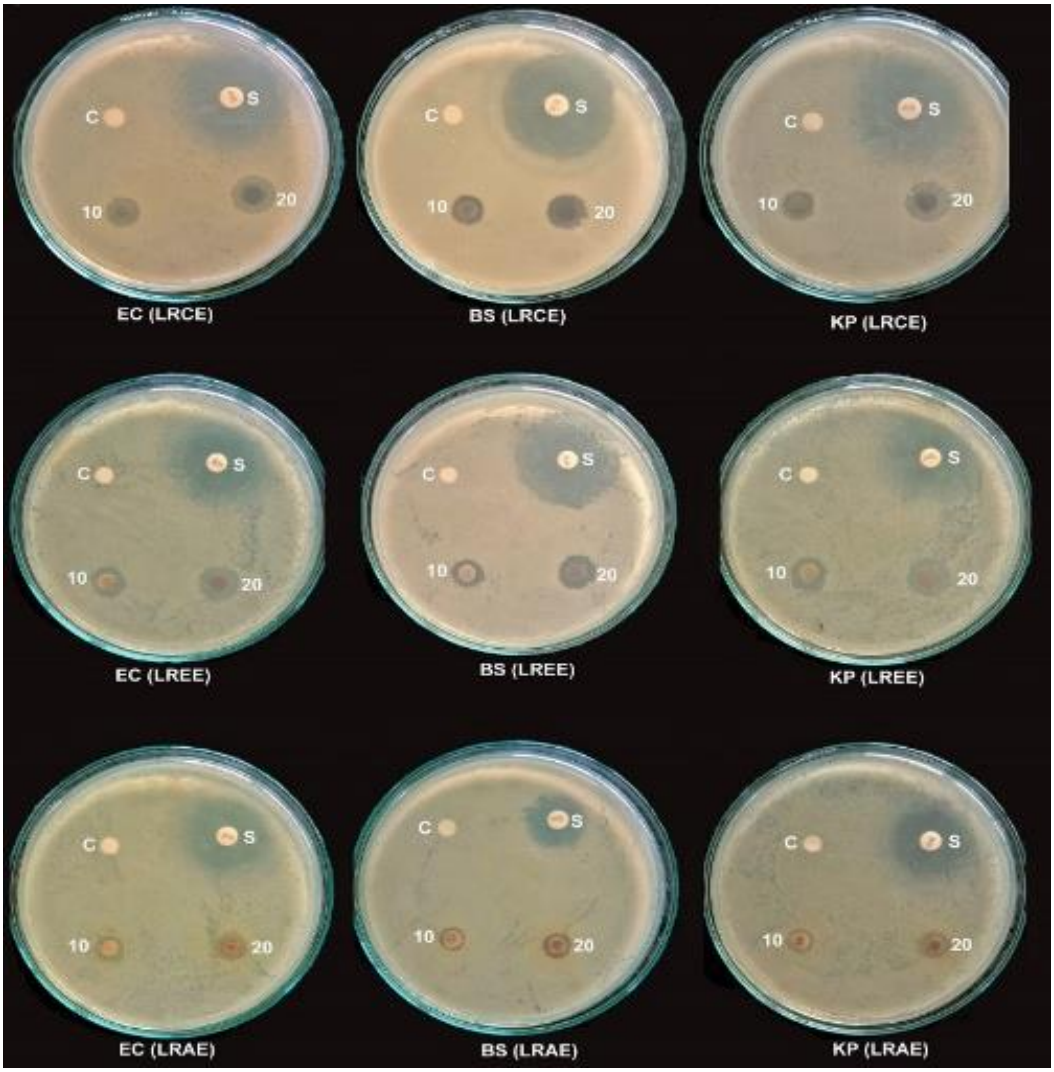


Figure 2.10: Antibacterial activity of LRCE, LREE and LRAE on different test microorganisms. EC-*Escherichia coli*, BS-*Bacillus subtilis*, KP-*Klebsiella pneumoniae*,LRCE- *Lindernia ruellioides* chloroform extract, LREE- *Lindernia ruellioides* ethanol extract, LRAE- *Lindernia ruellioides* aqueous extract, C-control, S-standard, 10-10 mg/ml extract, 20-20 mg/ml extract.

Minimum inhibitory concentration (MIC)

Our result (Table 2.1) showed that LREE is quite active even at low concentrations with an MIC value of 0.625 mg/ml on *E. coli*, *B. subtilis* and *K. pneumonia*. LRCE is also quite active on *E. coli* and *K. pneumonia* having MIC at 1.25 mg/ml and 2.5 mg/ml for *B. subtilis.* LRAE show MIC for *E. coli* at 2.5 mg/ml and for *B. subtilis* and *K. pneumonia* at 5 mg/ml.

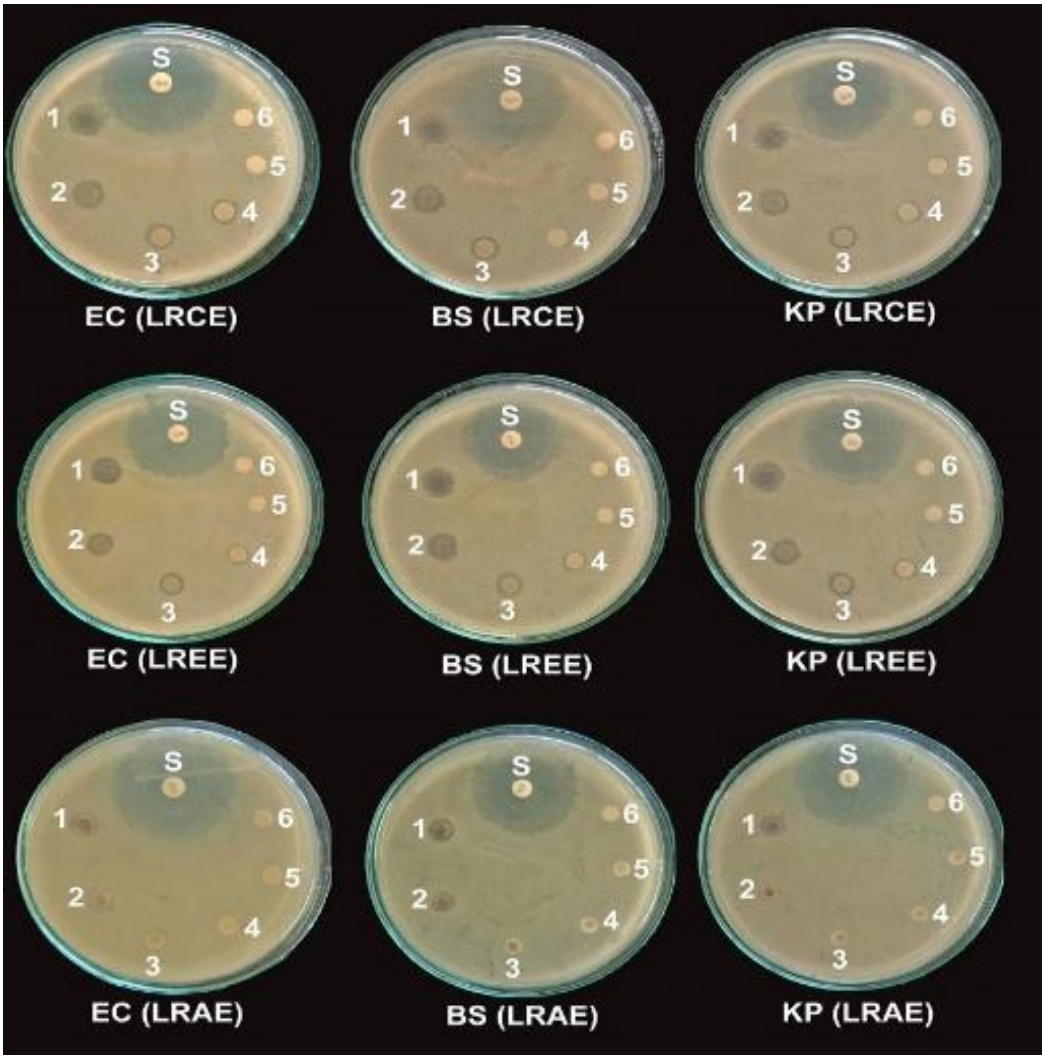


Figure 2.11: MIC of LRCE, LREE and LRAE on different test microorganisms. EC-*Escherichia coli*, BS-*Bacillus subtilis*, KP-*Klebsiella pneumoniae*, LRCE-*Lindernia ruellioides* chloroform extract, LREE- *Lindernia ruellioides* ethanol extract, LRAE- *Lindernia ruellioides* aqueous extract, S-standard, 1- 10mg/ml, 2- 5mg/ml, 3- 2.5mg/ml, 4- 1.25mg/ml, 5- 0,625mg/ml, 6- 0.3125mg/ml.

Table 2.1: Minimum inhibitory concentration (MIC) for different extracts of *L. ruellioides* on different test microorganisms (mg/ml).

Test organisms	MIC of LRCE	MIC of LREE	MIC of LRAE
Escherichia coli (MTCC- 40)	1.25	0.625	2.5
Bacillus subtilis (MTCC- 121)	2.5	0.625	5
Klebsiella pneumoniae (MTCC- 39)	1.25	0.625	5

MIC- Minimum inhibitory concentration; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract.

DISCUSSION

Oxidative processes constitute one of the principal factors of chemical deterioration, which leads to rancidity or impairment of nutritional content, color, flavor, texture and food safety (Frankel and Meyer, 2000; Carpenter *et al.*, 2007). An atom or molecule with an unpaired electron that is unstable is referred to as a free radical. When such unstable radical couples with biological macromolecules like proteins, lipids and DNA in healthy human cells, it has the propensity to become stable and then damage proteins and DNA (Gilgun-Sherki *et al.*, 2002). Free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are produced in our bodies by numerous endogenous mechanisms are highly reactive and potentially damaging transient chemical species, hence, to ensure that the body's physiological processes functioning properly, free radicals and antioxidants must be in equilibrium (Halliwell and Gutteridge, 1998). When free radicals are synthesized in excessive amounts, a condition known as "oxidative stress" emerges. Consequently, free radicals adversely affect distinctive biomolecules, including lipids, proteins and DNA, triggering various chronic diseases, such as coronary heart disease, atherosclerosis, cancer, along with aging (Halliwell, 1996; Madhavi *et al.*, 1996). Hence application of external sources of antioxidants may benefit in managing with oxidative stress.

Antioxidant action is dependent on the existence of its bioactive constituents, primarily polyphenols, carotenoids and vitamins E and C (Oktay *et al.*, 2003). This implies that demonstrating antioxidant activity depends on the concentration of the bioactive substances in the extract. Numerous substances are produced by plants as secondary metabolites, and a bunch of them possess antioxidant properties. Hence, in this study, the free radical scavenging activities of different extracts of *L. ruellioides* was determined on DPPH, ABTS and superoxide radicals. Every extract exhibited strong scavenging action, which increase as concentration emerged. Our findings are consistent with evidence that has been published previously (Pallerla *et al.*, 2019; Lalrinzuali *et al.*, 2015; Devi and Jagetia, 2017) and convey that the extract's chemical makeup and polyphenol content account for its antioxidant ability.

Antioxidants are hypothesized to have an influence on DPPH because of their propensity to donate hydrogen (Choi *et al.,* 2000). Radical scavenging initiatives are critical to prevent the detrimental effect of free radicals in various illnesses. DPPH free radical scavenging is a well acknowledged method for assessing the antioxidant capabilities of plant extracts. By adding the extract in a concentration-dependent manner, the violet DPPH solution in the assay is reduced to the yellow product, diphenylpicryl hydrazine. Because of how quickly it can be analysed, this approach has been widely utilized to predict antioxidant activity. Our findings showed that *L. ruellioides* scavenged DPPH in a dose dependent manner. DPPH radicals are scavenged by polyphenols due to their capacity to donate hydrogen (Duan *et al.,* 2007; Li *et al.,* 2009). According to our findings, every extract from *L. ruellioides* exhibited radical scavenging action through their capacity to donate hydrogen or transfer electrons. There is a strong correlation between total polyphenol concentration and antioxidant activity that scavenges radicals (Huang *et al.,* 2005).

