ENRICHMENT OF FLAVONOIDS FROM KENAF (*Hibiscus cannabinus* L.) USING MACROPOROUS ADSORPTION RESINS AND EVALUATION OF THEIR DIABETIC WOUND HEALING PROPERTIES ON ADULT ZEBRAFISH MODEL

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by

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Raghad Aljawarneh

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LIST OF ABBREVIATIONS

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin I-converting Enzyme
AgNP	Silver Nanoparticle
BC	Before Christ
ChCl	Choline Chloride
DPW	Days Post Wounding
DES	Deep Eutectic Solvent
DM	Diabetes Mellitus
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
H	High
H2O2	Hydrogen Peroxide
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
LC50	Half maximal inhibitory concentration
L	Low
IL-3	Interleukin-3
IL-12	Interleukin-12
KFEE	Kenaf Flavonoid Enrich Extract
KSB	Kenaf Seed Beverage
LC-MS-MS	Liquid Chromatography Tandem Mass Spectrometry
LDL	Low Density Lipoprotein

LSR	Liquid Solid Ratio
MAE	Microwave Assisted Extraction
MARs	Macroporous Adsorption Resins
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MPRs	Macroporous Polymer Resins
NaDES	Natural Deep Eutectic Solvents
NAR	Narirutin
NHEM	Normal Human Epidermal Melanocytes
PLE	Pressurized Liquid Extraction
SC	Sodium Caseinate
SDVB	Styrene Divinylbenzene
SFE	Supercritical Fluid Extraction
SPE	Solid Phase Extraction
T20	Tween 20
T2DM	Type 2 Diabetes Mellitus
TFC	Total Flavonoid Content
TNF-α	Tumor Necrosis Factor alpha
TPC	Total Phenolic Content
TPTZ	2,4,6-Tripyridyl-s-Triazine
UAE	Ultrasound-Assisted Extraction
YMC	Yeast and Mould Count

PENGAYAAN FLAVONOID DARIPADA KENAF (*Hibiscus cannabinus* L.) MENGGUNAKAN RESIN PENJERAPAN MAKROPORI DAN EVALUASI CIRI PENYEMBUHAN LUKA DIABETIK PADA MODEL ZEBRAFISH DEWASA

ABSTRAK

Diabetes mellitus adalah penyakit kronik yang menjejaskan jutaan orang di seluruh dunia, dengan kira-kira 340 juta kes yang didiagnosis. Satu komplikasi ketara adalah penyembuhan luka, yang menjejaskan hampir 25% pesakit diabetik. Rawatan konvensional, termasuk ubat-ubatan, pembalut, dan pembedahan, adalah mahal dan mungkin menyebabkan kesan sampingan. Oleh itu, alternatif semula jadi seperti flavonoid semakin mendapat perhatian kerana sifat antioksidan, anti-radang, dan antimikrobanya. Kenaf (*Hibiscus cannabinus*), sejenis tumbuhan yang kaya dengan flavonoid, mempunyai potensi terapeutik untuk penyembuhan luka diabetik. Kajian ini bertujuan untuk mengekstrak dan memperkayakan flavonoid daripada kenaf serta menilai ciri antioksidan, anti-radang dan penyembuhan luka mereka menggunakan model zebrafish dewasa bukan diabetik dan diabetik (*Danio rerio*). Flavonoid diekstrak daripada daun, bunga, dan kuntum kenaf menggunakan pengekstrakan dibantu ultrasonik dengan pelarut eutektik semula jadi (NaDES), diikuti dengan pengayaan melalui kromatografi berbantu resin makropori menggunakan resin HP-20, XAD-4, dan LXA-817. Penjerapan dioptimumkan pada pH 7, 338 K, dan kepekatan flavonoid awal 20 mg/mL, mencapai titik keseimbangan dalam masa 480 minit. Proses ini mengikut kinetik tertib pseudo-kedua dan isoterma Freundlich, menunjukkan penjerapan berlapis. Analisis termodinamik mengesahkan sifat endotermik penjerapan. Desorpsi menggunakan kecerunan etanol menghasilkan pengayaan

flavonoid sebanyak 11 kali ganda, 10 kali ganda, dan 5 kali ganda masing-masing untuk HP-20, XAD-4, dan LXA-817. Analisis HPLC-MS/MS mengenal pasti flavonoid utama, termasuk derivatif kaempferol, apigenin, kuersetin, dan mirisetin. Kedua-dua ekstrak kasar dan ekstrak diperkaya flavonoid menunjukkan aktiviti antioksidan yang kuat, dengan ekstrak diperkaya flavonoid kenaf (KFEE) menunjukkan kesan anti-radang yang lebih baik berbanding dengan ekstrak kasar dan ubat rujukan. Untuk menilai keberkesanan penyembuhan luka, zebrafish dirawat dengan KFEE. Ujian ketoksikan menentukan LC_{50} sebanyak 71 mg/mL, tanpa kematian di bawah 50 mg/mL. Zebrafish diabetik diinduksi menggunakan glukosa 2% selama empat minggu sebelum penciptaan luka. Zebrafish dibahagikan kepada kumpulan kawalan, kawalan positif (dirawat dengan allantoin), dos rendah (6.25 mg/L KFEE), dan dos tinggi (25 mg/L KFEE). Menjelang hari ke-30, zebrafish normal yang dirawat dengan KFEE pada dos tinggi dan rendah mencapai penutupan luka sebanyak masing-masing 95% dan 90%, mengatasi kawalan positif (85%) dan kumpulan zebrafish yang tidak dirawat (80%). Dalam zebrafish diabetik, kumpulan KFEE dos tinggi dan rendah menunjukkan penutupan luka sebanyak 80% dan 75%, melebihi kawalan positif (67%) dan negatif (60%). Walaupun penyembuhan luka lebih perlahan dalam zebrafish diabetik, rawatan dengan KFEE pada 25 mg/L meningkatkan penyembuhan sebanyak 1.18 kali ganda dalam kumpulan normal dan 1.34 kali ganda dalam kumpulan diabetik berbanding kawalan, menunjukkan potensinya sebagai terapi semula jadi untuk luka diabetik. Secara keseluruhan, kajian ini menunjukkan bahawa flavonoid yang diperoleh daripada kenaf, terutamanya dalam bentuk ekstrak diperkaya flavonoid, mempunyai ciri antioksidan, anti-radang, dan penyembuhan luka yang kuat, menyokong potensi mereka dalam penjagaan luka diabetik.

