

BINDURA UNIVERSITY OF SCIENCE EDUCATION

TITLE: EXTRACTION, THIN LAYER CHROMATOGRAPHY ISOLATION AND ANTIOXIDANT ACTIVITY DETERMINATION OF FLAVONOIDS FROM *PHYTOLACCA DIOICA L.* BERRIES

BY

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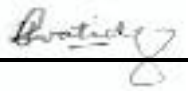
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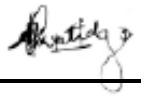
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ABSTRACT

Phytolacca dioica L. is a medicinal plant known to have various bioactive phytochemicals. This research aimed to extract, isolate, quantify and evaluate the antioxidant activity of the flavonoids obtained from ripe and unripe berries of *Phytolacca dioica* using ethanol and ethyl acetate as solvents. Dried berry powders were extracted by solvent extraction and the crude extracts were concentrated using a water bath at 40 ºC. Extraction yields were determined and flavonoid compounds were isolated using analytical and preparative thin-layer chromatography (TLC) in a n-butanol: acetic acid: water (2:2:6 v/v/v) solvent system. Flavonoid bands were visualized under the UV lamp after being sprayed with 1% aluminium chloride reagent. Total flavonoid content (TFC) was measured using the aluminium chloride colorimetric method with quercetin as a reference standard and the antioxidant activity was assessed using the hydrogen peroxide (H_2O_2). The highest extraction yield was obtained from ripe ethanol berry extract (10.6%), while the lowest was for unripe ethyl acetate berry extract (2.35%). TLC analysis confirmed the presence of flavonoids in all extracts by their characteristic yellow and yellow green fluorescent bands. The ripe berry ethanol extract showed the highest total flavonoid content, 658.55 mg QE/g, followed by ripe berry ethyl acetate with 499.24 mg QE/g, unripe berry ethanol extract with 426.87 mg QE/g and the lowest was recorded for unripe ethyl acetate berry extract (311.45 mg QE/g). At 100 µg/mL, ripe berry ethanol extract showed the highest scavenging activity (96.8%), followed by ripe ethyl acetate (88.8%), unripe ethyl acetate (72.9%) and unripe ethanol (61.7%). The ripe berry ethanol extract showed the strongest antioxidant activity, whilst the unripe berry ethanol extract showed the lowest activity.

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Abbreviations

ABTS: 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate)

DNA: Deoxyribonucleic Acid

DPPH: Diphenylpicrylhydrazyl

EGCG: Epigallocatechin Gallate

FRAP: Ferric Reducing Antioxidant Power

HAT: Hydrogen Atom Transfer

HPLC: High Performance Liquid Chromatography

LC-MS: Liquid Chromatography-Mass Spectrometry

ORAC: Oxygen Radical Absorbance Capacity

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SET: Single Electron Transfer

TLC: Thin Layer Chromatography

TFC: Total Flavonoid Content

UV: Ultra-Violet

CHAPTER ONE

1.1 BACKGROUND

Flavonoids are a varied family of polyphenolic molecules, widely distributed in plants and are essential bioactive ingredients in many nutraceuticals, pharmaceutical, therapeutical and cosmetic products. Flavonoids have health-promoting effects that come from their capacity to control important cellular enzyme activities by free radical scavenging, inhibiting or activating enzymes or signaling pathways (Talreja and Tiwari, 2024). Their ability to regulate most of the essential cellular enzyme activities, combined with their antioxidant, anti-inflammatory, anti-mutagenic, anti-microbial and anti-carcinogenic characteristic, are the reasons for flavonoids' beneficial impacts on health (Hassanpour and Doroudi, 2023)**.** By scavenging free radicals and reactive oxygen species (ROS) produced throughout regular metabolic activities, flavonoids shield biological systems therefore avoiding oxidative damage to lipids, proteins, and nucleic acids (Stachelska, Karpiński and Kruszewski, 2025). Given the growing worldwide burden of chronic and infectious diseases, together with the restrictions and adverse effects connected to synthetic pharmaceuticals, plant-derived flavonoids as safer and more effective treatment agents are getting renewed attention.

Long used in traditional medicine throughout several societies to treat inflammation, tumors, skin infections, and other metabolic issues, plants of the Phytolaccaceae family have great therapeutic value (Ma *et al.*, 2017)**.** Several species of the Phytolacca genus have been found to be anti-bacterial, anti-oxidant, anti-inflammatory, antimicrobial, and anticancer agents, therefore emphasizing their potential as rich sources of biologically active chemicals (Saleri *et al.*, 2017). These pharmacologic characteristics indicate the existence of secondary metabolites able to negate oxidative stress and associated pathological processes.

Phytolacca dioica L., is a member of the Phytolaccaceae family commonly known as the phytolacca (English), belombra (Spanish) and its trade name being ombú (Orwa *et al*, 2009). Prior phytochemical analysis of Phytolacca species has found flavonoids, phenolic acids, saponins and alkaloids—components that greatly influence their biological activity.

Research on extracts of Phytolacca plants from different parts have shown that they possess anti-inflammatory, anti-fungal, antibacterial and antioxidant properties, therefore suggesting their therapeutic promise (Ma *et al.*, 2017). Still, there is little scientific evidence on the flavonoid content and antioxidant action specifically pointing to *Phytolacca dioica* berries exists despite rising interest in natural antioxidants.

Many plant extracts' antioxidant activity is mostly attributed to flavonoids' capacity to donate hydrogen atoms or electrons and so stabilize free radicals (Do Socorro Chagas *et al.*, 2022)**.** Understanding their biological functions and possible uses thus depends on the separation and characterization of these substances. Commonly used for the separation, screening, and characterization of flavonoids in plant extracts, thin layer chromatography (TLC) is a fast, relatively low-cost, and easy analytic technique (Kowalska, 2022). TLC-based isolation offers insightful knowledge on the connection between chemical composition and biological activity when combined with antioxidant assays.

Because of the limited data on the antioxidant capabilities of flavonoids from *Phytolacca dioica* berries, research on their extraction, isolation, and biological analysis is required. Thus, using suitable solvent extraction and thin layer chromatography methods, this study aims to extract and purify flavonoids from dried *Phytolacca dioica L*. berries and assess their antioxidant activity using standard in vitro assays. The results of this study will enhance scientific knowledge about *Phytolacca dioica* as a possible natural source of antioxidant flavonoids and therefore encourage its possible use in medicine and nutraceutical applications.

1.2 PROBLEM STATEMENT

Despite the traditional use of *Phytolacca dioica L.* berries in medicine and its reported biological activities, no scientific study has isolated and characterized its flavonoid constituents or evaluated their antioxidant capacity using standardized assays hindering the understanding, validation and optimization of these berries phytochemical profile and therapeutical potential. Furthermore, there is an increasing demand for natural antioxidants

in food, pharmaceutical, and cosmetic industries highlighting the need to identify novel plant-based sources.

1.3 JUSTIFICATION

Phytolaccaceae are perennial plants globally distributed with different species belonging to a particular place. These plants are traditionally used in folk medicine for its anti-inflammatory, anti-microbial and wound healing properties. While preliminary studies on other Phytolaccaceae species suggests the presence of bioactive compounds, the specific flavonoid composition and antioxidant potential of *Phytolacca dioica* berries remain scientifically underexplored. This gap is of importance given that flavonoids are greatly recognized for their importance in human health due to various pharmacological activities. Flavonoids are known to have free-radical scavenging, anti- inflammatory, antibacterial, antiviral, ant carcinogenic and cardio protective properties (Roy *et al.*, 2022). Identifying these flavonoids from *Phytolacca dioica L.* and evaluating their antioxidant potential can provide an insight into the plants' phytochemical composition and antioxidant profile. This will also help promoting conservation and utilization of this plant species.

1.4 AIMS OF THE RESEARCH

To extract and isolate flavonoids from *Phytolacca dioica* berries using TLC and evaluate antioxidant potential using Hydrogen peroxide assays.

1.4.1 Objectives

- To extract flavonoids from *Phytolacca dioica* berries using ethanol and ethyl acetate solvents.
- To isolate flavonoids using analytical and preparative TLC
- To evaluate antioxidant activity using Hydrogen peroxide assays

1.5 SCOPE OF THE STUDY

The study will cover from the extraction of crude extracts from *Phytolacca dioica* berries by solvent extraction method using ethanol and ethyl acetate. Flavonoids will then be isolated from the crude extract using analytical and preparative Thin Layer Chromatography. UV light/ visualization spray is used to visualize the flavonoid bands and

R_f values are used to identify the flavonoids. Hydrogen peroxide assay will be used to evaluate the antioxidant potential of the isolated flavonoids found in *Phytolacca dioica* berries.