Stable free radical ABTS is produced when a potent oxidizing agent reacts with another substance with the ABTS salt which transforms ABTS into its radical cation. With its characteristic longwave absorption spectrum at 734 nm, this blue radical cation absorbs light. The level of decolorization indicates the effective inhibition of the $ABTS^{\bullet+}$ (Re *et al.,* 1999). The majority of antioxidants, such as phenols, thiols and vitamin C are reactive with $ABTS^{\bullet+}$. (Walker RB and Everette JD, 2009). The $ABTS^{\bullet+}$ scavenging activity could be because of high phenolic contents. The several *L. ruellioides* extracts used in our investigation demonstrated concentration-dependent suppression of ABTS radical generation. A comparable outcome has been noted with the extract of *Calpurnia aurea, Vernonia amygdalina, Lawsonia inermis, Croton macrostachyus* and *Withania somnifera* in earlier studies (Wasihun *et al.,* 2023; Olusola-Makinde and Bayode, 2021; Romha *et al.,* 2018). Since the FRAP assay is a quick and easy way to measure antioxidant activity (Hodzic *et al.,* 2009), it has been used in an earlier study in which it has been discovered that flavonoids from 19 distinct plants scavenge ABTS radicals and exhibit increased FRAP (Xia *et al.,* 2014).

In biological systems, superoxide is produced through cellular respiration in which oxygen molecules are consumed (Fujii *et al.,* 2022). In the presence of iron, they react to form the extremely reactive OH radical. Furthermore, by generating H2O2, •OH, peroxynitrite, or singlet oxygen, superoxide anions generated as a result of inadequate oxygen metabolism harm biomolecules either directly or indirectly (Lushchak, 2014; Kirkinezosa and Morae, 2001). Therefore, superoxide radicals must be suppressed in order to safeguard cells from oxidative stress as well as their deleterious effects (Yen and Duth, 1994). According to reports, the antioxidant effects of certain flavonoids behave efficiently primarily by means of scavenging superoxide radical (Robak and Gryglewski, 1998). Thus, the existence of flavonoids in *L. ruellioides* might be responsible for their scavenging activity. A number of plant extracts and some compound plant formulations were found to inactivate the subsequent generations of $O_2^{\cdot-}$ radicals in a dose dependent fashion (Wong *et al.,* 2006; Aparadh *et al.,* 2012).

Antibacterial agents play a crucial role in wound healing by preventing or addressing bacteria-related, which can impede the process of recuperation and generally aid the healing process by preventing infection, reducing the inflammatory response, promoting granulation and tissue formation (Mendame *et al.,* 2021; Norman *et al.,* 2016). Throughout the course of the past century or so, various unique chemicals with therapeutic potential have been developed from plants, serving as a complement or substitute for modern medical treatments (Agidew, 2022). Plants contain phytochemicals (Gebreslassie, 2019) and these phytochemicals have a significant inhibitory impact on the growth of pathogens (Melese *et al.,* 2019). Natural remedies are offering people solace in an era of escalating super illnesses and resistance strains (Breijyeh and Karaman, 2024). In recognition of the limited efficacy of current antimicrobials, numerous researchers have turned their attention to exploring natural products as potential sources of novel bioactive compounds (Recio and Rios, 1989; Silver and Bostian, 1993). Although their exact mode of action as antibacterial agents is still enigmatic, phenolic compounds are commonly used in traditional medicine. They seem to work differently from antibiotics, which simplifies the risk of cross-resistance thus

rendering them a promising agent against resistant pathogens (Cushnie and Lamb, 2005; Barbieri *et al.,* 2017; Cheesman *et al.,* 2017). In a meta-analysis study conducted by Wang *et al.,* 2023, the influence of polyphenol components on the eradication of bacterial infection was determined and discovered that the polyphenol compounds may have a favorable impact on bacterial removal, considering the overall removal rate of the bacteria was substantially greater in the polyphenol compounds group in comparison to the control group. Our current investigation discovered that different extracts of *L. ruellioides* inhibit the growth of all three bacteria species studied. Moreover, the ethanolic extract seemed to be the most potent as it has the lowest MIC value of 0. 625 mg/ml for *E. coli*, *B. subtilis* and *K. pneumoniae* which could be prompted by the presence of significant portion of polyphenol compounds.

CHAPTER- III

Wound healing evaluation of *Lindernia ruellioides* using excision and incision wound models in rats

INTRODUCTION

Wound can be described as the disruption of cellular and functional or anatomical integrity of living tissue and may be accompanied by disruption of the structure and function of underlying normal tissue (Mulkalwar *et al.,* 2015; Shrimanker *et al.,* 2013). Wounds frequently fall into two categories: acute wounds and chronic wounds (Jaswanth *et al.,* 2001). Although wound healing occurs naturally, an infection may prompt the healing process to be deferred (Subramoniam *et al.,* 2001). A multitude of processes are involved in the complicated (yet ordered) phenomena of wound healing. Upon an injury, a predictable sequence of processes occurs to mend what was damaged. The cells beneath the dermis, the deepest layer of skin, start producing more collagen (connective tissue) during the inflammatory response that follows an injury, progressing to the last stage of epithelial tissue regeneration (the outer layer of skin) (Martin, 1997). The process entails parenchymal cell regeneration, connective tissue and parenchymal cell migration and proliferation, extracellular matrix protein synthesis, connective tissue remodelling, and wound strength acquisition (Cotran *et al.,* 1994; Lazarus *et al.,* 1994; Bairy and Rao, 2001). Alterations to any of these phases may result in a delay in healing or perhaps the incapacity to recover fully (Akkol *et al.,* 2009). Despite their imprecise representation of human wound healing processes and associated clinical difficulties, animal wound healing models remain vital resources with the intention of creating alternative methods and techniques for therapy of wound healing (Ghosh, 2012).

There are plenty of wild plants that possess healing properties that have been stipulated by practitioners of indigenous herbal medicine (Saha *et al.,* 1997; Kumar *et al.,* 2007; Jaric *et al.,* 2007; Taranalli and Kuppast, 1996; Veerapur *et al.,* 2004; Rathi

et al., 2004). With regard to their antioxidant properties and ability to stimulate fibroblast growth, people have historically utilized plants to treat wounds (Houghton *et al.,* 2005). Countless natural and plant-based products that include active compounds like alkaloids and flavonoids and biomolecules (Chithra *et al.*,1995) have reportedly healing properties (Kumar *et al.,* 2007; Jaric *et al.,* 2007). Since the dawn of humanity, plants and their preparations are currently exploited to hasten the healing process of wounds (Schmidt *et al.,* 2009). Multiple research efforts have demonstrated that the phytochemical components found in medicinal plants aid in the process of wound healing (Nayak *et al.,* 2005; Haque *et al.,* 2003; Mesquita *et al.,* 2005; Nayak *et al.,* 2009). On top of that, according to reports, medicinal plants are exceptionally helpful in wound care given that they intensify the healing process and leave patients without any noticeable scars (Kumar *et al.,* 2007). Yet, there is no scientific proof of their effectiveness or understanding of the alleged active ingredients or their mode of action; instead, their use is purely customary. Recent years have seen an upsurge in the recreational consumption of medicinal plants as a means of addressing human health, along with an enormous urge to explore therapies that are less detrimental to the human body due to the ineffectiveness of some pharmaceuticals, high cost of currently accessible medications as well as severe side effects such as skin irritation, pain, allergic reactions and immune suppression (Bruning *et al.,* 2012; Oliveira *et al.,* 2022).

In view of this context, our focus evolves upon *L. ruellioides*, which has a strong traditional history in serving an assortment of ailments. The whole part of the plant can be used medicinally for treating detoxification, injuries, menoxenia, dysmenorrhea, and snake's and dog's bites. It is also commonly used for dressing cuts, wounds, boils, bruises, dysentery, jaundice, snakebite, urinary tract problems and accelerated healing of wounds when applied externally and the juices of the leaves are also used for massaging on strains. (Wei *et al.,* 2017; Sikdar and Dutta, 2008; Rai and Lalramnghinglova, 2010).

Regardless of the extensive use and compelling evidence of *L. ruellioides* as traditional medicine by the indigenous practitioners and traditional healers in

Mizoram, there is currently insufficient scientific evidence supporting their medicinal properties. As a consequence, the current study postulates a hypothesis that the traditional practice of *L. ruellioides* for improving wound healing correlates to its feasible bioactive substances that may constitute wound healing activity. Our expected outcome will confirm more than just the conventional claims but also enlightened the tactics that accelerate wound healing, that might be a stepping stone in the identification of novel therapeutic drugs in future.

MATERIALS AND METHODS

Plant extract

The ethanolic extract of *L. ruellioides* (LREE) was discovered to be the most potent among the three extracts from the preliminary studies and was used for the *in vivo* studies.

Experimental animals

Male inbred Wistar albino rats in an excellent health condition weighing 150-180 g were selected for the study. They had been maintained separately in regulated environments in propylene cages (temperature: 23 ± 2ºC, 12-hour light/dark cycle) and kept on a standard diet of food and water *ad libitum*. The rats were anesthetized both prior to and throughout the execution of experimental wounds using ketamine hydrochloride (10 mg/kg) and xylazine (7:1 ratio). Sterilization was maintained throughout the surgical interventions. The animals were periodically inspected for infections, and those that displayed symptoms were replaced and banished from the experimental group.

Animal Ethical Committee Approval

The ethical committee for animal experiments gave its approval to the project with clearance no: MZU/ZOOL/IAEC/2019-20/01.

Acute dermal toxicity

To assess the extract's acute dermal toxicity, the ointments were applied to the shaved back of rats. The research was carried out in compliance with OECD guidelines no. 402 (OECD guidelines, 1987). Rats were separated into different groups (n=6) and applied 5% LREE, 10% LREE and 20% LREE ointments on the rat's shaved back. Simple ointment was applied to the rats under control group. The rats were regularly monitored for the sign of acute toxicity throughout the first day and then once per day for 14 days.

Wound healing activity

Using excision and incision wound models, the ethanolic extracts of *L. ruellioides* were investigated for their capacity to promote wound healing in rats.

Four categories were used to group the animals, each consisting of six animals: Group 1 (Negative control) animals were treated with simple ointment base. Animals of Group 2 (Positive control) were topically treated with 5% povidine iodine (PVI). Group 3 and 4 are the test groups that received treatment with the ethanolic extract of *L. ruellioides*, 5% w/w in ointment and 10% w/w in ointment respectively. The pace of wound closure, the duration of epithelialization, antioxidant level, the strength of the wound breaking and histological analysis were employed to assess the impact of *L. ruellioides* on the rate of wound healing.

Excision Wound Model

Following anaesthesia, the rats were given surgical excision wounds in accordance with Morton and Malone, 1972 and Kamath *et al.,* 2003 recommendation. The animal's dorsal fur was painstakingly shaven with a medical blade and electric clipper. The intended wound area was outlined on the rat's back. Making use of a surgical blade, a full thickness excision wound of 300 mm^2 and 2 mm in depth was made along the markings. Making use of a cotton swab dipped in normal saline to dab at the wound, haemostasis was accomplished. The entire wound was remained uncovered. Every surgical operation was performed in a sterile setting.

Measurement of Wound Area

For the final analysis of data, the wound area was measured on days 3, 6, 9, 12, 15, 18 and 21 post-wounding days using translucent paper and graph paper. This allowed for the monitoring of the wound's progressive changes and the assessment of the wound contraction rate. The percentage reduction in the wound's region was utilized to compute the contraction of the wound (Werner *et al.,* 1994).

% Wound contraction = (Initial wound area - Specific day wound area) / Initial wound area X 100.

Period of epithelialization

The rate of epithelialization was defined as the number of days required for the eschar to fall off without rendering any raw wounds behind (Nayak *et al.,* 2007).

Incision Wound Model

According to Ehrlich and Hunt, 1968, an incision wound was inflicted. After the application of anaesthesia, the dorsal fur of the animals was shaved with the help of surgical blade and electric clipper. On the animal's back, a 6 cm long longitudinal paravertebral incision was created through the cutaneous muscle and skin. Using surgical thread and a curved needle, surgical sutures were firmly and constantly placed to the separated skin at intervals of 1 cm. The wounds were left undressed. The eighth post-wounding day saw the removal of the sutures, and the course of treatment was resumed. On the tenth day, the tensile strength of the injured skin was assessed using the Lee, 1968 method, which involves continuous and consistent water flow.

Determination of Tensile Strength

The increase in tissue tensile strength correlates significantly with the extent of wound healing. Tensile strength was assessed on day 10. A line was drawn both edges of the wound, 3 mm from the suture line, and the sedated animal was fastened to the table. A pair of forceps, one at either end opposite the other, were used to grasp the line on either side 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 volume of water was calculated and recorded. Three readings on average were made for a particular incision wound, and the group mean was used to calculate each breaking strength (Lee, 1968).

