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COMPARATIVE EVALUATION OF ULTRASOUND, ENZYME, MICROWAVE AND SOLVENT-ASSISTED EXTRACTION OF FLAVONOIDS IN *NEPHROLEPIS CORDIFOLIA (L.) C. PRESL*

BY

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APPROVAL FORM

The undersigned certify that they have supervised, read, and recommend to the Bindura University of Science Education for the acceptance of the research entitled:

Comparative evaluation of ultrasound-, enzyme-, microwave, and solvent-assisted extraction of flavonoids in *Nephrolepis Cordifolia (L.) C. PRESL*, submitted by **Bridget Subili** in partial fulfillment of the requirements for the Bachelor of Science Honors Degree in Chemical Technology.

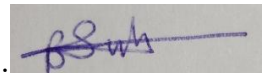
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DECLARATION

I, Bridget Subili, am officially declaring that this dissertation is a product of my original research and that I am the sole author of the work; as such, borrowed ideas are cited accordingly as references. This work has not been accepted elsewhere for the conferment of any academic entitlement and is not concurrently submitted in candidature for any such awards.

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DEDICATION

I dedicate this work to my amazing mother and my family, who offered me endless support and encouragement during the course of this work. You made it all possible. I appreciate the love and support.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Almighty God for the opportunity to be part of this research. I thank Him for the strength and wisdom that He offered during my studies and research. Secondly, I would like to express my gratitude to Dr. L Gwatidzo, my supervisor, for his academic guidance, support, and supervision. I also extend my gratitude to the Chemistry Department and laboratory personnel for assisting me during the analytical experiments that were conducted at the BUSE laboratory. I am also thankful to the gardeners who provided me with the plant that was used as the sample in the analytical experiments. I acknowledge the inspiration and guidance from all my educators and mentors. This project is a reflection of the knowledge and skills I have gained through their teachings. Lastly, I thank friends, family, and everyone who offered help in countless ways. I could not have done it on my own.

ABSTRACT

Flavonoids are bioactive secondary metabolites with diverse pharmacological properties, including antioxidant, anti-inflammatory, and antimicrobial activities. Their efficient recovery from plant sources is essential for pharmaceutical, cosmetic, and nutraceutical applications. This study compared four extraction techniques, namely, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), and conventional solvent extraction, for the isolation of flavonoids from the bulbils of *Nephrolepis cordifolia*. Ethanol was used as the extraction solvent, and yields were quantified as quercetin equivalents using UV-VIS spectrophotometry. TLC analysis confirmed flavonoid fractions, while antioxidant activity was assessed using the hydrogen peroxide scavenging assay. The results demonstrated significant differences among the methods: UAE yielded the highest flavonoid concentration (23.5 mg/L) and antioxidant activity (47.5%), followed by EAE (17.0 mg/L) and 36.3% antioxidant activity. MAE had (13.3 mg/L), and 31.3% antioxidant activity, and solvent conventional solvent extraction yielded (8.0 mg/L) and an antioxidant activity of 15%. The UAE, MAE, and EAE reduced solvent consumption by approximately 66.7% and isolated higher yields compared to conventional solvent extraction, underscoring their alignment with green chemistry principles. The null hypothesis was rejected, confirming that the extraction efficiency and bioactivity are method-dependent. This research establishes UAE as the most effective and sustainable technique for recovering flavonoids from *N. cordifolia*, combining high yield with enhanced antioxidant activity and reduced environmental impact. The findings contribute to the advancement of eco-conscious phytochemical extraction workflows and provide a methodological framework for industrial-scale applications in pharmaceutical and nutraceutical development.

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LIST OF ABBREVIATIONS

BUSE – Bindura University of Science Education

UAE-Ultrasound-assisted extraction

MAE-Microwave-assisted extraction

EAE- Enzyme-assisted extraction

SE-solvent extraction

TLC- Thin-layer chromatography

RF- Retention Factor

UV-VIS -Ultraviolet- visible spectrophotometry

BHT- Butylated hydroxytoluene

BHA- Butylated hydroxyanisole

SFE- Supercritical fluid extraction

PUAE – Pulsed Ultrasound-Assisted Extraction

UT-UAE – Ultra-Turrax Ultrasound-Assisted Extraction

IL-UAE – Ionic Liquid Ultrasound-Assisted Extraction

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

pH – Potential of Hydrogen

°C – Degrees Celsius

mg/L – Milligrams per Liter

% – Percentage

CHAPTER 1

1.0 BACKGROUND OF THE STUDY

Plants are a rich source of chemical compounds, some of which provide nutritional benefits, while others are non-nutritional. Non-nutritive substances, also known as secondary metabolites, are not involved in growth and development; instead, they play a defensive role against pathogens such as bacteria, viruses, and fungi (Tuladhar et al., 2020). The presence of these phytochemicals is also beneficial to humans, as they use these plants as herbal medicines, and researchers are using them as drug leads in the development of new medicines. *Nephrolepis cordifolia L.C. Presl*, a fern in the Nephrolepidaceae family, is also used in folk medicine due to its therapeutic effects (Dos Santos et al., 2024). The plant is native to northern Australia and Asia, but due to cultivation practices, it has spread across diverse habitats (Hovenkamp & Miyamoto, 2005). The fern propagates through rhizomes, stolons, tubers, and spores. It has bright green fronds that are usually 40-80 cm long and up to 10 cm wide (Oyeyemi et al., 2019). The plant has been used to treat indigestion, fever, and skin disorders, and also as a diuretic, contraceptive, and remedy for liver disorders (Sharma et al., 2025). Chemically, *N. cordifolia* is rich in secondary metabolites that include flavonoids, alkaloids, saponins, and terpenoids. These constituents are associated with biological activities such as antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, and cytotoxic effects (Twaij & Hasan, 2022). Among these secondary metabolites, flavonoids are essential in pharmaceutical research and drug development. Effective isolation and purification of flavonoids from natural sources is therefore, an important step for their study and application (Researchers & Professionals, 2025). Traditional sample preparation methods for extracting these compounds have been used, such as dissolving the plant material in water. This was a simple technique; however, for research purposes, conventional extraction methods are considered time-consuming and less effective (Liu et al., 2014; Patel et al., 2019). Modern extraction techniques, which emphasize green chemistry, have been applied to overcome limitations associated with conventional methods. Modern techniques offer shorter extraction time, reduced solvent use, and sustainability, especially for thermolabile compounds (Bitwell et al., 2023). These methods enhance the separation of target compounds from impurities, ensuring higher yields and more reliable analysis. Techniques such as microwave-assisted extraction (MAE), ultrasound-assisted

extraction (UAE), enzyme-assisted extraction (EAE), supercritical fluid extraction (SFE), membrane extraction, freeze-thaw assisted extraction, and pressure-assisted liquid extraction have been used in recent years (Goti & Dasgupta, 2023). This study aims to compare conventional solvent extraction techniques to these modern techniques, focusing only on MAE, UAE, and EAE in the isolation of flavonoids from the fern plant, and evaluate the antioxidant properties of the extracts.