ENRICHMENT OF FLAVONOIDS FROM KENAF (*Hibiscus cannabinus* L.) USING MACROPOROUS ADSORPTION RESINS AND EVALUATION OF THEIR DIABETIC WOUND HEALING PROPERTIES ON ADULT ZEBRAFISH MODEL

ABSTRACT

Diabetes mellitus is a chronic disease affecting millions worldwide, with approximately 340 million diagnosed cases. A significant complication is impaired wound healing, affecting nearly 25% of diabetic patients. Conventional treatments, including medications, dressings, and surgeries, are costly and may cause side effects. As a result, natural alternatives such as flavonoids are gaining interest for their antioxidant, anti-inflammatory, and antimicrobial properties. Kenaf, a plant rich in flavonoids, has potential therapeutic benefits for diabetic wound healing. This study aimed to extract and enrich flavonoids from kenaf and evaluate their antioxidant, anti-inflammatory and wound healing properties using both non-diabetic and diabetic adult zebrafish (*Danio rerio*) models. Flavonoids were extracted from kenaf leaves, flowers, and bulbs using ultrasonic-assisted extraction with natural deep eutectic solvents (NaDES), followed by enrichment via macroporous resin chromatography using HP-20, XAD-4, and LXA-817 resins. Adsorption was optimized at pH 7, 338 K, and 20 mg/mL initial flavonoid concentration, reaching equilibrium at 480 minutes. The process followed pseudo-second-order kinetics and the Freundlich isotherm, indicating multilayer adsorption. Thermodynamic analysis confirmed the endothermic nature of adsorption. Desorption using ethanol gradients yielded 11-fold, 10-fold, and 5-fold flavonoid enrichment for HP-20, XAD-4, and LXA-817, respectively. HPLC-MS/MS analysis identified key flavonoids, including kaempferol derivatives,

apigenin, quercetin, and myricetin. Both crude and enriched extracts demonstrated strong antioxidant activity, with KFEE exhibiting superior anti-inflammatory effects compared to the crude extract and the reference drug. To evaluate wound healing efficacy, zebrafish were treated with KFEE. Toxicity tests determined an LC_{50} of 71 mg/mL, with no mortality below 50 mg/mL. Diabetic zebrafish were induced using 2% glucose for 28 days before wound creation. Zebrafish were divided into control, positive control (allantoin-treated), low-dose (6.25 mg/L KFEE), and high-dose (25 mg/L KFEE) groups. By day 30, normal zebrafish treated with high and low-dose KFEE achieved 95% and 90% wound closure, respectively, outperforming the positive control (85%) and untreated groups (80%). In diabetic zebrafish, high-and low-dose KFEE groups exhibited 80% and 75% wound closure, exceeding positive (67%) and negative (60%) controls. While wound healing was slower in diabetic zebrafish, treatment with KFEE at 25 mg/L enhanced healing by 1.18-fold in normal and 1.34-fold in diabetic groups compared to untreated control group, demonstrating its potential as a natural therapeutic for diabetic wounds. Overall, this study demonstrates that kenaf-derived flavonoids, particularly in enriched form, possess potent antioxidant, anti-inflammatory, and wound healing properties, supporting their potential in diabetic wound care.

CHAPTER 1

INTRODUCTION

1.1 Research Background

An injury to the skin or underlying tissue is defined as a wound, which can result from physical or thermal damage and may disrupt normal anatomical structure and function. Wound healing is a complex and natural process that involves the replacement of damage or missing tissue with freshly produced tissue (Boateng and Catanzano 2015). The wound healing process consists of four distinct stages: haemostasis, inflammation, proliferation, and remodelling. The haemostasis stage immediately follows an injury, aiming to prevent blood loss by forming a clot. The inflammatory stage then works to clean the wound of any bacteria and debris that could cause an infection, while also preparing the wound area for new tissue growth. Next, the proliferative stage involves the formation of granulation tissue, angiogenesis, and epithelial cell migration to close the wound. Finally, the remodelling stage involves the maturation and organization of the new tissue to restore the original tissue structure (Young and McNaught 2011). Optimal wound healing requires the wound healing stages to occur in a precise order, with correct timing and intensity.

Diabetes mellitus (DM) is a major contributor to impaired wound healing, as it often disrupts the normal wound healing process. In fact, diabetes can reduce blood circulation, which impairs the delivery of oxygen, essential nutrients, and immune cells to the injured area, leading to delays in the healing process (Burgess et al. 2021). Globally, there are currently 340 million people living with diabetes, and approximately 25% of them experience impaired wound healing (Li et al. 2021). Various treatment options have been employed for diabetic wounds, including

antidiabetic drugs, dressings, growth factors, and hyperbaric oxygen therapy (Chakraborty et al. 2022). However, these approaches are often costly, may cause side effects and do not always lead to effective wound healing. Therefore, it is essential to develop natural, safe, affordable, and effective treatment method that enhances the healing of diabetic wounds. For this reason, there is growing attention towards the use of traditional medicinal plants in treating diabetic wounds.

Medicinal plants are important sources of bioactive compounds, including glycosides, steroids, essential oils, saponins and flavonoids. These compounds have a wide range of therapeutic potential and can be effective against several diseases. The presence of such compounds in plants has prompted researchers to investigate the role of medicinal plants in assessing their potential for diabetic wound healing. Several medicinal plants, including *Aloe vera* (Chithra et al. 1998), *Portulaca oleracea* (Rashed et al. 2003), *Leucas lavandulaefolia* (Saha et al. 1997), *Calotropis procera* (Rasik et al. 1999), and *Butea monosperma* (Sumitra et al. 2005), have demonstrated wound healing properties.

Kenaf, scientifically known as *Hibiscus cannabinus L*., is an annual herbaceous plant belonging to the Malvaceae family. It is widely distributed in Asia and is originally native to Africa. Kenaf is a versatile plant with a wide range of applications, including fibre production, food, animal feed, and traditional medicine. It is recognized as a cost-effective and eco-friendly fiber crop due to its rapid growth, high biomass yield, and adaptability. Kenaf fibres have various industrial uses, such as paper and pulp production, textiles, bio-composites, and absorbent materials (Alexopoulou et al. 2000). However, the extensive use of kenaf stems for fiber production has led to the underutilization of the plant's other parts (Vitrone et al. 2021). The plant also has

applications in food and animal feed production, with its leaves, flowers, and seeds utilized for several edible products (Giwa Ibrahim et al., 2019). In traditional medicine, kenaf has long been used to treat a range of ailments, including as Guinea worm disease, anemia, biliousness, bruises, coughs, throat issues, and diabetes (Ezzadin et al. 2022; Kujoana et al. 2023). In recent times, metabolites extracted from kenaf plant have been demonstrated to possess significant pharmacological effects including antioxidant (Yusri et al. 2012; Chan et al. 2014; Adnan et al. 2020), antimicrobial (Nilugal et al. 2016; Birhanie et al. 2021; Arulrajah et al. 2022), anticancer (Abd Ghafar et al. 2012; Foo et al. 2012; Abd Ghafar et al. 2013), anti-hypertensive (Zaharuddin et al. 2021b), anti-inflammatory (Lee et al. 2007; Nyam et al. 2015a; Shaikh 2016), and antidiabetic (Kumar et al. 2011; Elias 2020). However, their therapeutic potential in diabetic wound healing has not been explored yet and requires further investigation.