CHAPTER TWO: LITERATURE REVIEW

2.1 INTRODUCTION

Oxidative stress caused by reactive oxygen and nitrogen species (ROS/RNS) contributes significantly to aging and the development of chronic diseases such as cancer, diabetes, cardiovascular disorders, and inflammation (Balasaheb and Pal, 2015; Sari *et al.*, 2024). It is known to cause a significant risk factor contributing to the development of various chronic diseases (Sari *et al.*, 2024). Natural antioxidants, flavonoids and phenolic compounds, are able to neutralize free radicals through hydrogen donation, electron transfer, or metal chelation mechanisms. Flavonoids are polyphenolic molecules well known for their capacity to reduce oxidative stress. In plants, they function as antioxidants, antimicrobials, photoreceptors, visual attractants, feeding repellents, and light screens (Roy *et al.*, 2022). Flavonoids have been shown in numerous studies to have biological effects such as antiviral, anti-inflammatory, antifungal, antibacterial, vasodilating, and antiallergenic properties (Chávez-González *et al.*, 2020). Because of their variations in bioavailability, structural diversity, and metabolism, flavonoids have wide range of biological activities, especially their antioxidant and anti-inflammatory effects (Parmenter *et al.*, 2025).

2.2 PHYTOLACCA DIOICA

Phytolacca is a genus belonging to the family of Phytolaccaceae, comprising of approximately 35 species globally, with four species reported in the People's Republic of China (Ma *et al.*, 2017; Khalid, *et al*, 2025). Species of the Phytolaccaceae family have long been used both in traditional and modern medicines. The phytocompounds of the species in this family have a number of bioactive effects such as antibacterial, antifungal, antimalarial, molluscidal properties, anti-obesity, pro-oxidant effects and antitumor properties (Saleri *et al.*, 2017). Their therapeutic applications include the management of edema, inflammation, dermatitis, rheumatism, tumors and bronchitis, as well as applications as molluscicide agents (Iglesias *et al.*, 2016; Ma *et al.*, 2017; Khalid, *et al*, 2025). Phytochemical studies have shown that plants of the Phytolaccaceae family are rich in triterpenoid saponins, in addition to other bioactive constituents such as triterpenes,

flavonoids, phenolic acids, sterols and polysaccharides (Ma *et al.*, 2017; Trunjaruen, et al., 2022). A review by Di Liberto *et al.*, 2010, reported the presence of triterpenoid saponins in *Phytolacca dioica*. Species of the genus Phytolacca have a role in folk medicine in many different cultures. According to ethno pharmacological information, *Phytolacca dioica* is used to treat skin wounds (Ashafa, *et al*, 2010). These conventional uses point to the presence of bioactive components with anti-inflammatory, antifungal, antibacterial, antiviral and antioxidant effects.

2.2.1 Botanical Description



Figure 2.1: *Phytolacca dioica* plant (a) and berries (b)

Figure 2.1 shows the *Phytolacca dioica* plant and its berries. The *Phytolacca dioica L*. also known as ombú (Guarani), which means shade or big tree. *Phytolacca dioica* is a species of the Phytolaccaceae family which is native to the grasslands of South America and is exotic in Australia, India, Kenya, South Africa and United Kingdom. *Phytolacca dioica* is a spreading deciduous tree 6-10 m in height, with a domed crown. Its leaves are simple alternate appearing as whorls; smooth and oval. The flowers are small, creamy white with many stamens. The fruits of *Phytolacca dioica* are juicy from green to yellow to black berries as they mature, with 10 lobes

hanging in clusters irregularly produced after flowering. *Phytolacca dioica* is a tree for mild climates, undemanding in soil fertility and resistant to sea winds.

2.3 FLAVONOIDS: CHEMISTRY AND ANTIOXIDANT PROPERTIES

2.3.1 Fundamental Features and Structure

Flavonoids are a group of diverse phenolic structures found in natural sources. They are mostly found in fruits, vegetables, grains, bark, roots, stems, flowers and tea. The fundamental structure of flavonoids is a diphenyl propane (C6–C3–C6) structure, with two aromatic rings (A and B) linked by a three-carbon bridge that cyclizes forming an oxygenated heterocyclic ring (C) as shown in Figure 2.2 (Do Socorro Chagas *et al.*, 2022).

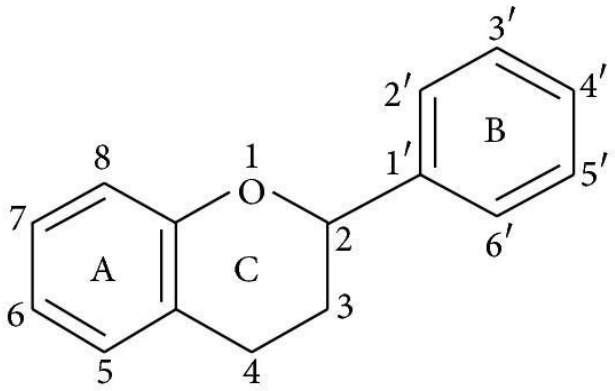


Figure 2.2: Flavonoid basic structure.

The core structure of flavonoids permits a wide range of diversity. Flavonoids are frequently found in plants as glycosides (conjugated to sugars) or aglycones (without sugar moieties) (Liga and Paul, 2023). Compared to their aglycone counterparts, glycosylation has a significant impact on their solubility, stability, bioavailability, and biological activity. The quantity and location of hydroxyl groups (-OH) on the flavonoids, particularly on the B-ring, as well as the presence of specific structural characteristics, such as the 2,3-double bond in conjunction with a 4-oxo group in ring C (found in flavonols, flavones) and the 3-OH group, are the main factors contributing to their classification.

2.3.3 Classification of Flavonoids

Flavonoids are divided into different subclasses according to the oxidation state and substitution patterns of the heterocyclic C-ring (Gupta, 2015). Variation in the oxidation state of ring C, the position of the ring B ring, and the number and arrangement of hydroxyl, methoxy and glycosidic substituents gives rise to flavonoid subclasses (Nicolescu, *et al,* 2025). These structural differences influence their biological functions. Flavonoid subclasses include flavanones, flavanols, flavonols, flavones, anthocyanidins and isoflavonoids. Figure 3 shows the flavone and flavanone skeleton.

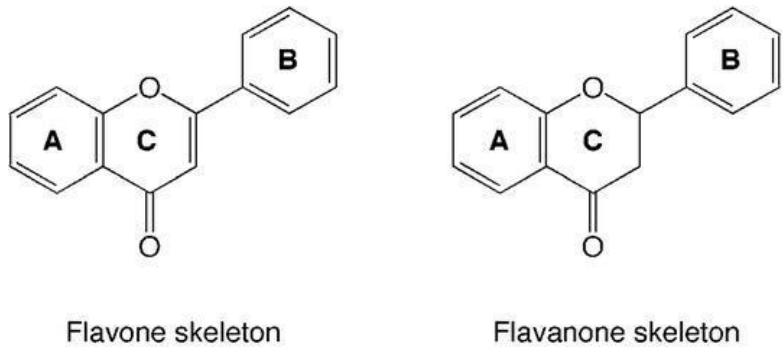


Figure 2.3: Flavone and flavanone basic skeletons

Flavanones are characterized by a saturated bond between C2 and C3 and the presence of a carbonyl group at C3 (Mutha, Tatiya and Surana, 2021). Flavanones are synthesized directly from chalcones through the action of chalcone isomerase and are particularly abundant in citrus fruits especially orange, lemons, and grapefruits. These flavonoids include naringenin and hesperidin (Kopustinskiene *et al.*, 2020).

Flavonols are similar to flavones with an additional hydroxyl group at C3, this 3-hydroxyl substitution increases antioxidant capacity when combined with a catechol arrangement (3',4'-dihydroxy substitution) on the B-ring (Kopustinskiene *et al.,* 2020). Flavonols are one of the most abundant dietary flavonoids commonly found in onions, apples, broccoli, berries and tea. Quercetin, kaempferol and myricetin are well known flavonols while rutin is a glycosylated form of quercetin.

Flavone are structurally different from flavanones by the presence of a double bond between C2 and C3 and a carbonyl group at C4, resulting in a more conjugated and planar system. They do not

possess a hydroxyl group at C3, thus have less hydroxyl groups than flavonol. This increased conjugation enhances their ability to stabilize free radicals, contributing to their antioxidant potential. Flavones are commonly found in herbs; parsley, celery, mint and chamomile (Kamboh, 2015). Representative flavone includes luteolin, apigenin, chrysin and tangeritin (Kopustinskiene *et al.*, 2020).

The structural arrangement of isoflavonoids gives them similarities to estrogen, allowing them to function as phytoestrogens (Roy *et al.*, 2022). Isoflavonoids are found in legumes and are abundant in soybeans (Kamboh, 2015), lentils, chickpeas. Examples include genistein and daidzein (Kopustinskiene *et al.*, 2020).

Flavanols are responsible for the stringent taste of many plant products. Their rich source includes green and black tea, cocoa, grapes, apples and certain berries. Examples are catechin, epicatechin and epigallocatechin gallate (EGCG) (Liga and Paul, 2023).

Anthocyanins represent a distinct class of flavonoids responsible for the red, purple and blue pigmentation observed in many fruits, vegetables and flowers (Liga and Paul, 2023). Structurally, they have a highly conjugated flavylium cation core. They occur as glycosides in nature, which enhances their stability and solubility. Rich sources include red grapes, blueberries, purple maize and red cabbage. Examples include cyanidin, delphinidin and pelargolin, responsible for red to magenta colors, purple to blue and orange to pink colors respectively (Silva, 2021).