1.1 PROBLEM STATEMENT

Herbal plants have long been valued in traditional medicine, yet most of the plants remain underexplored for therapeutic potential. In the food industry, synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are widely used to preserve products. However, these compounds have been associated with adverse health effects, including endocrine disruption and carcinogenic potential, raising concerns about their long-term safety for human consumption (Ren et al., 2024; FDA, 2026). This underscores the need for safer, plant-derived alternatives. *Nephrolepis cordifolia*, a fern species recognized for its medicinal and nutritional properties, has not been fully systematically investigated for its phytochemical composition, antimicrobial activity, and antioxidant potential. Conventional extraction methods have been employed to isolate phytochemicals, but these approaches are often criticised for being time-consuming, solvent-intensive, and environmentally unsustainable. Modern extraction techniques, including ultrasound-assisted, microwave-assisted, and enzyme-assisted methods, have emerged as promising alternatives, offering improved efficiency, selectivity, and reduced environmental impact. However, comparative studies evaluating these techniques against solvent extraction specifically for *N. cordifolia* are lacking. This knowledge gap creates uncertainty regarding the most effective method for maximizing yield, preserving bioactivity, and ensuring cost-effectiveness. Addressing this problem is essential to unlock the potential of *N. cordifolia* as a source of natural antioxidants, thereby supporting its application in health-related and commercial sectors. This study, therefore, aims to compare microwave-assisted extraction, ultrasound-assisted extraction, enzyme-assisted extraction, and conventional solvent extraction methods applied to the bulbils of *N. cordifolia*, to identify the most efficient and sustainable approach for phytochemical recovery.

1.2: AIM

To systematically compare ultrasound-assisted, microwave-assisted, enzyme-assisted, and conventional solvent extraction methods and to determine their relative efficiency, yield, and effectiveness in recovering flavonoids from the bulbils of *Nephrolepis cordifolia.*

1.3: OBJECTIVES

1. To extract flavonoids from bulbils using ultrasound-assisted, microwave-assisted, enzyme-assisted, and solvent extraction methods.
2. To determine the extraction yield for each method.
3. To compare the total antioxidant activity of the extracts from each of the extraction techniques.

1.4: RESEARCH QUESTIONS

1. Which extraction method yields the highest concentration of flavonoid compounds?

2. Which method is most sustainable and cost-effective?

3. What is the total flavonoid content obtained from the extracts of *N. cordifolia*?

1.5: SIGNIFICANCE OF THE STUDY

The study seeks to compare the efficiency of microwave-assisted, ultrasound-assisted, enzyme-assisted, and conventional solvent extraction techniques in isolating flavonoids from the bulbils of *N. cordifolia*. Flavonoids are known for their diverse pharmacological properties, including antioxidant, anti-inflammatory, and antimicrobial activities, and because of these properties, they are valuable in pharmaceutical, nutraceutical, and cosmetic applications. They are secondary metabolites that are widely distributed in plants; they are not artificially synthesized but are inherently present within plant tissues, requiring appropriate extraction methods to be isolated. However, the yield and quality of the bioactive compounds from plants are highly dependent on the extraction method employed. By systematically evaluating and contrasting these techniques, the study not only contributes to the optimization of flavonoid extraction from relatively underexplored fern species but also provides a methodological framework that can be applied to other medicinal plants. Furthermore, the findings have practical implications for industries seeking

a sustainable and cost-effective approach to bioactive compound recovery, as modern techniques such as microwave and ultrasound extraction often reduce solvent consumption, energy use, and processing time compared to conventional methods. Thus, the study advances phytochemical research, supports the development of standardized herbal formulations, and promotes environmentally conscious extraction practices, thereby bridging scientific innovation with industrial application.

1.6: DELIMITATIONS

The study is limited to the bulbils of *Nephrolepis cordifolia* as the plant material, excluding other parts such as the fronds, rhizome, or roots, which may also contain flavonoids. Secondly, only four extraction techniques are considered, while other advanced techniques are excluded. The study further confines itself to the quantitative assessment of flavonoid yield and extraction efficiency without extending to other bioactive compounds present in the plant.

1.7: LIMITATIONS

Firstly, the study is conducted under controlled laboratory conditions, which may not fully reflect the complexities and variability of industrial-scale extraction processes. The study does not account for seasonal, geographical, or environmental variations in plant material that may affect flavonoid content. Additionally, the availability and cost of specialized equipment, such as microwave and ultrasound extractors, may limit the scalability of the findings in resource-constrained settings. Finally, the study focuses on a single plant species, and therefore, the results may not be directly generalizable to other medicinal plants with different phytochemical compositions.

1.8: HYPOTHESIS

H(o): There is no significant difference in the yield and antioxidant activity of flavonoids extracted from the bulbils of *N. cordifolia* from ultrasound, microwave, enzyme, and solvent extraction methods

H (1): There is a significant difference in the yield and antioxidant activity of flavonoids extracted from the bulbils of *N. cordifolia* from ultrasound, microwave, enzyme, and solvent extraction methods

CHAPTER 2

LITERATURE REVIEW

2.0 INTRODUCTION

Flavonoids are a group of polyphenolic compounds found in natural sources with great diversity and wide distribution in plants. They are known for their antioxidant, anti-inflammatory, and antimicrobial properties, with applications across the pharmaceutical, nutraceutical, and cosmetic industries. Extraction of flavonoids from plants is an important step in assessing their bioactivity, and the selection of the extraction method strongly affects both yield and purity. *Nephrolepis cordifolia*, a medicinal fern, was previously reported for its bioactive constituents and pharmacological potential. But studies on comparative evaluation of various extraction methods for the isolation of flavonoids from this species are scarce. This chapter presents a summary of the principles, advantages, and limitations of four main extraction methods: microwave-assisted extraction, enzyme-assisted extraction, ultrasonic-assisted extraction, and conventional solvent extraction, focusing on previous research and factors affecting extraction efficiency.