Flavonoids, widely distributed in plants in nature, are considered secondary metabolites that belong to the category of polyphenolic compounds. Their chemical structure is based on a fifteen-carbon skeleton made up of C6 (A ring), C3 (C ring) and C6 (B ring). Ring A and ring B are linked by a heterocyclic pyrene ring (C) containing oxygen (Feng et al. 2017). Flavonoids are known for their potential health benefits and have been extensively studied for their medical properties. Modern pharmacological studies have demonstrated that flavonoids extracted from medicinal plants play a pivotal role in accelerating the diabetic wound healing process through various mechanisms. Flavonoids possess the ability to enhance the healing of diabetic wounds by exhibiting antioxidant, anti-inflammatory, antimicrobial, and angiogenic properties that are crucial for the regeneration of new tissues (Carvalho et al. 2021). Oxidative stress is a major factor contributing to the non-healing of diabetic wounds.

This oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the ability of cells to scavenge them. Therefore, the antioxidant properties of flavonoids play a vital role in improving the healing of diabetic wounds by decreasing the level of oxidative stress through the scavenging of ROS(Zulkefli et al. 2023). Flavonoids have also demonstrated potent anti-inflammatory capabilities, which can enhance the healing of diabetic wounds. This is achieved through their ability to reduce inflammatory cytokines, inhibit key enzymes, and promote anti-inflammatory cytokines by modulating important inflammatory pathways (Rajapaksha et al. 2020a). Moreover, flavonoids have been shown to exhibit antimicrobial properties that can help reduce the risk of microbial infection in diabetic wounds and enhance the healing process (Sychrová et al. 2022). Furthermore, flavonoids promote the expression of biomarkers associated with angiogenesis, which is essential for the formation of new blood vessels in the wound area (Liu et al. 2022a).

Despite the wide range of medicinal applications of flavonoids, their extraction and purification remain a significant challenge. Traditional extraction methods are often time-consuming, require large solvent volumes, and may result in the co-extraction of unwanted compounds (Martins et al. 2023). Additionally, the purification of flavonoids is crucial to ensure the desired therapeutic effects and minimize potential adverse reactions. In contrast, the use of natural deep eutectic solvents ultrasound-assisted extraction (NaDES-UAE) combined with macroporous resin purification, presents a new approach that can address these challenges and provide a sustainable solution for large-scale flavonoid production (Denga et al. 2023). Therefore, it becomes imperative to enhance the quantity, and the purity of flavonoids extracted from medicinal plants to improve their therapeutic efficacy in diabetic wound healing.

Mammalian animal models such as mice, rats, and rabbits have been widely used to examine diabetic wound healing. However, these models come with limitations including high cost, time-consuming nature, ethical concerns, and complex genetic backgrounds (Zain et al. 2021a). Recently, the zebrafish has emerged as a promising alternative model for studying diabetic wound healing (Caraguel et al. 2016; Richardson et al. 2016; Seo et al. 2017; Rajapaksha et al. 2020b; Zain et al. 2021a; Siregar et al. 2024). The zebrafish possesses several advantageous characteristics that make it an attractive model for investigating diabetic wound healing including lower maintenance cost requirements, easier to control experimental conditions, shorter testing and generation times and higher genetic similarities with humans (Lin et al. 2023). Also, zebrafish can develop diabetes faster than other animal models under the same experimental conditions. Additionally, zebrafish models allow for rapid high-throughput screening of novel therapeutics for diabetic wound healing, which can accelerate the drug discovery process (Naomi et al. 2021).

1.2 Problem Statement

Non-healing wounds, particularly in diabetic patients, represent a major health issue and therapeutic challenge. Despite of diversity of the available medications and treatment options, the successful treatment of diabetic wounds remains a significant therapeutic challenge. Furthermore, wound care contributes to a substantial economic burden, with global expenditure estimated at approximately USD 299.482 billion in 2019 (Queen and Harding 2023). Flavonoids extracted from different parts of the kenaf plant have demonstrated significant pharmacological activities, including antioxidant, antibacterial, anticancer, anti-hypertensive, anti-inflammatory, and antidiabetic

properties. However, their therapeutic potential in treating diabetic wound healing has yet to be explored.

Current research on kenaf flavonoids mainly examines the biological activities of flavonoids in crude plant extracts. These crude extracts often contain additional compounds, such as pigments, proteins, and carbohydrates which can potentially impact the biological activities of flavonoids. As a result, there is a pressing need to extract and enriched the flavonoids from kenaf to better understand their individual biological activities and enhance their therapeutic effectiveness in diabetic wound healing.

Numerous techniques, such as solid-liquid extraction (Aguilera 2003), liquid-liquid extraction (Liu et al. 2008), supercritical fluid extraction (He et al. 2012), gel column chromatography (Sun et al. 2014), sephadex LH-20 column chromatography (Mottaghipisheh and Iriti 2020), and preparative high-performance liquid chromatography (Wen et al. 2017), have been developed to purify flavonoids from various medicinal plants. However, these purification methods often have limitations, including excessive solvent consumption, lengthy processing times, high costs, and low recovery rates. In contrast, macroporous resins have emerged as an effective alternative for separating and purifying plant secondary metabolites, including flavonoids. These resins offer several advantages, such as cost-effectiveness, rapid adsorption capabilities, reusability, improved efficiency, and ease of use (Hou et al. 2019). Despite these advantages, the use of macroporous resins for the purification of flavonoids from kenaf has not been reported in the literature.

Therefore, this research aims to address these critical gaps by extracting and purifying flavonoids from different parts of the kenaf plant using various macroporous

adsorption resins. By optimizing the flavonoids purification process, this study will provide a more comprehensive evaluation of their biological activities, specifically their antioxidant, anti-inflammatory and wound healing properties. Furthermore, this research seeks to contribute valuable insights into the development of more effective, botanical-based therapeutic approaches for diabetic wound healing.

1.3 Research Objectives

This study aims to evaluate the potential use of different parts of kenaf as a source of flavonoids and evaluate their potential use in diabetic wound healing.

The specific objectives are:

1. To optimize the enrichment of total flavonoid content (TFC) from the kenaf extract (leaves, flowers, and bulbs) using ultrasonic-natural deep eutectic solvent assisted extraction integrated with macroporous resin.

