ASSESSMENT OF THE FREE RADICAL SCAVENGING, ANTIBACTERIAL AND WOUND HEALING ACTIVITIES OF *PILEA SYMMERIA* WEDDELL. IN SWISS ALBINO MICE.

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

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ASSESSMENT OF THE FREE RADICAL SCAVENGING, ANTIBACTERIAL AND WOUND HEALING ACTIVITIES OF *PILEA SYMMERIA* WEDDELL. IN SWISS ALBINO MICE.

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Submitted
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CERTIFICATE

I certify that the thesis entitled "**Assessment of the free radical scavenging, antibacterial and wound healing activities of *Pilea symmeria* Weddell. in Swiss Albino Mice**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **R.K. Lalremtluangi** is a record of the research work carried out during the period 2019 - 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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I, **R.K. Lalremtluangi**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do 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.

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GENERAL INTRODUCTION

A wound is a break or damage to the skin or other body tissue that occurs due to various factors such as physical trauma, chemical exposure, or biological agents (Agarwal *et al*., 2009). When the skin is injured, the body begins a complex healing process that unfolds in four main stages, Hemostasis, Inflammation, Proliferation, and Remodeling (Gilmore, 1991). These stages happen in a specific sequence and often overlap, each playing a crucial role in the recovery of the tissue. For wound healing to progress effectively, it is vital that these phases occur in the proper order and within a certain time frame (Mathieu *et al*., 2006). If any of these stages are disrupted or delayed, healing may not proceed as expected. Several factors can interfere with the normal process of healing, such as infections, hormonal fluctuations, aging, stress, and chronic conditions like diabetes (Guo *et al*., 2010). These can all lead to complications, causing the wound to heal more slowly or improperly.

Hemostasis

In the initial stages of wound healing, the body responds with a series of physiological events aimed at stopping blood loss and setting the stage for tissue repair. First, blood vessels constrict to reduce blood flow, while platelets gather at the site of injury to form an initial plug (Rumbaut & Thiagarajan., 2010). This aggregation is followed by coagulation, where fibrin, a protein, strengthens the platelet plug by creating a network (Scridon, 2022). A clot then develops as a result and the blood viscosity changes from liquid to more gel like consistency (Velnar *et al*., 2009). The clot acts as a barrier, trapping platelets and blood cells, effectively sealing the wound (Gale, 2011). Simultaneously, the wound and surrounding tissue release several important molecules, such as growth factors and pro-inflammatory cytokines, which play a crucial role in further healing (Broughton *et al*., 2006). These include epidermal growth factor (EGF), transforming growth factor beta (TGF-β), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGF-α), and platelet-derived growth factor (PDGF) (Gale, 2011; Campos *et al*., 2008). These

signalling molecules help initiate the next phase of healing by promoting inflammation and preparing the wound site for the subsequent stages of tissue repair (Schultz *et al*., 2011).

Inflammatory phase

The inflammatory phase of wound healing is primarily driven by neutrophils, monocytes, and macrophages, which are crucial for protecting the wound and initiating the healing process (Dovedytis *et al*., 2020). This phase typically begins within the first 24 hr following an injury and, in most cases, lasts up to two weeks in normal, healthy wounds (Mehra & Gupta, 2023). However, in chronic wounds that fail to heal properly, the inflammatory phase may persist much longer, complicating the healing process (Avishai *et al*., 2017). One of the hallmark features of the inflammatory phase is the presence of erythema (redness), oedema (swelling), heat (calor), and pain (dolor) (Hannoodee & Nasuruddin, 2024). These signs of inflammation occur as a direct result of vasodilation, which is the widening of blood vessels. This process allows more blood to flow to the site of injury, bringing immune cells and nutrients to aid in healing (Ramanlal & Gupta, 2023). Although inflammation is typically considered the second stage of wound healing, these characteristic symptoms appear almost immediately after the injury, marking the body's initial response to trauma (Landen *et al*., 2016). The primary purpose of the inflammatory phase is to defend the wound from infection, clearing away dead cells, and set the stage for the next steps in the healing process (Manoukian *et al*., 2016). Platelets release certain signals, triggering a response from neutrophils, the first immune cells to arrive at the scene. These neutrophils protect the wound by phagocytosing and breaking down bacteria, as well as clearing away any foreign particles or damaged cells (Margraf *et al*., 2021). In addition to this, neutrophils release signals like TNF-α and IL-1, which help attract and activate other important cells, like epithelial cells and fibroblasts, which are crucial for healing. They also release enzymes like collagenase and elastase, which break down damaged components of the extracellular matrix so that new, healthy tissue can form (Kolaczkowska & Kubes, 2013). Neutrophils also produce reactive oxygen species (ROS); powerful molecules that help destroy bacteria and prevent further infection

(Winterbourn *et al*., 2016). Together, these actions help clear the way for the wound to heal, while protecting it from microbial threats. ROS are important for fighting off infections, but too much production can end up damaging healthy tissue around the wound (Blass *et al*., 2013). This delicate balance is important for the healing process. After fulfilling their function, neutrophils typically undergo apoptosis and are subsequently cleared from the wound site within two to three days (Schultz *et al*., 2011). At this point, tissue monocytes, another type of immune cell, move in to take over. Around 24 hr after the injury, monocytes from the bloodstream migrate to the wound site, where they transform into activated macrophages in response to various signals, like chemokines, cytokines, and growth factors (Dash *et al*., 2024). Macrophages are crucial for managing the healing process. Like neutrophils, they help clean up the wound by removing bacteria, dead cells, and leftover neutrophils. They also play an essential role in driving the healing process forward (Ahmed *et al*., 2022). They release a variety of signaling molecules, including cytokines and growth factors like PDGF, IL-1, TGF-β, IL-6, TGF-α, IGF-1, FGF and TNF-α. These signals help orchestrate tissue repair by promoting cell growth, guiding cell migration, and stimulating the formation of new blood vessels and connective tissue (Duque & Descoteaux, 2014). Together, neutrophils and macrophages set the stage for the next phase of wound healing. Some of these soluble mediators help promote the growth of new blood vessels (angiogenesis), while others attract and activate fibroblasts, which are responsible for creating, depositing, and organizing the new tissue matrix (Bouchery & Harris, 2019). As neutrophils and macrophages decrease at the wound site, the inflammatory phase begins to wind down and the proliferative phase takes over. This shift in transitioning the inflammatory phase to proliferative phase is largely driven by macrophages (Landen *et al*., 2016).

Proliferative phase

Re-epithelialization, angiogenesis, matrix deposition, and collagen synthesis are the crucial processes that take place during this phase and result in the production of granulation tissue (Blass *et al*., 2013). The migration, proliferation, and differentiation of fibroblasts and epithelial cells are the main characteristics of this phase (Cialdai *et al*., 2022). Through a process called epithelialization, cells near the

edges of the wound lose their contact of inhibition and start to migrate into the wound area via epiboly, resulting in covering the wound (Tomic-Canic *et al*., 2018). The basal layer cells begin to multiply as this movement proceed; producing more epithelial cells (Pastar *et al*., 2014). Fibroblasts play a vital role as they migrate into the wound site in response to soluble signals released by platelets and macrophages (Darby & Hewitson, 2007). Once at the site, fibroblasts begin to multiply and produce collagen, elastin, and proteoglycans, key components of the extracellular matrix (Li *et al*., 2007). As part of this process, new blood vessels are formed through angiogenesis to replace damaged ones, and new granulation tissue rich in collagen and extracellular matrix is created (Tonnesen *et al*., 2000; Wilkinson & Hardman, 2020). The collagen matrix supports these newly formed blood vessels, contributing to the granulation tissue's characteristic uneven, bumpy texture (Alhajj & Goyal, 2022). Healthy granulation tissue usually appearing pink or red with a granular surface forms when the tissue received adequate supply of nutrients and blood through the blood vessels. On the other hand, black granulation tissue may indicate an infection or the presence of diseased tissue (Enoch & Leaper, 2008). Over time, fibroblasts mature into myofibroblasts, which are characterized by their ability to enhance collagen production while reducing cell proliferation (Bernardo & Fibbe, 2013). This increased collagen synthesis plays a key role in wound contraction, significantly contributing to the reduction of the wound's size as it heals (Ehrlich & Hunt, 2012).

Remodeling phase

During this stage, the granulation tissue transforms into a scar and its tensile strength increases (Wong *et al*., 2013). This strength is enhanced by changes in the type, amount, and structure of collagen, as well as by the cross-linking of collagen fibers (Singh *et al*., 2023). Type I collagen, the primary collagen found in the skin, is produced through the conversion of type III collagen, which is mostly present in granulation tissue (Amirrah *et al*., 2022). Additionally, the number of blood vessels in the affected area diminishes, and other cellular activities decrease as the healing process progresses (G. Schultz *et al*., 2005). The wound continues to contract, with the extent of contraction varying based on the depth of the injury. This contraction

process is driven by myofibroblasts (Li *et al*., 2007). The breaking strength of the wound increases, which is crucial for forming a strong, stable wound. However, scars are not as strong as healthy, uninjured skin tissue (Krzyszczyk *et al*., 2018).Top of FormBottom of Form

Free radicals

Free radicals are molecules that contain an unpaired electron in their outermost orbit (Cheeseman & Slater, 1993). Their instability and high reactivity drive them to extract electrons from other molecules. This chain reaction leads to the formation of additional free radicals, which can cause various harmful effects on living cells (Phaniendra *et al*., 2014). These radicals are naturally generated through the body's metabolic processes. When present in controlled amounts, they contribute positively by supporting cellular signaling pathways, senescence, facilitating cell growth, apoptosis, adhesion, and differentiation, and aiding in the body's defense against harmful microorganisms (Valko *et al*., 2006; Nordberg & Arner, 2001). However, an excess of free radicals can lead to oxidative stress, which occurs when there is an imbalance between free radicals and antioxidants in the body (Lobo et al., 2010). This oxidative stress has been linked to various diseases, including cancer, Parkinson's disease, Alzheimer's disease and Atherosclerosis etc. (Forman & Zhang, 2021). ROS such as superoxide radicals and hydroxyl radicals are generated as a result of tissue damage. This damage primarily stems from disruptions in normal cellular functions, the release of fragmented mitochondria from injured cells, or increased oxygen consumption during injury, which causes electrons to escape from the electron transport chain (Murphy, 2008; Turrens, 2003). After engulfing pathogens or damaged cells, phagocytes such as neutrophils and macrophages initiate an oxidative burst. During this process, the activated NADPH oxidase enzyme utilizes oxygen to generate superoxide anions, which can then be converted into other reactive species like hydrogen peroxide and hydroxyl radicals (Acosta & Alonzo, 2023; Andrés *et al*., 2023). Excessive UV radiation exposure, stressful situations, vigorous activity, and inadequate nutritional intake can also lead to an increase in the creation of free radicals (Jakubczyk *et al*., 2020).

Antioxidant

The human body naturally produces antioxidants to neutralize free radicals and minimize their harmful effects (Flieger *et al*., 2021). However, when free radicals accumulate excessively and the body's antioxidant defenses are insufficient, additional antioxidants must be sourced externally (Tumilaar *et al*., 2024). Many natural foods, including vegetables, fruits, herbs and spices, are rich in antioxidants (Yadav *et al*., 2016). These plant based foods contain essential vitamin and phytochemicals, such as phenolic compounds, which contribute to their antioxidant properties (Bansal *et al*., 2013; Jiang & Xiong, 2016; Lu *et al*., 2009). The antioxidant potential of various medicinal plants is closely linked to their therapeutic benefits (Suntar *et al*., 2012). This highlights the significance of studying medicinal plants that contain diverse beneficial compounds with antioxidant properties, which could help neutralize reactive free radicals and offer potential therapeutic applications.

Wound infection

Wounds can be categorized into acute and chronic wound. An acute wound follows a timely and orderly healing process, progressing through all the necessary stages efficiently (Ather & Harding, 2009). In contrast, a wound is classified as chronic if it takes an extended period to heal and fails to restore the normal anatomical and functional integrity (Garcia & Thomas, 2006). Factors such as infection, ischemia and trauma can contribute to the development of chronic wounds (Robson, 1997). Additionally, the presence of bacteria and their byproducts can further impede the wound healing process (Uberoi *et al*., 2024). When bacteria produce endotoxins, they can cause an increase in pro-inflammatory cytokines such as IL-1 and TNF-α (Zampini *et al*., 2009; Silva *et al*., 2009). If the inflammatory phase is prolonged or excessively intense, the wound healing process may be significantly delayed (Holzer-Geissler *et al*., 2022). Preventing wound infections is essential for accelerating the healing process and minimizing the risk of chronic complications. In response to rising antibiotic resistance, numerous medicinal plants

have been investigated, with several demonstrating significant antimicrobial activity. The widespread emergence of bacterial resistance to conventional antibiotics has intensified the search for alternative antimicrobial agents derived from natural sources (Anand et al., 2019). Therefore, increased attention to plant derived antibiotics is warranted, as they may offer safer and more sustainable options for managing wound infections and other illnesses linked to bacterial problems (Silva *et al*., 2010).

Medicinal plant

Medicinal plants are considered the cornerstone of modern medicine with their contents acting as a major source of pharmacological chemicals (Salmeron-Manzano *et al*., 2020). People experimented with many plants to treat health problems in the past, eventually discovering those with medicinal qualities (Kunle, 2012). A variety of therapeutic techniques have been created by many societies worldwide, drawing upon the local flora (Gurib-Fakim, 2005). Many plants are now grown around the world to extract important compounds for use in pharmaceutical and medical purposes (Kinghorn & Seo, 1996). People continue to use these therapeutic herbs because they are inexpensive, easily accessible, and have less negative effects than contemporary drugs (Salmeron-Manzano & Manzano-Agugliaro, 2020). Most modern wound care therapies are expensive and may cause medication resistance or adverse responses (Sai & Babu, 1998). Approximately 10% of the world's 50,000+ plant species are used in pharmaceuticals and medical goods, making medicinal plants widely disseminated (Huang, 2011; Rafieian-Kopaei, 2012). Since many potential health benefits of medicinal plants remain unexplored and require further scientific research to be fully understood, their future appears promising (Jamshidi-Kia *et al*., 2018). While these plants offer numerous health benefits, excessive consumption or use without sufficient scientific knowledge can pose serious risks. For example, *Momordica charantia* L. is commonly used to treat fever, but its unripe seeds can be harmful, potentially causing hypoglycemic coma by significantly lowering blood sugar levels (Manzano, 2020; Adoum, 2009). Therefore, conducting scientific research on the medicinal herbs found in nature is crucial to ensuring their safe and effective use.

Pilea symmeria* and an extensive overview of the ethnopharmacological characteristics of the plant genus *Pilea

Figure 1: *Pilea symmeria*

Classification

Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Rosales
Family: *Urticaceae*
Genus: *Pilea*
Species: *Pilea symmeria*
Distribution: Meghalaya, Nagaland, Sikkim, Uttarakhand, West Bengal, Mizoram, Tibet, Assam, Nepal, Bhutan, Arunachal Pradesh.

Habitat: They are found in shaded and moist environment.

Traditional use: Leaf paste has been used as an antiseptic for cuts and wounds in Mizoram, India (Rozika, 2005).

P. symmeria has long been used in Mizoram for healing wounds, but there is limited scientific research examining its full range of potential uses. Existing literature offers little information on its pharmacological properties, indicating that this plant remains largely unexplored in scientific research. However, several species within the *Pilea* genus have been investigated for their medicinal properties, and a summary of these studies is outlined below.

A study by Chahardehi *et al*. (2010) explored the antioxidant and antibacterial properties of *Pilea microphylla*, a plant with promising medicinal potential. Two different extraction techniques were used to analyze its bioactive compounds. The first method, Soxhlet extraction, involved solvents such as hexane, chloroform, ethyl acetate, and methanol. The second approach, known as partition extraction, utilized butanol, diethyl ether, ethyl acetate, and chloroform. Both methods revealed that the plant extracts contained significant amount of flavonoids and phenolic compounds, which are known for their antioxidant and antibacterial properties. The study also tested *P. microphylla* against a range of harmful bacteria, including both Gram-positive and Gram-negative strains. These included *Bacillus cereus, Bacillus spizizeni, Bacillus subtilis, Enterobacter aerogenes, Citrobacter freundii, Escherichia coli, Micrococcus species, Morganella morganii, Methicillin-resistant Staphylococcus aureus* (MRSA)*, Erwinia species, Salmonella paratyphi B, Serratia marcescens, Vibrio parahaemolyticus, Streptococcus salivarius,* and *Yersinia species.* The results suggested that *P. microphylla* not only has natural antioxidant and anti-aging properties but also has the potential to inhibit the growth of dangerous pathogens like MRSA and *B. cereus*, both of which are known to cause foodborne illnesses. This research sheds light on the possibility of extracting beneficial compounds from *P. microphylla* for use in developing new medications. With further study, this plant could play a significant role in combating foodborne diseases and contributing to advancements in natural medicine.

A study by Prabhakar *et al*. (2006) investigated the antioxidant and radioprotective properties of *P. microphylla*, revealing its potential as a natural protective agent against oxidative stress and radiation damage. The research found

that the plant's ethanolic extract effectively reduced iron-induced lipid peroxidation in phosphatidylcholine liposomes and demonstrated strong free radical scavenging activity by inhibiting DPPH, ABTS, and hydroxyl radicals. To further assess its radioprotective effects, the study conducted *in vivo* experiments using Swiss albino mice. The mice were exposed to whole body γ-rays at a source to skin distance of 64.2 cm, with a radiation dose rate of 1.66 Gy/min. The findings indicated that *P. microphylla* extract provided significant protection against radiation, with a dose reduction factor of approximately 1.12 and an impressive 80% protection rate at a dosage of 900 mg/kg. Additionally, the extract helped preserve essential antioxidants such as GSH, GST, SOD, catalase, and thiols, preventing their depletion in the liver of irradiated mice. Beyond liver protection, the study also highlighted its ability to safeguard the intestines and hematopoietic system from radiation induced damage. These results suggested that *P. microphylla* could be a promising natural radioprotective agent, potentially beneficial for reducing radiationrelated harm in medical and environmental exposures.

A study by Bansal *et al*. (2011) explored the potential of *P. microphylla* in managing metabolic disorders, specifically in a genetically engineered diabetic mouse model (C57BL/KsJ-db/db strain). The research aimed to evaluate the plant's effectiveness in improving diabetes related complications by administering its ethanolic extract orally and measuring various metabolic parameters. The findings revealed that the extract significantly reduced body weight and food efficiency ratio in the test subjects. Additionally, it played a role in regulating key metabolic markers boosting plasma insulin levels, inhibiting plasma dipeptidyl peptidase IV (DPP-IV), and leading to a marked decrease in plasma glucose (PG) and triglyceride (TG) levels. To further assess its impact, an oral glucose tolerance test (OGTT) was conducted on overnight fasted mice, showing that the extract helped lower blood glucose levels while stimulating glucose dependent insulin secretion. Histological analysis provided additional insights into its therapeutic potential. The extract was found to reduce lymphocytic infiltration in the islet region of diabetic mice, improve the connective tissue surrounding the intralobular ducts, and help preserve the overall structure of the islets. These promising results suggest that *P. microphylla* may offer

significant benefits in managing diabetes and related metabolic imbalances, potentially serving as a natural alternative for improving glucose regulation and pancreatic health.

Bansal *et al*. (2011) conducted a study to explore how phenolic compounds extracted from *P. microphylla* could protect cells from radiation induced DNA damage. The researchers identified six key compounds: quercetin-3-O-rutinoside, luteolin-7-O-glucoside, 3-O-caffeoylquinic acid, apigenin-7-O-rutinoside, apigenin-7-O-β-D-glucopyranoside, and quercetin. Among these, quercetin-3-O-rutinoside, 3-O-caffeoylquinic acid, luteolin-7-O-glucoside, and quercetin stood out for their strong antioxidant properties. They effectively neutralized harmful free radicals like DPPH, ABTS, and SOD while also preventing lipid peroxidation, a process that can damage cell membranes. The study further revealed that these compounds offered significant protection against DNA damage caused by hydroxyl radicals in calf thymus DNA. Pre-treatment with these phenolic compounds led to a reduction in Thiobarbituric acid reactive substances (TBARS), which are markers of oxidative stress. Additionally, when tested on V79 cells exposed to gamma radiation, these compounds helped improve cell viability and reduced DNA fragmentation. The comet assay results showed a decrease in tail length (TL) and Olive tail moment (OTM), indicating less DNA damage. Overall, this research highlights the potential of *P. microphylla* as a natural source of radioprotective compounds, which could be valuable in reducing radiation induced cellular damage and enhancing cell survival.

Chahardehi *et al*. (2013) explored the potential antidepressant effects of *P. microphylla* using a mouse model of depression. To assess its impact, they conducted two widely recognized behavioral tests: the Forced Swimming Test (FST) and the Tail Suspension Test (TST). Mice were administered specific doses of methanol, chloroform, and ethyl acetate extracts of the plant via intraperitoneal (i.p.) injection. The results showed a significant reduction in immobility time during both tests, suggesting an antidepressant like effect. Researchers attributed these positive outcomes to the plant's high flavonoid content, which is known for its neuroprotective and mood enhancing properties. These findings indicate that *P.*

microphylla may have potential as a natural alternative for managing depression, though further studies are needed to fully understand its mechanisms and therapeutic potential.

Choi *et al*. (2020) conducted a study to explore the potential benefits of *Pilea martini* for skin health, focusing on its anti-inflammatory and moisturizing properties. Using lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophage cells and human immortalized keratinocytes, they tested how the plant extract affected key inflammatory pathways. Their findings revealed that *P. martini* extract helped reduce the activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Activator Protein-1 (AP-1), proteins involved in inflammation, while also inhibiting the production of Prostaglandin E2 (PGE2) and nitric oxide (NO), both of which contribute to inflammation. Additionally, the extract decreased the activation of specific signaling proteins such as Extracellular Signal-Regulated Kinase (ERK), Jun N-terminal kinase (JNK), and P38 Mitogen-activated protein kinases (P38MAPK) and reduced the expression of genes linked to inflammation such as Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2). Beyond its anti-inflammatory effects, *P. martini* extract also supported skin hydration by increasing hyaluronic acid production and boosting levels of filaggrin and Serine Palmitoyltransferase (SPT), proteins essential for maintaining the skin barrier. It further suppressed the production of pro-inflammatory cytokines IL-6 and TNF-α and blocked NF-κB from binding to DNA in keratinocytes (HaCaT cells), limiting inflammation at a molecular level. Based on these diverse effects, the researchers concluded that *P. martini* possesses strong potential as a natural ingredient for soothing inflammation and enhancing skin hydration.

Alexander-Lindo *et al*. (2017) conducted a study using Sprague Dawley albino rats to investigate the potential of *Pilea elizabethae*, a plant traditionally used to manage diabetes. Their goal was to examine the blood sugar lowering effects of oleanonic acid, a natural compound extracted from the plant. To isolate this compound, they used hexane, ethyl acetate, and ethanol as solvents in the extraction process. To test the effectiveness of the extracts, the researchers conducted an oral

glucose tolerance test (OGTT), a standard method for assessing how well a substance can lower blood sugar levels. After testing all three extracts, they further refined their findings by using chromatography, which led to the identification of a pure hypoglycemic compound called R-E2Gii. This compound was confirmed to be oleanonic acid, a type of triterpenoid. The results were promising, when the rats were given R-E2Gii orally, their blood glucose levels dropped significantly. This demonstrated that the hexane-ethyl acetate extracts from *P. elizabethae* have antihyperglycemic properties, supporting its traditional use as a natural remedy for diabetes.

As mentioned above, several species within the genus *Pilea* have shown a variety of beneficial effects, such as anti-inflammatory, antibacterial, antidepressant, antidiabetic, radioprotective, and skin moisturizing properties. This connection prompted us to investigate *Pilea symmeria*, with the hope that it too may possess valuable pharmacological properties. Although a previous study evaluated the ethanolic extract of the root of *P. symmeria* for its antibacterial and DPPH radical scavenging activities (Subba & Basnet, 2014), no broader investigation has been conducted on the leaves or on its wider therapeutic potential. Our study aims to examine its phytochemical composition, antioxidant and antibacterial properties, effects on wound healing, and influence on gene expression. Through this research, we seek to gain important insights that will not only broaden scientific knowledge of this plant but also aid in the development of new treatments for wound care and related health issues.

The present thesis

This thesis explores the phytochemical composition and potential wound healing properties of *Pilea symmeria*. Extracts were prepared using ethanol, chloroform, and aqueous through sequential cold maceration. These extracts were analyzed for their phytochemical constituents, as well as their free radical scavenging and antibacterial activities through *in vitro* methods. Among them, the ethanol extract demonstrated the highest efficacy in these preliminary tests and was therefore selected for *in vivo* evaluation. Wound healing activity was assessed using Swiss

albino mice subjected to both excision and incision wound models. The impact of the ethanol extract on various wound healing parameters was then evaluated. A summary of the studies carried out is presented in the subsequent chapters.

Chapter 1: To determine the phytochemical content and free radical scavenging activity of *Pilea symmeria* in a cell free system.