2.1 THE PLANT

Nephrolepis cordifolia (L.).C Presl is a terrestrial fern that forms colonies by means of short rhizomes and small, scaly tubers at its roots. They are commonly identified as sword fern or ladder fern. People mostly use the plant to decorate their homes and in their flower gardens. Along its underground rhizomes, the plant produces small, round nodules that are called bulbils. The bulbils are responsible for nutrient storage; their other function is to help the plant grow vegetatively, and they are also rich in phytochemicals. These bulbils are edible, and people use them to ease stomach pains; for this reason, their pharmaceutical applications need to be fully understood. The diverse array of phytocompounds present in ferns plays a role in enhancing their ecological resilience, thereby enabling the plants to adapt and flourish across a wide range of environmental conditions (Chaves et al.,2020). Beyond their conventional recognition as ornamental species, the biochemical significance of the phytochemicals suggests a broader potential for human application. If isolated and harnessed, the phytochemicals from the ferns could provide valuable

contributions to various industrial sectors, thereby extending the relevance of ferns beyond their aesthetic appeal.



Figure 2. 1 The *Nephrolepis cordifolia (L.).C Presl* plant



Figure 2.2: Bulbils *Nephrolepis cordifolia (L.).C Presl* plant

2.2 FLAVONOIDS

Plants produce secondary metabolites that help them survive and adapt to threats that can be found in their ecological niches (Nabavi et al., 2018; Bitwell et al., 2023). When a plant is growing in unfavorable environmental conditions, it synthesizes biological molecules with insecticidal, antibacterial, or antiviral properties. These compounds contribute to pigmentation, UV protection, and pathogen defense(Rodríguez De Luna et al., 2020). These bioactive compounds, classified as terpenoids, alkaloids, phenolic compounds, and flavonoids, are also an important subgroup. The flavonoids are natural antioxidants due to their ability to eliminate free radicals and inhibit their formation. In addition, chelation of metal ions to inhibit lipid peroxidation is another characteristic of flavonoids(Fernando & Soysa, 2014). In this way, flavonoids could be used to treat some pathophysiological conditions that involve free radicals, such as cardiovascular and neurodegenerative diseases. Flavonoids are significant because they can be utilized as health supplements, food additives, cosmetics, and in pharmaceutical chemistry for potential treatment of diabetes and certain cancers(Rodríguez De Luna et al., 2020). Flavonoids are based on a fifteen-carbon flavanone skeleton (C6-C3-C6), consisting of two benzene rings A and B linked by an oxygen-containing heterocyclic pyran ring (C) (Chaves et al., 2020). Flavonoids are further divided into subgroups such as flavones, flavonols, flavanones, and anthocyanins (Tušek et al., 2022). Their biological activities include free radical scavenging, modulation of enzyme activity, and regulation of signaling pathways.

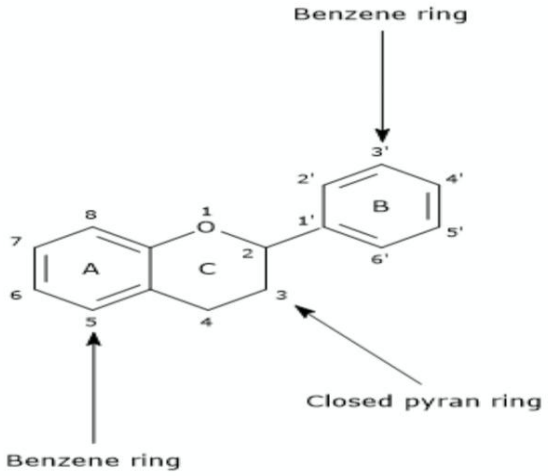


Figure 2.3: Basic structure of flavonoids

2.3 EXTRACTION TECHNIQUES

Extraction can be defined as a mass transfer process of components from one phase to another. In natural products, the sample to be extracted can be a solid material, and sometimes liquid samples are used (Goti & Dasgupta, 2023). Extraction methods are fundamental techniques in natural product research because they are used in the isolation of bioactive compounds from complex plant matrices. Biomolecules, such as flavonoids, alkaloids, and phenolics, are present in small quantities and require efficient recovery strategies to be studied and applied to the food, pharmaceutical, and cosmetic industries (Gokulakrishnan et al., 2025). Both conventional and modern extraction approaches have been developed to improve yield, selectivity, and sustainability (Chaves et al., 2020; Usman et al., 2023). Traditional solvent extractions remain widely used due to their simplicity, but modern techniques such as ultrasound-assisted, enzyme-assisted, and microwave-assisted extraction have emerged to overcome limitations, which include long processing times, high solvent consumption, and degradation of sensitive molecules (Chaves et al., 2020; Xu et al., 2017). Comparing these methods provides valuable insight into their efficiency, environmental impact, and suitability for different classes of bioactive compounds, thereby guiding researchers and industries toward more effective and eco-friendly practices (Chaves et al., 2020).

2.3.1 Solvent extraction

Conventional solvent extraction is a widely used technique in the isolation of phytochemicals from plant samples. It involves maceration or reflux with solvents such as methanol, ethanol, or acetone. In maceration, the homogenized plant sample is soaked in a solvent inside a closed container and left at room temperature, which softens and breaks down the cell walls of the plants, thereby releasing the phytochemicals that would be bound in the plant (Goti & Dasgupta, 2023). The solvent is then filtered to remove unwanted plant tissue (Singh, 2013). This method is commonly used in phytochemical and pharmaceutical research, as well as in food and herbal industries, where plant-derived compounds are needed for analysis or product formulation (Chaachouay & Zidane, 2024). Solvent extractions are a simple method, accessible and effective in the extraction of a wide range of compounds without the need for highly specialized equipment. Its limitations include: longer extraction times, consumption of larger volumes of solvent, and the elimination of the solvent from the product is difficult, in that it poses a threat to health and the environment (Eyube

et al., 2025). The method is generally less selective compared to advanced techniques, such as microwave-assisted extraction and ultrasonic extraction (Vina Nha Trang, 2025).