2. To fully characterize the metabolite profile of several optimized different kenaf flavonoid enriched extracts using the HPLC-ESI-MS/MS approach.

3. To evaluate the antioxidant, anti-inflammatory, normal and diabetic wound healing properties of the derived kenaf flavonoid enriched extracts using *in vitro* colorimetric assays and adult zebrafish.

1.4 Scope of the Study

This study focuses on extracting and enhancing total flavonoids from different parts of the kenaf plant and evaluating their antioxidant and wound healing properties. The scope of the study includes the extraction and fractionation of total flavonoids

from different parts of the kenaf plant (leaves, flowers, and bulbs) using NaDES-UAE method integrated with macroporous adsorption resin column chromatography method. It also involves metabolite profiling of both the crude and enriched flavonoid extracts using the HPLC-ESI-MS/MS method. *In vitro* evaluation of antioxidant and anti-inflammatory properties of the crude enriched flavonoid extracts was determined using standard assays including Ferric Reducing Antioxidant Power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and protein denaturation assay. Additionally, *in vivo* evaluation of normal and diabetic wound healing properties of the kenaf flavonoid enriched extracts was conducted on adult zebrafish model using the immersion technique.

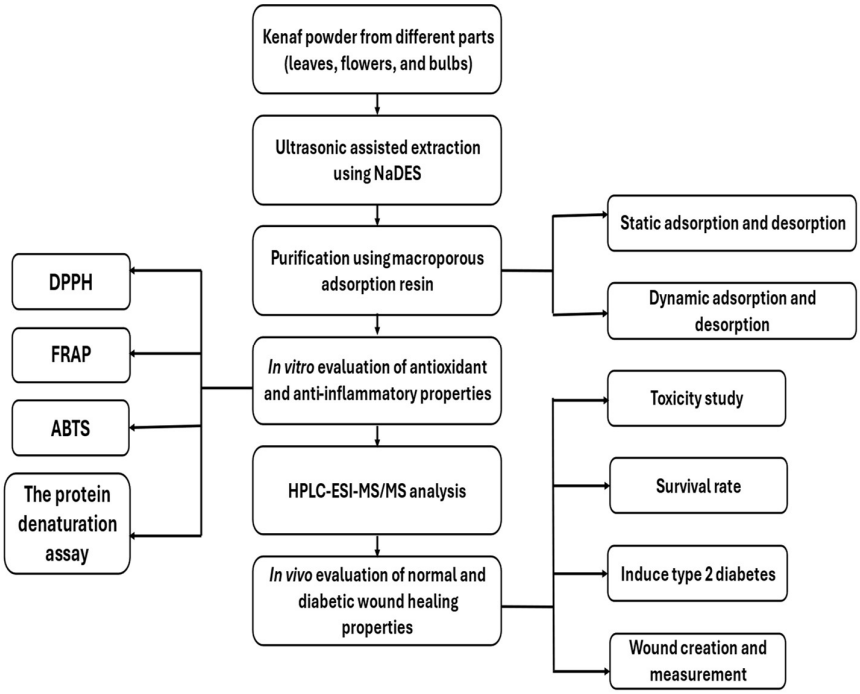

Figure 1.1 Scope of the study

1.5 Significant of the study

This study aims to evaluate the potential of flavonoid-rich extracts derived from different parts of the kenaf plant to enhance wound healing in a diabetic zebrafish model. By extracting and enriching flavonoids known for their antioxidant and antimicrobial properties, the research seeks to determine their efficacy in improving the healing process of diabetic wounds. The findings of this study could lead to the development of cost-effective, plant-based treatments for diabetic wound healing, offering promising alternatives to current therapeutic options and advancing the fields of wound care and regenerative medicine.

1.6 Organization of the Thesis

Chapter 1 provides an overview of the research background, highlighting the therapeutic potential of flavonoids extracted from medicinal plants, such as kenaf, for enhancing the healing of diabetic wounds. This chapter also outlines the problem statement, research objectives, the scope and significance of the study, and the organization of the thesis. Chapter 2 provides a comprehensive review of the kenaf plant, focusing on its botanical description, secondary metabolites and their biological activities. It also explores the role of macroporous resins in purifying flavonoids from medicinal plants, highlighting their efficiency in isolating bioactive compounds. The chapter further discusses the potential of flavonoids in enhancing diabetic wound healing. Finally, it reviews the use of the zebrafish model in studying diabetic wound healing, underscoring its advantages in assessing the biological effects of flavonoids *in vivo*. subsequently, Chapter 3 outlines the materials and methods used throughout the research. The chapter describes the experimental procedures in detail for the extraction, purification, and characterization of flavonoids from kenaf. Furthermore,

the chapter outlines the *in vitro* and *in vivo* assays used to evaluate the antioxidant, anti-inflammatory, and wound healing properties of these compounds. Next, Chapter 4 covers the results and discussion sections, presenting all the acquired results and explaining the findings in detail, followed by an in-depth discussion of the results. Lastly, Chapter 5 summarizes the key findings, addresses the implications of the research, and suggests potential areas for future research to further explore the therapeutic potential of kenaf derived flavonoids in diabetic wound healing.