Antioxidant assays

Granulation tissue was excised out on the 16^{th} day and the tissue homogenate was used to estimate glutathione (GSH), glutathione-s-transferase (GST), superoxide dismutase (SOD), and lipid peroxidation (LPO). The granulation tissues were homogenized in phosphate buffered saline (PBS, pH 7) using a glass homogenizer (10% w/v) at 4°C. After centrifuging the homogenates for 30 minutes at 12,000 rpm and 4°C, the supernatants were used right away to test for antioxidant activity.

Using the technique outlined by Lowry *et al.,* 1951, protein contents were determined.

Glutathione (GSH)

The method described by Moron *et al.,* 1979 was used to quantify glutathione (GSH) levels. 20 µl of 10 mM DTNB and 900 µl of 0.02 M sodium phosphate buffer were blended with 80 µl of the cell homogenate, and two min were spent incubating the combo at room temperature. At 412 nm, the sample's absorbance was measured in a UV-visible spectrophotometer against a blank. The standard graph was used to compute the GSH concentration, which was later represented in µmol/mg protein.

Glutathione-s-transferase (GST)

The method implemented by Beutler, 1984 was utilized to measure Glutathione-s-transferase (GST). In short, 850 µl of 0.1 M phosphate buffer (pH 6.5) was mixed with 50 µl of 20 mM CDNB, and incubation of the entire mixture lasted for 10 minutes at 37°C. Then, 50 µl each of 20 mM GSH and cell homogenate was incorporated to the mixture. For five minutes, the sample's absorbance was measured at 340 nm at one-minute intervals. The activity of GST was quantified as:

GST activity = (OD of test - OD of blank/9.6 × vol. of test sample) × 1000; where 9.6 is the molar extinction coefficient for GST in this instance.

Superoxide dismutase (SOD)

With couple of adjustments, Fried, 1975 was followed to estimate the SOD activity. 300 µL of 3.0 mM NBT and 200 µL of 780 µM NADH were combined with 100 µL of cell homogenate and 186 µM PMS. To halt the reaction, 1 mL of acetic acid and 4 mL of n-butanol were added complying with the mixture's 90 s incubation at 30°C. At 560 nm, the absorbance of the test sample and blank was measured, and the enzyme activity was presented in unit (1unit = 50% inhibition of NBT reduction)/mg protein.

% inhibition = (OD of blank - OD of test/OD of blank) × 100.

Lipid peroxidation (LPO) assay

The standard method proposed by Beuege and Aust, 1978 was used to measure lipid peroxidation (LPO). One helpful metric for evaluating the degree of lipid peroxidation reaction is malondialdehyde (MDA), a hazardous byproduct of the oxidation of fatty acids, incorporating polyunsaturated fatty acids (PUFA) and phospholipids. When MDA from LPO combines with TBA, a red fluorescent adduct is produced absorbing at 535 nm. Here, cell homogenate was added in a 1:2 ratio to a mixture of 0.8% TBA, 10% TCA, and 0.02 N HCl. After 10 minutes of boiling, the liquid was promptly allowed to cool prior to centrifugation at room temperature for 10 min at 1000 rpm. After gathering the supernatant, its absorbance at 535 nm was measured against a blank. The extinction coefficient of 1.56×10^6/M/cm was employed to ascertain the sample's MDA content.

Histopathological examination

For the histological analysis, a sample of tissue was extracted from each group of rat's healed skin (Bancroft and Gamble, 2008). Upon 16 days of treatment, cross-sectional full-thickness samples of the skin were acquired from each group in order to assess any histological changes. Here, 10% buffered formalin was used to fix the samples. Following a range of ethanol solutions with grades (70%, 90%, and 100%) for dehydration, prior to being embedded in paraffin wax, the tissues had been cleared applying xylene and then sectioned into 5 µm thick sections. The slides had poly-L-lysine applied to them to assure adequate adhesion, and tissue sections were spread out gradually within a water bath that had been maintained at 43ºC. The slides were deparaffinized using xylene, after which they were rehydrated for 10 min each using a sequence of ethanol concentrations. Hematoxylin and eosin stain (HE) were used to stain the sections with subsequent dehydration utilizing varying concentrations of ethanol. To enhance clarity, xylene was the agent employed to treat the slides in the final steps before being mounted with DPX. Finally, the slides were carefully examined and imaged.

Statistical analysis

Data are 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. SPSS software 27 and Graph Pad Prism 7 were used for statistical and graphical analyses. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Dermal acute toxicity

For the entire 14-day observation period, every animal involved in the acute dermal toxicity test showed sustained survival in compliance with OECD guideline 402. There were no alterations in the eyes, fur, somatomotor activity or behavioural patterns, or in the respiratory, circulatory, autonomic, central nervous system. Considering there was no discernible harm at the levels that were stipulated, the doses were deemed safe.

Antioxidant activity

Following treatment with varying doses of LREE, both the amount and activity of antioxidants and oxidants in the skin tissue of rats were assessed. Comparing the treatment groups with the untreated control group, the ethanolic extract of *L. ruellioides* increases antioxidant levels and activity. In comparison to the control group, LREE administration markedly raised glutathione content (Figure 3.1A), as well as GST (Figure 3.1B) and SOD (Figure 3.1C) activities in a dose-dependent manner. Lipid peroxidation (LPO) was assessed as a biomarker of oxidative stress in order to determine if LREE treatment affected intracellular oxidant levels. Following LREE treatment, there was a substantial reduction in oxidative stress levels, indicating decrease in tissue damage in contrast to the control group. It can be demonstrated that the reduction in LPO correlated with LREE treatment in a dose-dependent manner (Figure 3.1D).

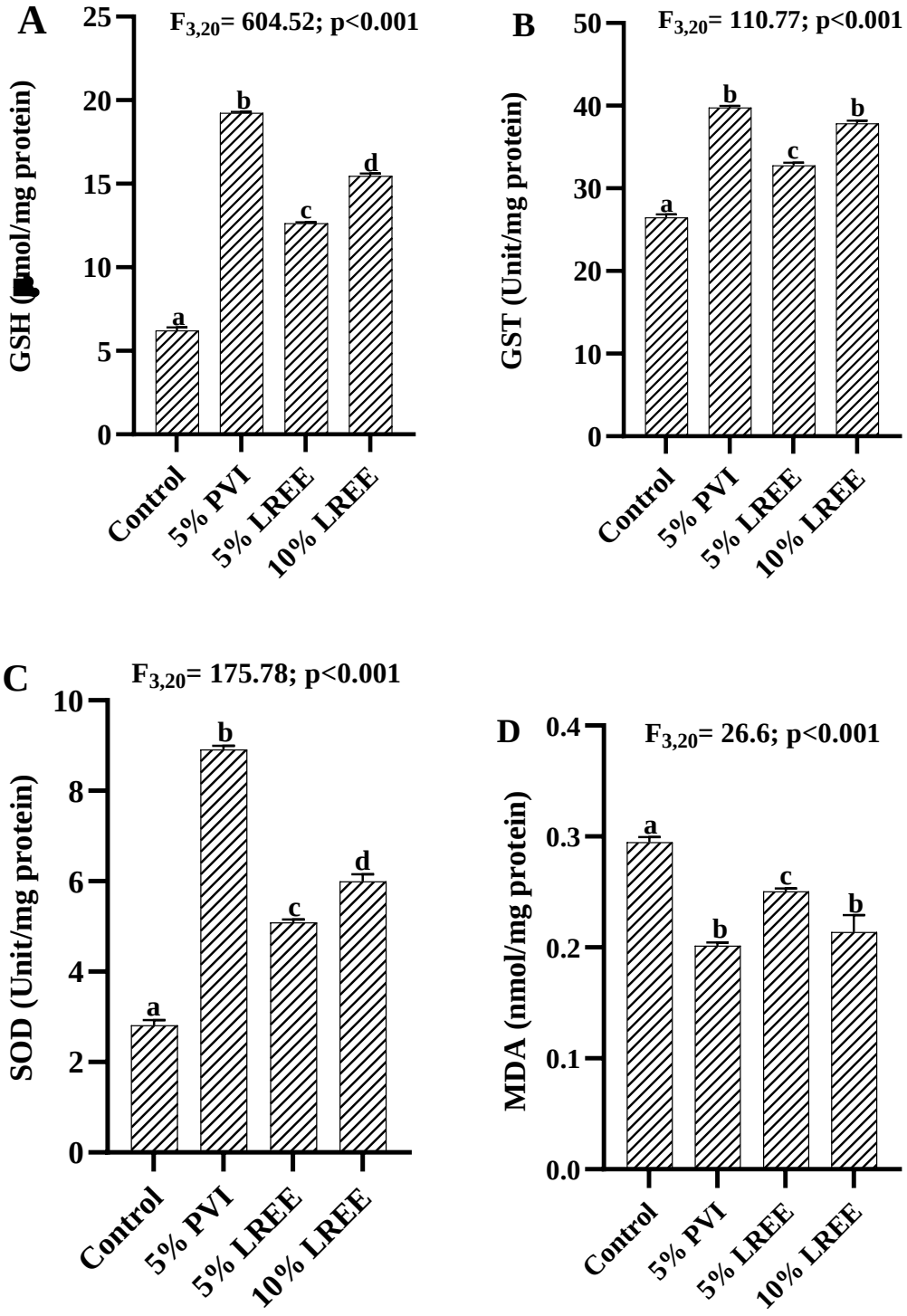
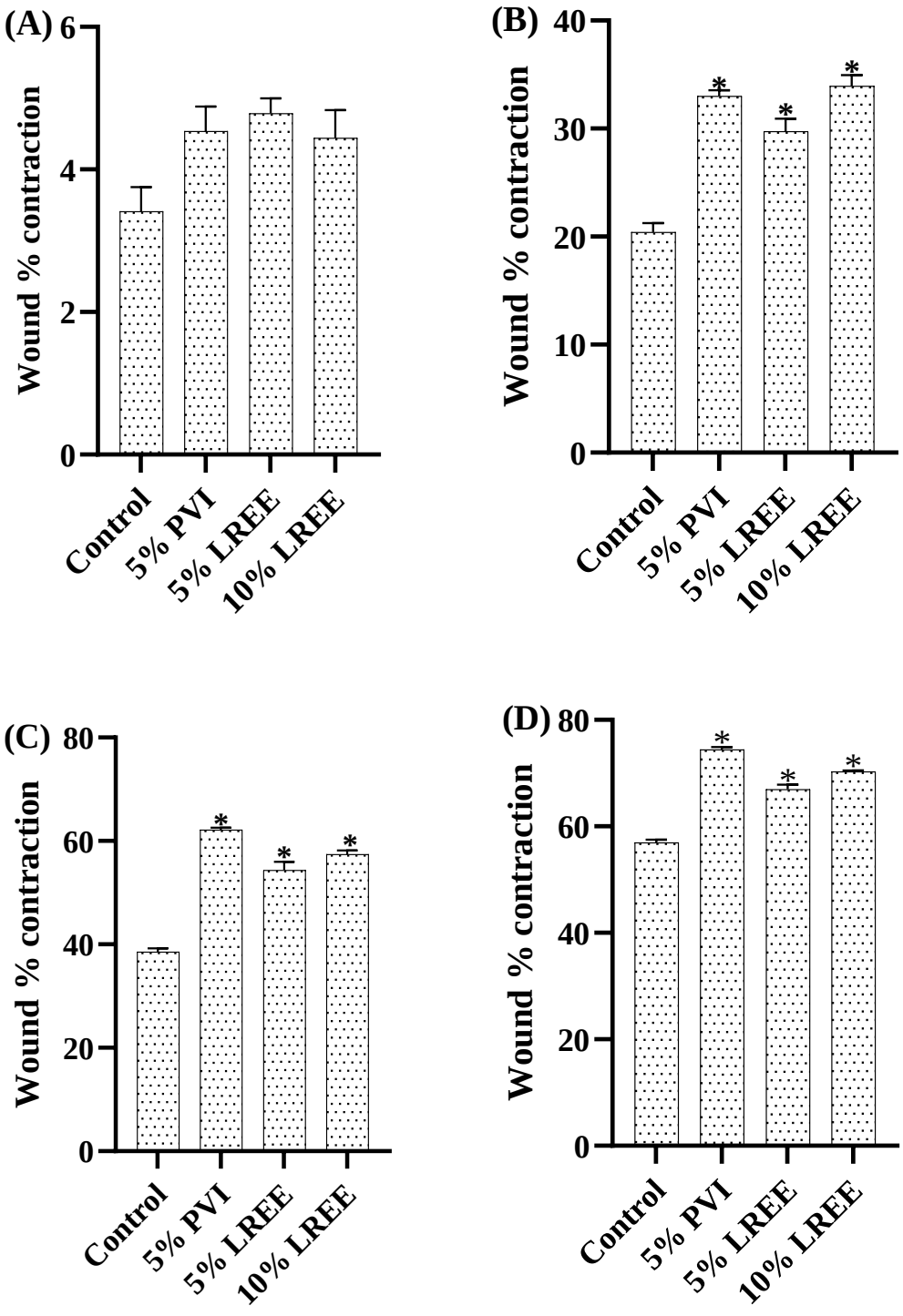


Figure 3.1: Effects of *L. ruellioides* ethanolic extract on (A) glutathione level (GSH); (B) glutathione-s-transferase activity (GST); (C) superoxide dismutase (SOD); (D) lipid peroxidation (LPO) expressed in malondialdehyde (nmol/mg protein) in the wounded skin tissue of rats.