2.3.2 Microwave assisted extraction

Microwave-assisted extraction (MAE) utilizes electromagnetic radiation to rapidly heat solvents and plant tissues. The use of microwaves for laboratory practices has been used for over 20 years(Majors, 2007). The localized heating enhances solvent penetration and disrupts plant cell walls, thereby releasing phytocompounds more efficiently. MAE operates through dipole rotation and ionic conduction, which enables the direct and uniform transfer to the extraction medium. MAE enhances extraction by heating solvent and material at frequencies between 300 MHz and 300 GHz, leading to energy conversion through dipolar rotations(Altemimi et al., 2017; Tušek et al., 2022). It offers shorter extraction times and higher yields compared to conventional methods. Microwave-assisted extraction also lowers solvent consumption and has lower energy requirements, which makes the method have a lower environmental footprint (Usman et al., 2023). The efficiency of MAE is particularly notable when extracting polyphenols, alkaloids, flavonoids, and essential oils. In a recent comparative analysis, MAE was shown to outperform ultrasound-assisted extraction (UAE) in complex plant matrices, such as traditional herbal formulations, due to its ability to rapidly rupture cell walls and enhance mass transfer rates (Ngamkhae et al., 2022). Furthermore, the versatility of microwave systems allows for integration with green solvents and closed-loop systems, further enhancing sustainability. For medicinal chemists, MAE has proven to be a reliable, reproducible, and scalable option for isolating bioactive compounds with high purity and minimal degradation, fostering a more efficient transition from plant-based inputs to pharmaceutical applications (Shen et al., 2023). Excessive microwave power may degrade heat-sensitive compounds. Careful optimization of parameters such as power, extraction time, and the choice of the solvent to be used in extraction is to be considered, as hydrocarbons cannot be used due to their high flammability (Leong & Doughty, 2024).

2.3.3 Enzyme-assisted extraction

Enzymes are biological molecules that speed up the rate of chemical reactions without being altered after the process. They are highly specific and effective (Xu et al., 2017). Each enzyme has an active site that binds to a substrate, causing conformational changes that facilitate the reaction process. The molecule to be catalysed is the substrate. In plants, the substrates are found in the cell

walls, which are composed of polysaccharide complexes such as cellulose, hemicellulose, pectin, and proteins (Rodríguez De Luna et al., 2020). These components are responsible for the structural stability of the walls of the plant, and when enzymes interact with them, they increase permeability and weaken their bonds, and this causes the cell walls to break, thereby releasing phytocompounds. This process enhances the extraction of oils, phenolics, flavonoids, carotenoids, and other bioactive compounds in the plant (Xu et al., 2017). Enzyme-assisted extraction (EAE) uses enzymes such as cellulase, pectinase, hemicellulose, and β-glucosidase to degrade plant cells, and this causes the bioactive compounds to be liberated into the solvent to form a solution (Zhang et al., 2018; Usman et al., 2023). Enzymes can be found in bacteria, fungi, vegetables, fruit extracts, or animal organs (Cheng et al., 2015; Liu et al., 2014). For efficient EAE, there must be careful optimization of conditions, including enzyme concentration, pH, incubation temperature, incubation time, and liquid-to-solid ratio (Parveen et al., 2025; Xu et al., 2017). Most extractions are performed under acidic conditions around pH 4, which stabilize hydrogen bonds and promote the release of bioactive compounds (Rodríguez De Luna et al., 2020). The optimum temperature for most enzymes is approximately 40°C, whereas temperatures above 60°C cause irreversible changes in enzyme structure, denaturing it (Rodríguez De Luna et al., 2020; Xu et al., 2017). EAE is an eco-friendly, selective, and often gives highly pure extracts. Its disadvantages include the high cost of enzymes and the need for precise control of experimental conditions.

2.3.4 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) is widely used in the extraction of bioactive compounds from natural products with applications in the food and cosmetic industries (Akbari,2019). UAE uses high-frequency sound waves to induce cavitation in the extraction medium, and this causes ultrasonic waves to generate microbubbles (Chaves et al., 2020). These bubbles collapse during compression-rarefaction, producing intense mechanical effects that reduce particle size and damage cell walls (Usman et al., 2023). This enhances solvent-sample interaction, increasing cell permeability and facilitating the mass transfer, resulting in higher yields within shorter extraction times (Um et al., 2018; Chaves et al., 2020). On bursting, cavitation bubbles can reach pressures of 100 MPa and temperatures of 5000 K, and these conditions break plant material and release intracellular compounds (Rodríguez De Luna et al., 2020). UAE operates at temperatures that are suitable for thermosensitive bioactive compounds, and it also preserves or minimizes oxidative

degradation (Eyube et al., 2025). Ultrasound-assisted extraction is applied in crude oil desulfurization, cell disruption, and the extraction of phenolic compounds from fruits, teas, vegetables, and flowers (Shirsath et al., 2012; Tabaraki et al., 2012). It is considered eco-friendly, requiring less solvent and energy (Altemimi et al., 2017). Common laboratory equipment, such as ultrasonic baths or probe systems, is used, with baths typically operating between 20-100 kHz (Chaves et al., 2020; Usman et al., 2023). Extraction efficiency depends on ultrasound frequency, intensity, temperature, and time (Altemimi et al., 2017; Xu et al., 2017). To further improve yields and reduce energy consumption, novel techniques such as pulsed UAE (PUAE), ultra-turrax-based UAE (UT-UAE), and ionic liquid-based UAE (IL-UAE) have been developed (Kazemi et al., 2016). However, scalability remains a challenge, and prolonged exposure may lead to oxidation of sensitive compounds. UAE has emerged as a versatile, efficient, and environmentally friendly extraction technique, offering high productivity with minimal solvent and time consumption, and proving to be suitable for molecules with high thermal sensitivity (Usman et al., 2023; Yusoff et al., 2016). Cai et al. (2016) used UAE for anthocyanins, total phenols, and flavonoids extraction from purple sweet potatoes and compared it with accelerated-solvent extraction and conventional extraction methods, and concluded that UAE is more convenient than other conventional methods. A key advantage of the UAE is its ability to operate at lower temperatures, preserving thermosensitive compounds and minimizing oxidative degradation (Eyube et al., 2025).

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

This chapter outlines the experimental methods used to assess and compare four different extraction techniques. The methods were applied to isolate flavonoids from the bulbils of the *Nephrolepis cordifolia L.C* plant.