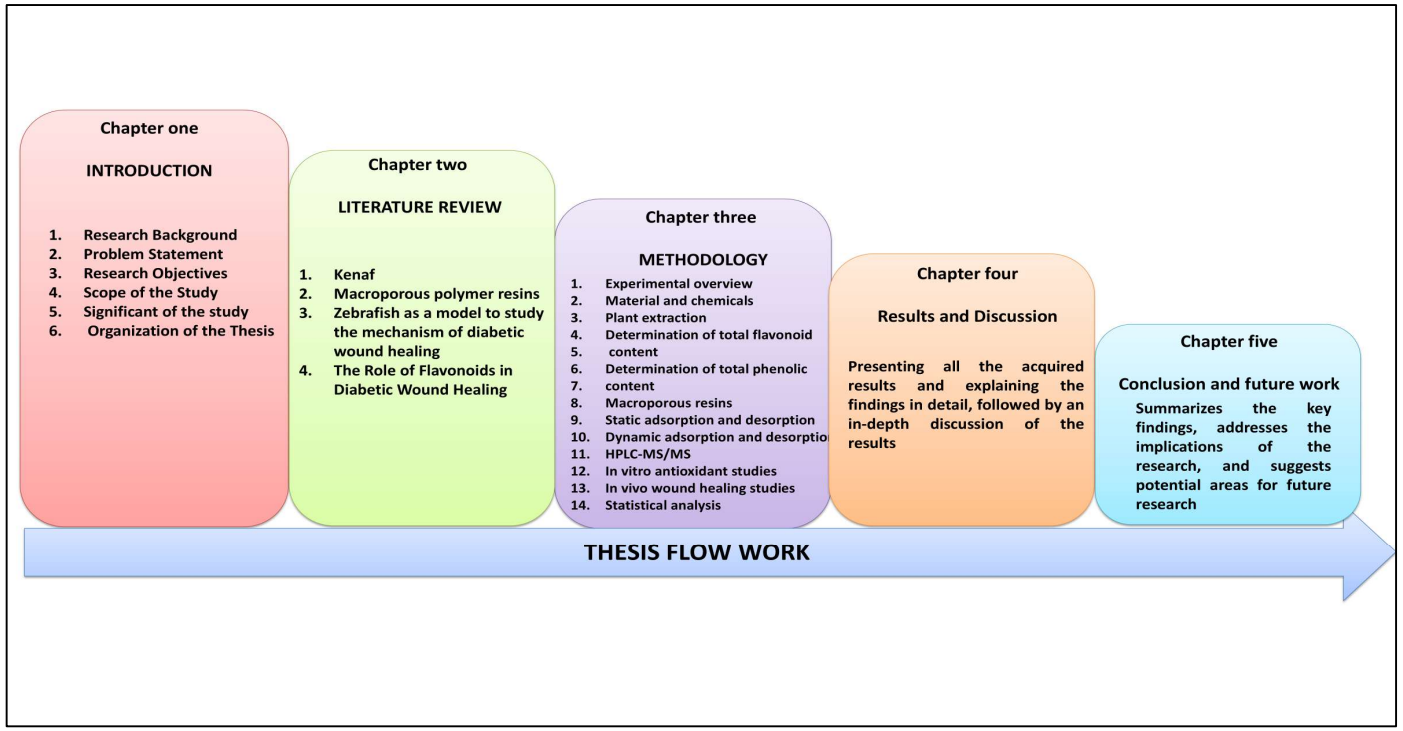

Figure 1.2 Organization of the thesis

CHAPTER 2

LITERATURE REVIEW

2.1 Kenaf

Kenaf is an annual herbaceous plant belonging to the Malvaceae family. This plant is closely related to several well-recognized species, such as cotton (*Gossypium hirsutum L.*), okra (*Hibiscus esculentus*), hollyhock (*Althaea rosea*), and roselle (*Hibiscus sabdariffa*) (Alexopoulou et al. 2013; Cheng et al. 2020). Originating in Africa around 4000 BC, kenaf has been cultivated for centuries across various regions, particularly in Asia. The plant was introduced to India approximately 200 years ago, to Russia in 1902, to China in 1935, and to the United States in 1950 (Webber III et al. 2002). Consequently, kenaf is known by diverse regional names depending on its geographic location, including Til in North Africa, Gambo in West Africa, Stokroos in South Africa, Mesta in India, Ambari in Taiwan, and Java Jute in Indonesia (Norhisham et al. 2023a). Kenaf is currently cultivated extensively in more than 20 countries worldwide, indicating its significance as an important and versatile crop.

Kenaf has a long history of various applications including fibre production, food, animal feed, and traditional medicine. Many countries recognize it as a cost-effective and eco-friendly fibre crop due to its rapid growth, high biomass yield, and adaptability to various growing conditions (Ayadi et al. 2017a). The high-quality fibres are obtained directly from the dried stem materials and have a wide range of industrial uses, such as paper and pulp production, textile manufacturing, bio-composite fabrication, and the creation of absorbent materials (Alexopoulou et al. 2000). Kenaf

has also found applications in food and animal feed production due to its high protein content (Giwa Ibrahim et al. 2019a). For instance, the leaves and flowers of kenaf are consumed as a Vegetable in some regions, kenaf seeds are utilized to produce edible oil, margarine, and flour (Giwa Ibrahim et al. 2019b) and dried kenaf powder is used as an ingredient in various food preparations. Furthermore, the stalks and leaves of kenaf are employed as livestock feed providing a cost-effective and readily available source of nutrition for animals (Popoola et al. 2024). In addition to its industrial and agricultural applications, Kenaf has a long history of traditional medicinal use in Africa and India (Ayadi et al. 2017b). In these regions, the plant has been employed to treat a variety of ailments. For example, kenaf leaves and stems have been utilized to treat conditions such as Guinea worm disease and anemia, while the flower and seed have been consumed to address biliousness and bruises (Kujoana et al. 2023). Furthermore, kenaf leaves have been employed to treat various diseases including coughs, throat issues, and diabetes (Ezzadin et al. 2022). Kenaf has also found applications beyond its traditional uses, including in pet care products like litter, shampoo, and flea/tick powder, as well as in home living items such as mattresses and air purifiers, and even in gardening applications (Norhisham et al. 2023b). As a result of its comprehensive utilization of its different parts across various sectors, kenaf is considered a zero-waste crop.

In recent years, kenaf has been recognized as a medicinal plant due to its rich profile of secondary metabolites, including phenolics, flavonoids, terpenoids, tocopherols, tocotrienols, and fatty acids, which have demonstrated potential health benefits (Ayadi et al. 2017c). Metabolites extracted from various parts of the kenaf plant have shown significant pharmacological effects, such as antioxidant, antibacterial, anticancer, antihypertensive, and antidiabetic properties (Sim and Nyam

2021c). However, there remains a notable gap in the literature regarding a comprehensive understanding of kenaf secondary metabolites and their associated bioactivities. This section aims to address this knowledge gap by providing a detailed overview of kenaf botanical characteristics, its secondary metabolites, extraction techniques and the pharmacological activities of its various components.

2.1.1 Taxonomy and Botanical Description of Kenaf

Kenaf is a vascular plant classified in the plant kingdom, within the subkingdom Tracheobionta. It is part of the superdivision Spermatophyta, which includes seed plants, and is categorized under the division Angiosperms, encompassing flowering plants. Within Angiosperms, kenaf is placed in the class Dicotyledonae (dicots) and the subclass Rosidae. The plant is further classified in the order Malvales and the family Malvaceae. Within this family, kenaf belongs to the genus Hibiscus and the species Hibiscus cannabinus (Alexopoulou et al. 2013; Islam 2019). The taxonomic status of the kenaf plant is presented in Figure 2.1.

Kenaf is a fast-growing and adaptable crop that performs well across a range of climates and soil types. Originating from tropical and subtropical regions, it is optimally suited to warm environments, thriving in temperatures between 20°C and 35°C. Under these ideal conditions, kenaf can attain heights of up to 5 meters (Vayabari et al. 2023a). Furthermore, while it prefers moderate rainfall, kenaf can tolerate drier conditions when provided with supplementary irrigation. In term of soil, Kenaf thrives in well-drained, loamy soils with a pH range of 6.0 to 7.0, and it grows best in soils that are rich in organic matter and have good water retention properties (Alexopoulou et al. 2000).