The leaves of *Pilea symmeria* were extracted through cold maceration using solvents of increasing polarity such as chloroform, ethanol, and aqueous. The resulting extracts were then analyzed through both qualitative and quantitative phytochemical assessments, along with Liquid Chromatography-Mass Spectrometry (LC-MS) analysis.

Chapter 2: *In vitro* evaluation of the antibacterial properties of *Pilea symmeria.*

This chapter focuses on the *in vitro* antibacterial activity of *Pilea symmeria* against three bacterial strains: *Escherichia coli*, *Bacillus subtilis* and *Klebsiella pneumoniae*. The antibacterial activity of the chloroform, ethanol and aqueous extract were tested using Disc diffusion method and the Minimum inhibitory concentration (MIC) and the Minimum bactericidal concentration (MBC) were also determined.

Chapter 3: Evaluation of the wound healing properties of *Pilea symmeria* using excision and incision wound models.

In this chapter, mice were subjected to excision and incision wound models. Wound contraction rate, epithelialization period, and histological evaluation of the excision wounds were assessed, while the tensile strength was measured for the incision wounds.

Chapter 4: Evaluation of the effect of *Pilea symmeria* on the antioxidant/oxidant status in a wound bearing Swiss albino mice.

In this chapter, the levels of antioxidant markers such as Glutathione (GSH), Glutathione S-Transferase (GST), and Superoxide Dismutase (SOD), along with the

extent of lipid peroxidation (LPO) in the granulation tissue of the excision wound were evaluated.

Chapter 5: To study the effect of *Pilea symmeria* on the gene expression involved in wound healing by using RT-PCR.

This chapter examines the impact of *Pilea symmeria* ethanol extract on excision wound healing at the gene expression level. The expression profiles of Vascular Endothelial Growth Factor (VEGF) and Tumor Necrosis Factor-alpha (TNF-α) in the granulation tissue were analyzed using RT-PCR.

Chapter 1

To determine the phytochemical content and free radical scavenging activity of *Pilea symmeria* in a cell-free system.

Abstract

The leaves of *P. symmeria* were subjected to cold maceration using chloroform, ethanol, and aqueous as a solvents. The phytochemical composition of the extracts was analyzed through both qualitative and quantitative phytochemical screening as well as Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. The antioxidant potential of the extracts was evaluated by assessing their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and superoxide ($O_2^{\bullet -}$) (SO) radicals. The extracts were found to contain a range of bioactive phytochemicals, such as terpenoids, tannins, flavonoids, cardiac glycosides, steroids, alkaloids, saponins, and phlobatannins. LC-MS analysis revealed the presence of 34 major compounds with diverse biological properties, including anti-inflammatory, antibacterial, and wound healing activities. Among the three extracts, the ethanol extract demonstrated the strongest free radical scavenging activity. It exhibited the highest phenolic and flavonoid content while also having the lowest IC_{50} value. These findings suggest that *P. symmeria* leaf extracts, enriched with bioactive phytochemicals such as flavonoids and phenolics, exhibit strong antioxidant potential, as demonstrated by their radical scavenging activity, supporting their potential for further pharmacological and therapeutic applications.

1. INTRODUCTION

The increasing interest in natural products, particularly medicinal plants with antioxidant properties, has become a significant trend in modern scientific research and healthcare (Chandimali *et al*., 2025). This growing fascination stems from the potential of plant derived compounds to offer protective benefits against various environmental stressors, including ultraviolet (UV) radiation, pollution, drought, and microbial pathogens (Gibson et al., 1998). Among these compounds, phytochemicals play a crucial role in enhancing plant survival and providing medicinal benefits to humans (Yang & Ling, 2024). These bioactive substances exhibit diverse biological activities, such as anti-inflammatory, antimicrobial, and anticancer effects, making them valuable in the field of medicine and pharmacology (Sharma *et al*., 2018; Jaeger & Cuny, 2016).

Phytochemicals are present in different parts of plants, including leaves, stems, fruits, roots, flowers, and seeds (Costa *et al*., 1999). Based on their function in plant metabolism, these compounds are categorized into primary and secondary metabolites (Erb & Kliebenstein, 2020). Primary metabolites, which are essential for plant growth and development, include sugars, amino acids, proteins, nucleic acids, purines, pyrimidines, and chlorophylls. Secondary metabolites, on the other hand, serve a defensive and adaptive role, comprising alkaloids, terpenes, flavonoids, lignans, steroids, saponins, curcumin, phenolics, and glucosides (Hahn, 1998; Ramawat *et al*., 2008). Among these, phenols and flavonoids are widely recognized for their potent antioxidant properties, which help mitigate oxidative stress and protect against various diseases associated with free radical damage, such as cardiovascular disorders, neurodegenerative diseases, and cancer (Andarwulan *et al*., 2021; Koche *et al*., 018). Flavonoids, in particular, are one of the most diverse groups of plant phenols and exhibit multiple pharmacological effects, including antimicrobial, cytotoxic, anti-inflammatory, and antitumor activities (Teiten *et al*., 2013; Shirsat *et al*., 2012; Dai & Mumper, 2010). To harness the therapeutic potential of phytochemicals, it is crucial to employ appropriate extraction techniques, as the efficiency of extracting these compounds depends on their solubility and stability in different solvents (Bitwell *et al*., 2023). Selecting the right solvent

significantly impacts the quality and potency of the extracted bioactive compounds, influencing their effectiveness in medicinal applications (Lobo *et al*., 2010).

Free radicals, though essential for normal cellular functions, can become harmful when their levels exceed the body's capacity to neutralize them (Fitzmaurice *et al*., 2011). These reactive oxygen species (ROS) are also produced by immune cells to combat infections, but an imbalance in their generation can lead to oxidative stress (Andres *et al*., 2022). This imbalance occurs when the body's antioxidant defenses fail to counteract the excess free radicals, resulting in cellular damage (Pizzino *et al*., 2017). The prolonged presence of free radicals can disrupt normal physiological processes and damage vital cellular components, including proteins, lipids, and DNA, ultimately contributing to chronic health conditions such as cancer, cardiovascular diseases, neurodegenerative disorders, and inflammatory ailments (Pham-Huy *et al*., 2008; Lobo *et al*., 2010).

Although the human body naturally produces antioxidants to combat oxidative stress, various factors, including illness and environmental influences, may lead to an insufficient antioxidant supply (Birben *et al*., 2012). Consequently, obtaining antioxidants from dietary sources or medicinal plants becomes necessary to restore balance and protect against oxidative damage (Xu *et al*., 2017). Scientific studies have demonstrated that plants with high antioxidant potential contribute significantly to human health by reducing oxidative stress and preventing disease progression (Muscolo *et al*., 2024).

Several analytical methods are employed to evaluate the antioxidant capacity of plant derived compounds, including the 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and superoxide (SO) assays (Foti, 2015). DPPH assay is commonly used to measure free radical scavenging activity. In this method, antioxidants donate hydrogen atoms to stabilize the DPPH radical, converting it from a deep violet to a pale yellow color (Yapıcı *et al*., 2021). This color change is measured spectrophotometrically, providing an indication of the compound's antioxidant strength (Bursal *et al*., 2013). Similarly, the ABTS assay assesses the ability of antioxidants to neutralize the ABTS radical cation

($ABTS^{\bullet+}$). The reaction between ABTS and potassium persulfate produces a green-blue chromophore, which loses its color in the presence of antioxidants (Benkhaira & Fikri-Benbrahim, 2021; Munteanu & Apetrei, 2021). The degree of decolorization is quantified through spectrophotometric analysis, allowing researchers to determine the extent of free radical inhibition. The superoxide assay evaluates the harmful effects of superoxide radicals, which can impair essential metabolic pathways by inactivating iron-sulfur cluster containing enzymes. The disruption of these enzymes releases free iron, which participates in the Fenton reaction, generating more harmful hydroxyl radicals (Wang *et al*., 2018). In the non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system, superoxide anions are produced through NADH oxidation, leading to the reduction of nitro blue tetrazolium (NBT) into a purple formazan compound (Hazra *et al*., 2008). Antioxidants mitigate this reaction by neutralizing superoxide anions, thereby reducing NBT absorbance at 560 nm, which serves as a measure of antioxidant activity (Athukorala *et al*., 2006).

In this study, *P. symmeria*, ethanol, chloroform, and aqueous extracts were analyzed to identify their phytochemical constituents using qualitative and quantitative techniques, including liquid chromatography-mass spectrometry (LC-MS). To further confirm their antioxidant potential, these extracts were tested for their radical scavenging ability using DPPH, ABTS, and SO radical scavenging assays.

2. METHODS

2.1 *Plant Collection and Extract Preparations*

P. symmeria was obtained from Tanhril, Aizawl district, Mizoram, India, during June-August 2020. Plant identification and authentication was done at Botanical survey of India (BSI), Shillong, and the herbarium was deposited at Natural History Museum Mizoram, Mizoram University, with the accession number NHMM-P/000161. The leaves were shade dried at 25°C and pulverized using an electrical grinder. Powdered leaves (100g) were soaked in solvents (500ml) such as ethanol, chloroform, and aqueous (sequential cold extraction) in continuous agitation

for 72 hr. Upon filtering employing Whatman filter paper No. 1, extracts were evaporated to dryness in a 40ºC oven. The extracted material for each solvent, i.e., *Pilea symmeria* chloroform extract (PSCE), *Pilea symmeria* ethanol extract (PSEE), and *Pilea symmeria* aqueous extract (PSAE), were stored at -20˚C till further usage.

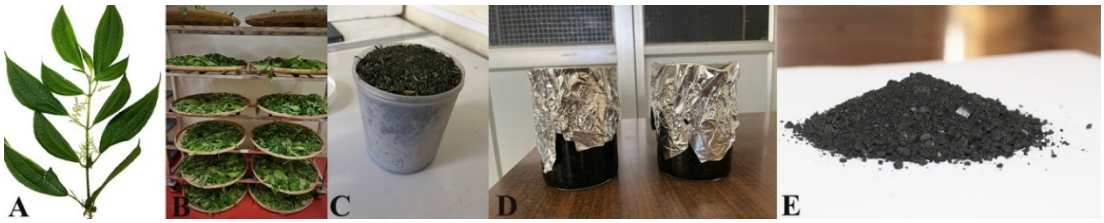

Figure 1.1 Plant collection and extraction processes; A) *Pilea symmeria*; B) Shade drying process C) Powdered leaves D) Extraction using cold maceration E) Extract.

2.2 Determination of Percentage (%) Yield of Extract

The percentage yield of the extract in different solvents (Chloroform, Ethanol and Aqueous) was calculated using the formula W_2-$W_1/W_0 \times 100$, where W_2 is the weight of the beaker containing the extract, W_1 is the weight of the empty beaker, and W_0 is the weight of the dried powdered leaves.

2.3 Preliminary Phytochemical Screening

Standard protocols were used for preliminary phytochemical analysis (Evans & Trease, 2002; Harborne 1998). Different extracts were evaluated for:

2.3.1 Alkaloids:

Dragendroff's reagent (0.5ml) was mixed with the extract (0.1g). Presence of a reddish-brown precipitate indicates the presence of alkaloids.

2.3.2 Cardiac glycosides:

Glacial acetic acid (2ml) with ferric chloride solution (a single drop) and concentrated H_2SO_4 (1ml) underlay were mixed with the extract (0.1g). Brown ring development at the interface indicates cardiac glycoside presence.

2.3.3 Saponins:

3 drops of olive oil were mixed with 0.1 g of extract. After mixing the mixture for a few minutes, a stable emulsion formed, indicating the presence of saponins.

2.3.4 Steroids:

0.1 g of the extract was dissolved in each solvent. Concentrated H_2SO_4 was added in a few drops. Steroid presence is indicated by the bottom layer turning red.

2.3.5 Tannins:

0.1 g of the extract was dissolved in each solvent. A solution of ferric chloride (0.1%) was added in a few drops. Brownish-green or blue-black color development indicates the presence of tannins.

2.3.6 Terpenoids:

5ml of each extract (0.1 g/ml) was mixed with chloroform (2ml). Concentrated H_2SO_4 (3ml) was then carefully added. A reddish-brown color appeared near contact, indicating the presence of terpenoids.

2.3.7 Phlobatannins:

1% aqueous HCl solution was used to boil 0.1 g of extract. Red precipitate indicates phlobatannins presence.

2.3.8 Flavonoids:

4 ml of 1N NaOH was mixed with 0.1 g of extract. The appearance of a dark yellow color indicates the presence of alkaloids.

2.4 Total Phenolic Content

Total phenolic content was estimated by applying the standard method (Singleton *et al*., 1999). A mixture was prepared by combining Folin-Ciocalteu's reagent (5ml) (diluted tenfold) with varied concentrations (ranging from 0.25-

8.0mg/ml) of *P. symmeria* extracts (1ml). Following a 5 min incubation period, the mixture received an additional sodium carbonate solution (4ml) with a 0.115mg/ml concentration. For 2hr (in the dark), the mixture was left to incubate at room temperature. Absorbance was then taken at 765nm. The calibration curve was created by combining a methanolic solution containing gallic acid (1mL, 0.25-4.0mg/mL) with the above reagents, and the absorbance was recorded. The experiment was conducted thrice. The total phenolic content was expressed as gallic acid equivalent to mg/g of the dried extract.

2.5 Total Flavonoid Content

Total flavonoid content was estimated by applying the standard method (Barreira *et al*., 2007). 0.25 ml of different *P. symmeria* extract concentrations (0.25 to 8.0 mg/ml) were combined with 5% sodium nitrite solution (75µl) and distilled water (1.25ml). After adding aluminum chloride (150µl) with a concentration of 10% (w/v), the solution was left to sit for 5min. Subsequently, 1M NaOH (0.5ml) was added to the mixture. By adding distilled water, the solution's volume was raised to 2.5ml. The reading at 510nm was taken to determine the absorbance. The standard quercetin solution (0.25ml, 0.25-4.0mg/mL) was mixed with the above reagents to create the calibration curve. The total flavonoid content of *P. symmeria* was expressed as quercetin equivalent to mg/g of the dried extract.

2.6 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

LC-MS was utilized to identify bioactive secondary compounds in chloroform, ethanol, and aqueous extracts of *P. symmeria*. An orthogonal ESI source was employed in the analysis, which was carried out with an ACCUCORE high-performance liquid chromatography (HPLC) system linked to a triple quadrupole tandem mass spectrometer. Within the m/z range 150-2000, mass acquisition spectra were acquired. For both ES+ and ES-, the source temperature was adjusted to 120 ºC, while the desolvation temperature was set to 350 ºC. Mobile phase A, including water with 0.1% formic acid, and mobile phase B, containing acetonitrile with 0.1% formic acid eluents combination, was utilized in linear gradient flow. 5µl volume injection was used. In contrast, the flow rate remained constant at 0.3ml/min during

the entire gradient. OpenLynx™ and MassLynx™ Software were used for analyzing the data.

2.7 Assessment of Free Radical Scavenging Activity

2.7.1 DPPH Radical Scavenging Activity

DPPH radical scavenging activity was performed following the standard protocol (Leong & Shui, 2002). 500 µl of *P. symmeria* extracts (20-1000 µg/ml) were mixed with 0.1mM DPPH (1ml) in absolute methanol, and the mixture was incubated for 30 min in the dark at room temperature, then absorbance was measured at 515nm. Methanol was utilized as blank; all reagents except extract samples were taken for control, and the reading was used to calculate the percent inhibition. Ascorbic acid was used instead of the extract for positive control. The scavenging activities were represented as a percentage and computed applying the formula:

$$\% \text{ Scavenging} = (Ac\text{-}As)/Ac \times 100$$

Here, As represented test sample absorbance, and Ac represented control absorbance.

2.7.2 ABTS Radical Scavenging Activity

A standard procedure was employed for testing the ABTS radical scavenging activity (Re *et al*., 1999). ABTS solution (7mM) and potassium persulfate solution (2.45mM) of the same amount were mixed to prepare the stock solution. The solution containing ABTS+ radicals turned dark after being incubated for 12 hr at room temperature in the dark. A freshly prepared ABTS solution was diluted with 50% methanol before each test to acquire an initial absorbance of 0.700 (± 0.02) at 745nm. Then, the ABTS (1.5ml) working standard was mixed with various concentrations (20-1000µg/ml) of extract (150µl). Immediately after the experiment, the reading was taken at 745nm. A total of 50% methanol was utilized as blank, and for control, all reagents except the extracted sample were taken, and the reading was used to calculate the percent inhibition. Each experiment was repeated thrice. Ascorbic acid was used instead of the extract for positive control. The scavenging activities were represented as a percentage and then computed applying the formula:

$$\% \text{ Scavenging} = (Ac-As)/Ac \times 100$$

Here, Ac represented control absorbance, and As represented test sample absorbance.

2.7.3 Superoxide Radical ($O2^{\bullet(-)}$) Scavenging Activity

Superoxide radical ($O2^{\bullet(-)}$) scavenging activity was done following the standard method (Hyland *et al*., 1983). NBT (1mg/ml in DMSO) (0.2ml) with different concentrations (20-1000µg/ml) (0.6ml) of extract were mixed. Alkaline DMSO (2ml) was prepared by mixing DMSO (1ml) in 5mM NaOH and was added to the reaction mixture to prepare a final volume of 2.8ml. At 560nm, absorbance was measured. Blank consists of pure DMSO; all reagents except the extract sample were taken for control, and the reading was used to calculate the percent inhibition. Ascorbic acid was used instead of the extract for positive control. The scavenging activities were represented as a percentage and then computed applying the formula:

$$\% \text{ Scavenging} = (As-Ac)/As \times 100$$

Here, Ac represented control absorbance, and As represented test sample absorbance.

2.8 Statistical Analysis

One-way ANOVA and Tukey multiple comparisons of means were used for the statistical analysis, which was performed employing SPSS software (Version 27). Values were expressed as mean ± SEM. GraphPad Prism software (Version 6) was used to plot the percent inhibition of free radicals versus the log dosages to determine the IC_{50} values. $p<0.05$ was considered statistically significant.

3. RESULTS

3.1 Percentage (%) Yield of the Extract

The percentage yield of the extract in different solvents (Chloroform, Ethanol and Aqueous) is shown in Table 1.1. Among the three solvents, ethanol has the highest extract yield, followed by aqueous and chloroform.

Table 1.1: Percentage (%) yield of various extracts of *P. symmeria*

Percentage (%) yield			
Plant name	**Chloroform**	**Ethanol**	**Aqueous**
Pilea symmeria	1.67 ± 0.33	6.67 ± 0.88	4.0 ± 0.58

3.2 Phytochemical Analysis

Preliminary phytochemical analysis revealed the presence of several types of phytochemicals in the ethanol, chloroform and aqueous extracts of *P. symmeria* as shown in Table 1.2.

Table 1.2: Phytochemical analysis of the leaf extracts of *P. symmeria*. The symbols (+) and (-) denote the presence and absence of phytochemicals, respectively. PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract.

Phytochemicals	Color indication	PSEE	PSCE	PSAE
Alkaloids	Reddish brown precipitate	+	_	_
Tannins	Brownish green or blue-black color	+	_	_
Flavonoids	Colorless	+	+	+
Terpenoids	Reddish brown	+	+	+
Saponins	Whitish emulsion	+	+	+
Steroids	Red color	_	+	+
Cardiac glycosides	Brown ring	+	+	_
Phlobatannins	Red precipitate	_	_	_

3.3 Total Phenolic Content

A concentration dependent increase in phenolic content was observed in all the extracts (Figure 1.3). Ethanol extract exhibits the highest phenolic content (750.89 ± 11.20 mg gallic acid equivalent/g dry extract), followed by aqueous extract (576.08 ± 4.89 mg gallic acid equivalent/ g dry extract) and chloroform extract (200.36 ± 2.85 mg gallic acid equivalent/g dry extract).

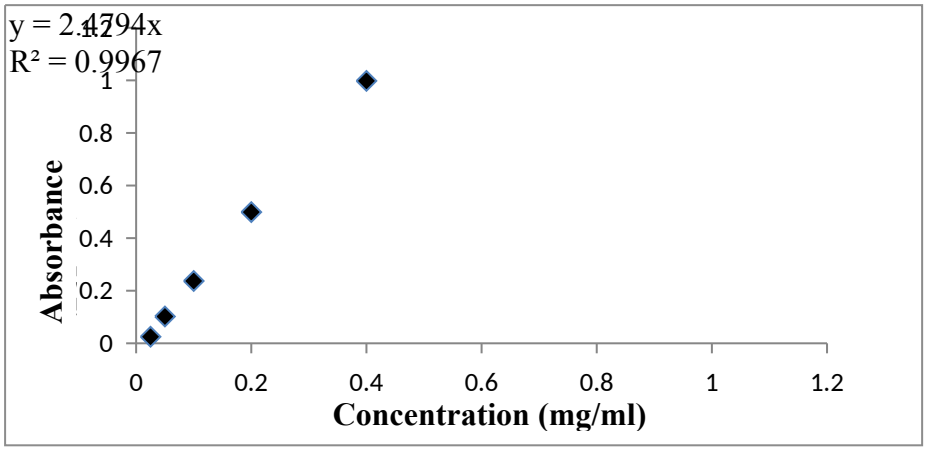

Figure 1.2: Standard graph of gallic acid.

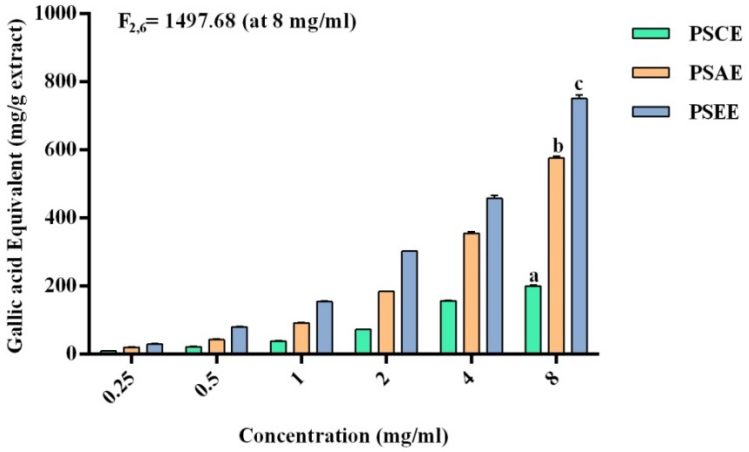

Figure 1.3: Total Phenolic Content of *P. symmeria* extracts; PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract; Values are expressed as Mean ± SEM. Different letters indicate significant variation at 8 mg/ml.

3.4 Total Flavonoid Content

The flavonoid content in ethanol, aqueous, and chloroform extracts increases significantly with an increase in concentration (Figure 1.5). The ethanol extract has a higher concentration of total flavonoid content (509.20 ± 5.23 mg quercetin equivalent/g dry extract) compared to that of aqueous extract (395.10 ± 5.01 mg quercetin equivalent/g dry extract) and chloroform extract (327.38 ± 3.57 mg quercetin equivalent/g dry extract).

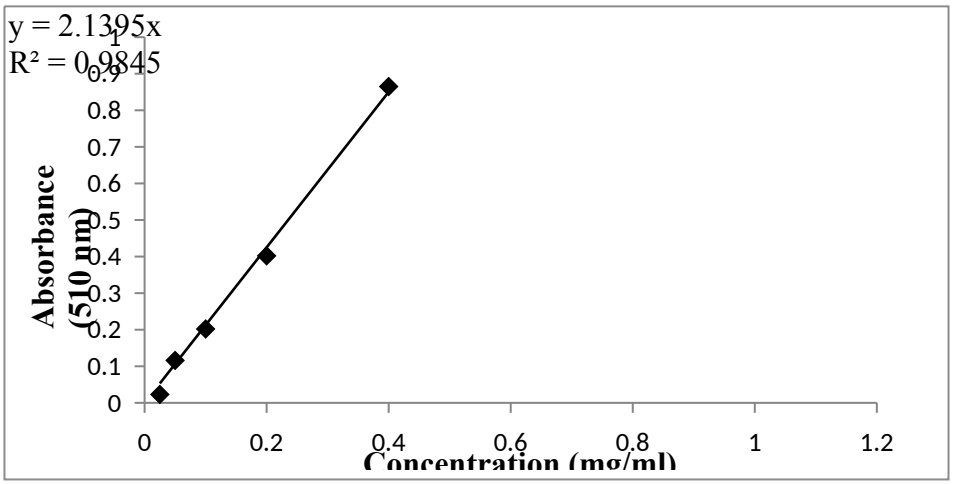

Figure 1.4: Standard graph of quercetin.