3.2 MATERIALS AND EQUIPMENT

Plant samples were obtained in January from the NASSA branch in Marondera, Zimbabwe. The specimens were submitted to the Harare Botanical Gardens for taxonomic verification, where they were formally identified as *Nephrolepis cordifolia (L.).C Presl.* Following identification, the bulbils of the plant were thoroughly washed to remove adhering soil and debris and subjected to sun drying for a period of one month. This drying process ensured the reduction of moisture content and preservation of phytochemicals. Upon completion of drying, the bulbils were pulverized using a laboratory blender to obtain a fine powder. The powdered material was stored in airtight containers under ambient laboratory conditions until required for the experimental analysis.

Chemicals and reagents were purchased from ACE chemicals and Glassworld. The following reagents were employed: ethanol, methanol, hexane, silica gel, ethyl acetate, aluminium chloride, enzyme pepsin, distilled water, hydrochloric acid, lead acetate, ferric chloride, phosphate buffer solution, hydrogen peroxide, sodium hydroxide, sodium acetate, quercetin, and ascorbic acid. The reagents were analytical reagent (AR) grade.

Apparatus used included: Analytical balance, microwave, ultrasonic bath, laboratory shaker, thermometer, pH meter, spectrophotometer, hot plate, vacuum pump, stop-watch, TLC plates, laboratory blender, and standard laboratory glassware.

3.3 SAMPLE PREPARATION

Fresh specimens of *Nephrolepis cordifolia L.C*. were uprooted, and the bulbils were collected. The samples were thoroughly cleaned to eliminate any soil and debris, followed by sun-drying for one month to reach a stable mass (Dzomba, 2012). The dried plant material was ground into powder using a pestle and mortar. Coarse fragments were re-crushed, and the remaining unusable parts were discarded. The powdered samples were sieved using a 75 mm sieve. This was done to obtain a homogenized powder.

3.4 EXTRACTION PROCESS

3.4.1 Solvent extraction

10 g of bulbils were precisely measured using an analytical balance. The samples were then transferred into a 250 ml conical flask, and 300 ml of 80% ethanol was added as the extraction solvent. The flasks were placed on a laboratory shaker and agitated at medium speed for 24 hours at room temperature to promote solvent penetration and compound extraction.

After 24 hours, the samples were retrieved, and any remaining plant material was removed using a vacuum filtration system. The resulting filtrates were placed in a water bath set at 50 °C to evaporate the solvent. The concentrated extracts were collected, weighed, and stored appropriately for future analyses.

3.4.2 Microwave-assisted extraction

10 g of the dried bulbils were weighed and put into a beaker. 100 ml of 80% ethanol was added to the volumetric flasks, and the flasks were put in a microwave for 30 minutes to absorb microwave radiation, thereby extracting phytocompounds (Rodríguez De Luna et al., 2020). The solution was cooled, and residual plant material was removed through filtration. The solution was then put on a water bath to evaporate the solvent; the water bath was maintained at 50 °C. After the evaporation, the extracts were collected, weighed, and stored for further analysis.

3.4.3 Ultrasound-assisted extraction

10 g of bulbils were submerged in 100 ml of 80%, and then they were sonicated for 30 minutes at 40 °C in an ultrasonic bath running at 40 kHz (Tušek et al., 2022; Goti & Dasgupta, 2023). After the sonication, the mixtures were cooled, placed on filter paper, and vacuum-filtered to eliminate any remaining plant material. After that, the extracts were concentrated in a fume hood using a water bath at 50 °C. The crude extracts were weighed and then refrigerated for further analysis.

3.4.4 Enzyme- assisted extraction

10 g of powdered plant material was suspended in 100 ml of 80% ethanol. Invertase was added at a concentration of 1% (w/v), and the mixtures were incubated in a water bath at 40 °C for an hour (Rodríguez De Luna et al., 2020). The pH was maintained around 5 because invertase is an acid-stable enzyme (Siddiqa & Amin, 2022). After the incubation time, the extracts were filtered under vacuum, concentrated by solvent evaporation at 50 °C, and stored under refrigeration for subsequent phytochemical analysis.

3.5 PHYTOCHEMICAL SCREENING TEST FOR FLAVONOIDS

2 ml of the extract was placed in a test tube, and a few drops of 10% ferric chloride solution were added. The solution was observed for any colour changes (Kumar, 2013).

3.6 PURIFICATION OF FLAVONOIDS

The concentrated crude extract was subjected to column chromatography for the purification of flavonoids.

A glass column was packed with silica gel using hexane as the packing solvent, and the extract was carefully loaded onto the column after being dissolved in a small amount of hexane. Elution was carried out using a gradient solvent system beginning with hexane to remove non-polar impurities, followed by increasing proportions of ethyl acetate in hexane (9:1,7:3,1:1), then pure ethyl acetate, and finally ethyl acetate: methanol mixtures (9:1,7:3) to elute flavonoid-rich fractions. Fractions of 10-20ml were collected and monitored by thin-layer chromatography using silica gel plates developed in ethyl acetate: methanol: water (100:13.5:10).

Visualization was performed under UV light (254 nm and 365 nm) and by spraying with aluminum chloride reagent, which produced characteristic fluorescence for flavonoids. Fractions showing

similar TLC profiles were pooled, concentrated, and weighed to determine the weight of the purified flavonoids. (Researchers & Professionals, 2025).

The percentage yield was calculated as follows:

$$\%Yield = \frac{mass\ of\ extract}{mass\ of\ plant\ sample\ measured\ for\ extraction} X\ 100$$

3.8 DETERMINATION OF TOTAL FLAVONOIDS BY SPECTROPHOTOMETRY

The flavonoids were evaluated by the aluminium chloride colorimetric technique (Bag et al., 2015; Bag G.C. et al., 2015)

A 1% aluminium chloride solution and 1 M potassium acetate were prepared in distilled water. 0.5 ml of the extract was mixed with 1.5 ml of 70% methanol, 0.1 ml of 1% aluminium chloride, and 0.1 ml of 1 M potassium acetate. Then, 2.8 ml of distilled water was added. The mixture was left to incubate for 30 minutes at room temperature. After incubation, absorbance was measured at 420 nm using a UV-Vis spectrometer. The flavonoid content was determined by the standard curve of quercetin. The outcome data were reported as mg/g of quercetin equivalents (mg QE/g) of the extract.

The quercetin standard and stock solutions were prepared by dissolving 10 mg of quercetin in 80% ethanol and diluting to 25, 50, and 100 µg/mL (Chang et al., 2002).