2.3.4 Antioxidant mechanisms of flavonoids

Oxidative stress is caused by an imbalance between the generation of reactive oxygen species (ROS) and the body's antioxidant defense systems (Munteanu and Apetrei, 2021). Reaction oxygen species such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2) can damage lipids, proteins and DNA (Kotha, et al, 2022; Hassanpour and Doroudi, 2023). Flavonoids are among the most important plant-derived antioxidants and play a significant role in protecting biological systems against oxidative damage (Abrahamse and George, 2024). Flavonoids exert antioxidant effects through mechanisms like metal ion chelation, direct free radical scavenging, inhibition of lipid peroxidation, enhancement of antioxidant enzymes and inhibition of pro-oxidant enzymes (Ullah *et al.*, 2020; Hassanpour and Doroudi, 2023).

The bioactivity of flavonoids depends on their chemical structure such as hydroxylation pattern and conjugation within the molecule (Ekalu, 2020). A high number of hydroxyl groups, the presence of a 3',4'-dihydroxy group in the B-ring, conjugation between the C2-C3 double bond and the 4-oxo group and the presence of a 3-hydroxyl group increases the antioxidant capacity of flavonoids (Wang, *et al*, 2018). Glycosylation may reduce the antioxidant activity.

2.3.4.1 Free Radical Scavenging Activity

The primary antioxidant mechanism of flavonoids is their ability to scavenge free radicals directly (Stachelska, *et al*, 2025). This mainly occurs through the hydrogen donation from their hydroxyl (-OH) groups to neutralize reactive species. Flavonoids contain phenolic hydroxyl groups that can donate hydrogen atoms and electrons to free radicals forming stable flavonoid radicals (Wang, *et al*, 2018). When a flavonoid donates hydrogen, the radical becomes stable, and the flavonoid forms a relatively stable radical due to resonance stabilization within its structure (Panche, *et al*, 2016).

2.3.4.2 Inhibition of Lipid Peroxidation

Lipid peroxidation is a process in which free radicals attack cell membrane lipids, leading to cell damage. Flavonoids help prevent this process by scavenging lipid peroxyl radicals, interrupting the chain reaction of lipid oxidation and protecting cell membrane integrity.

2.3.4.3 Metal Ion Chelation

Transition metals such as iron (Fe^{2+}) and copper (Cu^{2+}) catalyze the formation of highly reactive hydroxyl radicals. Flavonoids can chelate these metal ions, preventing them from taking part in radical-generating reactions thus reducing the formation of new ROS (Stachelska, *et al*, 2025). Metal chelation occurs at the 3',4'-dihydroxy group in the B-ring and the 3-hydroxy and 4-oxo groups in the C ring.

2.3.4.4 Inhibition of Pro-Oxidant Enzymes

Flavonoids reduce oxidative stress by inhibiting enzymes that generate reactive oxygen species, including Xanthine oxidase, NADPH oxidase and Cyclooxygenase (COX). Flavonoids decrease the production of free radicals in the body by suppressing these enzymes.

2.4 ISOLATION AND EXTRACTION METHODS

The methodology stages include isolation, extraction and identification of phytochemicals from plant materials. Firstly, there is sample preparation in which sample is dried, centrifuged or filtered. The second step is the extraction, isolation and purification of flavonoid compounds from plant material. Flavonoids are extracted using the processes like Soxhlet, boiling, reflux, soaking, hydro-distillation, maceration, microwave assisted extraction, ultrasound, etc. (Chaves *et al.*, 2020; Proestos *et al.*, 2023). In the final step, the purified extracts are used for identification, quantification, recovery of flavonoid compounds using chromatography techniques, for further evaluation processes.

2.4.1 Solvent Extraction

Generally, flavonoids extraction from plant material is solvent extraction using liquid-liquid or solid-liquid extraction processes. The nature of solvent used will affect the type of flavonoids to be extracted and will directly influence its biological activity. According to Yu, (2019) & Daud, (2017), ethanol and methanol, of the solvents tested, are the most widely used for extraction of flavonoids due to their higher yields in the recovery of flavonoids. And the polarity of the target flavonoids also determines the choice of solvent (aglycones are less polar than glycosides), which is the most important factor. Methanol (MeOH), ethanol (EtOH), acetone, ethyl acetate (EtAc), and water (H_2O) are common solvents, frequently used in mixtures of 70–80% aqueous MeOH or EtOH, 70% aqueous acetone to efficiently extract a wide variety of flavonoid glycosides while minimizing co-extraction of highly polar or non-polar substances. Time, particle size, type of solvents, the proportion of solid to solvent, temperature, number of extraction cycles, etc. are all parameters that affect extraction efficiency (Chávez-González *et al.*, 2020, Tomaz *et al*., 2016) . These must be optimized in order to maximize flavonoid output and consistency. Fractionation is necessary after the initial raw extraction to concentrate flavonoids and eliminate interfering substances, such as chlorophyll, lipids, sugars, and tannins. Fractionation greatly increases the specificity of TLC isolation, purification and antioxidant assays.

2.4.2 Isolation of flavonoids by Thin-Layer Chromatography (TLC)

Chromatography is an analytical separation technique applied in laboratories for analysis and quality control. It serves as a preparative tool for identification, purification and determination of contents of bioactive substances (Silver, 2020). Thin Layer Chromatography remains frequently used due to its simplicity, quick, inexpensive, reproducible and versatility in separating complex mixtures of organic components (Kowalska, 2022). It is applied for qualitative and quantitative analysis of drugs, isolated phytochemical components and natural raw materials. The technique is important in the investigation of plant secondary metabolites, including flavonoids, alkaloids, terpenoids and phenolic compounds. TLC has a wide range of applications, including the analysis of agricultural products, identification of plant and food components, screening of drugs, screening of vitamins, colorants and antioxidants (Kowalska, 2022; Zych and Pyka-paj, 2025).

Flavonoids contain multiple hydroxyl groups, which increase their polarity and affinity for polar stationary phases such as silica gel. Therefore, their separation is highly dependent on solvent polarity, making TLC an effective method for isolation of flavonoids. In this research, TLC is employed as an initial screening technique to confirm the presence of flavonoids in the crude extract and for isolation of flavonoids. TLC plays an important role in directing the isolation procedures by facilitating the selection of appropriate solvent systems and monitoring fraction purity.

2.4.2.1 Principle of Thin-layer chromatography

The separation of compounds in TLC is based on differences in polarity and distribution of active compounds between a stationary phase and a mobile phase (Nahari *et al.*, 2019). A TLC plate consists of a thin layer of adsorbent material coated onto a solid support such as glass, aluminium or plastic. A proper TLC analysis uses a sealed development chamber, allowing the solvent to equilibrate with its vapor (Silver, 2020). Separation occurs as a result of the differential partitioning between the mobile and stationary phases. Components with a strong affinity for the stationary phase travel more slowly, while those with greater solubility in the mobile phase travel further up the plate (Pahari *et al.*, 2021). The nature of these interactions include adsorption, partitioning, hydrogen bonding, dipole-dipole interactions and van der Waals forces.

The rate of movement of a compound is expressed by the retardation factor (R_f), defined as the ratio of the distance travelled by the compound to the distance travelled by the solvent front (Pahari *et al.*, 2021). The R_f value is a characteristic for a compound under specific experimental conditions and is influenced by factors including solvent composition, stationary phase type, layer thickness, temperature and saturation of the developing chamber. In flavonoid analysis, R_f values are commonly used as a comparative parameter for identification and purity assessment when reference standards are available (Pahari *et al.*, 2021).

2.4.2.2 Analytical TLC

Analytical TLC is primarily used for qualitative analysis and involves the use of tiny sample amounts, generally in the microgram range. Its uses are extensive, including monitoring extraction efficiency, assessing sample complexity, determining the number of components present in an extract, and evaluating compound purity. Analytical TLC is used in flavonoid research to rapidly screen plant extracts for the presence of flavonoid compounds by observing characteristic fluorescence or color reactions after visualization. This method enables identification of compounds by comparing R_f values and spot features with established flavonoid standards like quercetin**.**

2.4.2.3 Preparative TLC

Preparative TLC is mostly used for isolation and purification of compounds in larger quantities than those usually used in analytical TLC (Rabel and Sherma., 2017). The quantities are usually in the milligram range. Preparative TLC uses plates coated with a thicker layer of stationary phase, allowing greater sample loading capacity. The sample is applied as a continuous band rather than spots to maximize recovery of the target compound (Hayward, Mader and Trant, 2022).

After development, separated bands corresponding to individual compounds are viewed under UV light or after spraying with suitable reagents. The desired bands are then carefully scraped off the plate, and the compounds are recovered by elution with appropriate solvents. The isolated compounds can be concentrated and subjected to further purification or characterization. Preparative TLC provides an economical and efficient means of isolating bioactive compounds for biological evaluation, including the antioxidant activity assay.

2.4.2.4 TLC Phase Systems

Silica gel is the most commonly used stationary phase in TLC because of its high polarity, strong adsorptive properties, chemical stability and ability to give a luminescence under UV light (Nahari *et al.*, 2019). It contains surface silanol groups that readily form hydrogen bonds with polar functional groups making it suitable for separating flavonoids, which are rich in hydroxyl groups. Other stationary phases include alumina, cellulose and reversed- phase materials. The choice of stationary phase depends on the chemical nature of the sample, as well as the intended analytical or preparative purpose.