Excision wound model

Table 3.1 displays the area of wound contraction at 3, 6, 9, 12, 15, 18 and 21 days. In comparison to the control group, the animals topically treated with *L. ruellioides* extracts showed a significantly higher degree of compression of the wound starting from the 6^{th} day in comparison with the control. On day 21, The animal's wound area decreased at a pace of 98.91% and 97.56% for 10% and 5% LREE respectively in contrast to control (95.98%) and it was statistically significant ($P<0.01$). On top of that, it was found that the epithelization time was significantly ($P<0.001$) minimal for 10% LREE (16.61 ± 0.2) and 5% LREE (18.11 ± 0.48) as compared to control (23.44 ± 0.59). Interestingly, there was no notable differences between period of epithelization for 10% LREE (16.61 ± 0.2) and 5% PVI (15.66 ± 0.25) which implies they are comparable.


(A)
Wound % contraction
0
2
4
6
Control
5% PVI
5% LREE
10% LREE
(B)
Wound % contraction
0
10
20
30
40
*
*
*
Control
5% PVI
5% LREE
10% LREE
(C)
Wound % contraction
0
20
40
60
80
*
*
*
Control
5% PVI
5% LREE
10% LREE
(D)
Wound % contraction
0
20
40
60
80
*
*
*
Control
5% PVI
5% LREE
10% LREE

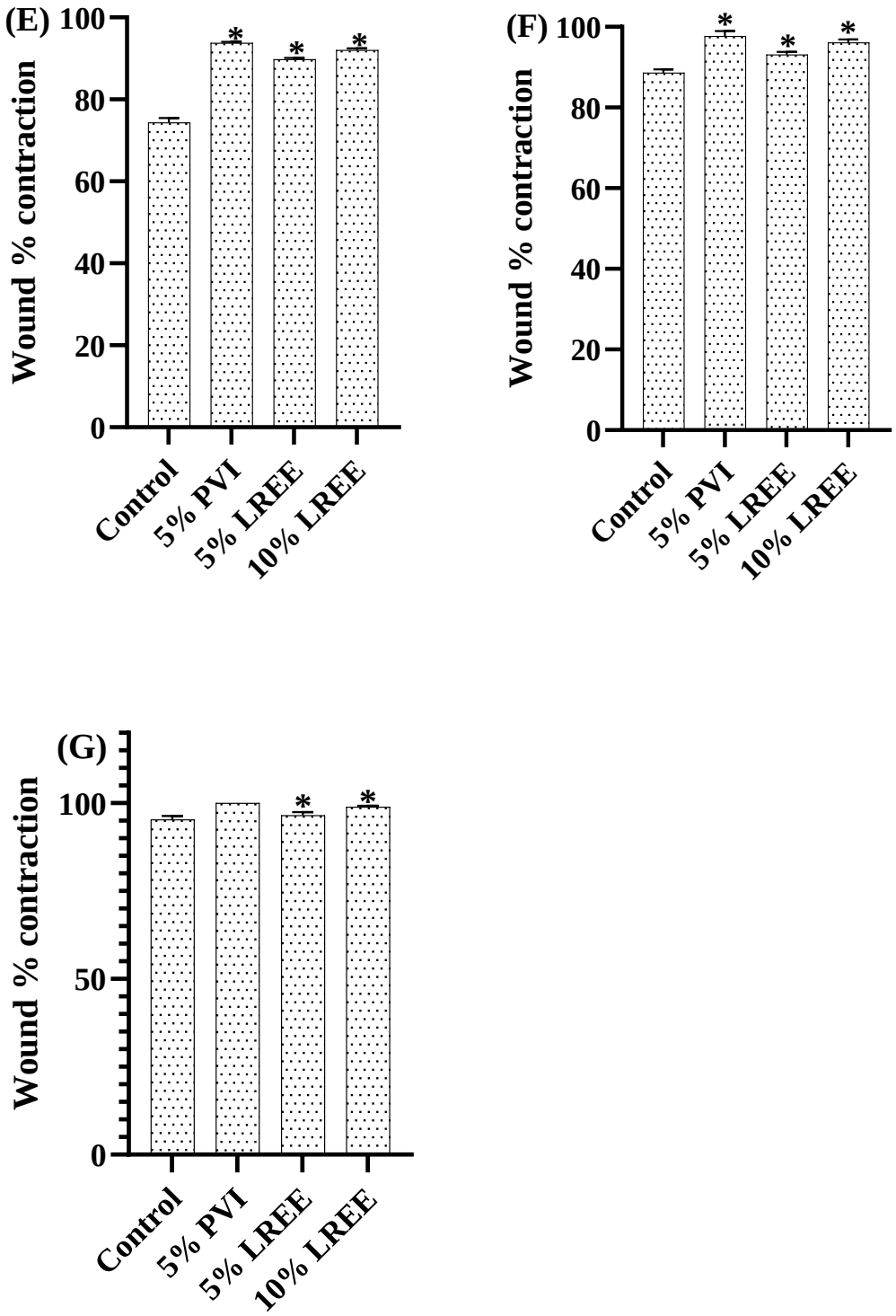


Figure 3.2: Graphical representation of wound closure rate in percentage on different days (A) Day 3, (B) Day 6, (C) Day 9, (D) Day 12, (E) Day 15, (F) Day 18, (G) Day 21). * Indicates significant variations in comparison with the control.

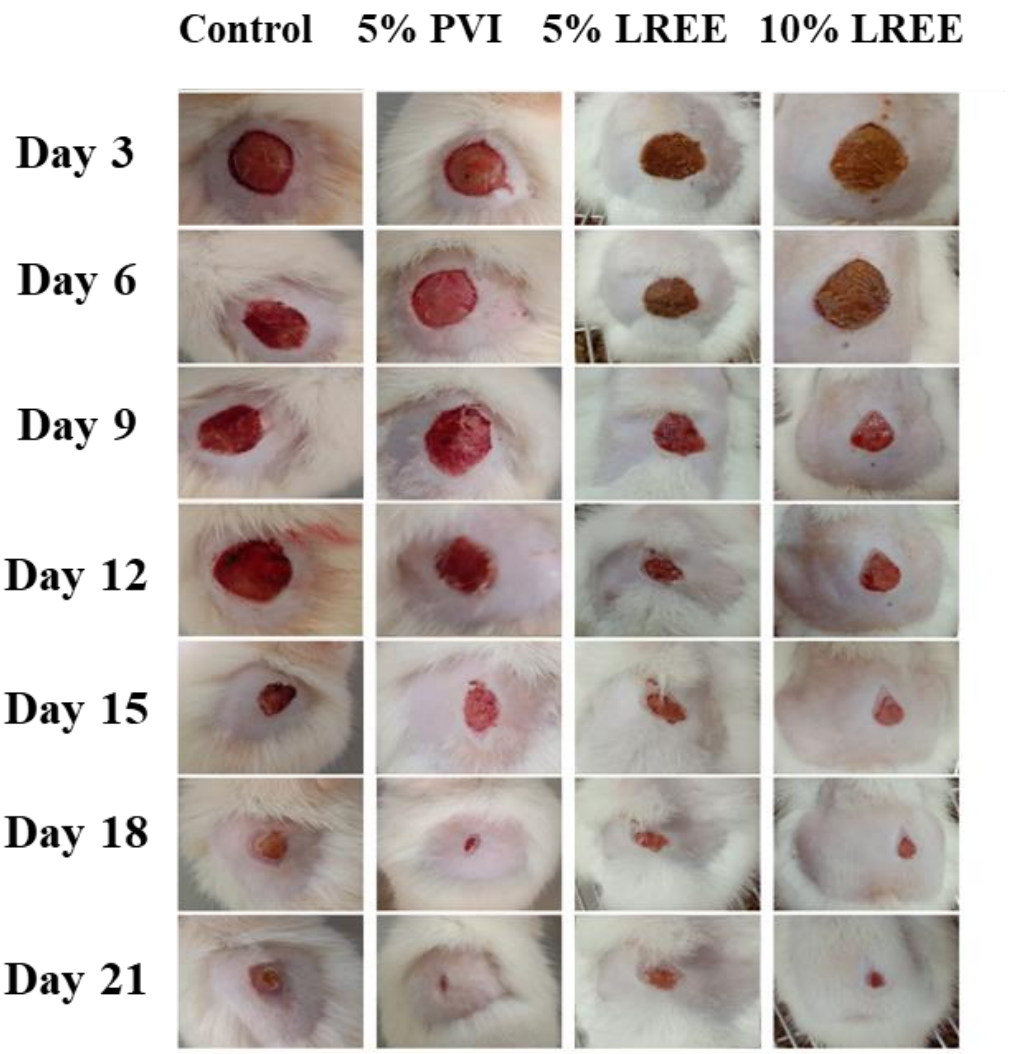


Figure 3.3: Photographic representation of wound closure rate at different days.

Table 3.1: Effect of ethanolic extract of *L. ruellioides* on reduction in wound area (mm^2).

Group/Treatment	**No. of days**							
	0 day	3rd day	6th day	9th day	12th day	15th day	18th day	21st day
Group 1 (Control)	298.56±0.75	288.38±0.51	237.63±0.82	183.35±0.69	128.44±0.53	76.47±1.67	32.99±0.89	11.99±0.61
Group 2 (5% PVI)	301.39±1.73	287.70±1.09	201.80±0.33*	114.13±1.10*	76.97±0.78*	18.74±0.52*	5.00±0.37*	0
Group 3 (10% LREE)	292.11±2.19	275.16±2.12	192.95±3.12*	124.39±1.42*	86.72±1.00*	23.22±0.52*	10.46±0.68*	3.18±0.58*
Group 4 (5% LREE)	288.89±0.94	276.08±1.91	202.98±2.61*	131.91±3.09*	95.38±1.71*	29.44±0.63*	18.99±0.36*	7.03±0.31*

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

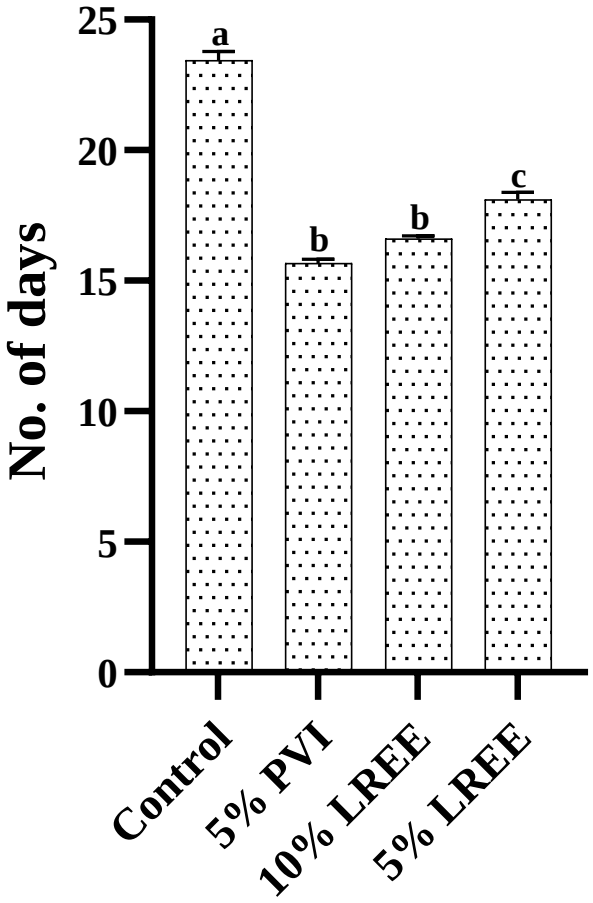


Figure 3.4: Comparison on period of epithelialization between control and LREE. Different letters indicate significant variations.