3.9 DETERMINATION OF ANTIOXIDANT ACTIVITY USING THE HYDROGEN PEROXIDE SCAVENGING METHOD

The ability of the extracts to scavenge hydrogen peroxide was determined according to the Ruch et al. (1989). A solution of hydrogen peroxide of 40 mM was prepared in a phosphate buffer of pH 7.4. Extracts (100 µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40 mM). 0.1 ml of sample extract was mixed with 3.4 ml of 0.1 M phosphate buffer and 0.6 ml of 40 mM H_2O_2. This mixture was incubated for 10 minutes at room temperature. The absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing phosphate buffer without hydrogen peroxide (Fernando & Soysa, 2015; Pleh et al., 2021). The percentage of hydrogen peroxide scavenging of the extracts from each of the extraction techniques and standard compounds was calculated using the formulae:

$$\%Scavenged = \frac{A_c - A_s}{A_c}$$

Where A_C is the absorbance of the control, A_S is the absorbance of the sample

CHAPTER 4

RESULTS AND DISCUSSION

4.0 INTRODUCTION

The results of the research are presented in this chapter and are displayed as tables and graphs. Ethanol was selected in this investigation due to its ability to dissolve both polar and non-polar compounds. According to Hyun et al. (2013), some solvents can be poisonous and carcinogenic; however, ethanol is safer and more desirable. Four extraction techniques: Ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE), and conventional solvent extraction were evaluated for their efficiency in the isolation of flavonoids, quantified as quercetin equivalents, from *N. cordifolia*. The calibration curve generated at 420 nm demonstrated excellent linearity (R^2 = 0.99947), thereby confirming the reliability of UV-VIS spectrophotometric quantification. Among the methods used, UAE yielded the highest flavonoid concentration (23.45 mg/L), followed by EAE (17.0 mg/L), MAE had a yield of 13.3 mg/L, while solvent extraction had the lowest yield of 8 mg/L.

4.1 PHYTOCHEMICAL SCREENING

A green colour change was observed upon the addition of the ferric chloride solution to the sample extract. Figure 4.1 shows the sample before and after the addition of the ferric chloride solution. The test tube on the left shows the sample before the ferric chloride solution was introduced and the one on the right shows the sample after the addition of the ferric chloride. The green colour change observed confirms the presence of flavonoids in the sample.

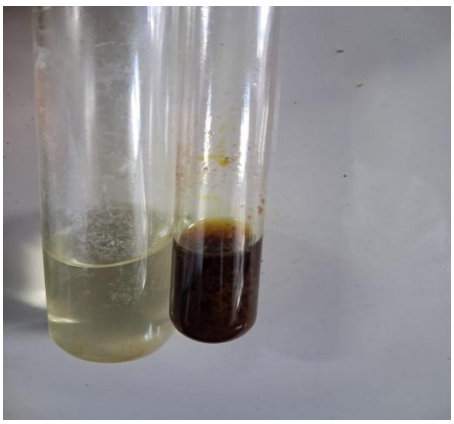


Figure 4. 1: Phytochemical screening of flavonoids

4.2 TLC R_f VALUES FOR FLAVONOID FRACTIONS

The R_f values of the flavonoid fraction were calculated using the equation below:

$$R_f = \frac{distance\ travelled\ by\ the\ compound\ (cm)}{distance\ travelled\ by\ solvent\ front\ (cm)}$$

Figure 4.1 shows a TLC separation of the flavonoid fraction.

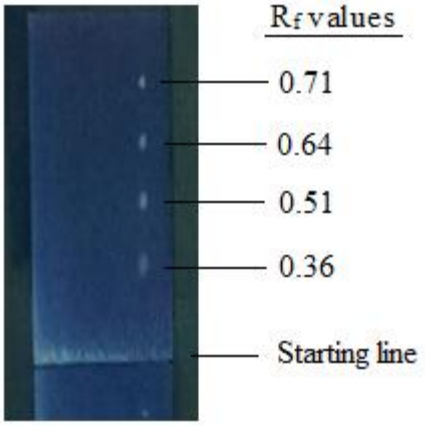


Figure 4. 2: TLC separation of flavonoid fraction

Table 4. 1: Results for flavonoid tests of TLC spots

R_f value	Test for flavonoids
0.36	Positive
0.51	Positive
0.54	Positive
0.75	Negative

Thin-layer chromatography was performed, and the results were recorded in Table 4.1. The fractions corresponding to R_f values of 0.36, 0.51, and 0.64 were confirmed as flavonoids by spraying with 1% ethanolic aluminum chloride reagent, which produced characteristic yellow-green fluorescence when visualized under UV light at 365 nm and this observation indicates the

presence of flavonoids. These fractions were pooled, concentrated, and weighed. The band at R_f 0.75 did not fluoresce and was excluded from pooling and yield calculations, thereby minimizing the inclusion of non-flavonoid impurities. The pooled fractions align with established flavonoid profiles, supporting their identification as flavonoid- rich components. The analysis by Gwatidzo et al. (2018) reported flavonoid TLC profiles with similar ranges in a different solvent system. Furthermore, the observed R_f values are consistent with literature reports for flavonoid compounds, for instance, quercetin standard in a TLC mobile phase solvent phase ratio of chloroform: methanol (1:1) showed an R_f value of 0.46 (Sambandam et al., 2016). In alignment with published chromatographic studies, flavonoids such as pelargodin, peonidin, malvidin, cyanidin, petunidin, and delphinidin have been reported to exhibit R_f values of 0.41, 0.39, 0.37, 0.31, 0.27, and 0.21, respectively (Jeffrey et al., 2013). These findings establish a consistent migration window for anthocyanidin-type flavonoids, generally ranging from 0.21 to 0.41 in conventional solvent systems. Therefore, this range serves as a benchmark for flavonoid identification in plant extracts. Bands migrating beyond 0.70 are rarely attributed to flavonoids. Recent studies emphasize that compounds in this zone require advanced chromatographic or spectroscopic validation through advanced techniques such as HPLC and LC-MS (Zych & Pyka-Pająk, 2025)

4.3 EXTRACTION YIELD

$$\%Yield = \frac{mass\ of\ extract}{mass\ of\ plant\ sample\ measured\ for\ extraction} \times 100$$

The fractions obtained from the chromatography were concentrated, weighed, and the percentage yield from each technique was recorded in Table 4.2.