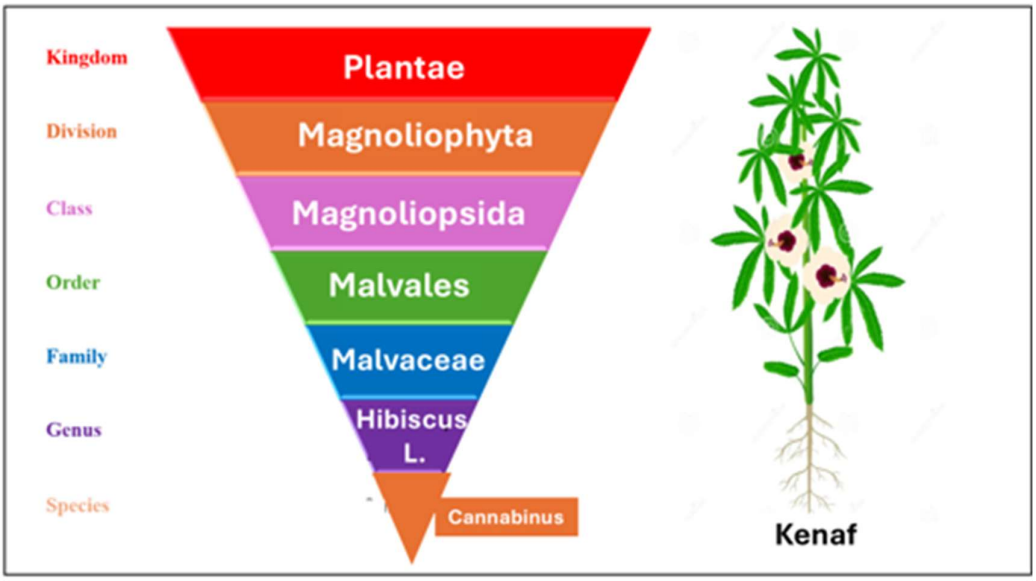

Figure 2.1 Taxonomic status of kenaf plant.

The kenaf plant is composed of six main parts: the stem, leaves, flowers, seeds, fruits and roots, as depicted in Figure 2.2. The kenaf stem, a primary source of the kenaf fibre, can achieve heights of 4 to 6 meters within a growth period of 4 to 6 months (Figure 2.2B). Its colouration varies from green to reddish, influenced by factors such as plant age, variety, soil conditions, and environmental factors (Pratik Satya and Ratikanta Maiti 2013a). The stem is characterized by small, variable-sized thorns distributed along its surface. Structurally, the stem comprises three distinct layers, namely, the bast, pith, and core. Each of these layers produces different types of fibres suited to various industrial applications. The bast fibres are noted for their length and strength, while the core and pith fibres are shorter and possess a woodier texture (Juliana et al. 2018).

Kenaf leaves exhibit a diverse range of shapes, sizes, and colours depending on the cultivar and maturity of the plant (Figure 2.2C and D). Generally, kenaf can produce leaves that are either simple or compound in structure, arranged in an alternating pattern along the stem. The leaves often have a palmate structure,

characterized by three to seven deeply divided lobes (Pratik Satya and Ratikanta Maiti 2013b). The leaf size can range from 10 to 20 cm in length and 8 to 15 cm in width. Kenaf leaves typically have a smooth and glossy surface, with a waxy coating that becomes increasingly rough and hairy on both sides as the leaves mature. The leaf colour can vary from a light green in the younger stages to a more intense green as the leaves age (Vayabari et al. 2023b). In addition to their traditional applications as sources of fibre, food, animal feed, and in folk medicine, recent research has revealed that kenaf leaves exhibit a range of pharmacological activities.

The kenaf plant produces large, bell-shaped flowers typically measuring 8-13 cm in diameter. These flowers have five petals and range in colour from light cream to dark purple (Figure 2.2F and G). They usually grow solitarily from the leaf axils along the stem (Liu 2005). While the individual kenaf flowers have a brief blooming period of just one day, the overall flowering period for the plant can vary from 3 to 4 weeks, depending on the cultivar and environmental conditions, often occurring during the summer months (Xu et al. 2020). Additionally, kenaf flowers are capable of both self-pollination and cross-pollination. Self-pollination occurs when the flower petals close in a twisting motion, while cross-pollination is facilitated by domesticated honeybees (Afzal et al. 2022).

The kenaf fruit has a distinctive hairy, rounded, and coarse segmented structure and each segment contains about 20-26 seeds (Figure 2.2H) (Chew and Nyam 2019a). The kenaf seeds are relatively small, about 2 to 4 millimetres in length and 0.1 to 0.2 grams weight (Asafa et al. 2024). They have a smooth, hard surface and a triangular shape similar to kidney beans, with sharp corners. Their colour ranges from light brown to dark brown with bright yellow dots (Vayabari et al. 2023b). Kenaf seeds

maintain viability for around eight months and can be used to grow new plants or utilized in various agricultural and industrial applications due to their durability and high oil content (Figure 2.2E) (Adebisi et al. 2014). Kenaf has a deep root system that can extend to more than 1 meter in depth. This deep root exploration helps the plant grow and effectively absorb water from deep soil, enabling it to thrive in diverse environments (Figure 2.2I) (Fernando 2013).