Incision wound model

The impact of *L. ruellioides* on wound healing in the incision wound model is shown in Table 3.2. Control group showed mean wound breaking strength of 290.44 ± 3.38 while 10% LREE and 5% LREE showed wound breaking strength of 478.72 ± 2.36 and 412 ± 0.96 respectively. Both the extract groups demonstrated significant increase (P<0.001) in wound breaking strength in comparison with control. Captivatingly, 10% LREE is not statistically significant in comparison to 5% PVI (487.39 ± 2.16), which infers they are indistinguishably beneficial at hastening wound healing.

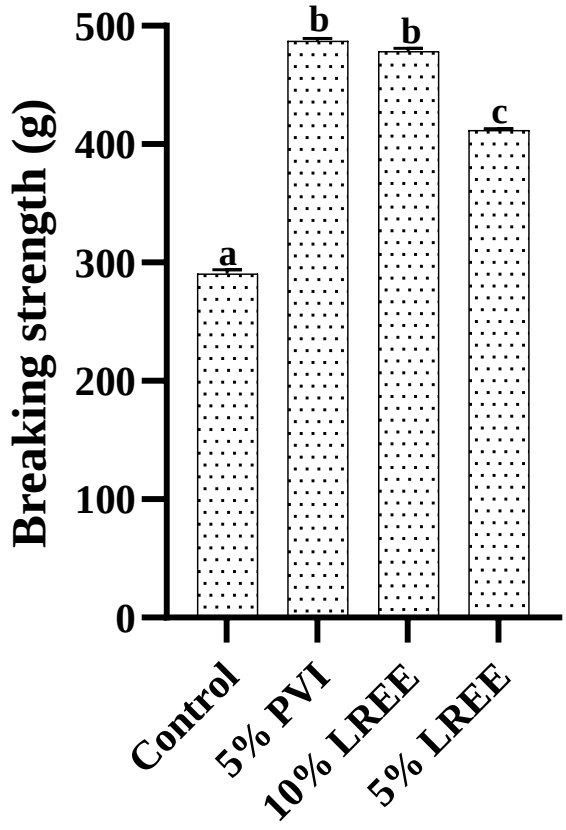


Figure 3.5: Effect of LREE on skin breaking strength. All groups are significant using ANOVA, $P<0.001$ when compared to control. Different letters indicate significant variations.

Histopathological evaluation

Fig. 3.6 displays histological photomicrographs of the treated and control group. Histopathological studies displayed an exponential increase in collagen deposition in 10% LREE treated group, which is an indication of increase mechanical strength of the skin. 10% LREE treated group also contain comparatively minimal number of inflammatory cells in comparison with the negative control group. In contrast to the extract-treated groups, the control group showed a delayed healing process and had more inflammatory cells and less collagen fibers and fibroblasts. As opposed to the control, faster remodelling was noticed in extracts treated groups. Notably, the histological examination revealed that the wound healing process of 10% LREE treated group were comparably close to 5% PVI (the positive control). According to histopathological observations, the extract's phytochemical content may be accountable for the collagen development during the proliferative stage, which is facilitated by an expansion in the quantity of fibroblasts. All of these studies highlight the potential benefits of *L. ruellioides* for wound healing, hence validating their promising function in enhancing wound healing.

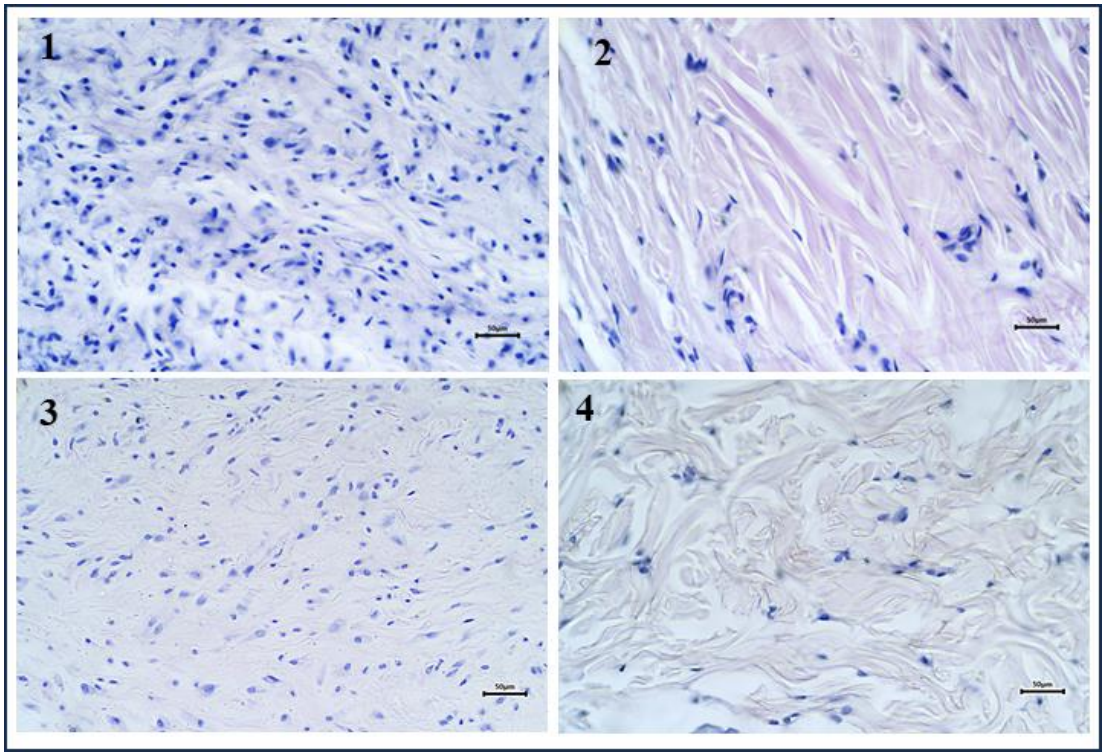


Figure 3.6: Photomicrographs showing the histopathology of the skin granulation tissues in both the experimental and control groups. Group 1 showcases the negative control, group 2 is the positive control (5% PVI) and the experimental groups, 3 and 4 correspond to the LREE treated groups at doses 5% and 10% respectively. Scale bar: 50 µm. Magnification: 20X.

DISCUSSION

Wound healing is a dynamic and intricate process that aims to return damaged tissue's cellular structures and layers of tissues as tightly as is feasible to their initial, normal form (Clark, 1985). The process of wound contraction starts at the fibroblastic stage, when the wound area shrinks, and continues throughout the healing phase (Chitra *et al.,* 2009). It takes a complex process encompassing matrix component synthesis, biomolecular interactions, migration, proliferation, interaction, and differentiation of several cell types (such as epidermal, dermal, and infiltrating inflammatory cells), as well as a complex signalling network, to reclaim the structural integrity of the damaged tissue (Koschwanez and Broadbent, 2011; Stephens *et al.*, 2013; Stojadinovic and Tomic-Canic, 2013). This study geared towards the wound healing abilities of *Lindernia ruellioides,* and the outcomes are on par with traditional long-standing practice. These results are in line with numerous reports asserting the wound healing potential in several plant extracts (Murthy *et al.,* 2013; Rasik *et al.*, 1999; Mukherjee and Suresh, 2000; Park and Chun, 2001; Nagappa and Cheriyan, 2001).

Remarkably, the investigation of antioxidants delivered results that were advantageous. In the case of increasing concentrations, reactive oxygen species (ROS) can seriously damage tissue by damaging cellular membranes, DNA, proteins and lipids, and even neoplastic changes, which further obstruct the healing process (Loft and Poulsen, 1996; Pryor, 1997). Consequently, removing ROS may be a crucial tactic in the treatment of chronic wounds (Dissemond *et al.,* 2002). Therefore, measuring antioxidants is important since they enhance the healing process of wounds by eliminating free radicals (Halliwell *et al.,* 1988). Consequently, a compound or plant extract with antioxidant capacity may be a useful therapeutic treatment for hastening the healing of wounds. The antioxidative potential as well as anyone of the identified phytochemical compounds witnessed in *L. ruellioides* could be in charge of the action that heals wounds. Research has demonstrated that phytochemical constituents present in plants (Nayak *et al.,* 2009; Okuda, 2005; Kähkönen *et al.,* 1999; Pulido *et al.,* 2000; Andrea *et al.,* 1998) are known to accelerate the process of wound healing mainly due to their astringent, free radical

scavenging and antibacterial properties which appear to be accountable for the accelerated pace of epithelialization and wound healing (Tsuchiya *et al.,* 1996).

Excision and incision wound models were utilized to evaluate wound area contraction, epithelialization duration and skin breaking strength which are the metrics of tissue cell rejuvenation, collagen capacity and skin mechanical strength (Shetty *et al.,* 2008). The rate of wound contraction, the duration of epithelialization and the strength of skin breaking were all increased by the ethanolic extract of *L. ruellioides*. One of the most frequently implemented models regarding wound healing is excisional wounds (Nauta *et al.,* 2013). Since this model is thought to mimic acute clinical wounds, it is possible to examine angiogenesis, remodelling, granulation tissue creation, re-epithelialization, inflammation and bleeding (Masson-Meyers *et al.,* 2020). Topical application of 10% LREE increased the wound contraction percentage and completed healing by 17^{th} day which highlight accelerated epithelization and collagenization. The rapid wound contraction may be driven by an upsurge of interleukin-8, an inflammatory α-chemokine which influences the recruitment and function of different inflammatory cells, including fibroblasts and keratinocytes. It may also enhance the gap junctional intracellular communication in fibroblast and induces increased granulation tissue maturation (Moyer *et al.,* 2002).

In incision wound study, the skin breaking strength increased in the extract-treated group. Increased in skin breaking strength indicated enhanced collagen maturation and stabilization of the fibers and obviously facilitating wound healing (Udupa *et al.,* 2008). At the wound site, the produced collagen molecules are deposited and cross-linked to create fibers. Both collagen remodelling and the creation of strong intra and intermolecular crosslinks contribute to wound strength. It may be concluded that the plant extract not only enhanced collagen synthesis per cell but also facilitated protein crosslinking because the incision wound treated with it had increased tensile strength. Consequently, it can be concluded that the extract is not only actively encouraging faster wound contraction but also functions as a powerful agent to support tissue granulation and remodelling.

CHAPTER- IV

Study of the expression pattern of *Tgf-β1* and *Vegf* using quantitative RT-PCR

INTRODUCTION

Our skin accomplishes a number of vital homeostatic tasks, including controlling thermostability and detecting external stimuli. It has been optimized to interact with the natural environment. (Wilkinson and Hardman, 2020). Notably, the skin serves as the body's main defence barrier, shielding internal structures from desiccation and injury from heat, chemicals, mechanical forces and photic agents. (Takeo *et al.,* 2015) Through an exquisitely tailored host-microbiota axis, this defence includes a complex immunological barrier response that guards against pathogenic infection while sustaining commensal microorganisms. (Naik *et al.,* 2015). The skin additionally established rapid and efficient techniques to seal off breaches in its barrier in a process so called as wound healing response.

Wound healing in the skin is an intricate biological mechanism, encompassing inflammation, chemotaxis, vascular, dermal and epidermal cell mitosis, neovascularisation, extracellular matrix proteins synthesis and scar tissue remodelling (Broughton *et al.,* 2006; Schultz and Mast, 1999). A competent integration of the intricate biological and molecular processes is vital for the optimal feasible restoration of a cutaneous wound (Wu *et al.,* 2007). Proteases, growth factors, and cytokines work seamlessly synergistically to initiate cascades of cellular and molecular processes which culminate in brimming normal tissue repair following damage (Singer and Clark, 1999) and this has given rise to the theory that these various molecular classes also control significant stages of wound healing *in vivo* (Bennett and Schultz, 1993). This hypothesis's key consequence is that chronic wound formation and maintenance are directly facilitated by the deficiency or asymmetry of growth factors, cytokines, proteases or hormones in wounds (Tarnuzzer and Schultz, 1996). Subsequently, growth factors and cytokines are good

candidates and indicators for determining the age or vitality of a wound (Kondo and Ishida, 2010).

Three overarching phases is usable for categorizing the processes that come into place following skin wound healing: inflammatory phase, proliferative phase and remodelling (Sengupta *et al.,* 2015). Each of these phases overlap significantly in time, and the process calls for several months for it to wrap up. The cell populations migrate, grow exponentially, and disintegrate at each phase. For the healing to stabilize and form a fully developed scar, proliferation and apoptosis must be synchronized. (Greenhalgh, 1998).