Table 4.2 Extraction yields of the flavonoids

Extraction method	**Yield (g)**	**%Yield**
UAE	0.625	6.25
EAE	0.413	4.13
MAE	0.293	2.93

Conventional solvent extraction	0.201	2.01

Table 4.2 shows the masses of the flavonoids obtained after purification of the crude extract, and the results indicate that UAE produced the highest yields (6.25%), followed by EAE (4.13), and MAE (2.93%). Conventional solvent extraction had the lowest yield of 2.01%, consistent with reports that highlight its longer extraction times, lower efficiency, and high solvent extraction (Chaves et al., 2020). These findings show how the modern extraction methods are improving efficiency and sustainability.

4.4 DETERMINATION OF FLAVONOIDS BY SPECTROMETRY

Figure 4.3 shows the quercetin calibration curve that was used as a standard in the determination of Total Flavonoid Content.

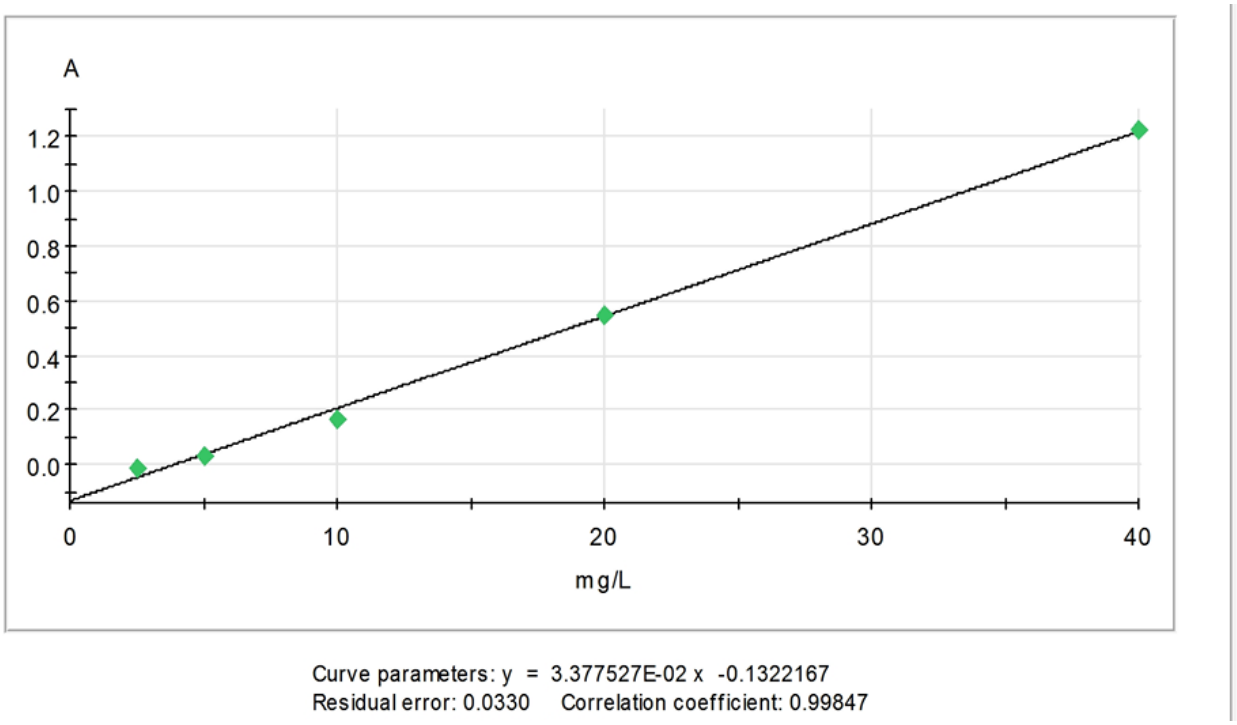


Figure 4. 3: Quercetin calibration curve

The Total Flavonoid Assay was performed on extracts from the different extraction techniques, and the mean absorbances and concentrations were recorded in Table 4.3.

Table 4. 3: Mean Absorbance and Concentration of Flavonoids obtained by the different extraction techniques

Extraction method	Mean absorbance	Mean concentration (mg/L)
UAE	0.660	23.48
EAE	0.441	16.96
MAE	0.319	13.35
Conventional solvent extraction	0.137	7.96

The results for the Total Flavonoid Content analysis performed were recorded in Table 4.3, showing clearly that ultrasound-assisted extraction (UAE) yielded the highest flavonoid content (23.45 mg QE/L), followed by enzyme-assisted extraction (17.0 mg QE/L), microwave-assisted extraction (13.3 mg QE/L) and solvent extraction (8.0 mg QE/L). These findings are consistent with previous reports that state that ultrasound-induced vibrations and cavitation enhance the capillary effect, accelerate mass transfer, reduce energy consumption, and minimize the amount of solvent that is used compared to conventional solvent extraction methods, resulting in improved extraction efficiency (Yang et al., 2024;Kazemi et al., 2016). EAE produced the second-highest yield, reflecting the enzyme's role in facilitating cell breakdown and promoting the diffusion of the solvent into the plant tissues, which enhances metabolite elution (Łubek-Nguyen et al., 2022). By degrading structural polysaccharides such as cellulose, hemicellulose, and pectin, the enzymes increase the porosity of the cell matrices, thereby enhancing metabolite elution and improving recovery of bound flavonoids (Usman et al., 2023). Although EAE outperforms conventional extraction techniques, it is an expensive technique and requires precise control of the reaction conditions; it is a challenging method (B. Suthar et al., 2023). MAE is widely reported to offer faster heating, selective energy dissipation, and improved heat and mass transfer, resulting in reduced working times and increased product yield (Lozano Pérez et al., 2024). In contrast, in this study, MAE produced the lowest yield among the modern extraction techniques, although it still outperformed the conventional solvent extraction method. The rapid heating mechanism of microwaves facilitates short extraction times; however, this advantage may also contribute to thermal degradation of flavonoids, thereby reducing yields compared to UAE and EAE. Elevated temperatures can induce uneven energy distribution within the extraction medium, leading to localized overheating and degradation of thermolabile compounds. S. Nithya et al (2023) similarly reported that MAE can compromise extract integrity under high temperature and pressure

conditions, underscoring the need for precise control of the operational parameters to preserve bioactive compounds. Although MAE remains an environmentally friendly and versatile technique, its operation is complex and requires precise control of extraction conditions to preserve bioactive compounds (Bitwell et al., 2023). Conventional solvent extraction produced the lowest yields in this study. Although it remains a simple and widely accessible technique, it has limitations. The method is time-consuming, has reduced efficiency, and requires the consumption of large volumes of solvents, raising concerns about both economic and environmental issues (Goti & Dasgupta, 2023b). These challenges underscore why modern extraction technologies such as UAE, EAE, and MAE have been developed. The modern techniques aim to overcome the limitations of conventional methods by reducing solvent use, shortening extraction time, and enhancing yield, thereby aligning with growing demand for sustainable and scalable approaches in pharmaceutical and nutraceutical applications. Overall, ultrasound-assisted extraction (UAE) demonstrated the highest flavonoid yield while requiring the least time and energy input during the extraction process. This efficiency underscores the UAE as a sustainable method, particularly when compared to conventional solvent extraction. Notably, the UAE used 66.7% less solvent than the traditional approach, aligning with green chemistry principles and contributing to reduced environmental impact.