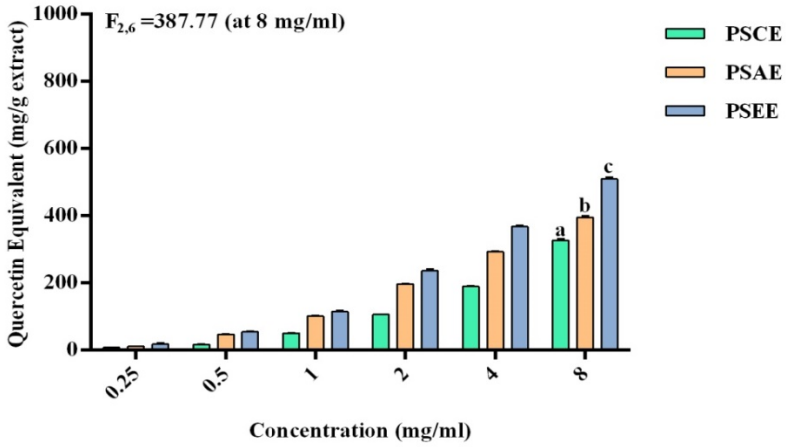

Figure 1.5: Total Flavonoid Content of *P. symmeria* extracts; PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract; Values are expressed as Mean ± SEM. Different letters indicate significant variation at 8 mg/ml.

3.5 Liquid Chromatography-Mass Spectrometry *(*LC-MS*) Analysis*

The various extracts of *P. symmeria* were analyzed using LC-MS. The results showed the presence of 34 major phytochemicals having various biological activities. The Peak area, retention time, molecular formula, molecular weight and biological activities corresponding to each compound are given in the Table 1.3-1.5.

Table 1.3: Compounds identified in PSEE by LC-MS.

Compound	Peak area	Retention time	Molecular formula	Molecular weight (Da)	Biological activity
(-)-Vestitone	1.63E+07	22.2899666	$C_{16}H_{14}O_5$	286.28	Antimicrobial activity (Guo *et al*., 1994)
Apigenin	4950586	23.9276009	$C_{15}H_{10}O_5$	270.24	Anti-inflammatory, antioxidant, antibacterial, and wound healing activities (Yan *et al*., 2017; Suntar *et al*., 2013; Xu *et al*., 2022)
Barbinine	468326.8	35.1232491	$C_{36}H_{46}N_2O_{10}$	666.8	Antimicrobial, antitumor and anti-inflammatory activities (Thawabteh *et al*., 2021)
Biochanin A	1194671	20.2746162	$C_{16}H_{12}O_5$	284.26	Anti-inflammatory, anticancer and antidiabetic activities (Yu *et al*., 2019; Sarfraz *et al*., 2021)
Biotin	1025798	28.1809158	$C_{10}H_{16}N_2O_3S$	244.31	Wound healing activity (Al-Akayleh *et al*., 2022)
Calafatimine	1.55E+07	34.4692841	$C_{38}H_{40}N_2O_7$	636.7	Antimicrobial and antiparasitic activities (Qing *et al*., 2017; Weber & Opatz, 2018)

Cassaine	16704869	32.8654671	$C_{24}H_{39}NO_4$	405.6	Anti-inflammatory and antiangiogenic activities (Zhong *et al*., 2022; Chen *et al*., 2022)
Cernuine	326512.3	31.5576496	$C_{16}H_{26}N_2O$	262.39	Anti-inflammatory, antibacterial and anticancer activities (Zhang *et al*., 2022)
Dihomo-gamma-linolenate	178202.8	34.314251	$C_{20}H_{34}O_2$	306.5	Anti-inflammatory activity (Silva *et al*., 2018)
Isobrucein A	3223732	21.9123001	$C_{26}H_{34}O_{11}$	522.5	Antincancer activity (Bagheri *et al*., 2018)
Ketoprofen	3552923	19.9645672	$C_{16}H_{14}O_3$	254.28	Anti-inflammatory activity (Yu *et al*., 2021)
Metocurine	201488.9	34.1930504	$C_{40}H_{48}N_2O_6^{+2}$	652.8	Unknown
Pheophorbide a	1227189.615	37.2061653	$C_{35}H_{36}N_4O_5$	592.7	Anti-inflammatory activity (Islam *et al*., 2013)
Pimelea factor P2	117718.8	30.5062676	$C_{37}H_{50}O_9$	638.8	Anti-inflammatory, antitumor and antibacterial activities (Saha *et al*., 2022)
Rescinnamine	11342853.9	36.5860825	$C_{35}H_{42}N_2O_9$	634.7	Antihypotensive activity (Cronheim *et al*., 1954)
Reserpine	1081749.525	31.5238495	$C_{33}H_{40}N_2O_9$	608.7	For the treatment of hypertension, cardiovascular disease and nervous disorder (Weiss & Fintelmann, 2000)
Rhodoxanthn	98000.48	37.6036148	$C_{40}H_{50}O_2$	562.8	Antioxidant activity (Fu *et al*.,

					2022)
Salidroside	5232889.85	26.9745503	$C_{14}H_{20}O_7$	300.3	Wound healing activity (Ariyanti *et al*., 2019)
Secologanate	129908.1	17.4841995	$C_{16}H_{22}O_{10}$	374.34	Anti-inflammatory (Li *et al*., 2019)
Soyasapogenol B 3-O-D-glucuronide	308863.5121	36.6396675	$C_{36}H_{58}O_9$	634.8	Anti-inflammatory, antimutagenic, anticarcinogenic, antimicrobial, hepato- and cardiovascular-protective activities (Guang *et al*., 2014)
Squalene	212612.7	32.5554161	$C_{30}H_{50}$	410.7	Anti-inflammatory and wound healing activities (Shanmugarajan *et al*., 2020)
taxifolin	86618.78	27.7158508	$C_{15}H_{12}O_7$	304.25	Antioxidant, anticancer, anti-inflammatory, antimicrobial, antitumor activities and for the treatment of cardiovascular and liver disorders (Park *et al*., 2023; Sunil & Xu, 2019)
Tingenone	163893.4	30.9713841	$C_{28}H_{36}O_3$	420.6	Analgesic and anti-inflammatory activities (Veloso *et al*., 2017)
Zeaxanthin	441835.9	33.4179344	$C_{40}H_{56}O_2$	568.9	Antioxidant activity (Murillo

					et al., 2019)

Table 1.4: Compounds identified in PSCE by LC-MS.

Compound	Peak area	Retention time	Molecular formula	Molecular weight (Da)	Biological activity
(-)-Vestitone	4.18E+07	22.2899666	$C_{16}H_{14}O_5$	286.28	Antimicrobial activity (Guo *et al*., 1994)
Apigenin	604509.5	23.9276009	$C_{15}H_{10}O_5$	270.24	Anti-inflammatory, antioxidant, antibacterial, and wound healing activities (Yan *et al*., 2017; Suntar *et al*., 2013; Xu *et al*., 2022
Biochanin A	1482551	20.2746162	$C_{36}H_{46}N_2O_{10}$	284.26	Anti-inflammatory, anticancer and antidiabetic activities (Yu *et al*., 2019; Sarfraz *et al*., 2021)
Cassaine	13366663	32.8654671	$C_{24}H_{39}NO_4$	405.6	Anti-inflammatory and antiangiogenic activities (Zhong *et al*., 2022; Chen *et al*., 2022
Epicatechin-(4beta->8)-ent-epicatechin	365248.1	23.9955158	$C_{30}H_{26}O_{12}$	578.5	Anti-inflammatory, antioxidant, free radical scavenging and wound healing activities (Bekoe *et al*., 2015)
Gambiriin C	125185.5	27.2848167	$C_{30}H_{26}O_{11}$	562.5	Anticancer, antidiabetic, anti-inflammatory, wound healing activities and for the treatment of cardiovascular diseases, neurodegeneration, allergies, and geriatric disorders (Nie & Sturzenbaum, 2018; Vilkickyte *et al*., 2022)
Isobrucein A	3211901	21.9123001	$C_{26}H_{34}O_{11}$	522.5	Antincancer activity (Bagheri *et al*., 2018)
Ketoprofen	3859513	19.9645672	$C_{16}H_{14}O_3$	254.28	Anti-inflammatory activity (Yu *et al*., 2021)
Pheophorbide a	318584	37.2061653	$C_{35}H_{36}N_4O_5$	592.7	Anti-inflammatory activity (Islam *et al*., 2013)
Salidroside	245591.3	26.9745503	$C_{14}H_{20}O_7$	300.3	Wound healing activity (Ariyanti *et al*., 2019)

Secologanate	228088.2	17.4841995	$C_{16}H_{22}O_{10}$	374.34	Anti-inflammatory (Li *et al*., 2019)
Tingenone	107825.1	30.9713841	$C_{28}H_{36}O_3$	420.6	Analgesic and anti-inflammatory activities (Veloso *et al*., 2017)

Table 1.5: Compounds identified in PSAE by LC-MS.

Compound	Peak area	Retention time	Molecular formula	Molecular weight (Da)	Biological activity
Biliverdin	65326.89	26.9065495	$C_{33}H_{34}N_4O_6$	582.6	Antioxidant activity and for the treatment of polymicrobial sepsis (Overhaus *et al*., 2006; Chowdhury *et al*., 2006)
Cassaine	22635809	32.8654671	$C_{24}H_{39}NO_4$	405.6	Anti-inflammatory and antiangiogenic activities (Zhong *et al*., 2022; Chen *et al*., 2022)
Cetylpyridinium chloride	297768.1	32.4001656	$C_{21}H_{38}CIN$	340	Antimicrobial and wound healing activities (Rembe *et al*., 2022)
Evocarpine	124371.9	20.4293995	$C_{23}H_{33}NO$	339.5	Antibacterial activity (Casciaro *et al*., 2020)
Glycerol	439316.7	27.0277672	$C_3H_8O_3$	92.09	Antibacterial and wound healing activities (Stout & McKessor, 2012)
Isobrucein A	3375756	21.9123001	$C_{26}H_{34}O_{11}$	522.5	Antincancer activity (Bagheri *et al*., 2018)
N-Arachidonyldopamine	94521.7	24.6150169	$C_{28}H_{41}NO_3$	439.6	Anti-inflammatory activity (Lawton *et al*., 2017)
Polhovolide	1023803	22.5321007	$C_{23}H_{32}O_8$	436.5	Anti-inflammatory, anticancer, antimicrobial, antioxidant activities and for the treatment of cardiovascular disease (Chadwick *et al*., 2013)
Reserpine	127492.5	31.5238495	$C_{33}H_{40}N_2O_9$	608.7	Hypertension, cardiovascular disease

					and nervous disorder (Weiss & Fintelmann, 2000)
Salidroside	4110341	26.9745503	$C_{14}H_{20}O_7$	300.3	Wound healing activity (Ariyanti *et al*., 2019)
Tingenone	216750.9	30.9713841	$C_{28}H_{36}O_3$	420.6	Analgesic and anti-inflammatory activities (Veloso *et al*., 2017)

3.6 Free radical scavenging activity

3.6.1 DPPH Radical Scavenging Activity

The extracts of *P. symmeria* exhibit significant dose dependent inhibition of DPPH (Figure 1.6), with ethanol (194.86 ± 3.87 µg/ml) being the most effective due to its lower IC_{50} value, followed by aqueous (266.66 ± 1.97 µg/ml) and chloroform (377.06 ± 3.09 µg/ml) extracts. The IC_{50} for the positive control, ascorbic acid, was 33.85 ± 0.34 µg/ml (Figure 1.7). The maximum scavenging activity for ethanol, aqueous, and chloroform extracts was 400 µg/ml, 500 µg/ml, and 800 µg/ml, respectively.

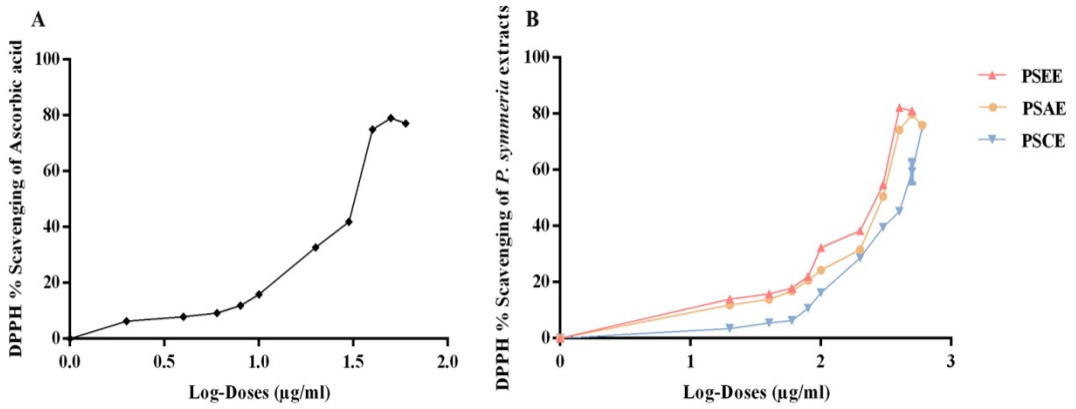

Figure 1.6: DPPH scavenging activity of A) Standard Ascorbic acid and B) *P. symmeria* extracts. Values were expressed as mean ± SEM, $p<0.001$. AA=Ascorbic Acid, PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract.

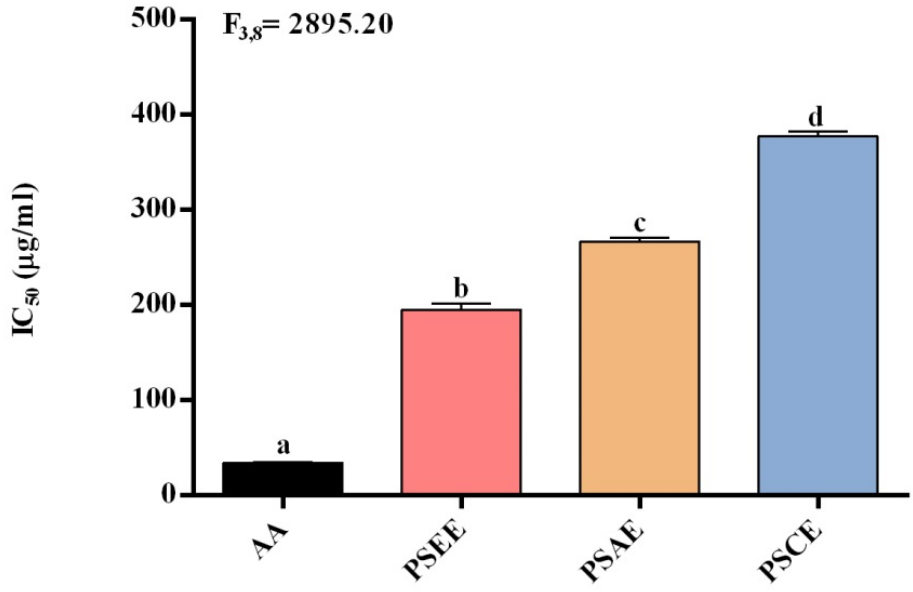

Figure 1.7: IC_{50} (µg/ml) for DPPH. Values were expressed as mean ± SEM, $p<0.001$. AA=Ascorbic Acid, PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract.

3.6.2 ABTS Radical Scavenging Activity

The ABTS radical scavenging activity also increases with increasing dosage. At concentrations of 400 µg/ml for ethanol and aqueous extracts and 700 µg/ml for chloroform extract, the highest scavenging activity was observed. The scavenging activities gradually declined after that (Figure 1.8). The ethanol extract has the lowest IC_{50} value (105.50 ± 4.59 µg/ml), followed by the aqueous extract (179.60 ± 5.90 µg/ml) and chloroform extract (398.73 ± 5.17 µg/ml). The IC_{50} for the positive control, ascorbic acid, is 15.71 ± 2.64 µg/ml (Figure 1.9).

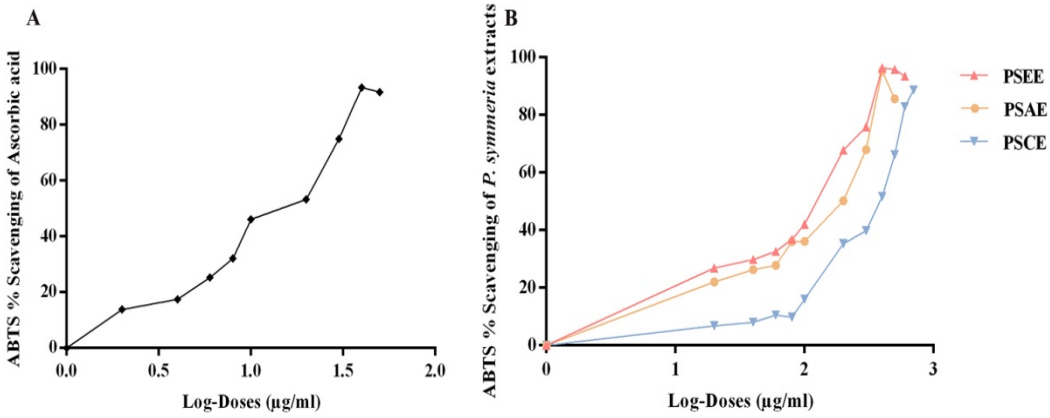

Figure 1.8: ABTS scavenging activity of A) Standard Ascorbic acid and B) *P. symmeria* extracts. Values are expressed as mean ± SEM, $p<0.001$. AA=Ascorbic Acid, PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract.

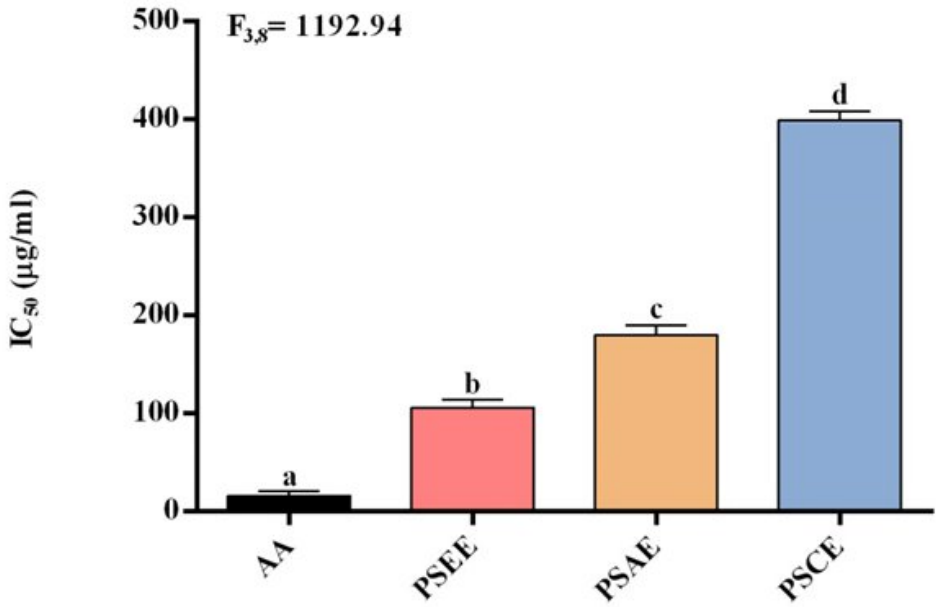

Figure 1.9: IC_{50} (µg/ml) for ABTS. Values were expressed as mean ± SEM, $p<0.001$. AA=Ascorbic Acid, PSEE= *P. symmeria* ethanol extract, PSCE= *P. symmeria* chloroform extract, PSAE= *P. symmeria* aqueous extract.

3.6.3 Superoxide ($O_2^{\bullet(-)}$) Radical Scavenging Activity

Superoxide (O2•(-)) Radical scavenging activity also increases in a concentration dependent manner (Figure 1.10). The maximum scavenging activity was observed for chloroform extract at 900 µg/ml, followed by aqueous extract and ethanol extract at 400 µg/ml. The IC_{50} of the positive control, ascorbic acid, was recorded at 19.65 ± 0.22 µg/ml. Among the three extracts, ethanol extract (182.83 ± 6.08 µg/ml) proved to be the most potent as it shows the lowest IC_{50} value, followed by aqueous extract (274.70 ± 5.40 µg/ml). Chloroform extract (389.73 ± 2.12 µg/ml) is the least effective, with an IC_{50} value at a higher dose (Figure 1.11).

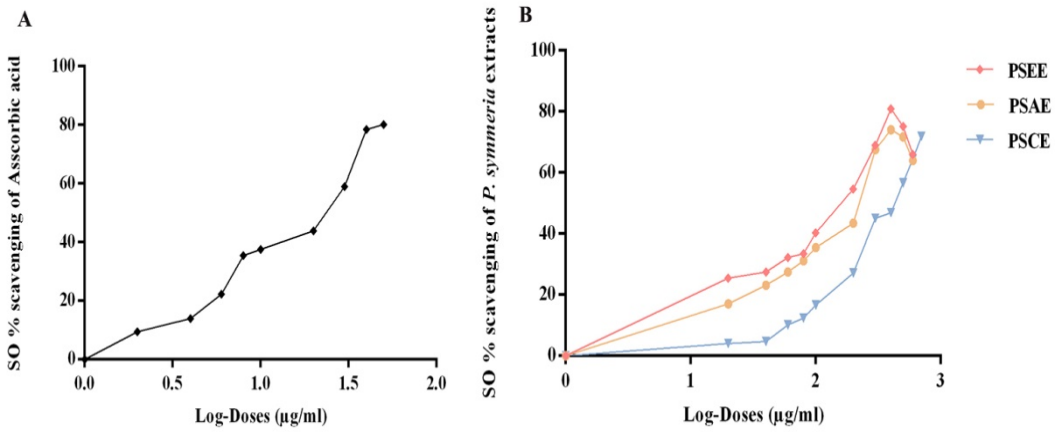

Figure 1.10: SO scavenging activity of A) Standard ascorbic acid and B) *P. symmeria* extracts. Values are expressed as mean ± SEM, $p<0.001$. AA= Ascorbic Acid, PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract.

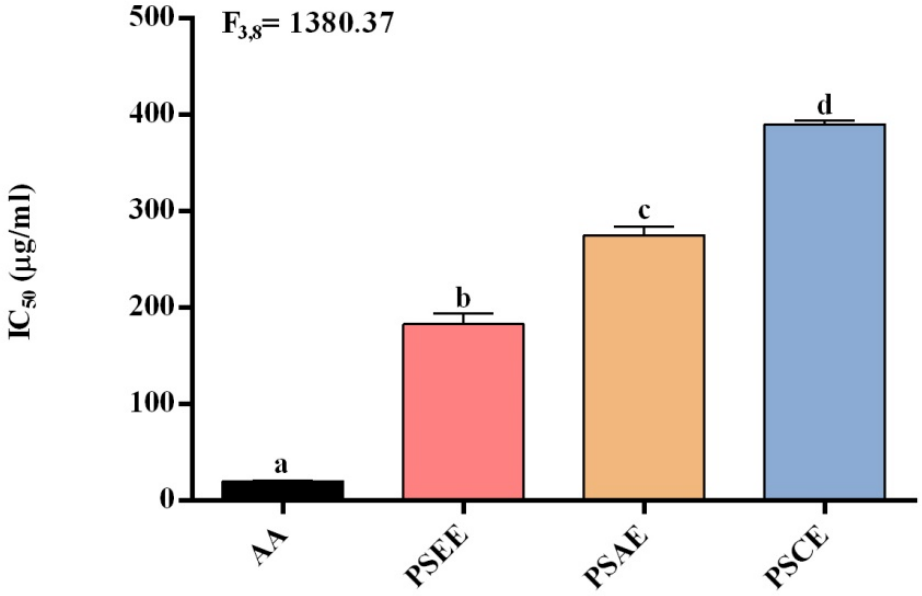

Figure 1.11: IC_{50} (µg/ml) for SO. Values were expressed as mean ± SEM, $p<0.001$. AA=Ascorbic Acid. PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract.

4. DISCUSSION

Herbal medicines are increasingly gaining popularity as an alternative approach for treating a wide range of disorders. This surge in interest can be attributed to several factors, including their affordability, effectiveness, and relatively fewer side effects compared to modern pharmaceutical drugs (Aghel *et al*., 2007). One of the primary reasons behind the therapeutic potential of herbal medicines is the presence of bioactive compounds, particularly polyphenols, which are derived from various plant sources (Nasim *et al*., 2022). These polyphenols are recognized for their significant role in the healing properties of medicinal plants (Pinto *et al*., 2021).

Studies conducted on *P. symmeria* extracts have revealed the presence of an array of phytochemicals that contribute to their medicinal value. Among these, phenols stand out as crucial plant components due to their ability to neutralize free radicals. This free radical scavenging ability makes phenols essential in evaluating the antioxidant potential of plants (Tosun *et al*., 2009). Another major class of phytochemicals found in *P. symmeria* extracts is flavonoids, which belong to a category of plant secondary metabolites. Flavonoids are well known for their strong antioxidant and metal chelating properties, further enhancing their biological significance (Shen *et al*., 2022). The considerable presence of both phenols and flavonoids in *P. symmeria* extracts suggests their possible role in exhibiting potent antioxidant activities. Additionally, LC-MS analysis has identified a diverse range of compounds within *P. symmeria* extracts, which possess various pharmacological properties, including anti-inflammatory, antibacterial, and wound-healing activities. Bioactive compounds such as Apigenin, Biotin, Salidroside, Squalene, Epicatechin-(4beta->8)-ent-epicatechin and Glycerol, detected in the LC-MS analysis are known to support wound healing.