The mobile phase in TLC consists of one or more solvents selected to achieve optimal separation of analytes. The use of solvent mixtures allows fine adjustment of polarity to achieve desired R_f values and resolution. Common mobile phase systems include mixtures such as ethyl acetate-methanol-water, n-butanol-acetic acid-water and toluene-ethyl acetate-formic acid (Pahari *et al.*, 2021). Addition of small amounts of acids, formic acid or acetic acid, help improve separation by reducing tailing effects caused by strong interactions between analyte and stationary phase.

In flavonoid analysis, solvent systems are usually chosen based on polarity considerations and empirical optimization.

2.4.3 Detection of zones in TLC

Visualization of separated compounds on a TLC plate is important for qualitative analysis and isolation. The process of identification involves comparing the retention factor (R_f) values and color reactions under UV/vis with those of standards that were tested on the same plate. TLC is an essential tool for initial profiling and directing subsequent isolation procedures. The methods used to visualize separated bands after development are classified into physical and chemical techniques.

2.4.3.1 Physical detection methods

UV Light is the most commonly used physical detection methods in TLC. UV detection is non-destructive, allowing subsequent recovery of compounds during preparative TLC and making suitable for isolating flavonoids intended for antioxidant activity assays. The bands on TLC plates are commonly examined under UV light at wavelengths of 254 nm and 366 nm (Zych and Pyka-paj, 2025). Flavonoids exhibit natural fluorescence or absorb UV radiation because of their

conjugated aromatic structures. Under 254 nm, many flavonoids appear as dark spots against a fluorescence background and under 366 nm, they exhibit natural fluorescence for example yellow, green, or blue fluorescence.

2.4.3.2 Chemical detection methods

Chemical detection involves spraying or dipping TLC plates in reagents that react with specific functional groups to produce colored or fluorescent spots. Aluminum chloride ($AlCl_3$) is widely used for flavonoid detection, as it forms complexes with flavonoids that enhance fluorescence under UV light. Aluminum Chloride forms yellow fluorescent complexes with flavonoids that have free 3-OH or 5-OH groups such as flavonols, flavones. Other reagents include natural product reagents such as diphenyl boric acid amino ethyl ester (NP/PEG), ferric chloride ($FeCl_3$), and vanillin-sulfuric acid (Pahari *et al.*, 2021). Diphenyl boric acid 2-aminoethyl ester, increases fluorescence and generates unique hues under 366 nm, such as yellow-orange for flavonols/flavones. Ferric Chloride produces blue, green, or purple hues when combined with phenolics. Vanillin-H_2SO_4 / Vanillin-HCl: Generates a wide range of colors for flavonoids and other phenolics (Zych and Pyka-paj, 2025).

Chemical detection improves sensitivity and selectivity, enabling the differentiation of flavonoid subclasses. However, some reagents may be destructive, limiting their use in preparative TLC.

2.5 EVALUATION OF ANTIOXIDANT ACTIVITY

Major antioxidant assays are classified into two groups, single electron transfer (SET) and hydrogen atom transfer (HAT) reaction-based assays, based on chemical reactions and reaction mechanisms involved (Gupta, 2015). DPPH radical scavenging assay, the ABTS radical cation decolorization assay, the oxygen radical absorbance capacity (ORAC) assay, and the ferric reducing antioxidant power (FRAP) assay are the most commonly used antioxidant evaluation assays. These assays differ in their reaction chemistry, sensitivity, and biological relevance, and therefore are often used together for comprehensive antioxidant profile. DPPH, FRAP and ABTS are SET based assays while ORAC is HAT based (Munteanu and Apetrei, 2021; Kotha, *et al*, 2022). While DPPH and ABTS are rapid screening tools, ORAC offers greater biological relevance, and FRAP provides valuable information on reducing power. In this study, some of

these assays will be used as an analytical approach for assessing the antioxidant potential of natural flavonoid isolates.

2.5.1 DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging assay

The DPPH assay measures the capacity of antioxidants based on the reduction of the stable purple nitrogen-centered DPPH• radical to its yellow non-radical form (DPPH-H) by donating hydrogen atoms or electrons. The DPPH radical is stable at 515 nm, which is the maximum absorbance used to measure the activity (Mustafa, Gu and Esra, 2016). It is the simplest and most rapid methods for measuring the free radical scavenging ability of antioxidants.

The antioxidant activity is usually expressed as:

- Percentage radical scavenging activity
- IC_{50} value (concentration required to inhibit 50% of radicals)

The radical scavenging DPPH• method is widely applied because it is fast, simple, inexpensive, and reproducible (Kowalska, 2022). According to Santos-sánchez *et al.* (2019), this method allows for reactions with any type of antioxidant due to the stability of DPPH•. DPPH assay can be used with both polar and nonpolar organic solvents to assess hydrophilic and lipophilic antioxidants. However, the stability of DPPH is affected by solvents with Lewis base properties and the presence of dissolved oxygen.

2.5.2 ABTS (2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) assay

The **ABTS assay** is another commonly used radical scavenging method that evaluates both hydrophilic and lipophilic antioxidant activity. It evaluates the ability to decolorize the preformed blue-green $ABTS^{+}$• radical cation. The $ABTS^{\bullet+}$ radical is produced through oxidation reaction of ABTS with potassium persulfate or other oxidizing agents. The radical solution absorbs strongly at 734 nm (Mustafa, Gu and Esra, 2016). When antioxidants are introduced, they reduce $ABTS^{\bullet+}$ back to its colorless neutral form, causing a measurable decrease in absorbance, which is used to quantify activity.

Results are often expressed as:

- Trolox equivalent antioxidant capacity (TEAC)(Kowalska, 2022).

The ABTS assay is advantageous because it can be used over a wide pH range and can measure antioxidant activity in complex plant extracts. However, like DPPH, it remains a non-biological radical model.

2.5.3 Ferric reducing antioxidant power (FRAP) assay

FRAP is a technique used to determine the total antioxidant activity of samples. This assay evaluates the ability of antioxidants to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), forming a blue complex (Santos-sánchez *et al.*, 2019). It is classified as a SET based assay; antioxidants donate electrons to the ferric ions as reducing agents. The method involves the reduction of the ferric-tripyridyl triazine complex:

$$Fe^{3+}\text{–TPTZ} \rightarrow Fe^{2+}\text{–TPTZ}$$

This reaction produces an intense blue color with maximum absorbance at 593 nm (Mustafa, Gu and Esra, 2016). Results are frequently expressed:

- Trolox equivalents (Mustafa, Gu and Esra, 2016)
- Fe^{2+} equivalents

FRAP assay is a simple, rapid, inexpensive and highly reproducible for measuring antioxidant capacity of biological sample (Munteanu and Apetrei, 2021). However, it only measures reducing capacity under acidic conditions and does not account for antioxidant mechanisms such as radical quenching through hydrogen donation or metal chelation.

2.5.4 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay is considered one of the most biologically relevant antioxidant assays because it measures antioxidant inhibition against peroxyl radicals, which are major propagators of lipid

oxidation in vivo (Munteanu and Apetrei, 2021). ORAC is classified as a hydrogen atom transfer (HAT)-based assay (Gulcin, 2020).

It uses:

- A radical generator such as AAPH
- A fluorescent probe such as ß-phycoerythrin fluorescein (Gulcin, 2020).

During the assay, peroxyl radicals generated by AAPH progressively quench the fluorescence signal. Antioxidants delay this fluorescence decay by scavenging radicals. The antioxidant capacity is determined by calculating the area under the fluorescence decay curve (AUC). Results are expressed in:

- Trolox equivalents (µmol TE/g)

ORAC provides an integrated measurement of both inhibition time and degree of radical scavenging, making it more physiologically meaningful. However, it requires specialized fluorescence equipment and is more time-consuming than DPPH or ABTS.

2.5.5 Hydrogen peroxide assay

Hydrogen peroxide is not very reactive, but it can be sometimes poisonous to cells, it may trigger the rise of hydroxyl radicals inside the cell (Esmaeili *et al.*, 2015). Human body are exposed to hydrogen peroxide through inhalation of vapor or mist and through eye or skin contact. Hydrogen peroxide decomposes into water, oxygen and this may form hydroxyl radicals (OH) that can initiate peroxidation and cause DNA damage in the body (Asiyabi *et al.*, 2023). H_2O_2 can pass through membranes rapidly once inside cells, H_2O_2 can react with Fe^{3+} and Cu^{2+}ions to also form OH radicals and this may be the source of many of its toxic effects (Asiyabi *et al.*, 2023). It is therefore important for cells to control the amount of H_2O_2 that is allowed to accumulate.

The ability of a compound to scavenge H_2O_2 helps predict its potential antioxidant activity. The hydrogen peroxide assay is determined using a UV-Vis spectrophotometer at 230 nm. The percentage of hydrogen peroxide scavenging is calculated using the equation:

$$\%Scavenging\ activity\ =\ \frac{[A_0\ -\ A_1]}{A_0} \times 100$$

Where,

A_0 is the absorbance of control

A_1 is the absorbance of sample

CHAPTER THREE: METHODOLOGY

3.1 MATERIALS

Ripe and unripe *Phytolacca dioica* berries, ethanol, ethyl acetate, formic acid, H_2O_2, $AlCl_3$, phosphate buffer, potassium permanganate, silica gel, distilled water, Filter papers, Quercetin, pulverizer, oven, horizontal laboratory shaker, heater, thermometer, water bath, UV lamp, UV-Vis spectrophotometer.