4.5 HYDROGEN PEROXIDE SCAVENGING ACTIVITY

The antioxidant capacity of the extracts was evaluated using the hydrogen peroxide scavenging assay. The percentage was calculated according to the equation:

$$\%Scavenged = \frac{A_c - A_s}{A_c}$$

Where A_C is the absorbance of the control, A_S is the absorbance of the sample

The equation quantifies the reduction in absorbance at 230 nm, thereby indicating the ability of the extracts to neutralize hydrogen peroxide radicals. The greater the reduction in absorbance compared to the control, the higher the percentage scavenging.

Table 4. 4 Hydrogen peroxide assay showing percentage antioxidant activity of the extracts

Extraction method	Concentration (mg/L)	Absorbance at 230 nm (AS)	% Scavenged hydrogen peroxide	Relative antioxidant activity
UAE	23.48	0.42	47.5	Highest
EAE	16.96	0.51	36.3	moderate
MAE	13.35	0.55	31.3	Moderate
Conventional solvent extraction	7.96	0.68	15	Lowest
Ascorbic acid	-	0.160	80	Reference
Control (hydrogen peroxide only)	-	0.800	0	-

From the results of the hydrogen peroxide assay recorded in Table 4.4, the control exhibited the highest absorbance (0.800), which corresponds to 0% scavenging. Ascorbic acid showed a lower absorbance (0.160) and 80% scavenging, thereby validating the assay. The extracts had absorbances of 0.42 for UAE, 0.51 for EAE, 0.55 for MAE, and 0.68 for the conventional solvent extraction. Lower absorbance indicated stronger hydrogen peroxide neutralization, the UAE extracts showed the highest relative antioxidant activity among the tested methods. The antioxidant assay showed that the extracts obtained from UAE > EAE >MAE >solvent extraction exhibited greater inhibition in the hydrogen peroxide scavenging assay. This hierarchy directly reflects the efficiency of each extraction method in obtaining bioactive flavonoids, with ultrasound-assisted extraction outperforming the other techniques. These outcomes highlight the potential of the UAE as a sustainable and scalable method for pharmaceutical and industrial applications, since it reduces solvent consumption, processing time, and energy whilst yielding high-quality extracts. The modern extraction technologies, such as UAE, MAE, and EAE, are being classified as green extraction methods because they require shorter extraction times, lower energy input, and reduced solvent volumes compared to conventional approaches (Chemat et al., 2019). By aligning high yield with enhanced antioxidant activity, UAE demonstrates both environmental and functional advantages, thereby making it suitable for integration into industrial-scale processes where sustainability and efficiency are paramount.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

This study demonstrated that ultrasound-assisted extraction yields significantly higher flavonoid recovery compared to enzyme-assisted extraction, microwave-assisted extraction, and conventional solvent methods. The superior performance of UAE, which was demonstrated in both absorbance and concentration values, translated into enhanced antioxidant activity, confirming a direct relationship between polyphenolic content and radical scavenging capacity. Thin-layer chromatography analysis further validated compound separation and pooling, ensuring reliable identification of flavonoid-rich fractions. These findings highlight the environmental and pharmaceutical significance of adopting green extraction technologies. UAE, MAE, and EAE reduce solvent consumption and extraction time, aligning with sustainable development goals and offering scalable potential for industrial applications. The antioxidant activity underscores the therapeutic promise of the flavonoids that are found in the N. cordifolia plant. This shows how the fern plant can provide flavonoids as natural drug candidates in formulations targeting oxidative stress-related disorders, which include endocrine disruption and certain types of cancers. Limitations, including solvent availability, equipment sensitivity, and the need for advanced analytical techniques such as HPLC or LC-MS for compound confirmation, should be acknowledged. Future research should explore clinical validation, optimization of extraction parameters across diverse plant matrices, and integration of UAE with complementary purification methods to enhance reproducibility and scalability. This work contributes to environmental science, nutraceutical and pharmaceutical technology by establishing ultrasound-assisted extraction as a superior, sustainable approach for flavonoid recovery. The integration of quantitative yield analysis, TLC profiling, and antioxidant assays provides a comprehensive workflow that advances green chemistry principles while supporting the development of natural therapeutic agents to replace the use of the synthetic antioxidants like BHT and BHA used in food industries, but are being used under regulated conditions since they have raised safety concerns, with BHA being classified as carcinogenic, while BHT has been linked to liver and kidney effects at high doses.

5.2 RECOMMENDATIONS

Future research should prioritise the optimization of ultrasound- assisted extraction parameters, particularly solvent ratios and sonication time, to enhance reproducibility and scalability. Microwave-assisted extraction and enzyme extraction techniques should also be systematically explored to broaden the efficiency of green extraction workflows and provide comparative insights into yield and bioactivity. Advanced analytical characterisation methods, including high performance liquid chromatography (HPLC) and liquid chromatography- mass spectrometry (LC-MS), are strongly recommended to confirm compound identity and purity beyond the preliminary resolution offered by thin layer chromatography. Expanding antioxidant evaluation to include diverse assays such the DPPH, FRAP and ORAC would generate a more comprehensive profile of flavonoid bioactivity and strengthen pharmaceutical relevance. Industrial feasibility studies should be undertaken to assess cost-effectiveness, equipment accessibility, and environmental impact in resource-constrained settings. By integrating green chemistry principles with advanced analytical validation, future work can establish UAE and complementary methods as standardized, eco-friendly workflows for phytochemical recovery in pharmaceutical, nutraceutical and cosmetic industries.

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