The antioxidant activity of *P. symmeria* extracts has been assessed through the DPPH radical scavenging assay, a widely used method for measuring free radical neutralization. The results demonstrated that the plant extract effectively scavenged free radicals at varying concentrations. This scavenging activity is visually noticeable, as the deep violet or purple DPPH solution undergoes a color change to

yellow or colorless after incubation with the plant extract. The color transformation occurs due to the donation of hydrogen atoms from antioxidants in the extract to the DPPH radical, leading to the reduction of its nitrogen-centered odd electron to a stable hydrazine form (Kedare & Singh, 2011). Further supporting this antioxidant potential, another study utilizing ethanol extracts of the roots of *P. symmeria* have consistently demonstrated DPPH radical scavenging activity (Subba & Basnet, 2014). These findings strongly indicate the capability of the plant to function as an effective free radical scavenger, reinforcing its potential therapeutic applications in combating oxidative stress related disorders. Another widely used method for evaluating antioxidant activity is the ABTS assay, which measures the ability of antioxidants to neutralize the ABTS free radical. The ABTS radical is generated from ABTS salt through its reaction with strong oxidizing agents such as potassium permanganate or potassium persulfate (Ilyasov *et al*., 2020). In this assay, the blue-green ABTS radical solution transitions to a colorless form upon interaction with an antioxidant, a change that can be quantified through spectrophotometric analysis at 734 nm (Dasgupta & Klein, 2014). *P. symmeria* extracts have been observed to effectively prevent the formation of ABTS free radicals, as evidenced by a rapid decrease in optical density due to the color shift from blue-green to colorless, confirming their strong antioxidant activity (Goldschmidt & Renn, 1922). Oxidative stress is further exacerbated by the generation of superoxide anion radicals ($O2^{\bullet(-)}$), which are byproducts of electron transport chain leakage (Nolfi-Donegan *et al*., 2020). These radicals can trigger a cascade of harmful cellular events, including apoptosis, aging, and senescence (Fujii *et al*., 2022). Moreover, superoxide radicals can react through the Haber-Weiss mechanism to generate more reactive free radicals, such as hydroxyl radicals ($OH^{\bullet}$), which further contribute to cellular damage and oxidative stress (Wong *et al*., 2017; Haber & Weiss, 1934). Therefore, inhibiting or neutralizing superoxide anion radicals can serve as a protective mechanism to reduce overall oxidative damage (Pisoschi & Pop, 2015). The current study has demonstrated the ability of all three *P. symmeria* extracts to scavenge superoxide radicals ($O2^{\bullet(-)}$), thereby suggesting their significant role in preventing oxidative stress related damage. Among the three extracts, the ethanol extract exhibited the lowest IC_{50} value, indicating its superior potency in scavenging various free radicals.

The collective evidence from this study supports the pharmacological potential of *Pilea symmeria*. Additionally, other species within the same genus have been reported to exhibit similar bioactivities (Modarresi Chahardehi *et al*., 2009; Chahardehi *et al*., 2010; Prabhakar *et al*., 2006; Bansal *et al*., 2011), further reinforcing its therapeutic relevance. The presence of a diverse range of phytochemicals in *P. symmeria*, particularly flavonoids and polyphenols, likely contributes to its strong free radical scavenging activity. These bioactive compounds are well known for their antioxidant properties, which enable them to neutralize reactive oxygen species and reduce oxidative stress, thereby enhancing its potential as a natural source of therapeutic agents.

Chapter 2

In vitro evaluation of the antibacterial properties of *P. symmeria*

Abstract

Various extracts of *Pilea symmeria* were evaluated for their antibacterial potential through *in vitro* studies. The antibacterial activity was determined using the disc diffusion method against three bacterial strains: *Escherichia coli (*ATCC strain 25922), *Bacillus subtilis* (ATCC strain 11447), and *Klebsiella pneumoniae* (ATCC strain 10031). The extracts were further analyzed for their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The findings revealed that the antibacterial activity of *P. symmeria* extracts was concentration dependent, with higher concentrations leading to greater inhibition of bacterial growth. Among the three tested extracts, both ethanol and chloroform extracts displayed similar antibacterial potency, effectively inhibiting the growth of the selected bacterial strains. However, the aqueous extract exhibited weaker antibacterial activity in comparison to the ethanol and chloroform extracts, suggesting that the choice of solvent plays a crucial role in extracting bioactive compounds with antibacterial properties. Therefore, this study reveals that *P. symmeria* possesses significant antibacterial properties and has the potential to combat wound infections and other bacterial infection related complications.

1. INTRODUCTION

When the skin barrier is damaged, microorganisms can contaminate the underlying tissue, leading to infection, slowed healing, and more tissue and organ loss (Uberoi *et al*., 2024). Infected wounds tend to heal more slowly and are more likely to leave scars (Robson, 1997). Bacteria often colonize these wounds, causing excessive inflammation and further delaying the healing process (Kalan *et al*., 2016). The interaction of the bacterial load, the local wound environment, and the host's resistance affects the incidence of wound infections (Hurlow & Bowler, 2022). The chance of getting chronic wounds might be significantly increased by age and health conditions (Falcone *et al*., 2021). For example, high blood sugar might negatively impact the host's innate immune response in diabetes patients, reducing their ability to fight against wound infections (Vaibhav *et al*., 2024). Chronicity may also develop from poorly treated wounds, which are defined by the insufficient and delayed clearance of bacteria or biofilm and necrotic tissue (Pilcher, 2016).

In wound infection, the microbial load, the kind of bacteria, and their phenotypic traits are also important factors (Caldwell, 2020). A microbial load of $\geq 10^{\wedge 6}$ viable cells per millilitre has been linked to an increased risk of infection (Bendy *et al*., 1964). The host's immune system efficiently controls inflammation in acute wounds, while microbial activity mostly governs this process in chronic wounds (Gurjala et al., 2011). The host's innate immune response is triggered during acute wound healing, which causes neutrophils to phagocytose invasive microorganisms. These pathogens are then eliminated by intracellular oxidative processes. Furthermore, neutrophil extracellular traps (NETs) released by neutrophils reduce the possibility of host cell injury by capturing and killing bacteria via a process called NETosis (Castanheira & Kubes, 2019). On the other hand, biofilm development is thought to be a major cause of infection in chronic wounds (Costerton *et al*., 1999). Biofilm is made up of a group of microorganisms, such as bacteria and fungus, and cellular debris that stick to the surface of the wound and are covered in a matrix that is mostly made of polysaccharides (Donlan, 2002). The ineffectiveness of antibiotics and antiseptics in treating wound infection is due to the fact that microorganisms in

biofilm form are more resistant to host defenses, systemic antibiotics, and topical antimicrobial agents (Zhao *et al*., 2023). The persistent biofilm causes chronic inflammation, which in turn causes an excess of NETs to be produced, further damaging the tissue (Castanheira & Kubes, 2019). Notwithstanding their inability to efficiently phagocytose the bacteria, neutrophils that are drawn to biofilms continue to generate enzymes and reactive oxygen species that harm the host tissues around them (Zhu *et al*., 2021). Biofilms also prevent granulation tissue development and wound re-epithelialization (Gurjala *et al*., 2011). Rather than eliminating biofilm, the host immune response has been shown to exacerbate damage to surrounding tissues by causing persistent inflammation and oxidative stress, elevation of pro-inflammatory cytokines, fibroblast senescence, and a lack of vital growth factors required for efficient wound healing (Moser *et al*., 2017; Wolcott *et al*., 2008; Kalan *et al*., 2016).

Among the most common bacteria that colonize chronic wounds are *Escherichia coli*, *Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis and Acinetobacter baumanii* (Di Domenico *et al*., 2017; Ding *et al*., 2022). The prevalence of bacterial resistance has increased since the introduction of antibiotics into medicine and is still rising (Brisson-Noel *et al*., 1989; Oteo *et al*., 2005). Self medication, antibiotic abuse, and the spread of bacterial strains are the main causes of this resistance (Dougnon *et al*., 2015). *Pilea microphylla, Colebrookea oppositifolia, Mussaenda macrophylla, Thysanolaena maxima, Mallotus philippensis, Celosia argentea, Pogostemon cablin, Flueggea virosa*, and *Argemone mexicana* are a few of the medicinal plants whose antibacterial qualities have been documented by researchers worldwide.

Since medicinal plants serve as a valuable source of natural antibiotics, researching and analyzing their antimicrobial properties is crucial for combating chronic wound infections (Budovsky *et al*., 2015). Therefore, this chapter examine *P. symmeria's* antibacterial qualities *in vitro* against three bacterial strains: *Klebsiella pneumoniae*, *Bacillus subtilis*, and *Escherichia coli*.

2. METHODS

2.1 Collection and Preparation of Bacterial Suspension

*Escherichia coli (*ATCC strain 25922) and *Bacillus subtilis* (ATCC strain 11447) were obtained from the Department of Botany, Mizoram University, Tanhril, Aizawl, and *Klebsiella pneumoniae* (ATCC strain 10031) was obtained from Pachhunga University College, Aizawl. The bacterial isolates were cultured on nutrient agar. Before the antibacterial testing, several colonies of the bacteria were scooped out from the nutrient agar and mixed with physiological saline (0.9%w/v NaCl) to create a diluted solution. Turbidity was then adjusted to a concentration of 0.5 McFarland standard.

2.2 *In Vitro* Assessment of the Antimicrobial Activity of P. symmeria

The disc diffusion method was employed to study *P. symmeria* antibacterial activity *in vitro* (Balouiri *et al*., 2015). Sterile paper discs (6mm diameter) impregnated with extracted samples at the desired concentration was placed on the surface of the agar medium inoculated with the target organisms. 5% DMSO was used for the negative control, while a standard antibiotic disc containing streptomycin (25µg) was used as a positive control. Plates were left for incubation at 37˚C for 24 hr. The diameter of the zone of inhibition was then measured.

2.3 Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined employing broth microdilution method with few minor modifications (CLSI, 2023; Datar and Datar, 2016). A twofold dilution of the extract was mixed with broth medium in a 96-well microtiter plate. The starting concentration is 5 mg/ml for chloroform and ethanol extracts and 20 mg/ml for aqueous extract. Each well is then inoculated with the microbial inoculum. Positive control well contained inoculated broth medium without the test sample, while negative control well contained non-inoculated DMSO supplemented media. Plates were incubated for 24 hr at 37˚C. After incubation, each well received 0.5% TTC dye (20µl) and was incubated again for 30 min at room temperature. The red color signifies bacterial growth, while the original color indicates inhibition of bacterial

growth by the extract. MIC was determined to be the extract's lowest concentration, displaying no observable growth.

2.4 Determination of Minimum Bactericidal Concentration (MBC)

MBC was determined following the standard method with a slight modification (CLSI, 1999). 20 µl of the sample was taken from each well with a concentration of MIC and above the MIC and spread onto nutrient agar fresh plates. The plates were then incubated for 24 hr at 37˚C. MBC was considered the plant extract's lowest dose that exhibited no bacterial growth on the agar plates.

2.5 Statistical Analysis

Statistical analysis was conducted using a one-way ANOVA followed by Tukey's multiple comparisons of means, using SPSS software (Version 27). Values were expressed as mean ± SEM. $p<0.05$ was considered statistically significant. GraphPad Prism software (Version 6) was used to plot the graph.

3. RESULTS

3.1: Antibacterial Susceptibility Tests

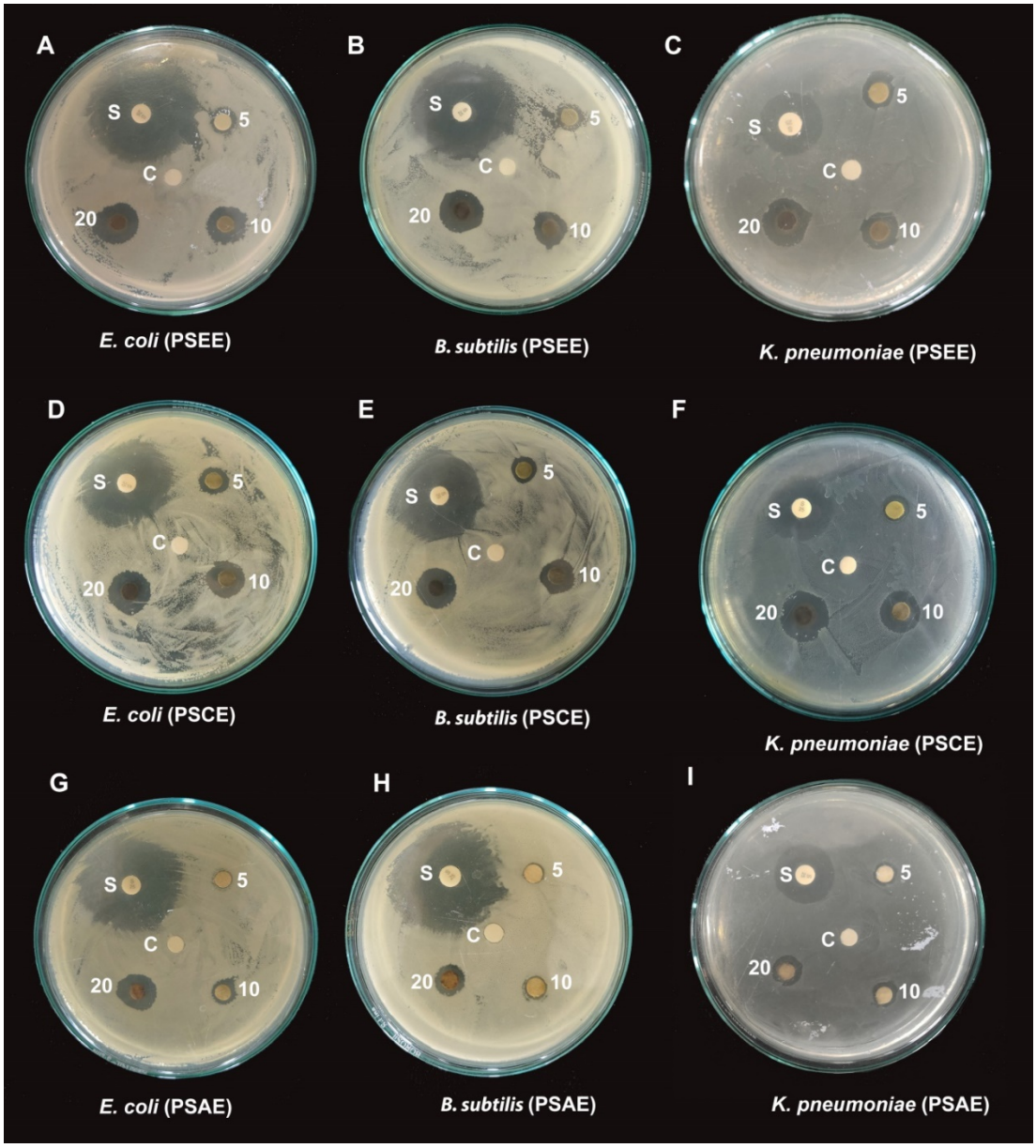

Figure 2.1: Zone of inhibition observed on nutrient agar plates indicating the antibacterial activity of *Pilea symmeria* extract against *Escherichia coli*, *Bacillus subtilis*, and *Klebsiella pneumoniae*.

Various concentrations of *P. symmeria* extracts (5, 10, and 20 mg/ml) were evaluated for their antibacterial properties against *E. coli* (ATCC-25922), *B. subtilis* (ATCC-11447), and *K. pneumoniae* (ATCC-10031) (Figure 4.1). The results from this study demonstrate that *P. symmeria* extract significantly inhibits the growth of the tested bacterial strains, showcasing its potential as an antimicrobial agent. The diameter of the zone of inhibition are given in Table 2.1.

Table 2.1: Diameter of zone of inhibition of the leaf extract of *P. symmeria* against the tested organisms.

Extracts (10µg/disc)	**Conc. (mg/ml)**	**Zone of Inhibition (mm)**		
		E. coli	***B. subtilis***	***K. pneumoniae***
Ethanol	5	8.20 ± 0.34	9.33 ± 0.57	10.86 ± 0.50
	10	12.16 ± 0.28	11.20± 0.34	11.53 ± 0.92
	20	14.86 ± 1.50	14.20± 0.34	13.96± 0.05
Standard	25µg	31.23 ± 0.40	31.06 ± 0.11	21.26 ± 0.46
Chloroform	5	9.06 ± 0.11	9.76 ± 0.32	10.86 ± 0.90
	10	11.30 ± 0.51	11.43 ±0.75	11.43 ± 0.75
	20	14.40 ± 0.69	14.53 ± 0.92	14.13 ± 0.23
Standard	25µg	29.10 ± 0.17	30.13 ± 0.23	22.20 ± 0.34
Aqueous	5	7.43 ± 0.75	7.23 ± 0.40	7.83 ± 0.44
	10	9.16± 0.28	8.53 ± 0.92	9.13 ± 0.23
	20	12.53 ± 0.92	10.20 ± 0.34	10.13 ± 0.23
Streptomycin	25µg	30.13 ± 0.23	31.26 ± 0.46	21.26 ± 0.46

3.1.1 Against *E. coli*

The antimicrobial activity of *P. symmeria* extract against *E. coli* exhibited a dose dependent increase in the zone of inhibition. The zones ranged from 7.43 ± 0.75 mm at the lowest concentration (5 mg/ml) to 14.86 ± 1.50 mm at the highest concentration (20 mg/ml). Among the different solvent extracts, the aqueous extract showed comparatively lower activity, resulting in smaller zones of inhibition compared to the ethanol and chloroform extracts, which exhibited similar and more pronounced antibacterial effects (Figure 2.2).

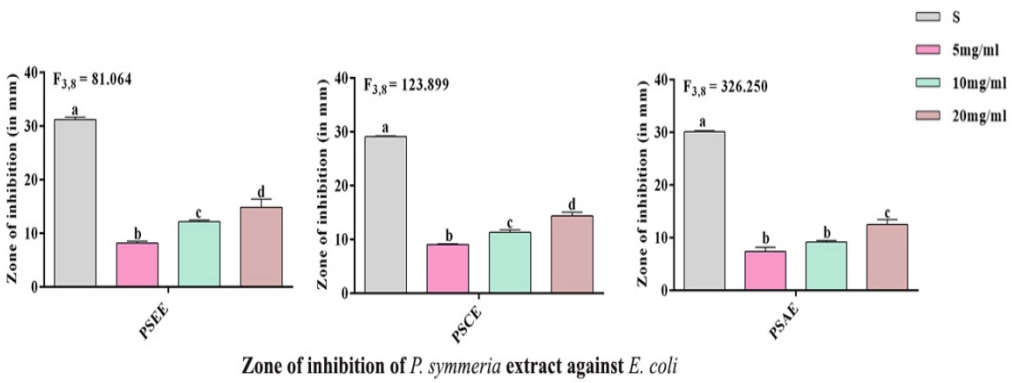

Figure 2.2: Diameter of zone of inhibition against *E. coli***.** PSEE= *P. symmeria* ethanol extract, PSCE= *P. symmeria* chloroform extract, PSAE= *P. symmeria* aqueous extract; Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.1.2 Against *B. subtilis*

A similar dose dependent trend was observed for *B. subtilis*, where the zone of inhibition increased with higher concentrations of *P. symmeria* extract. The zone of inhibition ranged from 7.23 ± 0.40 mm at the lowest concentration (5mg/ml) to 14.20 ± 0.34 mm at the highest concentration (20mg/ml). At the same concentration, the aqueous extract demonstrated a smaller zone of inhibition compared to the ethanol and chloroform extracts, which showed similar levels of antimicrobial activity (Figure 2.3).

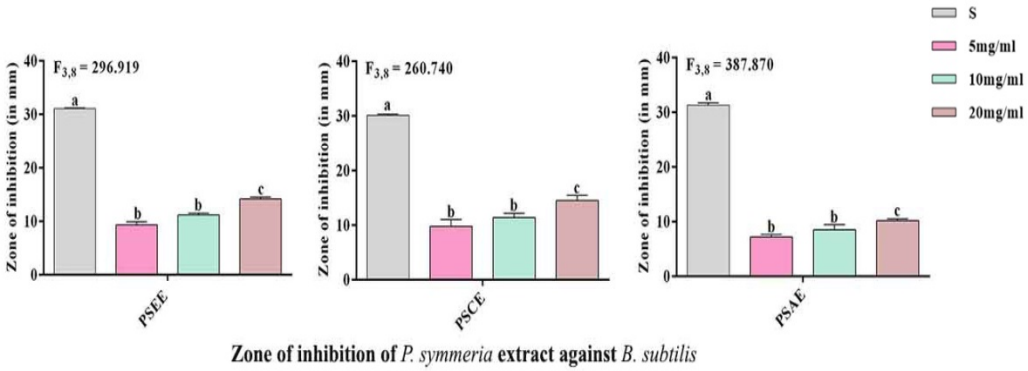

Figure 2.3: Diameter of zone of inhibition against *B. subtilis***.** PSEE= *P. symmeria* ethanol extract, PSCE= *P. symmeria* chloroform extract, PSAE= *P. symmeria* aqueous extract; Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.1.3 Against *K. pneumoniae*

The concentration of *P. symmeria* extract also had a direct effect on the inhibition of *K. pneumoniae*. As the concentration of the extract increased, the diameter of the zone of inhibition increased progressively, ranging from 7.83 ± 0.44 mm to 14.13 ± 0.23 mm. This indicate that the *P. symmeria* extracts effectively inhibit the growth of *K. pneumoniae*, with a stronger inhibitory effect at higher concentrations (Figure 2.4).

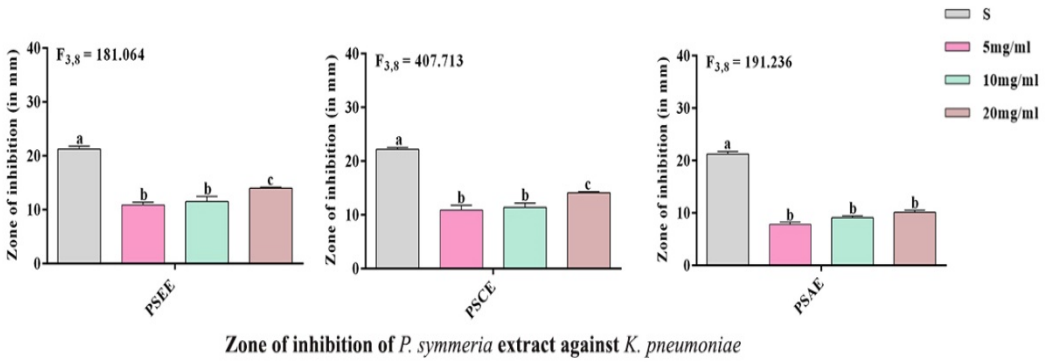

Figure 2.4: Diameter of zone of inhibition against *K. pneumoniae***.** PSEE= *P. symmeria* ethanol extract, PSCE= *P. symmeria* chloroform extract, PSAE= *P. symmeria* aqueous extract; Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.2 Minimum Inhibitory Concentration (MIC)

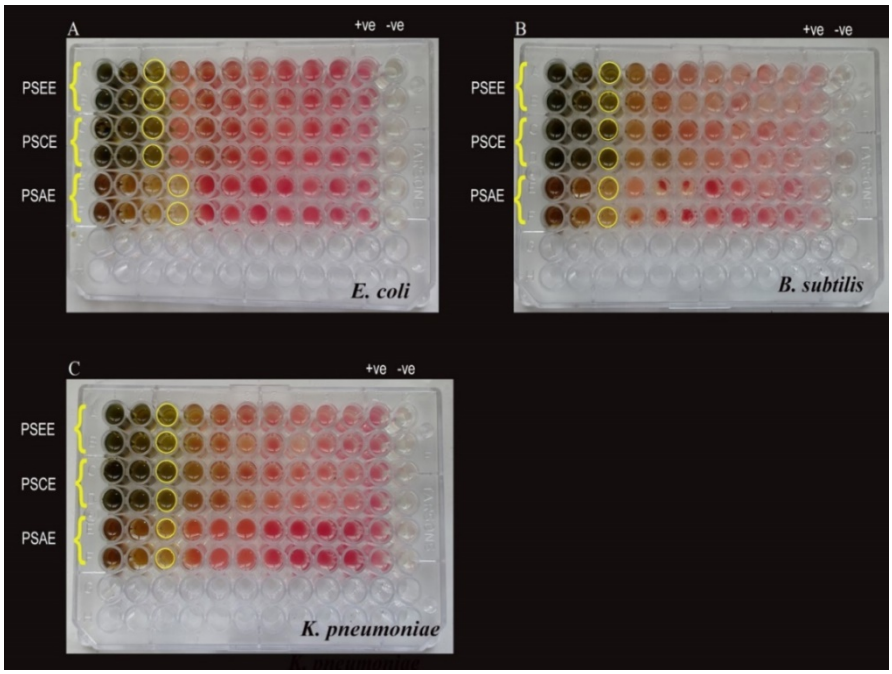

Figure 2.5: MIC of *P. symmeria* extracts against *E. coli* (A), *B. subtilis* (B) and *K. pneumoniae* (C) using Tetrazolium chloride as growth indicator. PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract. Yellow circle represents MIC.