3.2 SAMPLE COLLECTION AND PREPARATION

Ripe and unripe berries of *Phytolacca dioica* were collected from Harare Gardens, Harare province. The plant was authenticated by a qualified botanist at Harare Botanical Gardens. The berries were washed with distilled water and oven dried at 40°C for 24hrs to prevent degradation of heat sensitive phytochemicals. The dried ripe and unripe berries were pulverized using an electric grinder and passed through a sieve to obtain fine uniform particle size powder. The powdered material was stored in airtight containers reagent bottles, labelled and refrigerated until extraction.

3.3 SOLVENT EXTRACTION OF FLAVONOIDS

3.3.1 Ethanolic extraction

For flavonoid extraction, 10 g of powdered plant material was weighed and mixed with 50 ml absolute ethanol in a 250 ml Erlenmeyer flask. A horizontal shaker was used to shake the sample overnight at room temperature. The mixture was filtered using Whatman No.1 filter paper and the filtrate was concentrated using a water bath at 40 ºC to obtain a crude ethanolic extract. The extract was stored at 4 °C for further analysis.

3.3.2 Ethyl acetate extraction

The dried powdered plant material was weighed and extracted with ethyl acetate using the same procedure described above. Ethyl acetate was chosen for its intermediate polarity, suitable for isolating aglycone flavonoids.

After filtration, the solvent was evaporated using a hot water bath at 40 °C to yield the crude ethyl acetate extract. The dried extract was stored in airtight containers at 4 °C until use.

3.4.1 Analytical TLC

Four analytical TLC plates 10×1.5 cm were cut and used as the stationary phase. The plates were activated by heating at 100 °C for 10 minutes then allowed to cool to room temperature (Gwatidzo, Dzomba and Mangena, 2018). A pencil line was drawn 1.5 cm from the bottom edge on each plate. A flavonoid standard, quercetin was also prepared at the same concentration for comparison. Using capillary tubes, small spots of the extracts and standard were applied 1.5 cm above the base of the plate. The mobile phase consisted of n-butanol: acetic acid: water (2:2:6 v/v/v), commonly used for flavonoid separation. The TLC chamber was saturated with the solvent system for 30 minutes before development. The plate was placed in the chamber and allowed to develop until the solvent front travelled about 1 cm from the top end. After development, the plate was air-dried and observed under UV light at 254 nm and 366 nm. The plate was then sprayed with 1% ethanolic aluminum chloride reagent to enhance flavonoid fluorescence. Yellow or green spots were marked in their positions using pencil and retention factors were calculated (Gwatidzo, Dzomba and Mangena, 2018).

3.4.2 Preparative TLC

Glass TLC plates pre-coated with thick silica gel were used as stationary phase. The same mobile phase system was used. Each of the ethanol and ethyl acetate extracts from the berries sample was deposited as a concentrated band 1.5 cm from the edge of its TLC plate respectively and allowed to dry. The plates were then placed into the development chamber, closed and allowed to develop. The plates were removed, solvent front was marked and R_f values were calculated.

The retention factor (R_f) values were calculated using the equation:

$$R_f = \frac{Distance\ travelled\ by\ compound}{Distance\ travelled\ by\ solvent}$$

The bands that tested positive for flavonoids were scratched off, re-dissolved their respective solvents, filtered and stored for further analysis.

3.5 EVALUATION OF ANTIOXIDANT ACTIVITY

3.5.1 Hydrogen Peroxide (H_2O_2) Scavenging Assay

A 40 mM solution of hydrogen peroxide (H_2O_2) was prepared in phosphate buffer (pH 7.4). To 0.6 mL of H_2O_2 solution, 1.0 mL of extract solution was added. The mixture was incubated for 10 minutes at room temperature. Absorbance was measured at 230 nm using a UV–Vis spectrophotometer against a blank solution containing phosphate buffer without H_2O_2.

Percentage scavenging activity was calculated using the equation:

$$\%Scavenging\ activity\ = \frac{[A_0\ -\ A_1]}{A_0} \times 100$$

3.6 UV QUANTIFICATION OF FLAVONOID CONTENT

Total flavonoid content in the extracts of *Phytolacca dioica* was determined using a UV–Visible spectrophotometric method based on the aluminum chloride colorimetric assay. The plant extracts were prepared in ethanol at a concentration of 1 mg/mL. An aliquot of 0.5 mL of each extract was mixed with 1.5 mL ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL distilled water. The reaction mixtures were incubated at room temperature for 30 minutes to allow for complex formation between flavonoids and aluminum chloride. Absorbance was then measured at 415 nm using a UV–Vis spectrophotometer against a reagent blank. A calibration curve was constructed using standard solutions of quercetin, and the total flavonoid content was calculated from the regression equation and expressed as milligrams of quercetin equivalents (mg QE/g) per gram of extract.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 EXTRACTION YIELD

The extraction of flavonoids from *Phytolacca dioica* ripe and unripe berries was carried out using ethanol and ethyl acetate as solvents. A 40 ºC water bath was used to concentrate the filtered extract solutions. Percentage yield obtained from each extract was calculated based on the initial dry weight of the plant material used and the final weight of concentrated extract obtained after solvent evaporation. Table 4.1 shows the extraction yield and the percentage yield of each sample with their respective solvent used. The extraction yield results revealed that both berry maturity and solvent polarity significantly influenced the recovery of phytochemicals from *Phytolacca dioica* berries. Ethanol produced higher extraction yields than ethyl acetate in both ripe and unripe berries, while ripe berries consistently yielded more extract than unripe berries. The highest yield was obtained from the ripe ethanolic extract (10.6%), whereas the lowest yield was recorded for the unripe ethyl acetate extract (2.35%). These findings indicate that the phytochemical composition of *Phytolacca dioica* berries changes during maturation and that solvent polarity plays a crucial role in the extraction process.

Table 4.1: Percentage Yield of extracts from ripe and unripe *Phytolacca dioica* berries

Sample	**Solvent used**	**Weight of Plant material (g)**	**Weight of Extract (g)**	**Percentage Yield (%)**
Ripe berries	Ethanol	10	1.061	10.6
Ripe berries	Ethyl acetate	10	0.473	4.73
Unripe berries	Ethanol	10	0.690	6.90
Unripe berries	Ethyl acetate	10	0.235	2.35

The higher extraction yields observed in ripe berries may be attributed to biochemical and physiological changes that occur during fruit ripening. Ripening is associated with increased synthesis and accumulation of secondary metabolites, including flavonoids, phenolic acids and anthocyanins. These compounds are produced through the phenylpropanoid pathway and contribute to fruit pigmentation, defense against environmental stress and protection against oxidative damage. Similar observations have been reported by Giovanoudis *et al.* (2023), that

ripening results in profound biochemical changes associated with accumulation of bioactive compounds and increased fruit quality parameters. Giovanoudis *et al.*, 2023 observed physiological differences between ripe and unripe fruits, reflecting metabolic transformations.

Another factor that may explain the higher extraction yields in ripe berries is fruit softening. During ripening, degradation of cell walls components such as pectin, cellulose and hemicellulose weakens cellular structures and increases tissue permeability. Studies on blueberries have shown that ripe fruits possess lower firmness than unripe fruits due to the action of cell wall-degrading enzymes (Hwang, Young-Jun and Youngjae, 2020). This structural breakdown likely improves solvent penetration and mass transfer, facilitating the release of intracellular phytochemicals and resulting in greater extraction efficiency.

The better performance of ethanol compared with ethyl acetate can be explained by differences in polarity and viscosity of solvent. Ethanol is a polar solvent capable of dissolving a wide range of phenolic compounds, including flavonoid glycosides, which are commonly found in fruits. In contrast, ethyl acetate is less polar and preferably extracts moderately polar or less polar compounds. The higher extraction yield obtained with ethanol therefore suggests that the majority of extractable flavonoids in *Phytolacca dioica* berries occur in relatively polar forms. Furthermore, ethanol can penetrate plant tissues more effectively and extract a broader range of bioactive compounds due to its greater polarity and ability to solubilize flavonoid glycosides (Proestos *et al.*, 2023).

However, a higher extraction yield does not necessarily indicate a higher concentration of flavonoids. Ethanol may also extract other polar constituents such as phenolic acids, sugars, tannins and organic acids, which contribute to the overall mass of extract recovered. Therefore, determination of total flavonoid content was necessary to establish whether the higher extraction yield corresponded to a greater concentration of flavonoids.

Overall, these findings suggest that berry maturation enhances the accumulation of extractable phytochemicals and that ethanol is the most effective solvent for recovering flavonoid-rich extracts from *Phytolacca. dioica* berries. The extraction yield results therefore provide the first indication that ripe berries are likely to possess greater antioxidant potential than unripe berries.

4.2 THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography provides qualitative evidence for the presence of flavonoids in extracts. Therefore, it was used to separate and identify flavonoid compounds present in ripe and unripe *Phytolacca dioica* berry ethanolic extracts. Analytical TLC was carried out using the n-butanol: acetic acid: water (2:2:6, v/v/v) solvent system. Figure 4.1 shows the chromatograms obtained in the separation. Following development and spraying with aluminium chloride reagent, yellow and yellow-green fluorescent bands were observed under ultraviolet light.