Shortly after deterioration, the inflammatory phase commences when platelets clump together to cease bleeding from blood vessel damage. This initially begins the blood clotting process by triggering a proteolytic cascade, which eventually turns fibrinogen into fibrin (Zaidi and Green, 2019; Schaffer and Nanney, 1996; Falanga, 1998). Red blood cells and platelets become entangled as the fibrin molecules self-assemble into a net that resembles a web. Eventually, a collection of platelets, fibrin, and red blood cells is big enough to create a tampon, that assists in sealing up a damaged capillary and halt blood flow (Martin, 1992). On top of that, platelet degranulation imposed upon by blood coagulation causes an explosion of growth factors and pre-formed cytokines that are stored in alpha granules. Their primary function is to serve as a chemoattractant for leukocytes and circulating inflammatory cells, notably neutrophils and macrophages (Golebiewska and Poole, 2015; Falanga, 1998; Martin, 1992). Two significant processes, including the chemotaxis of inflammatory cells and the onset of tissue repair, are initiated by these cytokines and growth factors. In a normal wound, this inflammatory phase is episodic and lasts for 24 to 48 hours after the damage (Schaffer and Nanney, 1996).

The inflammatory phase is followed by the proliferative phase. With the intention for improved wound healing, the proliferative phase entails the proliferation of an assortment of cell types including fibroblasts, endothelial cells and keratinocytes (Nwomeh, 1998). This phase is characterized by fibroplasia, or the development of granulation tissue and the reconstruction of the dermal matrix

(Falanga, 1998). In response to many mediators, including growth factors, fibroblasts relocate into the wound approximately 48 hours after damage and manufacture important extracellular components, including collagens, proteoglycans, fibronectin and elastin. To restore damaged tissue, fibroblasts produce extracellular matrix, leaving behind a scar (Bennett and Schultz, 1993). As the key component of the extracellular matrix, collagen serves as a prerequisite for mending damage and reestablishing the composition and operation of the tissue (Nwomeh, 1998). At least a few days elapse during the proliferative stage of wound healing (Falanga, 1998; Martin, 1992).

The remodelling phase, the final and the pinnacle of tissue repair, may persist for years as the cellular constituents at the damage site endeavour to restore the matrix structure that prevailed before the injury (Lawrence and Diegelman, 1994). Molecules capable of disintegrating the cellular matrix are crucial during this stage (Lawrence, 1998). Proenzymes recognized as matrix-degrading metalloproteinases must be activated in order to function physiologically. This remodelling step encompasses three major families of enzymes such as stromelysins, collagenases and gelatinases. Normal tissue regeneration eventually leads to a balance between synthesis and breakdown, which is why it is crucial to govern the two processes (Falanga, 1998, Lawrence and Diegelman, 1994; Lawrence, 1998). Upon healing, the tensile strength has been foretold to be 70-80% of that of uninjured skin and never returns to preinjury values (Larson, 1990).

The recuperation process of wounds incorporates the extracellular matrix, cytokines, blood cells and growth factors. Growth factors are proteins which promote and expedite the healing process by triggering angiogenesis, gene transcription and cell proliferation, among other processes (Hom *et al.,* 2007). Among the growth factors the ones that are highly significant for the healing of wounds include epithelial growth factor (*Egf*), platelet-derived growth factor (*Pdgf*), transforming growth factor (*Tgf-β*), vascular endothelial growth factor (*Vegf*), fibroblast growth factor (*Fgf*) and insulin-like growth factor (*Igf*) (Joao De Masi *et al.,* 2015).

One particular growth factor that has been highlighted to be crucial in wound healing is *Vegf* (Werner and Grose, 2003). *Vegf* is secreted by a multitude of cells that aid in wound healing such as fibroblasts, smooth muscle cells, macrophages, endothelial cells, neutrophils and platelets (Jazwa *et al.,* 2006; Nissen *et al.,* 1998). This protein gets released in responses to inflammation and ischemia, boosting angiogenesis, development of granulation tissue, endothelium movement, chemotactic agent synthesis and proliferation (Bao *et al.,* 2009). A prominent angiogenic growth factor in skin wounds, *Vegf* advocates endothelial cell migration, proliferation, and differentiation (Johnson and Wilgus, 2014; Romano *et al.,* 2002). At the present time, placenta growth factor (PLGF), *Vegf* -A, *Vegf* -B, *Vegf* -C, *Vegf* -D and *Vegf* -E are members of the *Vegf* family (Saaristo *et al.,* 2006) and adhere to three alternative transmembrane tyrosine kinase receptors, acknowledged as *Vegf* receptor-1, *Vegf* receptor-2 and *Vegf* receptor-3 to haul out their biological activities (Karkkainen *et al.,* 2004; Gale and Yancopoulos, 1999).

The *Tgf-β* proteins have been witnessed in both vertebrates and invertebrates, alongside various species. Certain biological functions, which include development, tissue homeostasis and immune system modulation are profoundly regulated by the *Tgf-β* superfamily (Massagué *et al.,* 2000; Patterson and Padgett, 2000). Mammalian tissues exhibit three distinct isoforms of *Tgf-β* namely *Tgf-β1*, *Tgf-β2* and *Tgf-β3*. These isoforms differ in a number of amino acid sequences but contain exceptionally conserved regions. They are all mediated by analogous receptor signalling pathways (Cheifetz *et al.,* 1987; Mittl *et al.,* 1996).

Tgf-β1, the most prevalent and tremendously articulated isoform, was cloned from human term placenta mRNA (Derynck *et al.,* 1985) and takes precedence in cutaneous wound healing. *Tgf-β1* mRNA and/or protein was previously located within cartilage, endochondral and membrane bone, and skin in mouse development, implicating its involvement in these tissues' growth and differentiation (Dickinson *et al.,* 1990). They function by binding a heteromeric receptor complex built up of one type I and one type II receptor, both of which are serine-threonine kinases, and are generated by macrophages, fibroblasts, keratinocytes and platelets (Eppley *et al.,*

2004; Rolfe *et al.,* 2007; Mani *et al.,* 2002) Furthermore, they attach to a type III nonsignaling receptor that serves to deliver *Tgf-β* to the type II receptor. Following autophosphorylation, the receptors launch signalling molecules downstream that are representatives of the Smad transcription factor family (Liu *et al.,* 2005).

This chapter aims to investigate the expression pattern of *Tgf-β1* and VEGF in excision wound healing activity of *L. ruellioides* in experimental rats.

MATERIALS AND METHODS

Animal tissue harvesting

Male Wistar albino rats with an excellent health condition (150-180 g) were used for the study. They were separated into four groups containing five animals each. Five animals from each group were sacrificed on days 3, 7 and 11 post-wounding and the granulation tissue was carefully excised out. The tissue collected was immediately stored in RNA stabilization reagent (RNA*later*^{TM}, Qiagen, USA) at -80ºC for real time-PCR studies.

RNA isolation, cDNA synthesis, and quantitative (real-time) RT-PCR (qPCR)

Granulation tissue was subjected to RNA extraction by employing Tri reagent (Ambion AM9738, Grand Island, NY, USA) solution (Sangma & Trivedi, 2023). Quantification of RNA was done by using a spectrophotometer (ND-ONE NanoDrop one spectrophotometer; ThermoFisher Scientific, USA), and 1-µg RNA was used to make cDNA (iScript™ cDNA Synthesis Kit, BIO-RAD, U.S.A. 1708891) and studies of gene expression have involved the use of gene-specific primers (Sangma & Trivedi, 2023).

qPCR was carried out by using Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) with a reaction volume of 7 µl for every gene comprising 1 µl each of cDNA, gene-specific forward and reverse primers, 3 µl Power UpTM SYBR Green MasterMix (Applied Biosystem by Thermo Fisher Scientific; US, A25742) and 1µl of nuclease-free water (Ambion, AM9938; Borah *et al.,* 2022). The qPCR cycling conditions used to measure expressions of the target genes were 1 cycle at 95ºC (20 sec), 35 cycles at 95ºC (01 s), 60ºC (20 s), 95ºC (01 s), additional melt curve plot step included 1 cycle of 60ºC (20 s) and one cycle of 95ºC (01 s). The *Gapdh* gene served as a reference gene in our investigation to ascertain the relative expression levels of particular target genes (Sangma & Trivedi, 2023). Every sample was run twice along with non-template and negative RT controls (Borah *et al.,* 2022). The relative expressions of genes were determined by the ΔΔCt method (Livak & Schmittgen, 2001).

In the present study, the relative expression of *Tgf-β1* and *Vegf* genes in LREE treated groups and control group has been examined utilizing glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene expression as housekeeping gene.

Table 4.1. Sequences of the forward and reverse primers utilized for qPCR analysis.

Gene	**Forward Primer**	**Reverse Primer**
TGF-β1	5‘-TGCTCGCTTTGT ACAACAGC -3’	5’-TTGTTGCGGT CCACCATTAG -3’
VEGF	5’-ACGAAAGCGCA AGAAATCCC -3’	5’-ACGCGAGTCT GTGTTTTTGC -3’
GAPDH	5’-GCCTTCTCTTGT GACAAAGTGG -3’	5’-TGACTGTGCC GTTGAACTTG -3’

Statistical analysis

Graph pad prism version 10 was used for statistical analysis. Two-way ANOVA followed by Tukey’s Multiple Comparison Test was utilized to observe the impact of treatment, time and interaction of time and treatment amongst the groups. $P<0.05$ was considered as statistically significant.

RESULT

The variations in gene expression across various groups were analysed, and the expression of a target gene was normalized by *Gapdh*, a non-regulated reference gene. No variation was observed in the expression of the *Gapdh* gene among groups. Following this, this gene was employed as the control gene.

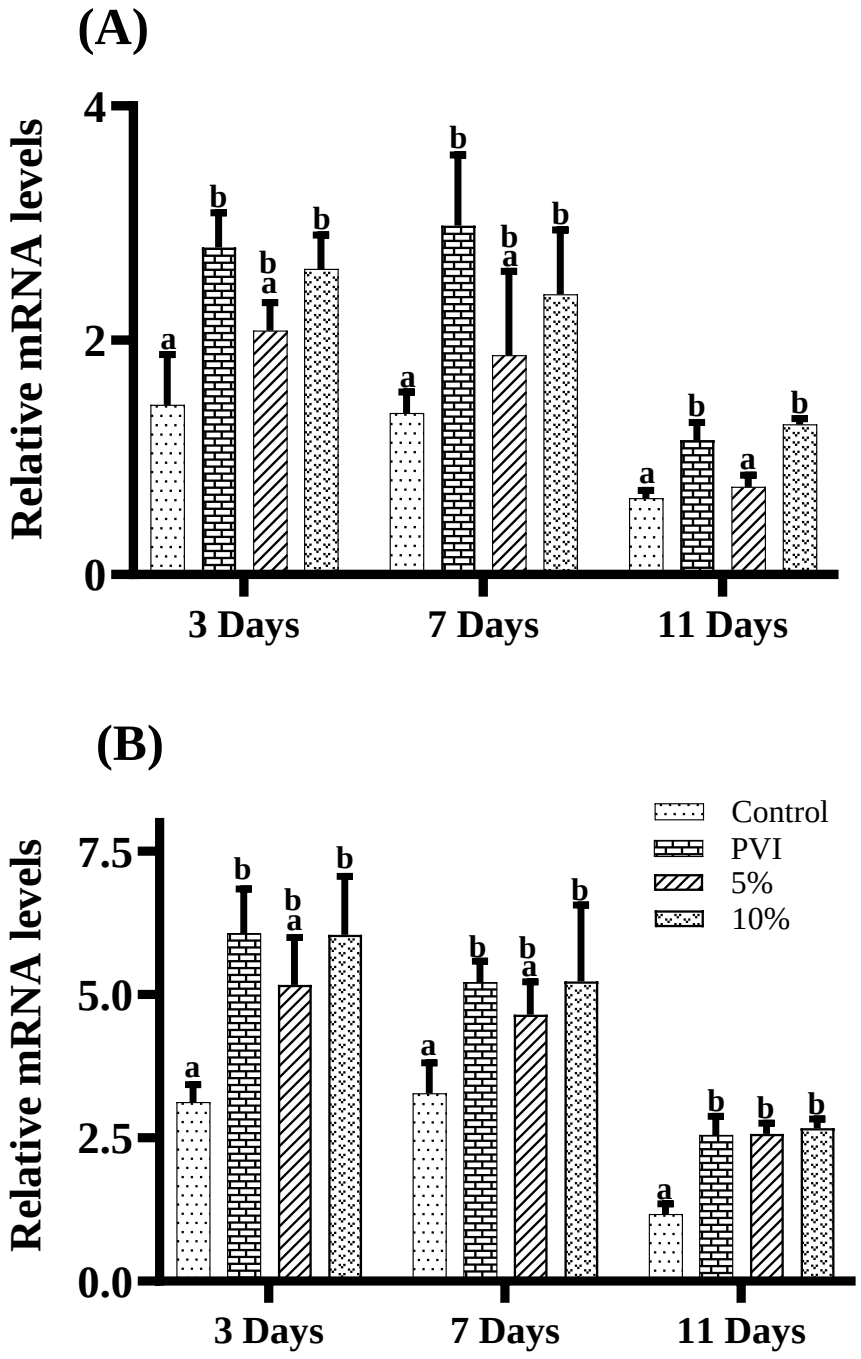


Figure 4.1: Relative changes in mRNA expressions of *Tgf-β1* (A) and *Vegf* (B) in LREE treated wounds in comparison to control. Data are expressed as mean ± SEM. Different letters indicate significant variations.