Results of the minimum inhibitory concentration (MIC) showed that *P. symmeria* chloroform and ethanol extracts have MIC value of 1.25 mg/ml against *E. coli*, *B. subtilis,* and *K. pneumoniae*, while aqueous extract shows MIC value of 2.5 mg/ml against *E. coli* and 5 mg/ml against *K. pneumoniae* and *B. subtilis* (Table 2.2).

3.3 Minimum Bactericidal Concentration (MBC)

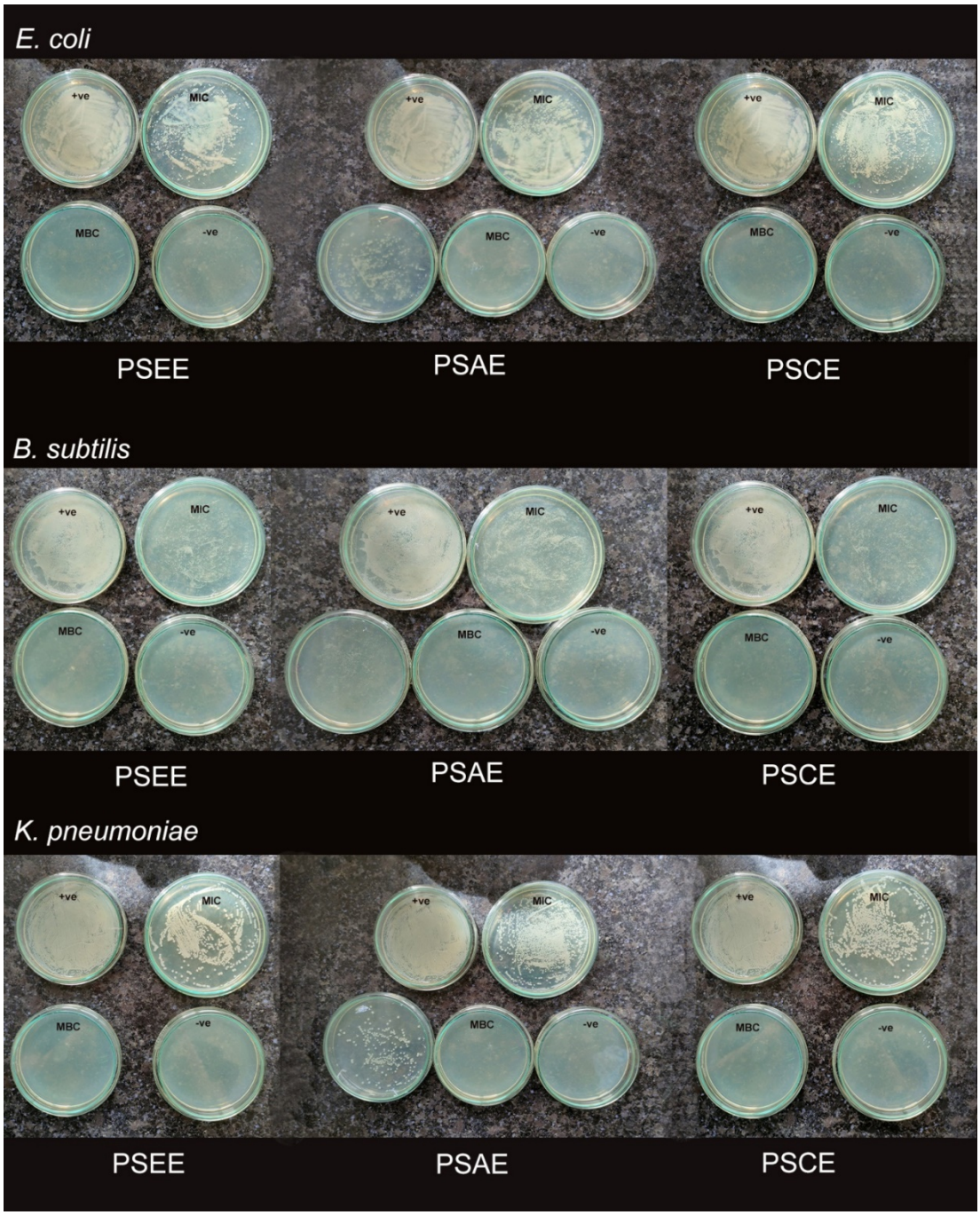

Figure 2.6: MBC of *P. symmeria* extracts against *E. coli, B. subtilis* and *K. pneumoniae.* PSEE= *P. symmeria* ethanol extract, PSAE= *P. symmeria* aqueous extract, PSCE= *P. symmeria* chloroform extract.

The growth of the tested bacterial strains sub-cultured from the well with concentrations of MIC and above the MIC on fresh nutrient agar was observed. The MBC was found to be 2.5 mg/ml for chloroform and ethanol extracts while aqueous extract, shows an MBC value of 10 mg/ml against *E. coli* and 20 mg/ml against *B. subtilis* and *K. pneumoniae* (Table 2.2).

Table 2.2: MIC and MBC of the leaf extract of *P. symmeria* against the tested ogranisms.

Extract	***E. coli***		***B. subtilis***		***K. pneumoniae***	
	MIC (mg/ml)	**MBC (mg/ml)**	**MIC (mg/ml)**	**MBC (mg/ml)**	**MIC (mg/ml)**	**MBC (mg/ml)**
Ethanol	1.25	2.5	1.25	2.5	1.25	2.5
Chloroform	1.25	2.5	1.25	2.5	1.25	2.5
Aqueous	2.5	10	5	20	5	20

4. DISCUSSION

Medicinal plants are rich sources of antimicrobial agents, making them valuable in fighting against infectious diseases (Ajaib *et al*., 2020). Plants have a variety of powerful defense mechanisms, such as the synthesis of secondary metabolites, to resist pests and pathogens before they can cause major damage (Chassagne *et al*., 2021). These secondary metabolites include alkaloids, flavonoids, tannins, saponins, terpenoids, and phenolic compounds, all of which contribute to the antimicrobial properties of medicinal plants (Anjali *et al*., 2023). These bioactive compounds function as natural antibiotics, targeting and inhibiting the growth of various pathogenic microorganisms, including bacteria, fungi, and viruses (Li *et al*., 2023).

Colonizing microorganisms are frequently introduced to the skin and underlying tissues during minor or major incidents that lead to breaks in skin surface integrity (Turner *et al*., 2014). Wounds, abrasions, burns, and cuts expose underlying tissue to a range of opportunistic and pathogenic microbes, which may result in infections (Sen, 2019). When microbial growth surpasses the host's immune defense mechanisms, it leads to inflammation, delayed healing, and, in severe cases, systemic infections (Jo, 2019). Skin abrasions and wounds can also be contaminated by microbes from external sources, such as water, soil, contaminated surfaces, and even from animal bites (Percival *et al*., 2011). The presence of these microbes can exacerbate wound healing complications and this requires immediate and effective treatment (Maheswary *et al*., 2021). Chronic wounds, such as diabetic ulcers, pressure sores, and venous leg ulcers, are particularly susceptible to microbial colonization and biofilm formation (Darwin & Tomic-Canic, 2018).

Infectious diseases remain a major global health concern, particularly with the rise of antibiotic resistant pathogens, which have significantly complicated treatment strategies (Nwobodo *et al*., 2022). Antibiotic abuse and misuse have resulted in the rise of multidrug resistant bacterial strains, worsening the problem (Mancuso *et al*., 2021). As a result, there is an imperative need to develop new antimicrobial compounds that can serve as an alternative or complementary therapeutic agent to combat microbial infections effectively (Andrea *et al*., 2019).

Medicinal plants have emerged as a promising source of such antimicrobial agents due to their diverse bioactive compounds, which exhibit broad spectrum activity against various pathogens (Anand *et al*., 2019). Given the global antibiotic resistance crisis and the decreasing effectiveness of existing treatments, the search for alternative antimicrobial agents is essential (Liu et al., 2020).

Infection in wounds can manifest in several ways, including delayed healing, increased exudate production, malodorous discharge, undermined wound borders, friable tissue, increased wound size, increased pain, and the presence of necrotic or sloughing tissue (Cutting & White, 2004). These symptoms indicate microbial overgrowth and require prompt intervention to prevent further complications.

A variety of laboratory methods can be used to evaluate or screen the *in vitro* antimicrobial activity of an extract or a pure compound. The most well known and basic methods include the disk-diffusion and broth or agar dilution methods (Balouri *et al*., 2015). The disk-diffusion assay, in particular, is widely used due to its simplicity, low cost, ability to test numerous microorganisms and antimicrobial agents simultaneously, and ease of result interpretation (Balouiri *et al*., 2015). This method provides valuable insight into the efficacy of plant derived antimicrobial compounds and their potential applications in clinical settings (Tenover, 2009).

This experiment demonstrated that *P. symmeria* extracts exhibit significant antibacterial properties. The various extracts of *P. symmeria* were shown to have a growth inhibitory effect against the tested bacterial strains, such as *E. coli*, *B. subtilis* and *K. pneumoniae*. The inhibitory effects observed in this study highlight the potential of *P. symmeria* as a potent antimicrobial agent with promising therapeutic applications. Another study also reported the antibacterial properties of the roots of *P. symmeria* ethanolic extract, further supporting its potential use in antimicrobial therapy (Subba & Basnet, 2014). Based on the results from current investigations and previous studies, *P. symmeria* is proven to be a potent antimicrobial agent with significant potential in wound management and infection control. The ability of *P. symmeria* extracts to inhibit bacterial growth suggests that they could serve as natural

alternatives to conventional antibiotics, particularly in the treatment of antibiotic resistant infections.

Although modern scientific research continues to validate the effectiveness of medicinal plants in treating microbial infection (Petrovska, 2012). The potency of the antimicrobial property can also be influenced by various factors, including plant species, extraction methods, solvent polarity, and the specific bioactive compounds present (Zouine *et al.,* 2024). Therefore, further research is needed to optimize the extraction and purification of antimicrobial compounds from medicinal plants to enhance their efficacy and ensure their safety for human use (Bitwell *et al*., 2023).

Chapter 3

Evaluation of the wound healing properties of *Pilea symmeria* using excision and incision wound models.

Abstract

The wound healing potential of various extracts from *Pilea symmeria* was evaluated using a mouse model to assess its therapeutic properties. In this experiment, mice were inflicted with both excision and incision wounds to simulate common types of injuries. The animals were divided into four distinct groups, each consisting of six animals. Group I, the negative control group, were treated with simple ointment base (SO), while group II, the positive control group, received 5% povidone iodine (PVI), which is widely recognized for its antiseptic and wound healing properties. Groups III and IV were treated with ointments containing 5% and 10% *Pilea symmeria* ethanol extract (PSEE), respectively, which were prepared using polyethylene glycol (PEG) as the base. The ointments were applied topically to the wounds till the day of epithelialization. The results demonstrated a significant ($p < 0.05$) increase in the rate of wound contraction in the group treated with *P. symmeria* extracts compared to the SO treated group. In addition, the time required for epithelialization, the process of skin regeneration and closure was notably shorter in the treatment groups, this was further confirmed by histopathological analysis. The breaking strength of the incision wounds was also significantly ($p < 0.05$) improved in the PVI 5%, PSEE 5%, and PSEE 10% treated groups when compared to the SO treated group. These findings suggest that *P. symmeria* extract facilitates wound healing by accelerating tissue regeneration and improving wound strength.

1. INTRODUCTION

The skin serves as a fundamental protective barrier, safeguarding the body against dehydration and the invasion of harmful microorganisms (Pereira *et al*., 2013). As the outermost layer of the body, it is constantly exposed to various environmental hazards, making it highly susceptible to different types of injuries. Among the most common reasons individuals seek medical attention are wounds and dermatological issues, which rank among the top five medical concerns worldwide (Ryan, 1992). When the skin sustains damage, it undergoes a highly regulated and systematic healing process, which consists of four distinct but overlapping stages: hemostasis, inflammation, proliferation, and remodeling (Schultz *et al*., 2011). These stages work collectively to restore the skin's normal structure and function (Bodeker *et al*., 1999). Wound healing is a highly intricate process involving a coordinated network of biochemical and cellular events (Wilkinson & Hardman, 2020). It requires the participation of extracellular matrix (ECM) components, platelets, inflammatory cells, cytokines, growth factors, and chemokines to ensure effective tissue repair (Xue & Jackson, 2013).

Wounds are broadly classified into two main categories: acute and chronic wounds, each differing in their pathophysiology and impact on the healing process (Chamgordani *et al*., 2023). Acute wounds typically follow an orderly and predictable healing trajectory, restoring the skin's structural and functional integrity within two to four weeks (Richardson, 2004). The initial response to tissue injury involves the activation of immune cells, primarily neutrophils and basophils, which play an essential role in initiating the repair mechanisms (Soliman & Barreda, 2022). Other immune cells, including T cells, B cells, macrophages, mast cells, and Langerhans cells, also contribute significantly to wound healing (Canedo-Dorantes & Canedo-Ayala, 2019). However, if the immune response becomes dysregulated, it can lead to the development of chronic wounds (Azevedo et al., 2020).

Chronic wounds, in contrast, are characterized by prolonged inflammation, persistent infections, and necrotic tissue formation, which significantly impair the healing process (Holzer-Geissler *et al*., 2022). Unlike acute wounds, which progress

through the healing phases in a timely manner, chronic wounds remain in a persistent inflammatory state, preventing proper tissue regeneration. In most cases, chronic wounds fail to heal within the expected timeframe, often taking more than four to six weeks to show significant recovery (Frykberg & Banks, 2015). This prolonged inflammatory response is associated with an imbalance in macrophage activity, proinflammatory macrophages are overly abundant, while there are low abundance of anti-inflammatory macrophages (Saleh *et al*., 2019; Krzyszczyk *et al*., 2018). Additionally, chronic wounds exhibit impaired clearance of apoptotic neutrophils, leading to an environment rich in inflammatory mediators such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Krzyszczyk et al., 2018). Macrophages in chronic wounds also secrete high levels of matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, which degrade the ECM and hinder the transition from the inflammatory to the proliferative phase of healing (Raziyeva *et al*., 2021).

In an effort to enhance wound healing and minimize complications associated with poor recovery, many traditional and natural remedies have been explored, often yielding promising results (Pereira *et al*., 2013). According to the World Health Organization (WHO), traditional medicine encompasses a comprehensive body of knowledge, skills, and practices that are rooted in indigenous cultural beliefs and experiences. These methods are employed to prevent, diagnose, and treat various health conditions, including skin and wound related ailments (Pereira & Bartolo, 2014). It is estimated that approximately 60–80% of individuals in developing countries rely on traditional medicine as their primary source of healthcare (Bodeker *et al*., 1999). Research into medicinal plants has demonstrated that herbal treatments are not only cost effective but often produce fewer adverse effects compared to synthetic pharmaceuticals (Kumar *et al*., 2007; Rhoads *et al*., 2012). Despite the increasing recognition of the therapeutic potential of traditional medicine in dermatology and wound care, there remains a considerable gap in research, clinical application, and policy support for these treatments (Karunamoorthi *et al*., 2012).

Scientific investigations into the efficacy of traditional medicinal plants in wound healing have been conducted through both *in vitro* and *in vivo* studies using human and animal models (Sami *et al*., 2018). The most frequently utilized models for

studying wound healing include humans, mice, rats, pigs, and rabbits (Parnell & Volk, 2019). Animal models have been widely employed to explore the mechanisms of skin disorders and evaluate potential treatment strategies (Zhang, 2017). *In vivo* models are particularly valuable for wound healing research, as they provide clinically relevant insights and serve as a crucial step before new treatments advance to human clinical trials (Stephens *et al*., 2013). While animal models share many similarities with human tissue regeneration mechanisms, it is important to acknowledge that no model can perfectly replicate human clinical conditions (Greenhalgh *et al*., 2005). Rodents, particularly mice and rats, are the most commonly used subjects in wound healing studies due to their availability, affordability, and small size, which facilitates efficient laboratory management (Dorsett-Martin & Wysocki, 2008; Martin *et al*., 2016). Two of the most widely employed wound models in scientific research are the excision and incision wound models.

Excisional wound models involve the removal of a portion of tissue, creating an open wound with a well defined outer border (Masson-Meyers *et al*., 2020). These wounds undergo an extensive proliferative phase characterized by granulation tissue formation, vascularization, and ECM remodeling. Eventually, reepithelialization occurs over the granulation tissue, leading to the closure of the full thickness wound (Rousselle *et al*., 2018). The incision model, on the other hand, is primarily used to assess the tensile strength of healed wounds over time. In this approach, a controlled incision is made through the skin and subsequently sutured or stapled. After a designated healing period, the wound breaking strength, measured by the force required to rupture the healed tissue is evaluated and compared between treated and control groups (Greenhalgh *et al*., 2005).

This chapter examines the impact of various *P. symmeria* extracts on wound healing using both excision and incision wound models in mice. After inducing the wounds, the animals are treated topically with an extract based ointment, and the healing progression is monitored. Several key parameters, including the rate of wound contraction, epithelialization time, and tensile strength of the repaired tissue,

are assessed to determine the efficacy of the extracts. Additionally, histological analysis of the excised wound tissues is conducted to further validate the results.

2. METHODS

2.1 Extract

Different extracts of *Pilea symmeria* were obtained through cold maceration, as described in Chapter I. Among them, *P. symmeria* ethanol extract, PSEE) exhibited the highest phenolic and flavonoid content and showed the strongest free radical scavenging activity. Therefore, it was selected for the formulation of the extract-based ointment for *in vivo* studies.

2.2 Ointment preparation

A simple ointment base was made using polyethylene glycol 400 (PEG-400) and polyethylene glycol 4000 (PEG- 4000) (20 g each) and melted in a water bath at 70-75 ºC. The mixture was stirred continuously until congealed; the extract was added and stirred at 40 ºC to make 5% and 10% extract ointment (Donkor *et al*., 2016).

2.3 Animals

Healthy Swiss albino mice of either sex, eight to ten weeks old, weighing 30-35 g, were obtained from North Eastern Hill University (NEHU). The animals were kept for acclimatization at Animal House, Department of Zoology, Mizoram University, for at least one week before the experiment. They were kept in a clean polypropylene cage with a temperature of 22 ± 3 °C and a 12 hr light-dark cycle; they were provided food and water *ad libitum*.

2.4 Animal Ethical Committee Approval

The study received ethical approval for animal experimentation under clearance number MZU/IAEC/2020/07.

2.5 Acute dermal toxicity

An acute dermal toxicity test was performed according to the OECD guideline no. 402. A total of five healthy female Swiss albino mice (30-35 g) were used for toxicity study. The animals were housed individually in a cage and acclimatized to the laboratory conditions for five days before the test. Before the experiment, the dorsal back of the mouse was shaved. A limit test dose of 2000 mg/kg of the 10% extract ointment was applied uniformly on the shaved area of two mice, no death or skin irritation was observed within 24hr, and then three additional mice were treated with the same dose of the extract ointment. At the end of the exposure, the test substance was removed and the animals were observed for the development of any skin irritations daily. Animals were observed with special attention given during the first 2 to 6 hr of exposure, and daily thereafter for 14 days. No deaths or signs of skin irritations were observed at the end of the study.

2.6 Grouping of animals

The animals were randomly divided into four groups of 6 animals each. Group I animals (negative control group) were treated with the vehicle of the simple ointment (SO). Group II animals (positive control group) were treated with povidone iodine (PVI) 5%. Group III and IV animals were treated with 5% and 10% (w/w) of *P. symmeria* ethanol extract ointment (PSEE), respectively.

2.7 Excision wound

Mice were inflicted with an excision wound following the standard method (Morton & Malone, 1972). Animals were anesthetized before and during the creation of the wounds, with intraperitoneal ketamine + xylazine (50 mg/kg). After anesthesia, the hair was removed by shaving the dorsal back of the mice. Ethanol (70%) was used as an antiseptic for the shaved region before making the wound. An excision wound was created by removing a full thickness of the skin 200 mm^2 circular area from a predetermined shaved area on the back of each animal. Each mouse was kept in a separate cage after the creation of the wound. All vehicles were applied once daily till the epithelization day. The wound closure rate was assessed by

tracing the wound on days 0, 4, 8, 12, 16, and 21 post-wounding using transparent paper and a permanent marker. The wound area recorded was used to calculate the percentage of wound contraction.

$$\% \text{ Wound closure} = (\text{Wound area on day } 0 - \text{Wound area on day } (n)) \times 100/\text{Wound area on day } 0$$

Where, n is the number of days.

2.8 The period of epithelialization measurement

The period of epithelialization was calculated as the days required for the dead tissue remnants to fall off without leaving any open wounds (Garg & Paliwal, 2011).

2.9 Incision wound

An incision wound was created following the standard method (Ehrlich & Hunt, 1968). Mice in each group were anaesthetized as mentioned earlier. An incision of about 3 cm was made on the paravertebral midline on the back of the mice. After the incision, the parted skin was stitched together with black thread at 0.5 cm intervals. The sutures were removed on day 8, and the tensile strength of the healed wound was measured on day 10 using a water flow system. The weight of water collected in the container was recorded and taken as a measure of the wound-breaking strength in grams.

2.10 Histological analysis

On day 16, the wound tissue was removed and fixed in 10% formalin. Histopathological examination was carried out following the standard procedure (Drury and Wallington, 1980). The prepared section was stained using hematoxylin and eosin (H&E), mounted with xylene, and carefully examined under a microscope for the evaluation of any histopathological changes.

2.11 Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey multiple comparison of means. Values were expressed as mean ± SEM. The analysis was carried out using SPSS (Version 27) and the graphs were plotted using GraphPad prism (Version 6). The value $p<0.05$ was considered to be statistically significant.

3. RESULTS

3.1 Excision wound

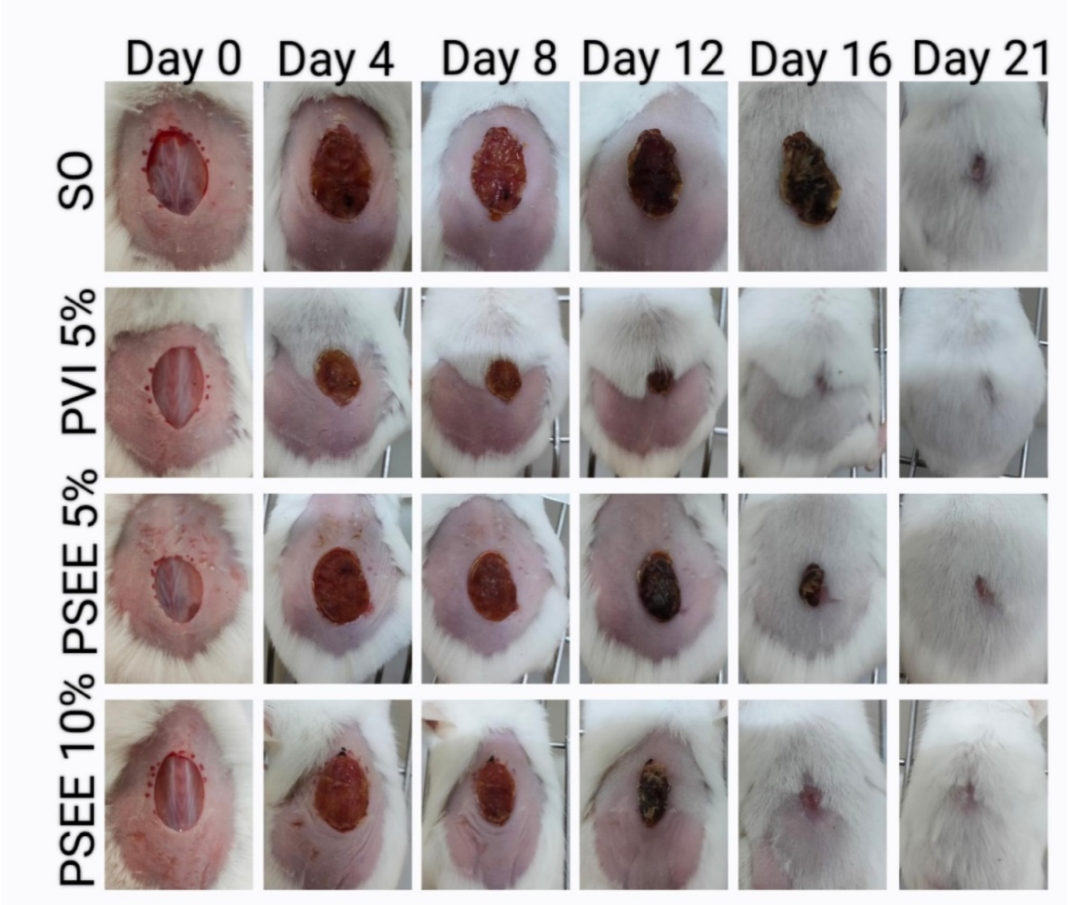

Figure 3.1: Photographic representation of the rate of wound contraction at different days of treatment with SO, PVI 5%, PSEE 5% and PSEE 10%. SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

Table 3.1: The average diameter of wound measured on post-wounding days. SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

Days	**Wound diameter in mm^2**			
	SO	**PVI 5%**	**PSEE 5%**	**PSEE 10%**
Day 0	195.83 ± 1.54	200.67 ± 0.42	197.50 ± 1.05	200.50 ± 1.40
Day 4	192.67 ± 1.76 (1.62%)	185.67 ± 0.84 (7.47%)	192.50 ± 1.18 (2.53%)	191.67 ± 1.28 (4.23%)
Day 8	178.17 ± 1.45 (9.00%)	154.33 ± 0.42 (23.08%)	164.33 ± 1.05 (16.78%)	164.00 ± 3.21 (18.22%)
Day 12	136.33 ± 0.67 (30.37%)	100.16 ± 1.97 (50.08%)	113.66 ± 3.89 (42.45%)	109.00 ± 3.08 (45.63%)
Day 16	89.00 ± 3.44 (54.48%)	1.50 ± 0.56 (99.25%)	4.83 ± 0.60 (97.63%)	2.33 ± 0.33 (98.84%)
Day 21	7.16 ± 0.70 (96.35%)	0.00 ± 0.00 (100%)	0.00 ± 0.00 (100%)	0.00 ± 0.00 (100%)

The data for wound contraction has been given in Table 3.1, and using the daiameter of the wound (mm^2), the percentage of wound contraction was calculated. The wound started to show significant reduction in the rate of wound contraction from day 4 in the PVI 5% and PSEE 10% treated groups. On day 16, PSEE 5%, PSEE 10% and the PVI 5% treated group showed 97.63%, 98.84%, and 99.25% of wound healing respectively, when compared with the SO treated group, which showed only 54.48% of wound healing. On day 21, PSEE 5%, PSEE 10% and the PVI 5% treated groups showed 100% of wound contraction, while the SO treated group showed 96.35% of wound contraction (Figure 3.2).