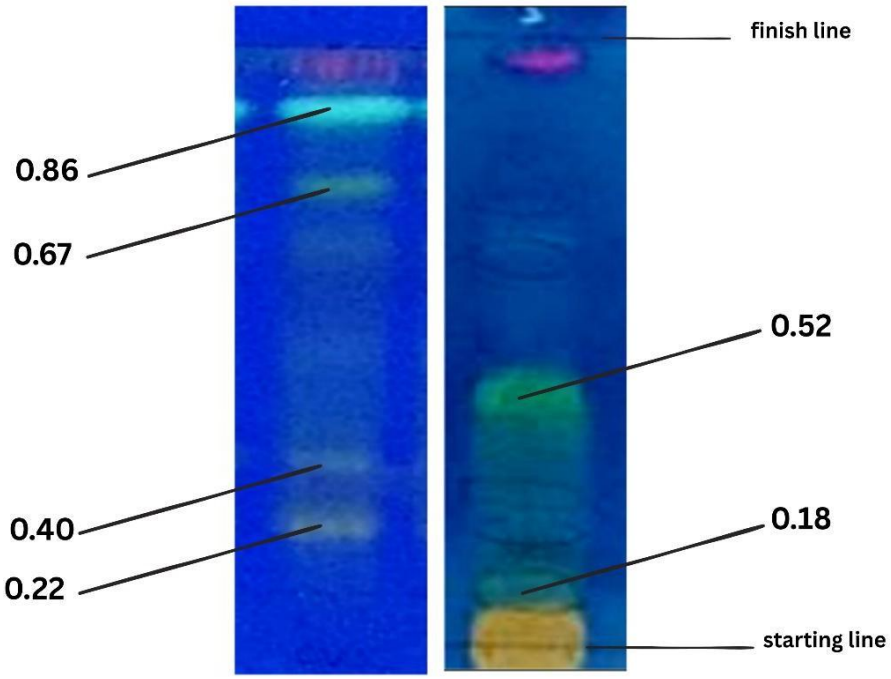


Figure 4.1: Analytical TLC chromatograms ripe berry extract (left side) and unripe berry extract (right side)

The ripe ethanolic extracts gave 4 spots which tested positive for flavonoids and they gave yellow and blue colors (R_f 0.22, 0.44, 0.67 = yellow; R_f 0.86 = blue). The unripe ethanolic extracts showed 2 spots and they both gave yellow-green colors (Mhini, 2021). This difference suggests that berry maturation increases not only the concentration but also the diversity of flavonoids present. The appearance of additional spots in ripe berries indicate activation of multiple branches of flavonoid biosynthesis during fruit development, resulting in the formation of new flavonoid derivatives as ripening progresses.

These fluorescent bands are characteristic of flavonoids, particularly flavones and flavonols, which form stable complexes with aluminium ions through their hydroxyl and carbonyl functional groups (Zych and Pyka-paj, 2025). The TLC results therefore confirmed the successful extraction of flavonoid compounds from both ripe and unripe *Phytolacca dioica* berries. The brighter fluorescence intensity observed indicate higher flavonoid concentrations. A number of spots with different R_f values were observed indicating the presence of several flavonoid constituents with varying polarities.

The observed R_f values ranged from low to high values, indicating the presence of compounds with varying polarities. Lower R_f values correspond to more polar compounds that interact strongly with the silica stationary phase, whereas higher R_f values represent less polar compounds with greater affinity for the mobile phase. This broad distribution of R_f values suggests that the extracts contain both polar flavonoid glycosides and less polar aglycone derivatives.

The greater number of bands observed in ripe berry extracts is consistent with reports that fruit maturation stimulates the synthesis of flavonoids, anthocyanins and other phenolic metabolites. For example, a studies on blueberries reported by Hwang *et al*, 2020 demonstrated that maturation increases anthocyanin and flavonoid accumulation due to enhanced activity of enzymes involved in phenolic biosynthesis. Therefore, the additional chromatographic bands observed in ripe *Phytolacca dioica* berries may represent newly synthesized flavonoid compounds associated with fruit maturation and colour development. Some of the compounds appearing during ripening may be anthocyanin-related flavonoids. Anthocyanins are synthesized from flavonoid precursors and are responsible for the red, purple and blue pigmentation of many fruits (Rajan and Bhat, 2016). Since ripening is often accompanied by increased anthocyanin production, some of the additional TLC bands observed in ripe berries may correspond to anthocyanin derivatives or related flavonoid metabolites.

Despite the usefulness of TLC as a rapid screening technique, it has its own limitations. R_f values are not unique identifiers and may vary depending on plate quality, solvent composition, temperature and sample loading. Different flavonoids may also migrate similarly and appear as a single band. Therefore, TLC can only provide tentative evidence for flavonoid presence and diversity.

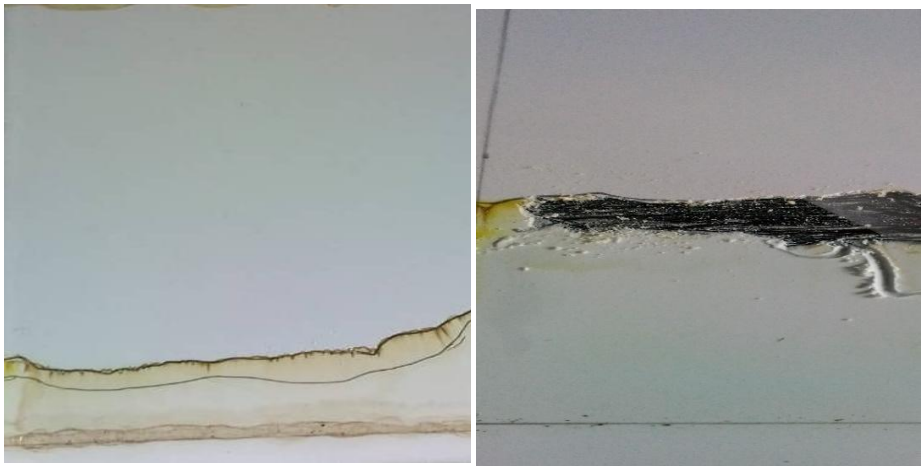


Figure 4.2: Preparative TLC

Preparative TLC was used to isolate flavonoid-rich fractions from the crude *Phytolacca dioica* berry extracts. Using the same solvent system as used in analytical TLC, distinct fluorescent bands were observed under UV light. As shown in Figure 4.2, the flavonoid positive bands displayed yellow after being sprayed with 1% aluminium chloride reagent. The flavonoid rich fractions were then scraped carefully from the silica gel plates for flavonoid recovery and subjected to spectrophotometric quantification and hydrogen peroxide scavenging assays. Similar studies have also reported the successful isolation of flavonoid-rich fractions prior to antioxidant evaluation (Hayward *et al*., 2022).

4.3 UV QUANTIFICATION: TOTAL FLAVONOID CONTENT

The total flavonoid content of the extracts was determined using the aluminium chloride assay and the absorbance was measured at 417 nm. The results are expressed as milligrams of quercetin equivalent per gram (mg QE/g) of the extract. A UV-Vis spectrophotometer was used to quantify the amounts of flavonoids in each extract using a calibration curve from a standard flavonoid which is quercetin. Figure 4.3 shows the calibration curve for the standard quercetin.

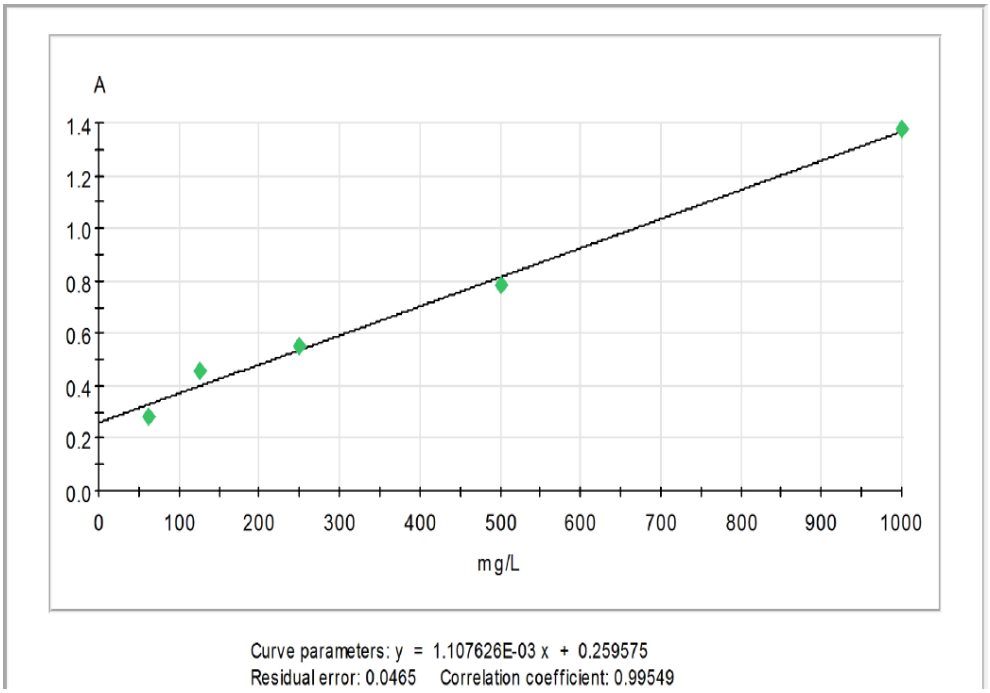


Figure 4.3: Calibration curve for the quercetin.