Two-way ANOVA analysis suggests that there is an effect of time ($F_{3,16}$= 35.28, P<0.0001) and treatment ($F_{3,16}$= 12.91, P= 0.0002) but not on interaction of time and treatment ($F_{3,16}$= 1.686, P= 0.1565) on the expression of skin *Tgf-β1* transcript. On Day 3, the expression of *Tgf-β1* was significantly upregulated in the 5% PVI and 10% LREE treated groups (P<0.05, Tukey's Multiple Comparison Test, Fig. 4.1 A) in comparison to Control. Nevertheless, no significant difference was noticed between control and 5% LREE treated groups (P>0.05, Tukey's Multiple Comparison Test, Fig. 4.1 A). Similarly, no significant difference was observed on skin *Tgf-β1* transcript between 5% PVI, 5% LREE and 10 % LREE treated groups (P>0.05, Tukey's Multiple Comparison Test, Fig. 4.1 A). On Day 7, the expression of *Tgf-β1* was significantly upregulated in the 5% PVI and 10 % LREE treated groups than control group (P<0.05, Tukey's Multiple Comparison Test, Fig. 4.1 A). Despite this, no significant difference was seen between control and 5% LREE treated groups (P>0.05, Tukey's Multiple Comparison Test, Fig 4.1 A). Further, no significant difference was observed on skin *Tgf-β1* transcript between 5% PVI, 5% LREE and 10% LREE treated groups (P>0.05, Tukey's Multiple Comparison Test, Fig. 4.1 A). On day 11, the expression of *Tgf-β1* was significantly downregulated in control and 5% LREE treated groups when compares with 5% PVI and 10% LREE treated groups (P<0.05, Tukey's Multiple Comparison Test, Fig. 4.1 A). However, there is no significant difference between control and 5% LREE treated groups and similarly between 5% PVI and 10% LREE treated groups (P>0.05, Tukey's Multiple Comparison Test, Fig 4.1 A).

Similarly, there is an effect of time ($F_{3,16}$= 85.25, P<0.0001) and treatment ($F_{3,16}$= 12.90, P= 0.0002) but not on interaction of time and treatment ($F_{3,16}$= 1.742, P= 0.1432) on the expression of skin *Vegf* transcript. On Day 3, the expression of *Vegf* was significantly upregulated in the PVI and 10% LREE treated groups (P<0.05, Tukey's Multiple Comparison Test, Fig. 4.1 B) in comparison to control. Even so, no significant difference was found between control and 5% LREE treated groups (P>0.05, Tukey's Multiple Comparison Test, Fig. 4.1 B). Similarly, no significant difference was observed on skin *Vegf* transcript between 5% PVI, 5% LREE and 10% LREE treated groups (P>0.05, Tukey's Multiple Comparison Test,

Fig. 4.1 B). On Day 7, the expression of *Vegf* was significantly upregulated in the PVI and 10 % extract treated groups than control group ($P<0.05$, Tukey's Multiple Comparison Test, Fig. 4.1 B). However, no significant difference was found between control and 5% LREE treated groups ($P>0.05$, Tukey's Multiple Comparison Test, Fig. 4.1 B). Further, no significant difference was observed on skin *Vegf* transcript between PVI, 5 % and 10 % extract treated groups ($P>0.05$, Tukey's Multiple Comparison Test, Fig. 4.1 B). On day 11, the expression of *Vegf* was significantly downregulated in control group when compares with 5% PVI, 5% LREE and 10% LREE treated groups ($P<0.05$, Tukey's Multiple Comparison Test, Fig. 4.1 B).

DISCUSSION

For decades, complementary and alternative medicines, increasingly referred to as herbal medicines, have been extensively implemented to mitigate illnesses while boosting wellbeing; in fact, at least 80% of people worldwide are contingent upon them for some aspect of their basic healthcare (Ekor, 2014). Exploring the possibility of plants as potential wound-healing agents has now become one section of the modern biomedical sciences. Enhancing wound healing and maximizing tissue function restoration continue to be major clinical care priorities (Esmaeili *et al.,* 2014). Studies perpetually sought for approaches which could mitigate healing process limitations like infection, colloidal fractures, and crippling scarring (Savari *et al.,* 2019). Numerous studies and techniques have been implemented in this area, including the incorporation of pharmaceutical medications, herbal remedies, homeopathic remedies, physical treatments including laser therapy and other traditional techniques (Dovi *et al.,* 2003). Untreated wounds on the skin may lead to local infections and eventually cancer. In light of this reality with the importance of wound healing, researchers put a lot of effort on creating novel pharmaceuticals with minimal or no adverse effects. In a variety of pharmacological scenarios, some medicinal plants including *Achiella millefolium*, *Althaea officinalis*, *Calendula officinalis*, *Matricaria chamomilla*, *Aloe vera*, *Curcuma longa*, Eucalyptus, pomegranate, Jojoba, pine, Inula, plantain and green tea have been scientifically screened to evaluate their capacity to heal wounds (Albahri *et al.,* 2023; Kumar *et al.,* 2007). Despite this, many plants still have untapped potential.

Healing encompasses complex cascade of responses and cellular interactions involving inflammatory mediators and cell growth liaisons (Wrotniak *et al.,* 2007; Yazawa *et al.,* 2003; Vick *et al.,* 2006) by stimulating the production of countless factors and processes including phagocytosis, chemotaxis, mitogenesis, angiogenesis and apoptosis (collagen and extracellular matrix components) (Bennett and Schultz, 1993; Schaffer and Nanney, 1996). Wound healing is influenced by an assortment of internal and external factors and several commercial items are accessible that strive to prevent or promote the healing process (Mandelbaum *et al.,* 2003). The immune system and immunomodulatory functionality work in tandem

with the link between nutrition and the wound process of healing (Campos *et al*, 2008). The impact of growth factors namely *Vegf* and *Tgf-β1* on the healing process was addressed in the present study. Immune cells like neutrophils, which are triggered by platelets, migrate at the forefront of the healing process. This is followed by macrophages, which are linked to the release of *Vegf* and *Tgf-β1*, which aid in angiogenesis and fibroplasia.

Over the preceding twenty years, the healing process of wounds has been enhanced through the integration of growth factors, skin transplants or skin substitutes (Schaffer and Nanney, 1996). Following an injury, the recuperation process is triggered by the release of cytokines along with varying growth factors encompassing epidermal growth factor (*Egf*), transforming growth factor beta (*Tgf-β*), fibroblast growth factor (*Fgf*), insulin-like growth factor (*Igf*), vascular endothelial growth factor (*Vegf*) and platelet-derived growth factor (*Pdgf*) and their receptors expression may altered during wound healing (Bennett and Schultz, 1993; Servold, 1991; Greenhalgh, 1996; Steed, 1997).

Immediately following injury, platelets accumulate a significant amount of *Tgf-β1* (Assoian *et al.*, 1983). This preliminary setup of active *Tgf-β1* from platelets act as a chemoattractant upon neutrophils, macrophages and fibroblasts and these cell types elevate the amounts of *Tgf-β1* in numerous cell types. More than just active forms, latent *Tgf-β*s are also generated and stored in the wound matrix, enabling proteolytic enzymes to expel them gradually. The blend of temporary storage and several cellular sources guarantee a steady flow of *Tgf-β* during the healing process (Roberts and Sporn, 1996). It was anticipated that *Tgf-β* in the granulation tissue would be crucial for effective healing considering that it has been demonstrated to foster matrix deposition, angiogenesis, fibroblast proliferation and myofibroblast differentiation (Desmouliere *et al.*, 1993; Roberts *et al.*, 1986). Numerous research conducted in Countless animal experiments have provided evidence in favor of this assumption, demonstrating that exogenous *Tgf-β* facilitates wound healing through the enhancement of both the rate of healing and the integrity of the healed wound (Roberts and Sporn, 1996).

An imperative growth factor, *Tgf-β1*, that is secreted by multiple cells play a role in wound healing by an assortment of techniques including inflammation, angiogenesis, fibroblast proliferation, collagen production and the remodelling of new extracellular matrix (Roberts *et al.,* 1986; Nall *et al.,* 1996). Having a multitude of functions in the healing process, *Tgf-β1* is a fundamental growth factor modulator of cell development and differentiation (Sporn *et al.,* 1986). Early in the healing process, *Tgf-β* is released, which causes inflammatory cells to be drawn from the bloodstream and migrate to the injured area and results in granular tissue formation, angiogenesis and synthesis of collagen (Barrientos *et al.,* 2008). On top of that, *Tgf-β* minimizes collagen proteases while simultaneously encouraging cells to produce more extracellular matrix proteins (El Gazaerly *et al.,* 2013). It also contributes to the upregulation of VEGF, an angiogenic growth factor (Riedel *et al.,* 2007). *Tgf-β1* might possess both stimulatory and inhibitory effects (Roberts and Sporn, 1990). Despite heightened *Tgf-β1* production facilitates quicker re-epithelialization, over-expression of *Tgf-β1* at later phases of wound healing brings about hypertrophic scarring and keloid development (Pakyari *et al.,* 2013). The level of *Tgf-β1* in the current study impart evidence suggesting that *L. ruellioides* can increase *Tgf-β1* expression in wound healing during the early proliferative phase, promoting fibroblast activity and improved extracellular matrix (ECM) deposition and granulation tissue development.

One of the key characteristics of *Vegf* is the stimulation of angiogenesis which in turn is the primary characteristic of the proliferative phase in wound healing (Bao *et al.,* 2009). The results of our research revealed that *Vegf* mRNA levels increased considerably in the 10% LREE treated group at all time periods compared to the control, which implies that *L. ruellioides* has a pro-healing action and angiogenic potential in the skin. During angiogenesis, the number of blood vessels in the wound area briefly increases (Johnson and Wilgus, 2014). Inconsistencies in wound healing have been linked to reduced expressions of the *Vegf* gene or mRNA, along with its rapid degradation (Swift *et al.,* 1999). In the final stage of wound healing, *Vegf* promotes scar formation (Nissen *et al.,* 1996).

In this present study, *Tgf-β1* and *Vegf* genes were discovered to be upregulated in 10% LREE treated groups. Considering the findings of both *Tgf-β1* and *Vegf* gene expression, there is most likely a strong correlation between the expression of these two genes and the healing of wounds in rats. *Tgf-β1* is released instantaneously upon wounding by damaged platelets which stimulates inflammatory macrophages, the primary source of *Vegf* synthesis. The significant expression of these two growth factors is likely to be due to the fact that *Tgf-β1* regulates *Vegf* expression and accelerates the healing process by producing granulation tissue and angiogenesis. Consequently, it can be claimed that the ethanolic extract of *L. ruellioides* accelerates the healing activities by boosting the production of these two noteworthy growth factors.

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HSLC	2010	Mizoram Board of School Education	General	Distinction	75.6%
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B. Sc	2015	Mizoram University	Zoology	First	76.42%
M. Sc	2017	Mizoram University	Zoology	First	66.48%
GATE	2024	IITs/IISc	Life Sciences	NA	NA

PAPERS PRESENTED

International Seminar

1. International Conference on "Recent Advances in Animal Sciences (ICRAAS)" organized by the Department of Zoology, Pachhunga University College, Aizawl, India held during 6-8 November, 2019 on the topic, '**Qualitative and quantitative phytochemical content of *Lindernia ruellioides* (colsm.) Pennell**'.