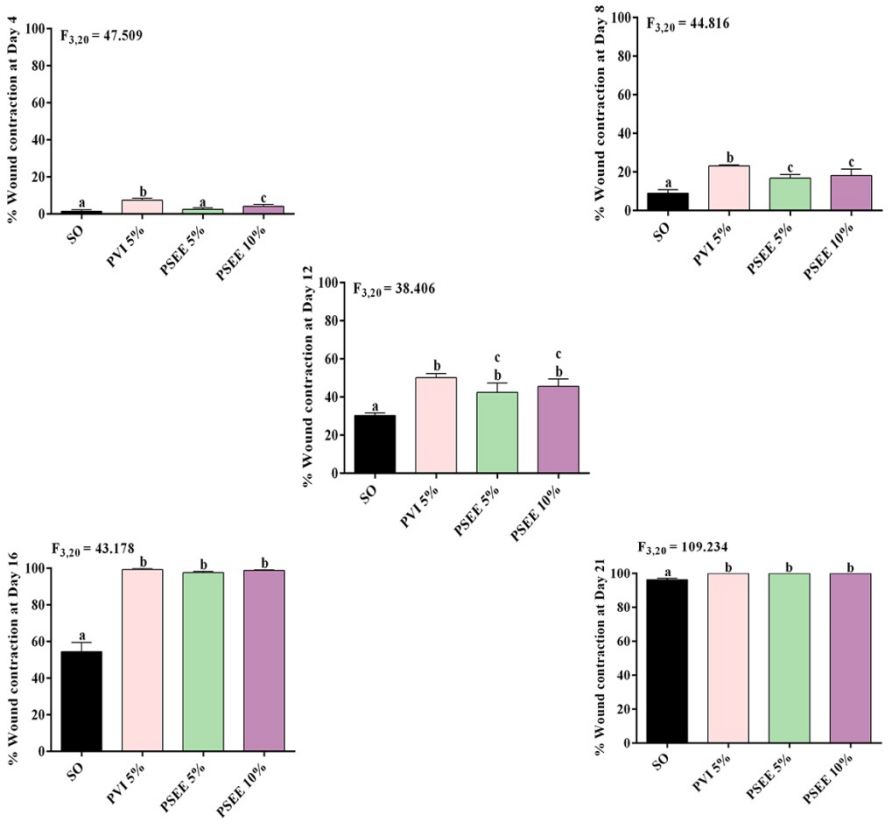

Figure 3.2: Graphical representation of percentage wound contraction at different days of treatment with SO, PVI 5%, PSEE 5% and PSEE 10%. A) Day 4. B) Day 8. C) Day 12. D) Day 16. E) Day 21. Different letters indicate significant variations. $p<0.001$ when compared with SO treated group. SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

3.2 Period of epithelialization

The results showed a significant ($p<0.001$) reduction in the period of epithelialization in the treatment groups when compared with the SO treated group (19.00 ± 0.45 days). The positive control group that received PVI 5% (14.00 ± 0.26 days) had the shortest duration of epithelialization, followed by the PSEE 10% (15.00 ± 0.52 days) and PSEE 5% (17.00± 0.45 days) treated groups. However, no significant difference was observed between PSEE 10% and PVI 5% treated groups.

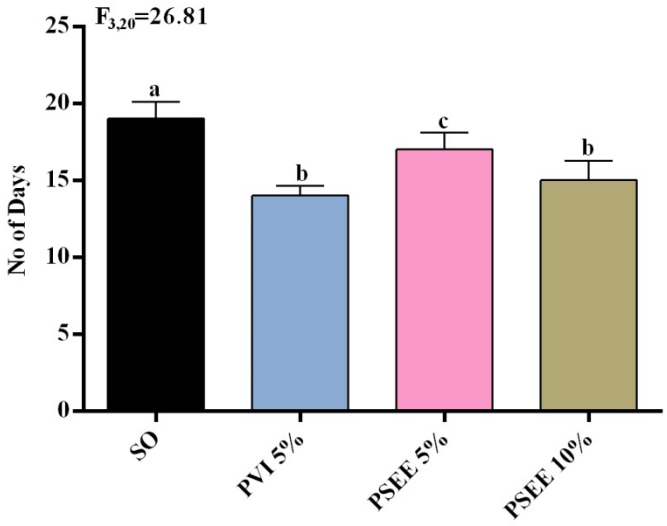

Figure 3.3: Period of epithelialization. Values were expressed as mean ± SEM, $p<0.001$ when compared with SO treated group. SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

3.3 Incision wound

A significant ($p<0.001$) increase in wound tensile strength was observed in the animal treated with PVI 5% (361.68 ± 10.58), PSEE 5% (373.53 ± 8.90), and PSEE 10% (325.01 ± 6.16) when compared with the SO treated group (270.41 ± 9.62). However, the results of the PSEE 10% shows a comparable results with the PVI 5% treated group (Figure 3.4)

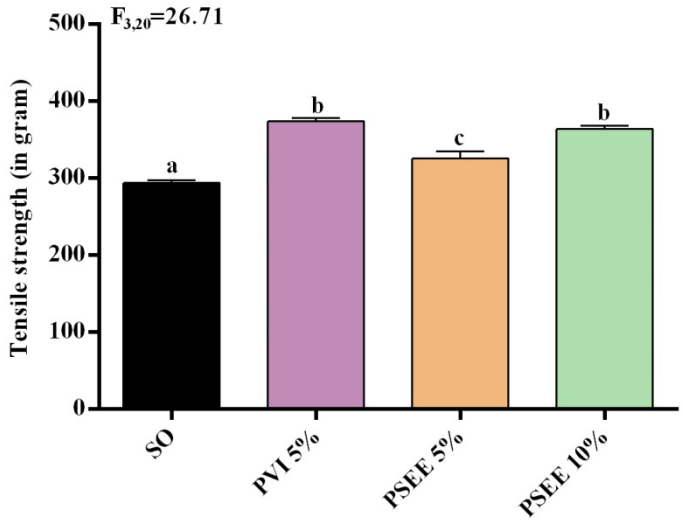

Figure 3.4: Effect of topical application of SO, PVI 5%, PSEE 5% and PSEE 10% on tensile strength of mice wound incision. $p<0.001$ when compared with SO treated group. SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

3.4 Histology

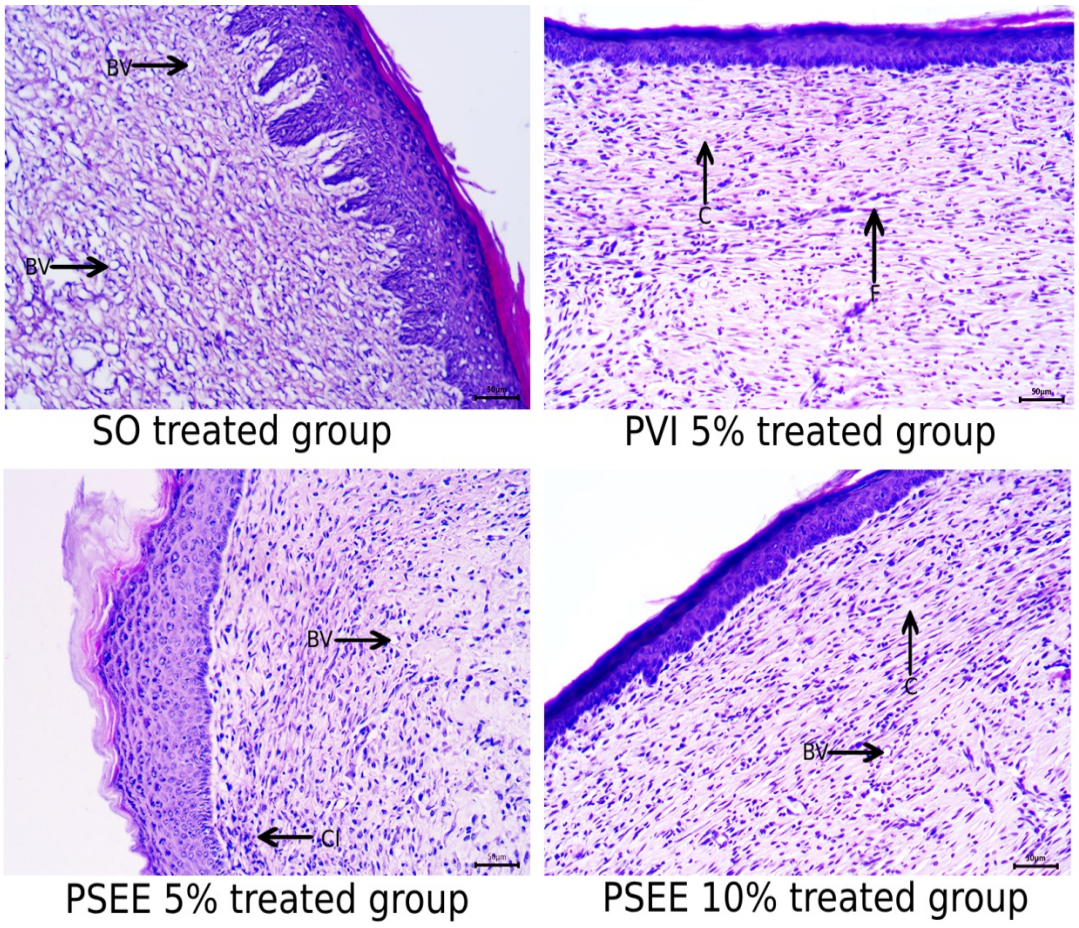

Figure 3.5: Histological slides showing the effect of topical application of *P. symmeria* extract ointment on excision wound model in mice at day 16. A) SO treated group; B) PVI 5% treated group; C) PSEE 5% treated group; D) PSEE 10% treated group. Abbreviations: BV- blood vessel; CI- cellular infiltration; C- collagen; F- fibroblast; SO- simple ointment; PVI- povidone iodine; PSEE - *Pilea symmeria* ethanol extract.

Histological examination revealed that animals treated with PSEE 10% showed significant improvement in wound healing. This was evidenced by abundant collagen deposition, reduced vascularization, and the presence of a thin, well-organized, mature epidermis comparable to that of normal skin. Similarly, animals treated with PVI 5% exhibited a thin layer of mature epidermis and abundant collagen, suggesting accelerated wound healing. The healing pattern in the PVI 5% treated group closely resembled that of the PSEE 10% treated group. In contrast, while animals treated with PSEE 5% showed substantial collagen deposition, the presence of a thicker epidermis and some cellular infiltration indicated incomplete healing. Animals treated with the SO exhibited a thicker epidermis, greater vascularization, and lower collagen deposition, all of which are indicative of delayed wound healing (Figure 3.5).

4. DISCUSSION

Wound healing processes encompass various cellular and biochemical processes that work together to restore tissue integrity after an injury (Karodi *et al*., 2009). Plant derived bioactive compounds have been extensively studied for their significant role in enhancing the wound healing process by promoting cell migration, proliferation, and tissue regeneration (Bass *et al*., 2021). Excision and incision wound models allow researchers to evaluate the efficacy of various treatments in enhancing wound closure and tissue repair (Nayak *et al*., 2009). Numerous experimental observations have shown that animals treated with plant based extracts exhibit improved wound healing, as evidenced by increased wound contraction and a shorter epithelialization period (Farahpour *et al*., 2012; Rub *et al*., 2009; Demilew *et al*., 2018; Agarwal *et al*., 2009). This suggests that phytochemicals can accelerate the natural repair mechanisms of the body, leading to faster tissue regeneration (Vitale *et al*., 2022).

The application of *Pilea symmeria* ethanol extract ointments led to a significant enhancement in wound contraction compared to the SO treated group. Among the treatment groups, animals receiving PVI 5% and PSEE 10% exhibited the most notable improvement in wound healing, with a marked increase in wound closure rates. The outcomes of these two treatments were comparable; indicating that a higher concentration of bioactive compounds in the PSEE 10% formulation may contribute to a more pronounced healing effect. In contrast, the PSEE 5% group showed a moderate improvement, though the rate of contraction was relatively slower than that observed with PVI 5% and PSEE 10%. In the case of incision wounds, extract treated wounds exhibited higher wound breaking strength compared to the SO treated group. This increased tensile strength is indicative of enhanced collagen synthesis and better tissue integrity, which are essential for effective wound healing. Additionally, histological examination of the excision wound model further corroborated these findings. Microscopic analysis revealed enhanced epidermal remodeling, abundance of collagen deposition and absence of newly formed blood vessels, all of which are crucial markers of proper wound healing (Qureshi *et al*., 2019). Previous studies investigating the wound healing efficacy of traditional

medicinal plants have reported findings that are consistent with our results (Qureshi *et al*., 2019; Mulisa *et al*., 2015; Demilew *et al*., 2018).

Although scientific evidence supports the ability of several traditional plant remedies to enhance tissue repair (Yuan *et al*., 2016). However, slight variations in the rate of healing observed in different studies may be attributed to multiple factors, including the health status of the animals, their diet, genetic differences, environmental conditions, and variations in the phytochemical composition of plant extracts (Avishai *et al*., 2017). The concentration of bioactive compounds in medicinal plants can fluctuate based on factors such as soil quality, harvesting season, and extraction methods, which may influence the effectiveness of the treatment (Abubakar & Haque, 2020). Even within the current experiment, minor differences in the wound healing rates were observed among animals receiving the same treatment. These variations suggest that the rate of wound healing is influenced by a complex interplay of biological and environmental factors.

In conclusion, the findings of this study underscore the promising role of *P. symmeria* extract in enhancing wound healing through its bioactive compounds. Experimental models have demonstrated that the extract significantly improves wound contraction, collagen deposition, and tissue remodeling, indicating its potential as a natural therapeutic agent. While the study provides compelling evidence supporting the potential of *P. symmeria* extract in promoting wound healing, further research is required to gain a comprehensive understanding of its therapeutic effects, additional research is necessary to isolate and analyze these compounds in detail. Identifying the specific phytochemicals responsible for the healing effects will help researchers assess their individual roles in the wound healing process. This knowledge will ultimately contribute to the creation of safer, more effective, and widely accessible treatments for wound care.

Chapter 4

Evaluation of the effect of *P. symmeria* on the antioxidant/oxidant status in a wound bearing swiss albino mice.

Abstract

Various extracts of *Pilea symmeria* were tested for their antioxidant properties in mice with excision wounds. The mice were inflicted with excision wound and divided into four groups, each consisting of six animals. Group I (negative control) animals were treated with simple ointment base (SO); group II (positive control) received 5% povidone iodine (PVI); groups III and IV were treated with 5% and 10% *Pilea symmeria* ethanol extract (PSEE) ointments respectively. The ointments were applied topically to the wounds each day, and the wound tissue was removed on day 16. To assess the antioxidant status of the excision wound tissue, several key biomarkers were measured, including reduced glutathione (GSH), glutathione S-transferase (GST), superoxide dismutase (SOD), and malondialdehyde (MDA). The results revealed that, compared to the SO treated group, the groups treated with PVI 5%, PSEE 5%, and PSEE 10% showed a significant ($p<0.05$) increase in the levels of antioxidant markers such as GSH, GST, and SOD and a significant ($p<0.05$) decrease in MDA levels . Our findings indicate that *P. symmeria* extracts effectively boost antioxidant defenses in the wound tissue. Additionally, the marked reduction in MDA levels points to a decrease in oxidative stress and lipid peroxidation.

1. INTRODUCTION

Antioxidants are essential compounds that play a crucial role in neutralizing free radicals and reactive oxygen species, thereby protecting the body from oxidative damage (Abuajah *et al*., 2014). They have a preventive role against the harm that free radicals do to the body (Aher *et al*., 2010). These substances function through multiple mechanisms, including donating electrons, chelating metal ions, acting as co-antioxidants, and regulating gene expression (Krinsky, 1992). Antioxidants are thought to work via two main pathways (Rice-Evans & Diplock, 1993). The first mechanism, referred to as the chain breaking mechanism, entails giving free radicals electrons. In the second method, the antioxidant eliminates free radicals by quenching chain initiating catalysts (Lobo *et al*., 2010).

One of the most significant endogenous antioxidants is glutathione (GSH), a non-protein thiol that is abundant in mammalian tissues. It plays a vital role in maintaining cellular redox balance and defending against oxidative damage (Xu *et al*., 2017). Within eukaryotic cells, glutathione is primarily distributed in the cytosol (80–85%), with smaller fractions found in the mitochondria (10–15%) and the endoplasmic reticulum (Baiano & Del Nobile, 2015; Cai *et al*., 2004; Shan *et al*., 2005). GSH is also essential for regulating cellular functions, including apoptosis, necrosis, and cell division (Yuan & Kaplowitz, 2008; Pallardo *et al*., 2008). It aids in detoxifying harmful substances by conjugating with xenobiotics, facilitating their removal from the cell (Boyland & Chasseaud, 1969). Glutathione S-transferases (GSTs) belong to the phase II detoxification enzyme family and are crucial for neutralizing harmful substances. These enzymes catalyze the conjugation of GSH with various hydrophobic and electrophilic compounds, thereby reducing their toxicity and expediting their elimination (Mazari *et al*., 2023). Beyond detoxification, GSTs are involved in cell signalling, post translational modifications, and resistance to chemotherapy drugs (Mannervik *et al*., 1988). When xenobiotic enter the body, phase I enzymes, such as cytochrome P450s, initiate their metabolism. In phase II, GSTs conjugate these metabolites with GSH, and in phase III, they are expelled from the cell through multidrug resistance associated proteins (MRPs) (Hayes *et al*., 2004). Superoxide dismutase (SOD) is another critical enzyme

that helps protect cells from oxidative stress by catalyzing the conversion of superoxide radicals into hydrogen peroxide and oxygen (Wang *et al*., 2018). SOD plays a fundamental role in antioxidant defense, and any dysregulation in its activity is linked to various diseases (Marcus *et al*., 2006; Robbins and Zhao, 2014). Through their dismutase activity, SODs facilitate the interaction between the superoxide anion ($O2^{\bullet-}$) and itself, producing oxygen and hydrogen peroxide (H_2O_2) (Fridovich, 1997). Although they have a low level of reactivity, superoxides are categorized as free radicals that have the ability to produce additional reactive species (Stamler *et al*., 1992; Beckman and Koppenol, 1996). Superoxide dismutases (SODs) also modify the concentrations of free radicals by controlling the amounts of $O2^{\bullet-}$ (Zheng *et al*., 2023).

Lipid peroxidation is one of the processes that free radicals may manifest their consequences inside the body. Free radicals attack polyunsaturated fatty acids in cell membranes during this process (Ayala *et al*., 2014). They produce a lipid radical by removing hydrogen atom from the fatty acid. A chain reaction of lipid peroxidation is then started when this lipid radical combines with molecular oxygen to form a lipid peroxyl radical, which then proceeds to extract hydrogen atoms from nearby fatty acids (Repetto *et al*., 2012). Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), two very reactive metabolites of lipid peroxidation, may interact with proteins and DNA to impair their activity (Barrera *et al*., 2018).

In the context of wound healing, antioxidants play a significant role by mitigating oxidative stress, which can hinder tissue repair. While excessive free radicals can damage cells, a controlled presence of reactive oxygen species is essential for initiating and regulating the wound healing process (Beckman & Ames, 1998). At low concentrations, these reactive species protect cells from infections and promote tissue repair by activating survival signaling pathways (Rodriguez *et al*., 2008; Sanchez *et al*., 2018). However, when reactive oxygen species accumulate beyond the body's antioxidant capacity, oxidative stress occurs, leading to cellular damage and delayed healing (Ponugoti *et al*., 2013; Johnson *et al*., 2021).

Evaluating endogenous antioxidant levels serves as a key indicator of cellular health and helps determine whether the application of the extract based ointment promotes the wound healing process. In this chapter, excised wound tissues from experimental animals are analyzed for antioxidant markers such as GSH, GST, and SOD, alongside measuring lipid peroxidation levels through MDA concentration. These evaluations help determine the effectiveness of the treatments, including those derived from medicinal plants, in enhancing the wound healing process by reducing oxidative damage and supporting cellular repair mechanisms.

2. METHODS

2.1 Grouping of animals

The animals were randomly divided into four groups of 6 animals each. Group I animals (negative control group) were treated with the vehicle of the simple ointment (SO). Group II animals (positive control group) were treated with povidone iodine (PVI) 5%. Group III and IV animals were treated with 5% and 10% (w/w) of *P. symmeria* ethanol extract ointment (PSEE), respectively.

2.2 Processing of wound tissue

The wound tissue was excised at day 16 and immediately used to make 5% (w/v) tissue homogenate using 1xPBS. The homogenates were centrifuged at 10,000 rpm at 4˚C for 30 min. The supernatant was collected and stored at -80˚C until further use. The protein content of the supernatant was determined following the standard method (Lowry et al., 1951).

2.3 Antioxidant assay

2.3.1 Glutathione (GSH) assay

GSH content was measured using the standard method (Moron *et al*., 1979). GSH was tested by reacting it with 5,5'-dithiobis(2- nitrobenzoic acid) (DTNB), resulting in a molecule that absorbed at 412 nm (Ellman's method). Each test tube contained 2 ml of 0.6 mM DTNB in 0.2 M sodium phosphate, pH 8.0, 100 μL of the sample, and 0.2 M phosphate buffer to give a final volume of 3 ml. The reference

cuvette contained 100 µL of 5% trichloroacetic acid instead of the sample solution. GSH concentration was then calculated from the standard graph and expressed in µmol/mg protein.

2.3.2 Glutathione S-transferase (GST) assay

GST was measured following the standard method with minor modifications (Beutler *et al*., 1984). To 100 µL of 0.1M phosphate buffer (pH 6.5), 20 µL of 20 mM CDNB and 1.76 mL of distilled water were added. The mixture was incubated at 37 ºC for 10 min. Then, 60 µL 20mM GSH and tissue homogenate were added to the mixture. For the blank, distilled water was added instead of the tissue homogenate. The absorbance was then measured at 340nm with a 1 min interval. The GST activity was then calculated using the following formula:

GSH activity = (OD of test – OD of blank/9.6 × volume of test sample) × 1000

where OD is the optical density of the tested sample.

2.3.3 Superoxide dismutase (SOD) assay

SOD activity was tested following the standard protocol with slight modifications (Sun *et al*., 1988). A sample of 10 µL was mixed with 15 µL distilled water, 25 µL phenazine methosulfate (PMS), 75 µL nitro-blue tetrazolium (NBT), and 50 µL nicotinamide adenine dinucleotide (NADH). The mixture was incubated for 90 secs at 30 ºC. Then, 250 µL of acetic acid and 1000 µL of butanol were added to the mixture. The mixture was vortexed for 2-3 secs and let stand for 10 min. It was then centrifuged for 30 secs. For blank, distilled water was added instead of the tissue homogenate. The absorbance was then taken at 560 nm. The enzyme activity was expressed in unit/mg protein.

2.3.4 Lipid peroxidation assay

Lipid peroxidation activity was tested using the standard method with slight modifications (Ohkawa *et al*., 1979). To 125 µL of the tissue homogenate, 125 µL of 15% TCA was added and centrifuged at 10000 rpm for 10 min. The supernatant was taken out and kept in a clean and dry test tube. Twice the amount of the present

thiobarbituric acid (0.8%) solution was added to the test sample. Test tubes were then kept in a 95 ºC water bath for 45 min and the formation of colored complexes was observed. For the blank, distilled water was added instead of the tissue homogenate. After cooling, the absorbance was read at 532nm.