The calibration curve used for quantification showed excellent linearity (R^2 = 0.995), demonstrating good reliability of the analytical method. Therefore, the differences observed among extracts are likely to reflect true differences in flavonoid content rather than analytical variability

Table 4.2: Estimated flavonoid concentrations per quercetin equivalent

Sample	**Solvent Used**	**Mean Absorbance**	**Total Flavonoid Content (mg QE/g)**
Ripe berries	Ethanol	0.989	658.55
Ripe berries	Ethyl acetate	0.813	499.24
Unripe berries	Ethanol	0.732	426.87
Unripe berries	Ethyl acetate	0.605	311.45

The total flavonoid content results demonstrated significant effects of both berry maturity and extraction solvent on flavonoid recovery. The ripe ethanolic extract exhibited the highest flavonoid content (658.55 mg QE/g), followed by ripe ethyl acetate extract (499.24 mg QE/g), unripe ethanolic extract (426.87 mg QE/g) and unripe ethyl acetate extract (311.45 mg QE/g). These findings indicate that flavonoid accumulation increases during fruit maturation and that ethanol is more effective than ethyl acetate for extracting flavonoids from *Phytolacca dioica* berries. A positive relationship was observed between absorbance and flavonoid concentration, as higher absorbance values corresponded to higher calculated flavonoid contents. Higher absorbance values observed in ripe berry extracts, 0.989 and 0.813, showed a greater concentration of flavonoid compounds compared to unripe berry extracts. According to Rajan and Bhat, (2016), high concentrations of flavonoids and flavonols were high in ethanolic extracts, which is in agreement with the results obtained.

The flavonoid contents obtained in this study (311.45 to 658.55 mg QE/g) are considerably higher than values reported for many berries. For example, blueberry flavonoid contents reported by Hwang *et al*. (2020) ranged approximately from 39 to 103 mg CE/100 g fresh weight depending on cultivar and stage maturity. The exceptionally high total flavonoid content values recorded in this study may be explained by several factors. Firstly, results were expressed on an extract basis (mg QE/g extract) rather than on a fresh-weight basis, which generally produces larger numerical values. Secondly, concentration of the extracts prior to analysis may have enriched the flavonoid fraction. Thirdly, some non-flavonoid phenolics capable of interacting with aluminium chloride may have contributed slightly to the measured absorbance. However, the consistent trend across all extracts strongly indicates genuine differences in flavonoid abundance.

The higher flavonoid content observed in ripe berries is consistent with the physiological changes that occur during fruit ripening. Ripe fruits are often associated with increased synthesis and accumulation of secondary metabolites, including flavonoids, anthocyanin, and other phenolic compounds (Chang and Zhang, 2017). Maturation is accompanied by increased activity of enzymes involved in the phenylpropanoid pathway, including phenylalanine ammonia lyase, chalcone synthase and flavonol synthase (Chang and Zhang, 2017; Hwang *et al*, 2020). These

enzymes promote the synthesis of flavonoids and anthocyanins, which contribute to pigmentation, antioxidant defense and protection against environmental stress.

The highest flavonoid recovery achieved with ethanol further supports the importance of solvent polarity. Many naturally occurring flavonoids are present as glycosides, which contain sugar moieties that increase their polarity. Such compounds are more soluble in ethanol than in ethyl acetate, explaining the consistently higher total flavonoid content values obtained from ethanolic extracts.

The positive relationship between extraction yield and flavonoid content suggests that the higher extraction yields observed for ethanol were largely due to greater recovery of flavonoid compounds. However, it is important to note that the aluminum chloride assay measures flavonoids in terms of quercetin equivalents rather than absolute concentrations. Therefore, the reported values represent relative estimates based on the response of flavonoid compounds compared with quercetin.

4.4 ANTIOXIDANT ACTIVITY ASSAY

The antioxidant activity of the flavonoid extracts from ripe and unripe *Phytolacca dioica* berries was evaluated using the hydrogen peroxide assay at 230 nm wavelength. The hydrogen peroxide scavenging ability of ascorbic acid and the berry extracts are shown in Table 4.3.

Among the extracts, the ripe ethanolic extract exhibited the strongest antioxidant activity (96.8%), followed by the ripe ethyl acetate (88.8%), unripe ethyl acetate (72.9%) and unripe ethanol extract (61.7%). These results are presented graphically in Figure 4.5. These results indicate that both berry maturity and solvent extraction influence the antioxidant potential of the extracts, likely due to changes in the quantity and composition of antioxidant phytochemicals during ripening and differences in solvent polarity affecting flavonoid recovery.

Table 4.3: Hydrogen peroxide scavenging activity (% inhibition)

Concentration (µg/mL)	Ripe ethanol	Ripe ethyl acetate	Unripe ethanol	Unripe ethyl acetate	Ascorbic acid
20	38.6	66.3	16.4	10.4	38.8
40	56.8	71.8	26.1	15	57.2
60	72.5	75.5	38.4	36.6	72.7
80	86.4	85.9	50.3	59.6	86.8
100	96.8	88.8	61.7	72.9	99.6

Figure 4.4 shows a graph of ascorbic acid, which is used as a positive control in antioxidant assays because it is a well-known antioxidant with strong hydrogen peroxide scavenging ability. The results show that ascorbic acid exhibited the highest antioxidant activity 99.6% at 100 µg/mL, confirming the validity of the assay and providing a benchmark against which berry extracts could be evaluated.