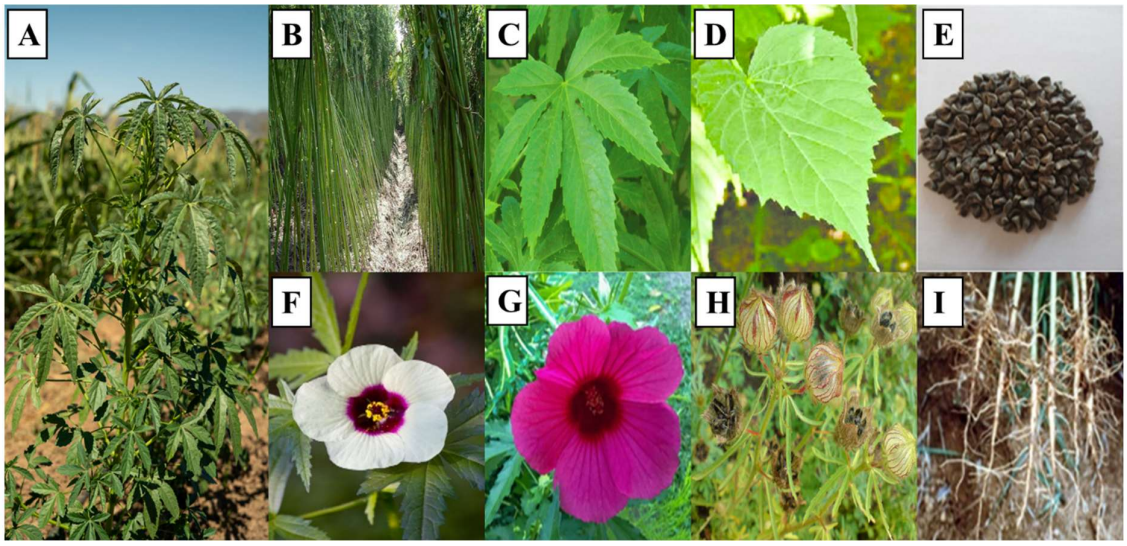

Figure 2.2 Kenaf (A) whole plant. (B) stem. (C) lobed leaves. (D) unlobed leaves. (E) seeds. (F) light creamy flower. (G) dark purple flower. (H) kenaf fruit. (I) kenaf root.

2.1.2 Extraction technologies of kenaf bioactive compounds

Plants produce a wide range of organic molecules called bioactive compounds which are not essential for their primary growth, development, or reproduction. Instead, these compounds play a crucial role in plant defence, aiding in responses to environmental stresses and interactions with other organisms (Böttger et al. 2018). Additionally, bioactive compound hold significant therapeutic potential for human health, offering various pharmacological benefits such as antioxidant, anti-inflammatory, anti-cancer, antifungal and antimicrobial effects.

Extracting bioactive compounds from plants is essential for the pharmaceutical, food, and chemical industries. Conventional extraction methods, such as reflux, maceration, soaking and Soxhlet, are widely used due to their simplicity and low equipment cost. However, these methods have several limitations, including the use of large amounts of organic solvents, significant environmental impact, long extraction times, and lower yields of the desired compounds (Rasul 2018). As a result, there is an increasing need to explore advanced extraction techniques that can overcome these challenges and improve the recovery of valuable bioactive compounds. These methods include UAE (Liu et al. 2022b), microwave-assisted extraction (MAE) (Du et al. 2009), solid-phase extraction (SPE) (Zhu et al. 2023), supercritical fluid extraction (SFE) (Mariod et al. 2011), pressurized liquid extraction (PLE) (Zgórka 2009), and enzyme-assisted extraction (Maier et al. 2008). Several factors, such as the extraction method, raw materials, extraction solvent, and experimental conditions, can influence the efficiency of bioactive compound extraction. Hence, it is crucial to adopt efficient extraction techniques that maximize yield, ensure high product quality, and balance cost-effectiveness with environmental sustainability.

Modern analytical studies have demonstrated that the kenaf plant is a rich source of secondary metabolites, including phenolics, flavonoids, terpenoids, tocopherols, tocotrienols**,** and fatty acids. These metabolites are often found in complex mixtures that may contain undesirable or potentially harmful substances. Therefore, it is crucial to separate these secondary metabolites to maximize their effectiveness and safety for pharmaceutical applications. Researchers have employed various extraction methods to isolate bioactive compounds from the kenaf plant. These methods include conventional techniques such as, Soxhlet, soaking, and reflux and

solvent extraction, as well as innovative techniques like ultrasound-assisted extraction and supercritical fluid extraction. Table 2.1 summarizes the extraction methods, solvents used, and experimental conditions for extracting bioactive compounds from different parts of the kenaf plant. Solvent extraction has been a widely used method for extracting bioactive compounds from Kenaf. However, the extensive use of solvents, including toxic ones, has limited the widespread adoption of this approach (Kobaisy et al. 2001; Nyam et al. 2009; Chew et al. 2017; Ryu et al. 2017; Sulaiman et al. 2024). In contrast, UAE has emerged as the most commonly employed technique for extracting secondary metabolites from kenaf. This method is preferred for its efficiency, cost-effectiveness, environmental sustainability, and its ability to enhance compound yield (Mohamed et al. 1995; Mariod et al. 2012; Wong et al. 2014a; Pascoal et al. 2015a; Park et al. 2020; Chew et al. 2021a; Zaharuddin et al. 2021a; Duan et al. 2024). Additionally, the supercritical fluid extraction technique has been utilized to extract secondary metabolites from Kenaf, likely due to its efficiency in extracting compounds with minimal solvent use, thereby offering a more sustainable and selective extraction process (Mariod et al. 2011; Foo et al. 2012; Abd Ghafar et al. 2013).

The choice of solvent is also crucial in determining the solubility and extraction efficiency of bioactive compounds from kenaf plant (Lezoul et al. 2020). Several studies have shown that polar solvents, such as methanol and water, are optimal for extracting polar compounds, while non-polar solvents, like hexane, are more effective for extracting non-polar compounds from different part of the plant (Wong et al. 2014b; Sim et al. 2019; Adnan et al. 2020). Additionally, extraction time and temperature are critical factors for maximizing yields. Higher temperatures enhance permeability, solubility, and mass transfer, enabling faster compound extraction.

However, excessively high temperatures can reduce recovery or degrade compounds, negatively impacting overall yield and quality (Oreopoulou et al. 2019). Furthermore, the type, maturity, and specific plant parts of the kenaf material can significantly impact the concentration and composition of its bioactive compounds (Norhisham et al. 2023c). For example, younger plants may exhibit different phytochemical profiles when compared to mature plants, while the leaves, stems, and seeds can vary in their bioactive compound content (Shukri et al. 2022). Finally, the specific characteristics of the bioactive compounds themselves, such as their polarity, solubility, stability, and molecular weight, also influence the effectiveness of the extraction process (Bubalo et al. 2018) Thus, optimizing the extraction method and conditions is essential for maximizing the yield of bioactive compounds from kenaf, ensuring efficient recovery and maintaining compound quality.

Table 2.1 Extraction methods and optimal extraction parameters of kenaf bioactive compound.