2.4 Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey multiple comparison of means. Values were expressed as mean ± SEM. The analysis was carried out using SPSS (Version 27) and the graphs were plotted using GraphPad prism (Version 6). The value $p<0.05$ was considered to be statistically significant.

3. RESULTS

3.1 Antioxidant levels

3.1.1 Glutathione (GSH) assay

GSH is an important antioxidant that plays a vital role in reducing oxidative stress and protecting cells from damage. The results indicate a significant increase in the levels of GSH in animals treated with *P. symmeria* extract ointment at various concentrations (PSEE 5%, and PSEE 10%) compared to the control group treated with SO. The elevated GSH levels in the treated groups suggest that the *P. symmeria* extract promotes antioxidant activity at the wound site, which is crucial for protecting the tissue from oxidative damage and supporting the healing process. The increase in GSH levels directly reflects a favorable biochemical environment, which supports cellular regeneration and accelerates the healing of the wound tissue (Figure 4.1).

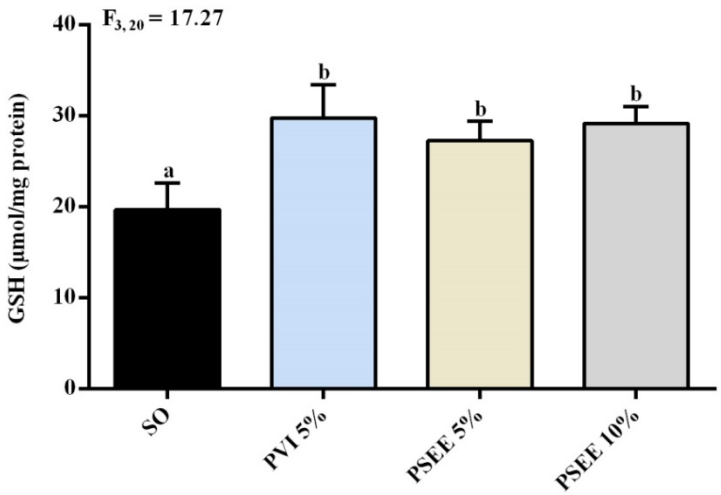

Figure 4.1: Effect of topical application of SO, PVI 5%, PSEE 5% and PSEE 10% on GSH levels of excision wound in mice at day 16. Means not sharing the same letters are statistically significant ($p<0.05$). Abbreviations: SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

3.1.2 Glutathione S-transferase (GST) assay

Glutathione S-transferase (GST) is an enzyme involved in the detoxification of reactive oxygen species (ROS) and other harmful substances in the body. Elevated levels of GST indicate an enhanced ability to neutralize oxidative stress and improve the overall healing process. In this study, the animals treated with *P. symmeria* extract ointment (PSEE 5%, and PSEE 10%) showed a significant increase in GST levels compared to the SO treated group. This suggests that the extract may not only boost antioxidant defenses by enhancing GSH levels but also promote detoxification processes by increasing GST activity. The increase in GST levels supports the idea that *P. symmeria* extract plays a role in modulating oxidative stress and promoting tissue recovery during wound healing (Figure 4.2).

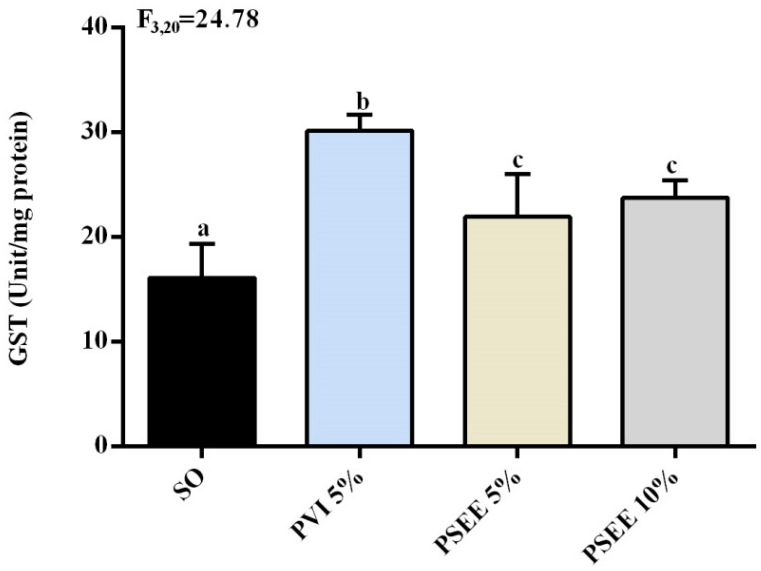

Figure 4.2: Effect of topical application of SO, PVI 5%, PSEE 5% and PSEE 10% on GST levels of excision wound in mice at day 16. Means not sharing the same letters are statistically significant ($p<0.05$). Abbreviations: SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

3.1.3 Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) is another important antioxidant enzyme that plays a crucial role in protecting cells from oxidative damage by converting superoxide radicals into less harmful molecules. The results showed a significant increase in SOD levels in the groups treated with *P. symmeria* extract at various concentrations (PSEE 5%, and PSEE 10%) when compared with the SO treated group. This finding further supports the hypothesis that *P. symmeria* extract has a potent antioxidant effect, enhancing the body's natural defense mechanisms against oxidative stress at the wound site. The elevated SOD levels suggest that *P. symmeria* extract may be contributing to the reduction of oxidative damage and improving the overall wound healing process by enhancing enzymatic antioxidant defences (Figure 4.3).

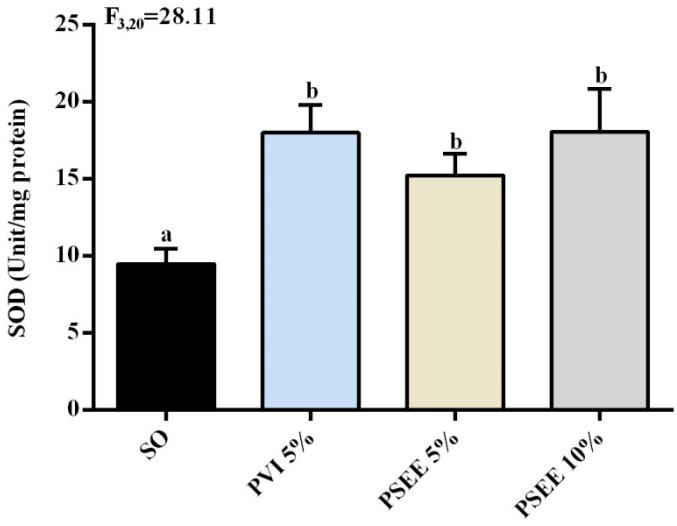

Figure 4.3: Effect of topical application of SO, PVI 5%, PSEE 5% and PSEE 10% on SOD levels of excision wound in mice at day 16. Means not sharing the same letters are statistically significant ($p<0.05$). Abbreviations: SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

3.1.4 Lipid peroxidation assay

Malondialdehyde (MDA) is a byproduct of lipid peroxidation, which occurs when cell membranes are damaged due to oxidative stress. Elevated MDA levels are often associated with increased cellular damage and delayed healing. In this study, the groups treated with *P. symmeria* extract ointment (PSEE 5%, and PSEE 10%) exhibited a significant decrease in MDA levels compared to the SO treated group. The reduction in MDA levels suggests that the *P. symmeria* extract effectively minimizes lipid peroxidation and oxidative damage in the wound tissue. This decrease in MDA levels is a strong indicator that the treatment helps to prevent further tissue damage and promotes a more efficient healing process (Figure 4.4).

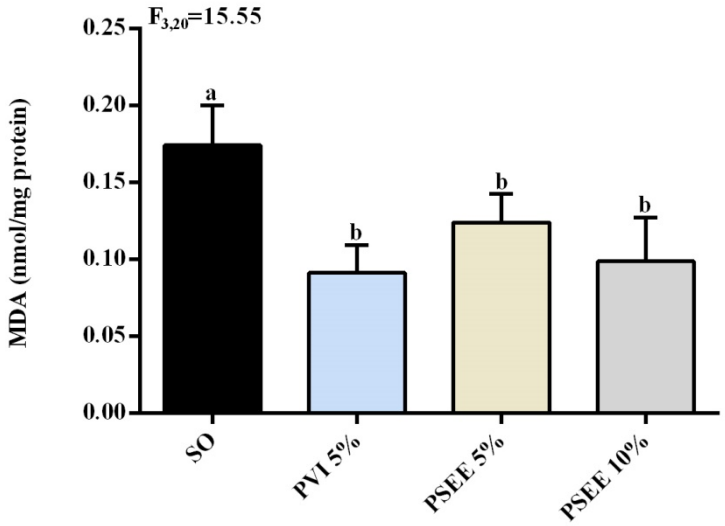

Figure 4.4: Effect of topical application of SO, PVI 5%, PSEE 5% and PSEE 10% on MDA levels of excision wound in mice at day 16. Means not sharing the same letters are statistically significant ($p<0.05$). Abbreviations: SO- simple ointment; PVI- povidone iodine; PSEE- *Pilea symmeria* ethanol extract.

4. DISCUSSION

Reactive oxygen species (ROS) are continuously generated as byproducts of normal cellular metabolism and play essential roles in cell signaling and homeostasis (Lennicke & Cocheme, 2021). However, when produced in excess, ROS can overwhelm the body's antioxidant defenses, resulting in oxidative stress and subsequent cellular damage (Juan *et al*., 2021). Uncontrolled ROS can interact with key cellular macromolecules such as, lipids, proteins, and DNA, leading to structural and functional impairment (Droge, 2002). This oxidative damage has been implicated in the development and progression of various chronic and degenerative diseases, including cardiovascular diseases, neurodegenerative disorders such as Alzheimer's and Parkinson's, diabetes, arthritis, chronic inflammation, pulmonary diseases, and multiple forms of cancer (Barnham *et al*., 2004; Noreen *et al*., 2017).

The ability of medicinal plants to combat oxidative stress through their antioxidant constituents has garnered considerable scientific interest. Natural antioxidants, especially those derived from plants, are considered safer alternatives to synthetic compounds due to their biocompatibility and multifaceted bioactivity (Mueed *et al*., 2023). Among these, polyphenols stand out as major contributors. These plant secondary metabolites are abundant in fruits, vegetables, teas, and wines and are widely recognized for their potent antioxidant capacity (Pandey & Rizvi, 2009; Hano & Tungmunnithum, 2020). Polyphenols exert their antioxidant effects primarily through free radical scavenging, metal ion chelation, and upregulation of endogenous antioxidant defense systems (Stadtman, 1992; Maxwell, 1995). Beyond direct antioxidant actions, polyphenols influence cellular signaling pathways involved in inflammation, apoptosis, and immune modulation, further supporting their protective role against oxidative damage (Yahfoufi et al., 2018).

Epidemiological and clinical evidence strongly supports the health benefits of polyphenol rich diets, which are associated with reduced risks of chronic disease (Zhang & Tsao, 2016). Specific polyphenol subclasses such as flavonoids, phenolic acids, lignans, and stilbenes, have been identified as key agents in promoting these protective effects (Lang *et al*., 2023).

In addition to dietary antioxidants, endogenous antioxidant systems are crucial in defending against oxidative damage. Glutathione (GSH), a tripeptide composed of glutamine, cysteine, and glycine, serves as a major intracellular antioxidant. It protects cells by directly scavenging ROS, regenerating other antioxidants, and participating in detoxification processes (Townsend *et al*., 2003; Averill-Bates, 2023). The enzyme glutathione S-transferase (GST) plays a vital role in detoxification by catalyzing the conjugation of GSH to electrophilic substrates, facilitating their neutralization and elimination (Sheehan *et al*., 2001). Superoxide dismutase (SOD), another key antioxidant enzyme, serves as the first line of defense against oxidative stress. It catalyzes the conversion of superoxide radicals ($O_2^{\bullet -}$) into molecular oxygen and hydrogen peroxide, thereby preventing the formation of more reactive species like hydroxyl radicals (Ighodaro & Akinloye, 2017; Maurya & Namdeo, 2021). SOD activity is essential for maintaining cellular redox balance and protecting tissues from oxidative injury (Yoonus, 2018; Kangralkar *et al*., 2010).

One of the most detrimental consequences of oxidative stress is lipid peroxidation, wherein ROS attack polyunsaturated fatty acids in cell membranes, initiating a chain reaction that compromises membrane integrity (Valgimigli, 2023). Malondialdehyde (MDA), a major end product of lipid peroxidation, is a widely accepted biomarker for oxidative damage (El-Aal, 2012; Gaweł *et al*., 2004). Elevated MDA levels are often observed in conditions such as atherosclerosis, neurodegeneration, and cancer (Jena *et al*., 2023).

In the present study, the topical application of *P. symmeria* extract based ointment resulted in significant upregulation of key endogenous antioxidants such as GSH, GST, and SOD, in the wound tissues. This enhancement indicates the extract's strong antioxidant potential and its ability to reinforce the body's natural defense mechanisms against oxidative stress. The observed increases in these enzymatic and non-enzymatic antioxidant markers demonstrate that the plant extract aids in neutralizing ROS, thereby minimizing oxidative damage at the cellular level.

Moreover, treatment with the plant extract led to a marked decrease in MDA levels, signifying a reduction in lipid peroxidation. This reinforces the protective

effect of the extract in preserving membrane integrity and preventing ROS induced cellular dysfunction. The extract's ability to scavenge free radicals and inhibit oxidative damage positions it as a potent natural antioxidant agent with therapeutic potential.

Medicinal plants have a long standing history in traditional medicine systems for treating oxidative stress related and inflammatory conditions (Basu *et al*., 2023). Modern research has validated many of these traditional uses, identifying the bioactive constituents responsible for their efficacy. The findings of this study further support the therapeutic relevance of such plants. The results of this study demonstrate that the plant extract used in the formulation possesses antioxidant properties and by enhancing the activity of key antioxidant enzymes (GSH, GST, and SOD) and reducing lipid peroxidation (MDA levels), the extract effectively combats oxidative stress and promotes tissue repair. These outcomes highlight the extract's potential role in maintaining redox homeostasis and protecting against oxidative damage. Furthermore, this study contributes to the growing body of evidence supporting the use of medicinal plants as a source of natural antioxidant therapies. Incorporating such plant derived compounds into therapeutic or dietary regimens may offer a practical approach to disease prevention, tissue protection, and overall health enhancement.

Chapter 5

To study the effect of *P. symmeria* on the gene expression involved in wound healing by using RT-PCR.

Abstract

Wound healing is a dynamic and highly regulated process involving a cascade of cellular and molecular events coordinated by cytokines, growth factors, and chemokines. This study aimed to evaluate the effects of *Pilea symmeria* ethanol extract (PSEE) on mice excision wound healing by analyzing the gene expression of *Tnf-α* and *Vegf*, two key regulators involved in inflammation and angiogenesis, respectively. The wounds were topically treated with PSEE (5% and 10%) and compared to animals treated with the standard antiseptic povidone iodine (PVI 5%), serving as the positive control group, and those treated with simple ointment (SO) alone, serving as the negative control group. Granulation tissues were collected on days 4, 8, and 12 post wounding, and gene expression was analyzed using real-time PCR. The results showed significant downregulation of *Tnf-α* in the PSEE 5%, 10% and PVI 5% treated groups compared to the SO treated group, suggesting early resolution of inflammation. *Vegf* expression was significantly upregulated at days 4 and 8 in the PSEE 5%, PSEE 10% and PVI 5% treated groups, peaking during the proliferative phase, followed by downregulation at day 12, indicating accelerated angiogenesis and vascular stabilization. In contrast, *Vegf* expression remained elevated at day 12 in the SO treated group, suggesting delayed healing. These findings demonstrate that *Pilea symmeria* extract promotes wound healing by regulating the expression of key genes involved in inflammation and angiogenesis, supporting its potential as a natural therapeutic agent for enhancing tissue repair.

1. INTRODUCTION

Wound healing is a complex, multi phase process that relies on the coordinated interactions of various growth factors, cytokines, and chemokines (Dehkordi *et al*., 2019; Inan *et al*., 2006). This process involves the combined actions of multiple cell types, including keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets, which secrete signaling molecules to facilitate tissue repair (Barrientos *et al*., 2008; Kangal & Regan, 2023). Key signaling molecules involved in wound healing include interleukin (IL)-1β, transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet derived growth factor (PDGF) (Rumalla & Borah, 2001; Barrientos *et al*., 2008; Nakamichi *et al*., 2016; Kirwan et al., 2009).

Angiogenesis, the formation of new blood vessels to restore blood flow to damaged tissue, is a crucial step in wound healing. Failure of this process can lead to chronic wounds (Goswami *et al*., 2022). VEGF is a key regulator of angiogenesis, with its expression increasing throughout this process (Nissen *et al*., 1998). In addition to angiogenesis, VEGF plays a significant role in epithelialization and collagen deposition (Stojadinovic *et al*., 2007). The angiogenesis process consists of multiple steps, including vasodilation, degradation of the basement membrane, endothelial cell migration, and subsequent proliferation (Chaplain, 2000). VEGF enhances blood vessel permeability, allowing immune cells, nutrients, and other growth factors to access the wound site, thereby promoting tissue healing and waste elimination (Brem & Folkman, 1993). VEGF also stimulates endothelial cells to produce and release matrix metalloproteinases (MMPs), which break down basement membrane components to facilitate cell migration (Unemori et al., 1992; Amirrah *et al*., 2022). It promotes endothelial cell migration through chemotaxis by creating a concentration gradient that directs movement toward angiogenic signals (Pellet-Many, 2022). Additionally, VEGF induces vasodilation by stimulating nitric oxide (NO) production, increasing blood flow and permeability, which further supports endothelial cell migration (Bao *et al*., 2008). VEGF induced endothelial cell

proliferation occurs through the activation of VEGFR-2, triggering the MAPK/ERK and PI3K/Akt pathways, which promote cell cycle progression, enhance survival, and inhibit apoptosis (Abhinand *et al*., 2016). It also upregulates cyclins and increases NO production, creating a favorable environment for cell growth (Hood *et al*., 1998). Moreover, VEGF promotes epithelialization by stimulating keratinocyte migration and proliferation, enhancing angiogenesis for nutrient supply, increasing vascular permeability for growth factor delivery, and facilitating basement membrane remodeling for efficient wound closure (Shaw *et al*., 2024). It also enhances collagen deposition by activating fibroblasts and upregulating TGF-β expression (Shams *et al*., 2022; Savari *et al*., 2019).

Another key factor involved in wound healing is TNF-α. While widely recognized for its role in tumor necrosis, it also plays a crucial role in the early phases of wound healing (Jiang *et al*., 2017). It is produced primarily by neutrophils and macrophages, and aid in recruiting inflammatory leukocytes to the injured tissues and initiating the inflammatory phase (Mast and Schultz, 1996; Jang *et al*., 2021). TNF-α is critical for regulating inflammation, recruiting immune cells, stimulating angiogenesis, and supporting tissue remodeling (Jang *et al*., 2021; Wei *et al*., 2023; Serandour *et al*., 2005). TNF-α exerts its effects by binding to two receptors, TNFR1 and TNFR2, activating signal transduction pathways that mediate various cellular responses, including survival, differentiation, proliferation, and apoptosis (Jang *et al*., 2021). It induces the expression of MMP-9 in keratinocytes through NF-κB signaling, highlighting its role in wound re-epithelialization (Banno *et al*., 2004). Additionally, TNF-α, particularly in conjunction with IL-17, facilitates neutrophil recruitment during inflammation by synergistically activating endothelial cells, increasing the expression of adhesion molecules essential for neutrophil migration (Griffin *et al*., 2012). Furthermore, TNF-α stimulates the secretion of pro-angiogenic and inflammatory factors, including VEGF, which is crucial for endothelial cell proliferation and migration (Dergilev *et al*., 2023; Sainson *et al*., 2008).

TNF-α levels peak during the early stages of wound healing, as neutrophils, its primary producers are abundant in the initial inflammatory phase. This underscores its role in initiating inflammation and triggering the healing response (Feiken *et al*., 1995). However, excessive activation of TNF-α signaling can lead to prolonged inflammation and contribute to various clinical complications (Mateen *et al*., 2016; Bradley, 2007). Elevated systemic and local TNF-α levels have been observed in patients with chronic ulcers, suggesting a link between sustained inflammation and impaired healing (Ashcroft *et al*., 2011).

Therefore, this chapter focuses on evaluating the effects of *P. symmeria* extract ointment on excision wound healing in Swiss albino mice by analyzing the gene expression levels of *Vegf* and *Tnf*-α.

2. METHODS

2.1 Animal tissue harvesting

Healthy adult male Swiss albino mice (weighing between 30-35 g) were selected for this study. The mice were divided into four groups, each consisting of five animals. On days 4, 8, and 12 following wounding, five animals from each group were euthanized, and the granulation tissue was carefully removed. The collected tissue samples were immediately preserved in RNA stabilization reagent (RNAlater™ from Qiagen, USA) and stored at -80ºC for subsequent real-time PCR analysis.

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

RNA extraction from granulation tissue was carried out using Tri reagent (Ambion AM9738, Grand Island, NY, USA) solution (Sangma & Trivedi, 2023). The RNA concentration was quantified using a spectrophotometer (ND-ONE NanoDrop One, ThermoFisher Scientific, USA), and 2 µg of RNA was used to synthesize complementary DNA (cDNA) using the iScript™ cDNA Synthesis Kit

(BIO-RAD, USA, 1708891). Gene-specific primers were employed for gene expression analysis (Sangma & Trivedi, 2023).

Quantitative PCR (qPCR) was performed using the QuantStudio 5 (Applied Biosystem by Thermo Fisher Scientific, USA) with a 7 µL reaction volume for each gene. The reaction mix contained 1 µL of cDNA, 1 µL each of gene-specific forward and reverse primers, 3 µL of Power Up™ SYBR Green MasterMix (Applied Biosystem by Thermo Fisher Scientific, USA, A25742), and 1 µL of nuclease-free water (Ambion, AM9938; Borah *et al*., 2022). The qPCR cycling conditions involved an initial cycle at 95°C for 20 secs, followed by 35 cycles of 95°C for 1 sec, 60°C for 20 secs, and 95°C for 1 sec. A melt curve step was included with one cycle at 60°C for 20 secs, followed by one cycle at 95°C for 1 sec. GAPDH was used as the reference gene to normalise the expression levels of target genes (Sangma 7 Trivedi, 2023). Each sample was run in duplicate, alongside non-template and negative reverse transcription (RT) controls (Borah et al., 2022). The relative gene expression levels were calculated using the ΔΔCt method (Livak & Schmittgen, 2001).

In this study, the relative expression of the *Vegf* and *Tnf-α* genes was examined in the *P. symmeria* ethanol extract treated groups and the control group, with glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene expression serving as the housekeeping gene.

Table 5.1. Sequences of the forward and reverse primers used for qPCR analysis.

Gene	Forward Primer	Reverse Primer
Vegf	5’-ACG AAA CG CAA GAA ATC CC-3’	5’-ACG CGA GTC TGT GTT TTT GC-3’
Tnf-α	5’-CGG CAT GGA TCT CAA AGA CAA C-3’	5’-AGC CTT GTC CCT TGA AGA GAA C-3’
Gapdh	5’-AAC GAC CCC TTC ATT GAC CTC-3’	5’-ACT GTG CCG TTG AAT TTG CC-3’

2.3 Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Bonferroni post hoc test in GraphPad Prism (Version 10). The values were expressed as mean ± SEM. The value $p<0.05$ was considered statistically significant.

RESULTS

Tnf-α

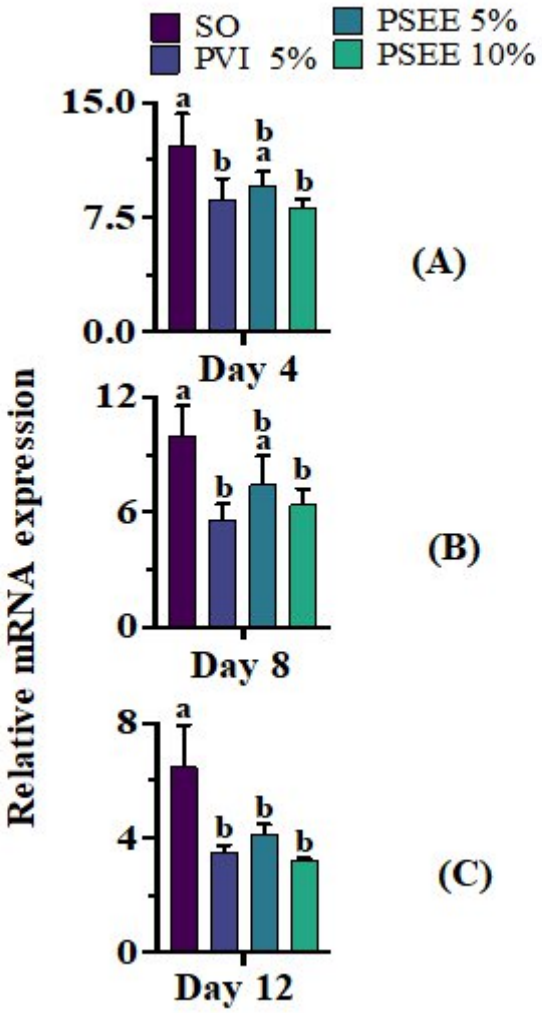

Figure 5.1: Relative changes in mRNA expressions of *Tnf-α* in PSEE treated wounds in comparison to control at (A) Day 4, (B) Day 8 and (C) Day 12. PSEE= *P. symmeria* ethanol extract; Values are expressed as Mean ± SEM. Different letters indicate significant variation ($p<0.05$).