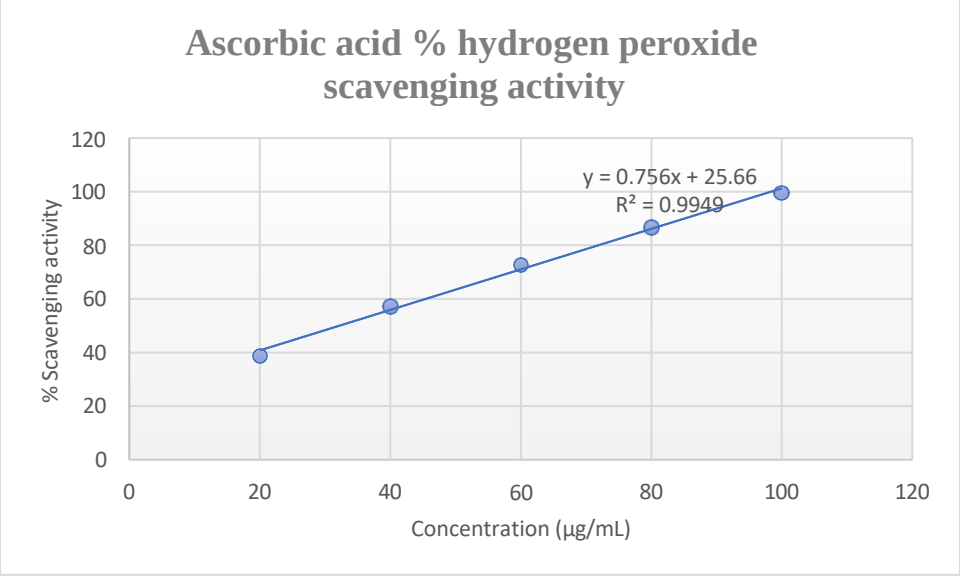


Figure 4.4: Standard curve of ascorbic acid of H_2O_2 scavenging assay

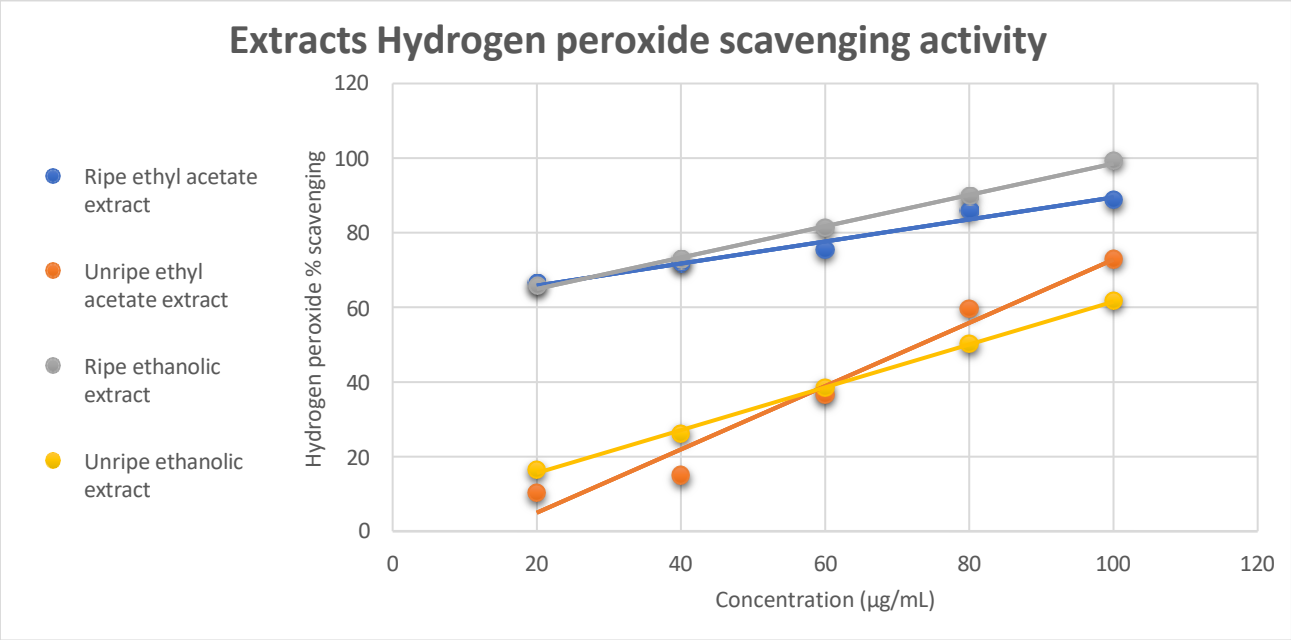


Figure 4.5: H2O2 scavenging activity of ripe and unripe berry extracts of Phytolacca dioica, extracted using ethanol and ethyl acetate solvents.

The results showed that percentage scavenging activity increases with increasing extract concentration, indicating a concentration dependent antioxidant response. This shows that flavonoids present in each extract were capable of neutralizing hydrogen peroxide. As shown in Figure 4.5, all extracts demonstrated hydrogen scavenging activity; however, the degree of action varied with concentration.

A Pearson correlation analysis was performed using Microsoft Excel to evaluate the relationship between total flavonoid content and hydrogen scavenging activity. The analysis yielded a strong positive correlation coefficient (r = 0.777). The close agreement between flavonoid concentration and antioxidant activity provides strong evidence that flavonoids are major contributors to the antioxidant properties of *Phytolacca dioica* berries and support its potential use as a natural source of bioactive compounds. The coefficient determination (R^2 = 0.604) indicates that approximately 60.4% of the variation in antioxidant activity may be due to differences in flavonoid concentration. The correlation plot and statistical output are presented in Figure 4.6. The observed relationship between flavonoid content and antioxidant activity is consistent with numerous studies

demonstrating positive correlations between phenolic content and antioxidant capacity in berry fruits. Similar trends have been reported by Hwang *et al*, (2020) **,** where increased flavonoid and phenolic concentrations were associated with enhanced antioxidant activity.

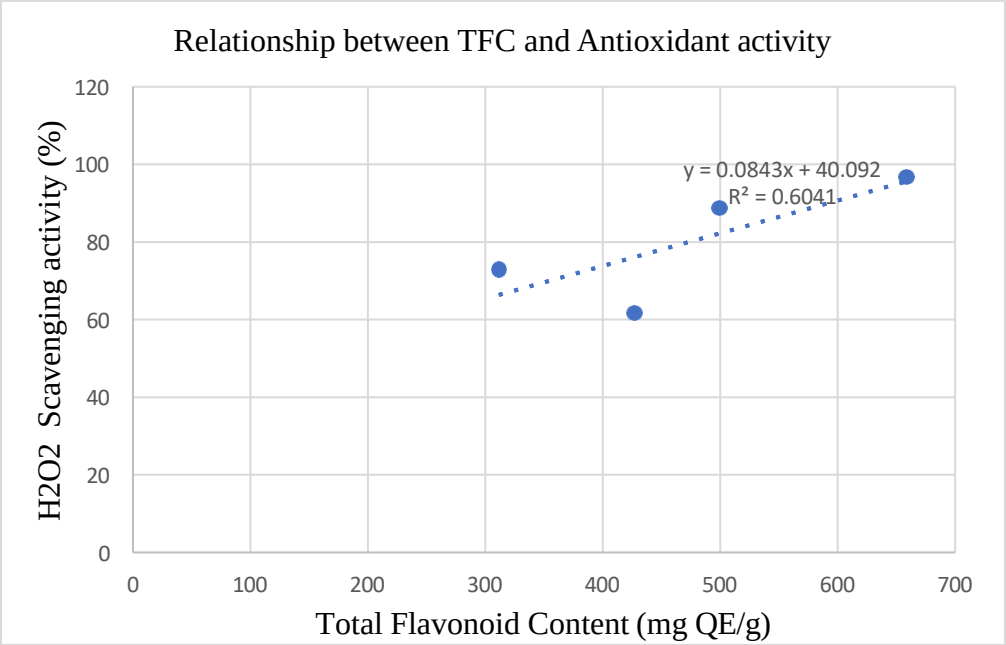


Figure 4.6: Correlation between Total Flavonoid Content and Hydrogen Peroxide Scavenging Activity of Phytolacca dioica berry extracts

Hydrogen peroxide is a relatively stable reactive oxygen species; however, in the presence of transition metals such as iron and copper, it can generate highly reactive hydroxyl radicals through Fenton-type reactions. These free radicals can start a chain reaction that eventually causes lipid peroxidation, protein oxidation and DNA damage leading to many degenerative diseases (Rabiu, Dandare and Bello, 2018). Flavonoids help prevent such damage through several mechanisms, including direct scavenging of reactive oxygen species, donation of electrons or hydrogen atoms and chelation of transition metals that catalyze radical formation (Silva, 2021; Stachelska *et al*, 2025).

The higher antioxidant activity of the ripe ethanolic extract may therefore be attributed not only to its higher flavonoid concentration but also to the presence of flavonoid-like structures possessing multiple hydroxyl groups capable of effective radical stabilization. However, in some studies, unripe fruits showed the highest antioxidant activity. For example, in a study reported by (Tinrat,

2014) the unripe fruit methanol extract exhibited the highest antioxidant activity, this shows that there is a possibility of solvent limitations.

An additional interpretation is that increased flavonoid accumulation during ripening may serve a physiological role in protecting fruits against oxidative stress. Ripening is associated with increased respiration and metabolic activity, both of which generate reactive oxygen species. Accumulation of flavonoids during this period may therefore represent a natural protective mechanism that helps maintain cellular integrity.

Although flavonoids appear to be the major contributors to antioxidant activity, it is unlikely that they are the only compounds involved. Other phytochemicals such as phenolic acids, tannins, anthocyanins and vitamin-like antioxidants may also contribute to hydrogen peroxide scavenging. Synergistic interactions among these compounds may enhance the overall antioxidant capacity of the extracts.

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

This research successfully extracted, isolated, and evaluated flavonoids from ripe and unripe berries, addressing the knowledge gap regarding the antioxidant properties of *Phytolacca dioica L* derived flavonoids. The results demonstrated that both berry maturity stage and solvent type significantly influenced flavonoid yield, flavonoid content, and antioxidant activity. Among all extracts, the ripe berry ethanol extract exhibited the highest extraction yield (10.6%), the highest total flavonoid content (658.55 mg QE/g), and the strongest hydrogen peroxide scavenging activity. However, the unripe berry ethyl acetate extract showed the lowest performance in all evaluated parameters. Preparative TLC analysis confirmed the presence of flavonoid compounds in extracts. The observed fluorescent yellow bands under UV light supported the successful isolation of flavonoid compounds. The hydrogen peroxide scavenging assay revealed that antioxidant activity increased with increasing extract concentration, demonstrating a concentration-dependent response. A positive relationship was observed between total flavonoid content and antioxidant activity, suggesting that flavonoids are major contributors to the antioxidant potential of *Phytolacca dioica* berries. The study therefore confirms the potential of *Phytolacca dioica* berries as a valuable source of bioactive compounds with possible pharmaceutical, nutraceutical, and food preservation applications.

5.2 RECOMMENDATIONS

Based on the findings of this study, *Phytolacca dioica L*. flavonoids are a potential natural antioxidant source and ethanol is recommended as the preferable solvent for the extraction of flavonoids from the berries because of its higher extraction yield, total flavonoid content and highest antioxidant activity compared to ethyl acetate. Ripe berries having exhibited the higher flavonoid concentrations and stronger hydrogen peroxide scavenging activity is also recommended as the most suitable plant source for natural antioxidants. Further studies should focus on the identification and characterization of the isolated flavonoid compounds using advanced analytical techniques such as HPLC and LC-MS. The hydrogen peroxide scavenging assay has limitations. It is an in vitro chemical assay and may not fully represent biological antioxidant activity within

living systems. In addition, extract colour, turbidity and the presence of other reducing substances may influence absorbance measurements. Therefore, while the results demonstrate substantial antioxidant potential, further evaluation using additional assays such as DPPH, ABTS, FRAP and ORAC, together with compound identification studies, would provide a more comprehensive assessment of antioxidant properties. Furthermore, optimization of extraction conditions such as solvent concentration, extraction time, temperature, solvent-to-sample ratio should be studied to maximize flavonoid recovery. Evaluation of the safety and toxicity of the *Phytolacca dioica L*. berry extracts is recommended to assess their potential applications in pharmaceutical, nutraceutical and food industries as a natural antioxidant source.

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