Extraction methods	Kenaf part	Solvent	Extraction condition	References
Ultrasonic assisted extraction	Leaves	Ethanol	Concentration: 100 mg/ml, Time: 3 min, Temperature: 18°C.	(Sim et al. 2019)
	Seeds	Ethanol	Concentration: 10 mg/ml, Time: 15 min, Temperature: 25°C	(Wong et al. 2014b)
	Seeds	Methanol	Concentration: 0.1 mg/ml, Time: 1 hr, Temperature: 25°C	(Mariod et al. 2012)
	Leaves, Stem	Methanol	Concentration: 25 mg/ml, Time: 60 min, Temperature: 40°C	(Duan et al. 2024)
	Seeds	Ethanol	Concentration: 100 mg/ml, Time: 15 min, Temperature: 25°C	(Wong et al. 2014a)
	Leaves, Flower	Methyl alcohol	Concentration: 66.66 mg/ml, Time: 1h, Temperature: 25°C	(Park et al. 2020)
	Leaves	Methanol	Concentration: 33.33 mg/ml, Time: 2h, Temperature: 25°C	(Pascoal et al. 2015a)
	Seeds	Hexane	Concentration: 0.125 mg/ml, Time: 1h, Temperature: 45°C	(Zhang et al. 2020)
	Seeds	Hexane-isopropanol	Concentration: 100 mg/ml, Time: 1min, Temperature: 25°C	(Mohamed et al. 1995)
	Leaves	Ethanol	Concentration: 0.1 mg/ml, Time: 3 min, Temperature: 22°C	(Sim and Nyam 2021a)
	Leaves	Ethanol	Concentration: 0.1 mg/ml, Time: 3 min	(Chew et al. 2021a)
	Seeds	Petroleum ether	Concentration: 0.33 mg/ml, Time: 30 min, Temperature: 28°C	(Zaharuddin et al. 2021a)
	Seeds	Hexane	Concentration: 100 mg/ml, Time: 1.5 h, Temperature: 25°C	(Kai et al. 2015a)
Solvent extraction	Leaves, Stem	Methanol, Water, Ethanol, Chloroform.	Concentration: 93.3 mg/ml, Time: 8 h, Temperature: 25°C	(Sulaiman et al. 2024)
	Leaves, Stem, Flower	Water, Methanol, Ethanol, Chloroform	Concentration: 200 mg/ml, Time: 24 h, Temperature: 25°C	(Ryu et al. 2017)
	Leaves	Water	Concentration: 400 mg/ml, Time: 10 days, Temperature: 25°C	(Kobaisy et al. 2001)
	Seeds	Hexane	Time: 3 h, Temperature: 60°C	(Chew et al. 2017)
	Seeds	Petroleum ether	Concentration: 0.1 mg/ml, Time: 8 h, Temperature: 60°C	(Nyam et al. 2009)
	Seeds	Hexane	Time: 3 h, Temperature: 60°C	(Chu and Nyam 2020)

	Leaves	Methanol	Concentration: 40 mg/ml, Time: 12 h, Temperature: 75°C	(Shaikh 2016)
Reflux extraction	Seeds	Ethanol	Concentration: 66.66 mg/ml, Time: 3 h, Temperature: 25°C	(Chan et al. 2014)
	Leaves	Ethanol	Concentration: 0.1 mg/ml, Time: 2 h, Temperature: 85°C	(Birhanie et al. 2021)
Soaking extraction	Seeds	Ethanol	Concentration: 200 mg/ml, Time: 5 days, Temperature: 25°C	(Adnan et al. 2020)

2.1.3 Bioactive compounds of kenaf extracts

2.1.3(a) Phenolics

Phenolic compounds are essential for both human health and plant defense, offering a range of therapeutic benefits, including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. These compounds have been vital in the development of widely used drugs, such as such as aspirin and salicylate (Mahdi et al. 2013). Phenols, the simplest class of phenolic compounds, are characterized by an aromatic benzene ring with one or more hydroxyl groups attached (Vuolo et al. 2019). They are primarily classified into two main types: hydroxycinnamic acids and hydroxybenzoic acids. Hydroxycinnamic acids, such as caffeic acid and ferulic acid, have a benzene ring linked to a three-carbon chain with a carboxyl group. In contrast, hydroxybenzoic acids, including salicylic acid and gallic acid, have a benzene ring with one or more hydroxyl groups (da Silva et al. 2023). Numerous studies have reported the presence of phenolic acids such as gallic acid, caffeic acid, vanillin, syringic acid, tannic acid, and *p*-hydroxybenzoic acid in different parts of the kenaf plant, all of which exhibit a wide range of bioactive and pharmacological properties. Kenaf leaves exhibit the highest phenolic content, with 592.1 mg/100g, followed by the flower at 310.6 mg/100g, the seed at 188.90 mg/100g, and the stem at 81.57 mg/100g (Sulaiman et al. 2024). Similarly, another study reported that kenaf leaves contain the highest phenolic content, with 555.9 mg/100g, while the flower, seed, and stem had 308.5 mg/100g, 162.70 mg/100g, and 84.80 mg/100g, respectively (Ryu et al. 2017). The total phenolic content (TPC) in kenaf leaf extract has been reported as 192.08 mg/g, comprising 4.13 mg/g of tannic acid, 117.84 mg/g of catechin hydrate, and 70.11 mg/g of caffeic acid(Sim and Nyam 2021c). Another study indicated that kenaf leaves contain the phenolic chlorogenic acid at 23.4 mg/100g and caffeic acid at

76.4 mg/100g (Ryu et al. 2017) Kenaf seeds have been shown to contain syringic acid at 1.094 mg/100g, chlorogenic acid at 0.533 mg/100g, hydroxybenzoic acid at 0.319 mg/100g, and gallic acid at 0.085 mg/100g (Mariod et al. 2012) Furthermore, a study revealed that kenaf seed extract contains various phenolic compounds, including gallic acid, tannic acid, catechin, benzaldehyde, benzoic acid, syringic acid, sinapic acid, ferulic acid, naringin acid, and protocatechuic acid, with tannic acid at 2302.20 mg/100g and sinapic acid at 1198.22 mg/100g being the predominant (Wong et al. 2014b). Similarly, kenaf seeds have been reported to contain *p*-coumaric acid at 109.17 mg/g, caffeic acid at 33.17 mg/g, catechin at 2.8 mg/g, and gallic acid at 0.804 mg/g (Chan et al. 2014)

The chemical characteristics and polarities of the phenolic compounds extracted from different kenaf plant parts are diverse, meaning that they may not be readily soluble in a single solvent. Consequently, the choice of solvent, its polarity, and the extraction conditions and techniques can significantly impact the yield and composition of the extracted phenolic compounds. Several researchers have investigated the phenolic profile of kenaf plants using various extraction solvents and conditions. The TPC extracted from kenaf samples increased significantly, from 34.44 mg/g when using methanol to 146.46 mg/g when n-butanol was employed as the extraction solvent (Chan et al. 2014). Another study reported that the phenolic content in kenaf seed extracts ranged from 2.16 to 18.87 mg GAE/g when using various solvents such as hexane, chloroform, methanol, and water (Yusri et al. 2012). The study also indicated that polar solvents, such as water and methanol, were more efficient in extracting phenolic compounds from kenaf seeds compared to non-polar solvents like hexane and chloroform. Consistent with these findings, other studies also reported that water resulted in the highest TPC for all the different parts of the kenaf