One-way ANOVA revealed significant differences in *Tnf-α* expression across all groups at each time point ($F_{3,16}$= 6.766; p=0.0037; One Way ANOVA; Figure 5.1 (A)). On day 4**,** *Tnf-α* expression was significantly downregulated in the PVI 5% and PSEE 10% treated groups compared to the SO treated group (p<0.05; Bonferroni's post hoc test). No significant difference was observed between the PSEE 5% and SO groups, and similarly, no significant differences were found among the three treatment groups (PVI 5%, PSEE 5%, and PSEE 10%) (p>0.05; Bonferroni's multiple comparison test). A similar pattern was observed on day 8 ($F_{3,16}$= 6.376; p=0.0048; One Way ANOVA; Figure 5.1 (B)), where *Tnf-α* was significantly downregulated in the PVI 5% and PSEE 10% treated groups relative to SO treated group, with no significant variation among the treatment groups (p>0.05; Bonferroni's multiple comparison test). By day 12, *Tnf-α* expression continued to be markedly downregulated in all treatment groups (PVI 5%, PSEE 5%, and PSEE 10%) compared to SO treated group ($F_{3,16}$= 8.512; p=0.0013; One Way ANOVA; Figure 5.1 (C)), with no significant differences observed among the treatment groups. Notably, *Tnf-α* expression remained significantly upregulated in the SO treated group when compared with the treatment groups (p>0.05; Bonferroni's multiple comparison test).

Vegf

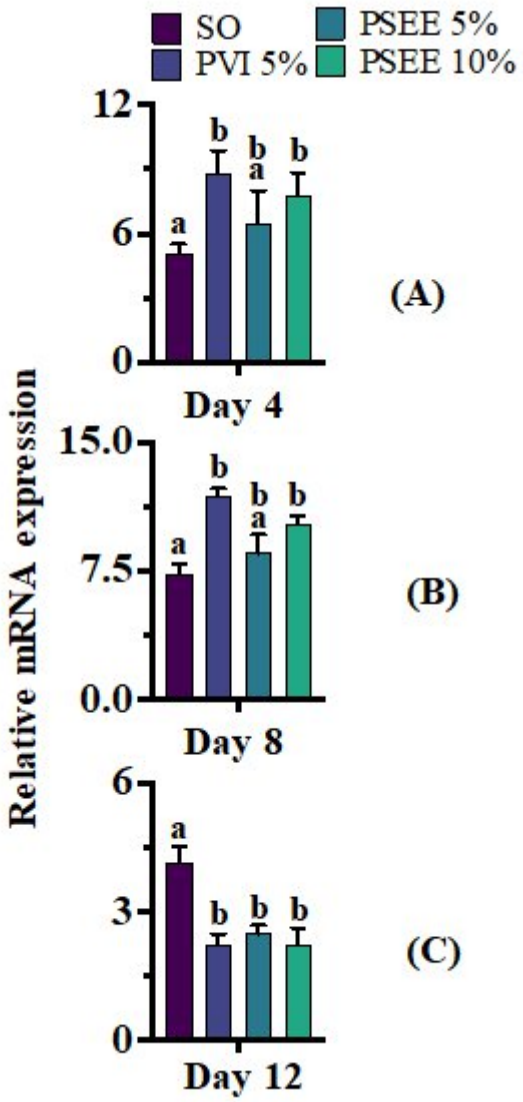

Figure 5.2: Relative changes in mRNA expressions of *Vegf* in PSEE treated wounds in comparison to control at (A) Day 4, (B) Day 8 and (C) Day 12. PSEE= *P. symmeria* ethanol extract; Values are expressed as Mean ± SEM. Different letters indicate significant variation ($p<0.05$).

A significant upregulation in the expression of *Vegf* was observed in the PVI 5% and PSEE 10% treated groups on day 4 compared to the SO treated group ($F_{3,16}$= 7.204; p=0.0028; One Way ANOVA; Figure 5.2 (A)). No significant difference was found between the PSEE 5% and SO treated groups, nor among the treatment groups (PVI 5%, PSEE 5%, and PSEE 10%) (p>0.05; Bonferroni's multiple comparison test). A similar expression profile was observed on day 8 ($F_{3,16}$= 12.56; p=0.0002; One Way ANOVA; Figure 5.2 (B)) with *Vegf* expression reaching a relatively higher level compared to other time points in the study (p>0.05; Bonferroni's multiple comparison test). By day 12, *Vegf* expression was significantly downregulated in all the treatment groups, with no significant differences among them ($F_{3,16}$= 13.93; P=0.0001; One Way ANOVA; Figure 5.2 (C)). However, the SO treated group continued to exhibit significantly higher *Vegf* expression compared to the treatment groups (p>0.05; Bonferroni's multiple comparison test).

3. DISCUSSION

Growth factors, chemokines, and cytokines are thought to play a crucial role in controlling the rate at which wounds heal (Abdulkhaleq *et al*., 2018; Behm *et al*., 2011). The depletion or addition of these molecules has been shown to have a significant effect on wound healing (Koh & DiPietro, 2011). For example, changes in the levels of cytokines like TNF-α, which play a dual role in wound healing, can either accelerate or delay the repair process depending on the context and concentration present at the wound site (Zins *et al*., 2010).

In acute wounds, the inflammatory response is necessary to combat infection and remove debris, but in chronic wounds, this inflammatory response becomes dysregulated, leading to a prolonged inflammatory phase (Soliman & Barreda, 2022). Chronic wounds are often characterized by excessive leukocytosis, increased production of cytokines and chemokines, and enhanced degradation of extracellular matrix (ECM) components (Ashcroft *et al*., 1999). The imbalance between matrix degradation and deposition can lead to delayed or impaired wound healing, as the ECM plays a crucial role in providing structural support and guiding cellular migration during repair (Xue & Jackson, 2013).

Angiogenesis is critical during wound healing because it increases blood circulation to the wound site, providing the necessary nutrients and oxygen required for cell proliferation and tissue regeneration (Nazarnezhad *et al*., 2022). It also facilitates the removal of metabolic waste products, which is important for maintaining a healthy wound environment (Zins *et al*., 2010). A study has demonstrated that increased gene expression of VEGF during the wound healing process is associated with enhanced angiogenesis, collagen deposition, and improved epithelialization (Romagnani *et al*., 2004).

Among the various cytokines involved in wound healing, TNF-α is an important factor that contributes to both the beneficial and detrimental phases of the healing process (Ashcroft *et al*., 2012; Feldmann *et al*., 2009; Chan *et al*., 2002). It acts as an initial trigger for the inflammatory response, promoting the recruitment of leukocytes and other immune cells to the wounded tissue (Mast & Schultz, 1996).

This process is essential for preventing infection and clearing away damaged tissue (Wilkinson & Hardman, 2020). Besides TNF-α inducing the production of MMPs leading to the excessive degradation of ECM, impairing tissue integrity and delaying the repairing process (Szabo *et al*., 2004). It also decreases the expression of tissue inhibitors of metalloproteinases (TIMPs), which normally regulate MMP activity and prevent excessive matrix degradation (Brew & Nagase, 2010). When there is an imbalance between MMPs and TIMPs, it results in the destruction of the ECM, preventing the normal progression of wound healing (Barrientos *et al*., 2008).

In the early stages of wound healing, TNF-α plays a protective role by initiating the inflammatory phase and preventing infection (Popa *et al*., 2007). However, when TNF-α level remain elevated for a prolonged period, they can disrupt the delicate balance between inflammation and tissue repair (Bhol et al., 2024). The excessive presence of TNF-α in chronic wound interferes with the formation of new tissue and proper wound remodeling, ultimately contributing to delayed healing (Bryan *et al*., 2005). Indeed, studies have consistently shown that elevated TNF-α level are associated with chronic, non-healing wounds, implicating this cytokine in their pathophysiology (Bryan *et al*., 2005). While inflammation is a necessary and beneficial component of the healing process and TNF-α is crucial for defending against pathogens, excessive or prolonged inflammation can hinder wound resolution and promote chronicity (Holzer-Geissler *et al*., 2022; Ashcroft *et al*., 2011; Schäfer & Werner, 2008).

Key cytokines such as TNF-α and VEGF play central roles in regulating inflammation, angiogenesis, and tissue remodeling during wound healing (Jeong *et al*., 2020). In the present study, *Tnf-α* expression was significantly downregulated in the PVI 5%, PSEE 5%, and PSEE 10% treated groups compared to the SO treated group. This suggests that the extract facilitates early resolution of inflammation in the treatment group. In contrast, persistent high *Tnf-α* expression observed in the SO treated group on day 12 reflects a delayed healing response relative to the treated groups.

For *Vegf*, a significant increase in expression was observed on day 4 in all treated groups compared to the SO treated group. By day 8, corresponding to the peak of the proliferative phase, *Vegf* expression reached its highest level across all time points. The significant decline in *Vegf* expression by day 12 in the PVI 5%, PSEE 5%, and PSEE 10% groups indicate that the extract promotes timely angiogenesis and stabilization of newly formed vessels, in contrast to the SO treated group, where *Vegf* remained elevated, suggesting delayed vascular remodeling.

Phytochemicals found in plants have garnered significant attention for their potential role in promoting wound healing. These bioactive compounds such as sterols, phenols, triterpenoids, tannins, and flavonoids, possess well documented antioxidant, anti-inflammatory, and antimicrobial properties, which contribute to the modulation of gene expression involved in key processes like collagen synthesis, angiogenesis, and cellular proliferation during tissue repair (Al-Taee *et al*., 2022).

Studies have demonstrated that phytochemicals can enhance angiogenesis by modulating vascular function and increasing the expression of pro-angiogenic factors such as *Vegf*, thereby improving blood supply to the wound site and accelerating tissue regeneration (Hashad *et al*., 2025; Hoeben *et al*., 2004). Additionally, these compounds influence essential cellular responses including proliferation, migration, and differentiation, by activating key molecular pathways involved in wound repair (Addis *et al*., 2020; Thangapazham & Sharad, 2014).

These findings highlight the therapeutic potential of plant derived bioactive compounds and reinforce the relevance of *Pilea symmeria*, as treatment with its extract modulated the expression of key genes involved in wound healing, such as *Tnf-α* and *Vegf*. This supports the growing interest in developing phytochemical based formulations as safer and effective alternatives to synthetic agents for enhancing and accelerating the wound repair process.

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ABBREVIATIONS

PSEE	*Pilea symmeria* ethanol extract
PSAE	*Pilea symmeria* aqueous extract
PSCE	*Pilea symmeria* chloroform extract
EGF	Epidermal growth factor
TGF-β	Transforming growth factor beta
FGF	Fibroblast growth factor
VEGF	Vascular endothelial growth factor
TGF-α	Transforming growth factor alpha
PDGF	Platelet-derived growth factor
ROS	Reactive oxygen species
IL-1	Interleukin-1
UV	Ultraviolet
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
GSH	Glutathione
GST	Glutathione S-transferase
SOD	Superoxide dismutase
DPP-IV	Plasma dipeptidyl peptidase IV
PG	Plasma glucose

TG	Triglyceride
OGTT	Oral glucose tolerance test
TBARS	Thiobarbituric acid-reactive substances
TL	Tail length
OTM	Olive tail moment
FST	Forced Swimming Test
TST	Tail Suspension Test
i.p.	Intraperitoneal
LPS	lipopolysaccharide
PMS	Phenazonium Methosulphate
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
AP-1	Activator Protein-1
PGE2	Prostaglandin E2
ERK	Extracellular Signal-Regulated Kinase
JNK	Jun N-terminal kinase
iNOS	Inducible Nitric Oxide Synthase
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
LC-MS	Liquid chromatography-mass spectrometry

H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
HPLC	High-performance liquid chromatography
DMSO	Dimethyl sulfoxide
SPSS	Statistical Package for the Social Sciences
ANOVA	Analysis of Variance
SEM	Standard Error of the Mean
IC_{50}	Half-maximal inhibitory concentration
MMPs	Matrix metalloproteinases
ECM	Extracellular Matrix
WHO	World Health Organization
NEHU	North Eastern Hill University
PEG	Polyethylene Glycol
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate-Buffered Saline
Hr	Hours
min	Minutes
secs	Seconds
g	Gram
w/v	Weight per volume

w/w	Weight per weight
mg	Milligram
kg	Kilogram
n	Number
mm^2	Square millimeters
cm	Centimetre
M	Molar
OD	Optical density
µL	Microliter
nm	Nanometer
H&E	Hematoxylin and Eosin
SO	Simple ointment
PVI	Povidone iodine
O2•−	Superoxide anion
MDA	Malondialdehyde
DTNB	5,5'-dithiobis(2- nitrobenzoic acid)
CDNB	1-chloro-2,4-dinitrobenzene
NETs	Neutrophil extracellular traps
ATCC	American Type Culture Collection
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration

RT-PCR	Real time-polymerase chain reaction
qPCR	Quantitative PCR
cDNA	Complementary DNA
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase

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Educational Qualifications

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HSSLC	2013	Mizoram Board of School Education	Science	Second	57.2%
B. Sc	2016	Mizoram University	Zoology	First	90.08%
M. Sc	2018	Mizoram University	Zoology	First	78.86%

LIST OF PAPERS PRESENTED

International

1. International Conference on "Biodiversity, Biogeochemistry and Ecosystem Sustainability in Changing Environment" jointly organised by Department of Forestry, MZU and Indian Ecological Society during 14-16th June, 2023.

National

2. National conference on "4th Mizoram Science Congress" organised by Mizoram Science, Technology, and Innovation Council (MISTIC) in collaboration with Mizo Academy of Sciences (MAS), Mizoram Science Society (MSS), Science Teachers' Association, Mizoram (STAM), Geological Society of Mizoram (GSM), Mizoram Mathematics Society (MMS), Biodiversity and Nature Conservation Network (BIOCONE) and Mizoram Information & Technology Society (MITS) during 24-25th November, 2022.

LIST OF SEMINAR/WORKSHOP ATTENDED

1. 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).
2. Webinar on "Nutraceuticals and Wound Healing" organised by Department of Food Technology, Mizoram University on 23th September, 2020.
3. 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.
4. International conference on "Current Trends in Biological Sciences" organised by the School of Life Sciences, Mizoram University from 18-20th March, 2024.

LIST OF PUBLICATIONS

Lalremtluangi, R. K., Lalthansangi, C., Lalhmingliani, E., & Vabeiryureilai, M. (2025). *Pilea symmeria*: A natural ally in antioxidant defense and wound repair in mice. *Journal of Herbmed Pharmacology. 14*(1), 97-103.

Lalremtluangi, R. K., Lalthansangi, C., Lalhmingliani, E., & Vabeiryureilai, M. (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.

Lalthansangi, C., **Lalremtluangi, R. K**., Lalhmingliani, E., & Vabeiryureilai, M. (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.

Lalthansangi, C., **Lalremtluangi, R. K**., & Lalhmingliani, E. (2025). Chemical composition and wound healing potential of *Lindernia ruellioides* (Colsmann) Pennell from Mizoram, India. *Asian Pacific Journal of Tropical Biomedicine*. *15*(4): 169-176.

Book Chapter

Lalthansangi, C., **Lalremtluangi, R. K**., Lalhmingliani, E., & Vabeiryureilai, M. (2023). Medicinal Plants With Antibacterial And Wound Healing Activity: A review. In P. Shankarishan, Deepti, S, Seshadri, & B. Pankhaniya (Eds.), *Futuristic Trends in Biotechnology* (pp. 192-208). IIP Series.

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DEPARTMENT: ZOOLOGY

TITLE OF THE THESIS: ASSESSMENT OF THE FREE RADICAL SCAVENGING, ANTIBACTERIAL AND WOUND HEALING ACTIVITIES OF *PILEA SYMMERIA* WEDDELL. IN SWISS ALBINO MICE.

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ABSTRACT

ASSESSMENT OF THE FREE RADICAL SCAVENGING, ANTIBACTERIAL AND WOUND HEALING ACTIVITIES OF *PILEA SYMMERIA* WEDDELL. IN SWISS ALBINO MICE.

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

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JULY, 2025

ASSESSMENT OF THE FREE RADICAL SCAVENGING, ANTIBACTERIAL AND WOUND HEALING ACTIVITIES OF *PILEA SYMMERIA* WEDDELL. IN SWISS ALBINO MICE.

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CONSOLIDATED ABSTRACT

Wounds are a common issue encountered in daily life, and appropriate treatment is essential to prevent their development into chronic, non-healing conditions. One major complication associated with wounds is infection, which can significantly delay healing. While modern antibiotics are widely used to manage wound infections, the growing problem of antibiotic resistance has created an urgent need for safer and more effective alternatives, particularly from natural sources. Oxidative stress, another key factor that contributes to delayed wound healing and various life threatening diseases, also warrants serious attention. Research has shown that medicinal plants are rich in phytochemicals with antioxidant properties, which may help mitigate oxidative stress and support the healing process. Given India's rich biodiversity and the abundance of medicinal plants used in traditional practices, there is great potential to explore and scientifically validate these natural remedies. Although several medicinal plants have already been studied, many remain uninvestigated despite long-standing traditional claims of their therapeutic value. *Pilea symmeria* is one such plant traditionally used for wound healing in the state of Mizoram. However, scientific evidence supporting its medicinal properties is currently lacking. This study aims to bridge that gap by systematically evaluating the plant's wound healing potential. This thesis is broadly divided into five main objectives to provide a comprehensive scientific assessment of *Pilea symmeria*.

Chapter 1 outlines the extraction method of *Pilea symmeria*, along with the analysis of its phytochemical components and its ability to scavenge free radicals.

Chapter 2 explores the *in vitro* antibacterial activity of *Pilea symmeria* against three bacterial strains: *Escherichia coli*, *Bacillus subtilis*, and *Klebsiella pneumoniae*.

Chapter 3 presents the *in vivo* evaluation of the wound healing potential of *Pilea symmeria* using excision and incision wound models in Swiss albino mice.

Chapter 4 investigates the impact of topically applied *Pilea symmeria* ethanol extract on the antioxidant profile within the excision wound tissues of the treated mice.

Chapter 5 examines the effect of *Pilea symmeria* ethanol extract on gene expression related to wound healing in excision wounds following topical application.

Chapter 1: To determine the phytochemical content and free radical scavenging activity of *Pilea symmeria* in a cell-free system.

Abstract

The leaves of *P. symmeria* were subjected to cold maceration using chloroform, ethanol, and aqueous as a solvents. The phytochemical composition of the extracts was analyzed through both qualitative and quantitative phytochemical screening as well as Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. The antioxidant potential of the extracts was evaluated by assessing their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and superoxide ($O_2^{\bullet -}$) (SO) radicals. The extracts were found to contain a range of bioactive phytochemicals, such as terpenoids, tannins, flavonoids, cardiac glycosides, steroids, alkaloids, saponins, and phlobatannins. LC-MS analysis revealed the presence of 34 major compounds with diverse biological properties, including anti-inflammatory, antibacterial, and wound healing activities. Among the three extracts, the ethanol extract demonstrated the strongest free radical scavenging activity. It exhibited the highest phenolic and flavonoid content while also having the lowest IC_{50} value. These findings suggest that *P. symmeria* leaf extracts, enriched with bioactive phytochemicals such as flavonoids and phenolics, exhibit strong antioxidant potential, as demonstrated by their radical scavenging activity, supporting their potential for further pharmacological and therapeutic applications.

Chapter 2: *In vitro* evaluation of the antibacterial properties of *P. symmeria*

Abstract

Various extracts of *Pilea symmeria* were evaluated for their antibacterial potential through *in vitro* studies. The antibacterial activity was determined using the disc diffusion method against three bacterial strains: *Escherichia coli (*ATCC strain 25922), *Bacillus subtilis* (ATCC strain 11447), and *Klebsiella pneumoniae* (ATCC strain 10031). The extracts were further analyzed for their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The findings revealed that the antibacterial activity of *P. symmeria* extracts was concentration dependent, with higher concentrations leading to greater inhibition of bacterial

growth. Among the three tested extracts, both ethanol and chloroform extracts displayed similar antibacterial potency, effectively inhibiting the growth of the selected bacterial strains. However, the aqueous extract exhibited weaker antibacterial activity in comparison to the ethanol and chloroform extracts, suggesting that the choice of solvent plays a crucial role in extracting bioactive compounds with antibacterial properties. Therefore, this study reveals that *P. symmeria* possesses significant antibacterial properties and has the potential to combat wound infections and other bacterial infection related complications.

Chapter 3: Evaluation of the wound healing properties of *Pilea symmeria* using excision and incision wound models.

Abstract

The wound healing potential of various extracts from *Pilea symmeria* was evaluated using a mouse model to assess its therapeutic properties. In this experiment, mice were inflicted with both excision and incision wounds to simulate common types of injuries. The animals were divided into four distinct groups, each consisting of six animals. Group I, the negative control group, were treated with simple ointment base (SO), while group II, the positive control group, received 5% povidone iodine (PVI), which is widely recognized for its antiseptic and wound healing properties. Groups III and IV were treated with ointments containing 5% and 10% *Pilea symmeria* ethanol extract (PSEE), respectively, which were prepared using polyethylene glycol (PEG) as the base. The ointments were applied topically to the wounds till the day of epithelialization. The results demonstrated a significant ($p < 0.05$) increase in the rate of wound contraction in the group treated with *P. symmeria* extracts compared to the SO treated group. In addition, the time required for epithelialization, the process of skin regeneration and closure was notably shorter in the treatment groups, this was further confirmed by histopathological analysis. The breaking strength of the incision wounds was also significantly ($p < 0.05$) improved in the PVI 5%, PSEE 5%, and PSEE 10% treated groups when compared to the SO treated group. These findings suggest that *P. symmeria* extract facilitates wound healing by accelerating tissue regeneration and improving wound strength.

Chapter 4: Evaluation of the effect of *P. symmeria* on the antioxidant/oxidant status in a wound bearing swiss albino mice.

Abstract

Various extracts of *Pilea symmeria* were tested for their antioxidant properties in mice with excision wounds. The mice were inflicted with excision wound and divided into four groups, each consisting of six animals. Group I (negative control) animals were treated with simple ointment base (SO); group II (positive control) received 5% povidone iodine (PVI); groups III and IV were treated with 5% and 10% *Pilea symmeria* ethanol extract (PSEE) ointments respectively. The ointments were applied topically to the wounds each day, and the wound tissue was removed on day 16. To assess the antioxidant status of the excision wound tissue, several key biomarkers were measured, including reduced glutathione (GSH), glutathione S-transferase (GST), superoxide dismutase (SOD), and malondialdehyde (MDA). The results revealed that, compared to the SO treated group, the groups treated with PVI 5%, PSEE 5%, and PSEE 10% showed a significant ($p<0.05$) increase in the levels of antioxidant markers such as GSH, GST, and SOD and a significant ($p<0.05$) decrease in MDA levels . Our findings indicate that *P. symmeria* extracts effectively boost antioxidant defenses in the wound tissue. Additionally, the marked reduction in MDA levels points to a decrease in oxidative stress and lipid peroxidation.

Chapter 5: To study the effect of *P. symmeria* on the gene expression involved in wound healing by using RT-PCR.

Abstract

Wound healing is a dynamic and highly regulated process involving a cascade of cellular and molecular events coordinated by cytokines, growth factors, and chemokines. This study aimed to evaluate the effects of *Pilea symmeria* ethanol extract (PSEE) on mice excision wound healing by analyzing the gene expression of *Tnf-α* and *Vegf*, two key regulators involved in inflammation and angiogenesis, respectively. The wounds were topically treated with PSEE (5% and 10%) and compared to animals treated with the standard antiseptic povidone iodine (PVI 5%), serving as the positive control group, and those treated with simple ointment (SO)

alone, serving as the negative control group. Granulation tissues were collected on days 4, 8, and 12 post wounding, and gene expression was analyzed using real-time PCR. The results showed significant downregulation of *Tnf-α* in the PSEE 5%, 10% and PVI 5% treated groups compared to the SO treated group, suggesting early resolution of inflammation. *Vegf* expression was significantly upregulated at days 4 and 8 in the PSEE 5%, PSEE 10% and PVI 5% treated groups, peaking during the proliferative phase, followed by downregulation at day 12, indicating accelerated angiogenesis and vascular stabilization. In contrast, *Vegf* expression remained elevated at day 12 in the SO treated group, suggesting delayed healing. These findings demonstrate that *Pilea symmeria* extract promotes wound healing by regulating the expression of key genes involved in inflammation and angiogenesis, supporting its potential as a natural therapeutic agent for enhancing tissue repair.