2. International Conference on "Biodiversity, Biogeochemistry and Ecosystem Sustainability in Changing Environment" jointly organized by Department of Forestry, MZU and Indian Ecological Society during 14-16 June, 2023 on the topic, '**Experimental evaluation of ethanolic extract of *Lindernia ruellioides* (Colsmann) Pennell. for its wound healing activity using excision and incision wound models in Wistar albino rats**'.

National Seminar

1. National Seminar on "Emerging Trends in Biological Sciences: A North East India Perspective" organized by the Dept. of Biotechnology and Bioinformatics, NEHU, Shillong in collaboration with BRDC, Shillong and BioNEST Bio incubator Facility, NEHU, Tura Campus during 28th February to 1st March, 2023 on the topic, '**Evaluation of the free radical scavenging and antibacterial activity of *Lindernia ruellioides* (Colsm.) Pennell**'.

SEMINAR/TRAINING/WORKSHOP ATTENDED

1. UGC Stride Workshop on "Basic and Advanced Molecular Techniques" held from 1st June, 2021 to 5th June, 2021 at the Mizoram University, Aizawl, Mizoram.

2. One week online National workshop on "Data Analysis in Academic Research Using Statistical Tools" organised by Dept. of Economics and Central Library, Nanda Nath Saikia College, Jorhat, Assam from 5th to 10th June, 2023.

3. Workshop on "Microbial Screening of Food & Drugs Samples" organised by IBThub, RIPANS, funded by the Department of Biotechnology, Govt. of India held during 19-21 June, 2019.

4. International Conference on "Chemistry & Environmental Sustainability (ICCES-2019)", organized by Department of Chemistry, Mizoram University during February 19-22, 2019.

5. 4th Mizoram Science Congress organised by Mizoram Science, Technology and Innovation Council (MISTIC) held during 24-45 November, 2022.

6. The 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) & International Conference on BEHIET organised by School of Life Sciences, Mizoram University and ABAP, India, during 12-14 November, 2018.

7. 2nd Annual Convention of "North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology" during 16th-18th November 2020 (Virtual) organized by NEAST, Mizoram University, Aizawl-796004, Mizoram (India).

LIST OF PUBLICATIONS

1. **C Lalthansangi**, RK Lalremtluangi, Esther Lalhmingliani, M Vabeiryureilai (2024). Evaluation of the free radical scavenging activities and antibacterial activities of the extracts of *Lindernia ruellioides* (Colsmann) Pennell. *Current Trends in Biotechnology and Pharmacy*. 18 (4): 2036-2047.
2. **C Lalthansangi**, RK Lalremtluangi, Esther Lalhmingliani. Chemical composition and wound healing potential of *Lindernia ruellioides* (Colsmann) Pennell from Mizoram, India. *Asian Pacific Journal of Tropical Biomedicine*. 15 (4): 169-176.
3. RK Lalremtluangi, **C Lalthansangi**, Esther Lalhmingliani, M Vabeiryureilai (2025). *Pilea symmeria*: A natural ally in antioxidant defence and wound repair in mice. *Journal of Herbmed Pharmacology*. 14 (1).
4. RK Lalremtluangi, **C Lalthansangi**, Esther Lalhmingliani, M Vabeiryureilai (2025). Assessment of the phytochemical profile, free radical scavenging and antibacterial effects of *Pilea symmeria* from Mizoram, India. *Iranian Journal of Pharmaceutical Sciences*. 21 (1): 48-60.

BOOK CHAPTER PUBLISHED

1. **C Lalthansangi**, RK Lalremtluangi, Esther Lalhmingliani, M Vabeiryureilai (2023). Medicinal Plants with Antibacterial and Wound Healing Activity: A Review. Futuristic Trends in Biotechnology. ISBN: IIP Series, Vol. 3.

PARTICULARS OF THE CANDIDATE

NAME OF THE CANDIDATE: LALTHANSANGI

DEGREE: DOCTOR OF PHILOSOPHY

DEPARTMENT: ZOOLOGY

TITLE OF THE THESIS: EVALUATION OF WOUND HEALING AND ANTIBACTERIAL ACTIVITY OF *LINDERNIA RUELLIOIDES* (COLSM.) PENNELL EXTRACTS IN WISTAR ALBINO RATS.

DATE OF ADMISSION: 01.08.2018

APPROVAL OF RESEARCH PROPOSAL

1. **D.R.C.** : 03.05.2019
2. **B.O.S.** : 08.04.2019
3. **SCHOOL BOARD** : 17.05.2019

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ABSTRACT

EVALUATION OF WOUND HEALING AND ANTIBACTERIAL ACTIVITY OF *LINDERNIA RUELLIOIDES* (COLSM.) PENNELL EXTRACTS IN WISTAR ALBINO RATS

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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SCHOOL OF LIFE SCIENCES

MARCH 2025

ABSTRACT

EVALUATION OF WOUND HEALING AND ANTIBACTERIAL ACTIVITY OF *LINDERNIA RUELLIOIDES* (COLSM.) PENNELL EXTRACTS IN WISTAR ALBINO RATS

BY

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Department of Zoology

Name of supervisor

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Submitted

In partial fulfillment of the requirement for the degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl

ABSTRACT

Wound healing is a sophisticated physiological process that encompasses a multitude of coordinated procedures which restore both functionality and structural tissue integrity. The intention of promoting natural remedies has prompted the screening of several plants and herbs for their abilities to heal wounds, with the results demonstrating the improvement of several components of the healing process. For decades, complementary and alternative medicines, frequently known as herbal medicines, have been adopted to alleviate health problems while boosting well-being. Investigating the potential of plants as wound-healing agents is a burgeoning subject in modern biomedical sciences. Despite this, the enormous potential of several plants is still unexplored. In this research, we chose *Lindernia ruellioides,* one of the most extensively utilized traditional medicinal plants among the natives of Mizoram. The molecular mechanisms underlying the wound healing potential of *L. ruellioides* was subjected in this thesis. There are four main chapters in this thesis. **Chapter 1** describes about the collection of *L. ruellioides,* extraction process, qualitative and quantitative phytochemical analysis as well as analysis of bioactive compounds using LC-MS. **Chapter 2** describes the free radical scavenging activity and antibacterial activity of *L. ruellioides*. In **Chapter 3**, the wound healing activity of ethanolic extract of *L. ruellioides* (LREE) was studied using excision (wound contraction rate, epithelialization period, antioxidant assay, histological examination) and incision (tensile strength) wound models in Wistar albino rats. **Chapter 4** describes the expression pattern of two growth factors *Tgf-β1* and *Vegf* by employing quantitative RT-PCR in an excision inflicted granulation tissue.

Chapter I: Preparation of *Lindernia ruellioides* extracts, phytochemical analysis and identification of bioactive compounds using LC-MS.

Lindernia ruellioides was collected, washed and allowed to shade dried at room temperature and then powdered. It was then subjected to sequential cold maceration using different solvents such as chloroform, ethanol and distilled water. Qualitative phytochemical analysis as well as quantitative analysis such as total phenolic and total flavonoid contents was carried out using standard procedures to identify the

constituents for all the extracts. The chloroform extract, ethanol extract and aqueous extract were further subjected to LC-MS analysis in order to identify the bioactive compounds present in them. The preliminary phytochemical screening revealed the presence of various naturally occurring phytochemicals like alkaloids, saponins, flavonoids, tannins, phlobatannins and terpenoids in the ethanolic extract (LREE), alkaloids, flavonoids, tannins and steroids in aqueous extract (LRAE) and alkaloids, saponins and tannins in the chloroform extract (LRCE). Quantitative analysis of total phenolic and total flavonoid contents revealed that the ethanolic extract (LREE) has significantly highest total phenolic (327.97 ± 1.77 mg GAE /g of dry extract) and flavonoid (264.95 ± 0.71 mg quercetin equivalent/g of dry extract) contents when compared with the aqueous extract (LRAE) and chloroform extract (LRCE). Furthermore, the result of LC-MS revealed the presence of several bioactive compounds having various pharmacological activities. From our results, we found that *L. ruellioides* contains several phytochemicals and a significant amount of both phenolic and flavonoid contents which indicates various possible pharmacological activities of *L. ruellioides.*

Chapter II: Free radical scavenging activities and antibacterial activities of various extracts of *Lindernia ruellioides*.

When free radicals are produced at moderate concentration, they exert beneficial effects. However, overproduction of free radicals can cause damage to biomolecules such as lipids, proteins and DNA which can leads to several diseases. At this point, antioxidant that are found naturally in several plants play a significant role in protecting our body from this free radical damage. This led to the increase research with naturally occurring antioxidants to prevent free radical associated diseases. This study aims to determine the free radical scavenging activity as well as the antibacterial activity of different extracts of *L. ruellioides.* The scavenging activity of various extracts of *L. ruellioides* against DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) and $O_2^{\bullet-}$ (superoxide anions) was determined following standard protocols. And from the results, we found that different extracts of *L. ruellioides* scavenged DPPH, ABTS and $O_2^{\bullet-}$ radicals in a concentration dependent manner. Among the three extracts, the ethanolic extract was

found to be the most potent in scavenging DPPH and ABTS radicals with an IC_{50} of 158.0 ± 4.82 µg/ml and 112.9 ± 6.47 µg/ml respectively. However, aqueous extract was found to inhibit $O_2^{\bullet-}$ radical most efficiently with an IC_{50} of 118.1 ± 7.88 µg/ml. Different extracts of *L. ruellioides* were also analyzed for their antibacterial activity against three bacteria species such as *Escherichia coli*, *Bacillus subtilis* and *Klebsiella pneumoniae* and all the three extracts were found to inhibit the growth of these bacteria species with a significant degree of inhibition. Moreover, from the result of minimum inhibitory concentration (MIC) the ethanolic extract was found to be active even at a very low concentration. The results of our study suggests that different extracts of *L. ruellioides* significantly possess free radical scavenging activity as well as antibacterial activity which implies *L. ruellioides* as a potential candidate for having various pharmacological activity including wound healing agent.

Chapter III: Wound healing activity of *Lindernia ruellioides* using excision and incision methods.

In this study, the wound healing potential of *L. ruellioides* ethanolic extract (LREE) was determined using standard protocols. To guarantee the safety of the plant extract, an acute dermal toxicity test was conducted prior to the experiment. Every *in vivo* investigation was performed in compliance with OECD guidelines. Excision and incision wounds were inflicted following standard protocols. Our analysis disclosed that LREE significantly accelerated wound healing in experimental rats. Notably, we observe a dose-dependent increase antioxidant levels while there was a substantial reduction in oxidative stress levels, indicating decrease in tissue damage in extract treated groups. The activity of wound healing in excision wound model as well as incision wound model also show significant increase in wound contraction starting from day 6 (P<0.01). On day 21, wound area significantly decreased at a pace of 98.91% and 97.56% for 10% and 5% LREE respectively when compared with control (P<0.01). Faster time frame for epithelialization, for 10% LREE (16.61 ± 0.2) and 5% LREE (18.11 ± 0.48) was noticed when compared with control (23.44 ± 0.59) and the skin breaking strength significantly elevated in LREE treated group when compared with the control. In addition, the histological examination shows encouraging results. The granulation tissue from the extract treated animals depicted increase collagen

deposition while a decrease in inflammatory cells which are the results of tissue regeneration. LREE exhibited an impressive impact on wound healing conversely with those in the control group. Accordingly, our study validates the possible pharmacological activity of *L. ruellioides* as wound healing agent.

Chapter IV: Study of the expression pattern of transforming growth factor beta 1 (*Tgf-β1)* and vascular endothelial growth factor (*Vegf)* using quantitative RT-PCR.

Immediately following an injury, the release of cytokines and other growth factors initiates wound healing. Among those growth factors, *Tgf-β1* and *Vegf* has been proven to play a significant role in wound healing. This study intends to gain insight how LREE alters the expression of *Tgf-β1* and *Vegf* upon wound healing in experimental rats. The control group was studied using glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene expression as housekeeping gene. Male Wistar albino rats were used for the study and inflicted with excision wound following standard protocol. The animals were sacrificed on days 3, 7 and 11 post-wounding and the granulation tissue was utilized for real time-PCR studies. Following LREE treatment, the mRNA expression levels of the two marker genes *Tgf-β1* and *Vegf* were significantly upregulated ($P<0.05$). From our results, we found that, there is a significant relationship between the expression of these two genes (*Tgf-β1* and *Vegf*), and the rate of wound healing in experimental rats.