

***Phytochemical Profiling, Antioxidant Potential, and Antimicrobial Assessment
of the Essential Oil Extracted from Moringa oleifera***

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A thesis submitted to the **Department of Mathematics and Natural Sciences** as a partial fulfillment of the requirements for the degree of **Bachelor of Science in Biotechnology**

Department of Mathematics and Natural Sciences

BRAC University

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Declaration

It is hereby declared that:

1. The thesis report submitted is my/our own original work while completing degree at Brac University.
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3. The report does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Abstract

Since antibiotic resistance is still on the rise, the search for safe plant-based bioactives keeps growing in importance. The "Miracle Tree," *Moringa oleifera*, also has immense potential since it contains a vast pool of compounds and is also said to be capable of curing a vast pool of diseases as well. Phytochemical content, antioxidant activity, and antibacterial activity of *Moringa oleifera* leaf extracts in ethanol, ethyl acetate, and chloroform solvents were investigated comprehensively in this study. Four fractions: crude ethanol, non-partitioned ethyl acetate, organic chloroform, and aqueous chloroform, were subjected to Thin Layer Chromatography (TLC), DPPH antioxidant assay, and Kirby-Bauer antibacterial test.

Thin Layer Chromatography (TLC) of *Moringa oleifera* leaf extracts exhibited solvent-dependent variation in bioactive compound profiles. The most compositionally rich was ethanol extract, with several flavonoid bands ($R_f \approx 0.55\text{--}0.93$), alkaloid bands ($R_f \approx 0.51\text{--}0.88$), anthraquinone bands ($R_f \approx 0.57\text{--}0.64$), phenolics, and proteins, showing efficient extraction of mid-polar and polar compounds. Moderate alkaloids, flavonoids, and anthraquinones bands ($R_f \approx 0.24\text{--}0.75$) were shown by the organic chloroform fraction, while the ethyl acetate extract showed weak bands of phenolics and alkaloids ($R_f \approx 0.78\text{--}0.93$). No bands were seen by aqueous chloroform extract, showing low yield of phytochemicals.

Overall, ethanol was the most suitable solvent since it had extracted the broadest range of bioactive compounds for the antimicrobial and antioxidant activity of *Moringa oleifera* leaves. DPPH assay for antioxidant assay showed that extracts had dose-dependent radical scavenging activity. Ethanol extract was superior, with an IC_{50} value of $56.54\text{ }\mu\text{g/mL}$. Ethyl acetate, aqueous chloroform, and organic chloroform extracts were inferior with IC_{50} values $159.40\text{ }\mu\text{g/mL}$, $300.52\text{ }\mu\text{g/mL}$, and $580.25\text{ }\mu\text{g/mL}$, respectively.

Compared to the control ascorbic acid ($IC_{50} = 6.1\text{--}10.47\text{ }\mu\text{g/mL}$), the ethanol extract was around 5–9-fold lower. Yet it was greater than 90% scavenging activity at 1000 $\mu\text{g/mL}$ and therefore was highly active against antioxidants owing to the presence of flavonoids and phenolics. No zone of inhibition was found in well diffusion and disk diffusion antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Shigella flexneri*, *Vibrio cholerae*, and *Enterococcus faecalis*. Inhibition of activity because of small quantity of the extract (30 mg/disc), inability of lipophilic molecules to diffuse via agar, and ultimate break-down of heat labile molecules.

Moringa oleifera leaf extracts, particularly the ethanol fraction, were rich in phytochemicals and showed vigorous antioxidant activity with poor antibacterial inhibition under the assay conditions.

The research highlights the solvent polarity as a driving force for phytochemical recovery and antioxidant activity and as a foundation for its future optimization and for improved nano-formulation approaches to antimicrobial delivery.

Dedication

This is dedicated to our loving parents, our respected teachers and our friends for their unwavering support and encouragement.

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List Of Acronyms

ft	Feet
cm	Centimeter
<i>M. oleifera</i>	<i>Moringa oleifera</i>
TLC	Thin Layer Chromatography
%	Percentage
°C	Degree celsius
ATCC	American Type Culture Collection
MHA	Muller-Hinton agar
NA	Nutrient Agar
µg	Microgram
mg	Milligram
ml	Milliliter
mm	Millimeter
DCM	Dichloromethane
µl	Microliter
CFU	Colony Forming Unit
UV	Ultra-Violet
nm	Nanometer
DPPH	2,2-Diphenyl-1-picrylhydrazyl
RSA	Radical Scavenging Activity
EA	Ethyl acetate
CHCl ₃	Chloroform
MeOH	Methanol
AST	Antibiotic Susceptibility Test

Chapter 1

Introduction

1.1 World wide demands of *Moringa Oleifera* consumption and usages

Moringa oleifera, a drought-resistant tree that is native to the Indian subcontinent, is a fast-growing member of the *Moringaceae* family. *Moringa oleifera*, also known as the drumstick tree or miracle tree, has garnered significant attention as a potential source of bioactive compounds that could be used to treat various diseases. It is widely cultivated for the variegated use of its young seed pods and green leaves as vegetables and for medicinal purposes. It is beneficial to increase the blood antioxidant level and decrease the level of blood sugar and sustained inflammation (Islam et al., 2021). Originating from Bangladesh, India, Afghanistan, and Pakistan, this adaptable tree is now flourishing in tropical and subtropical regions worldwide. Research shows that households in these regions cultivate it in close proximity to their residences (Hirao et al., 2025). The global consumption trends indicate that *M. oleifera* is widely used because nearly all parts of the tree are utilized for their therapeutic properties and essential nutrients. India is the largest producer, producing 1.1 to 1.3 million tons of tender pods annually. The nation exported them at a value of \$4.7 million between 2013 and 2015 (Islam et al., 2021).

1.2 Objective of the study

The main objective of this study is to find out the antibacterial properties, antioxidant capacity, and phytochemical composition by TLC of essential oil extracted from *Moringa oleifera* leaves. The study aims to extract and analyze the essential oil to identify its primary bioactive components, such as flavonoids, phenolics, and terpenoids. The objective is to assess the antioxidant activity of the essential oil through the DPPH radical scavenging assay and to study its antibacterial efficacy against particular pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Shigella spp.*, through well diffusion and minimum inhibitory concentration (MIC) techniques. The study aims to discover a correlation between the phytochemical profile of the essential oil and its observed antioxidant and

antibacterial properties, thereby improving the understanding of *Moringa oleifera* as a potential natural source of therapeutic agents.

1.3: Taxonomy of *Moringa Oleifera*

- Kingdom: Plantae
- Subkingdom: Tracheobionta
- Division: Magnoliophyta
- Class: Magnoliopsida
- Order: Brassicales
- Family: Moringaceae
- Genus: *Moringa*
- Species: *Moringa Oleifera*

1.4: Description:

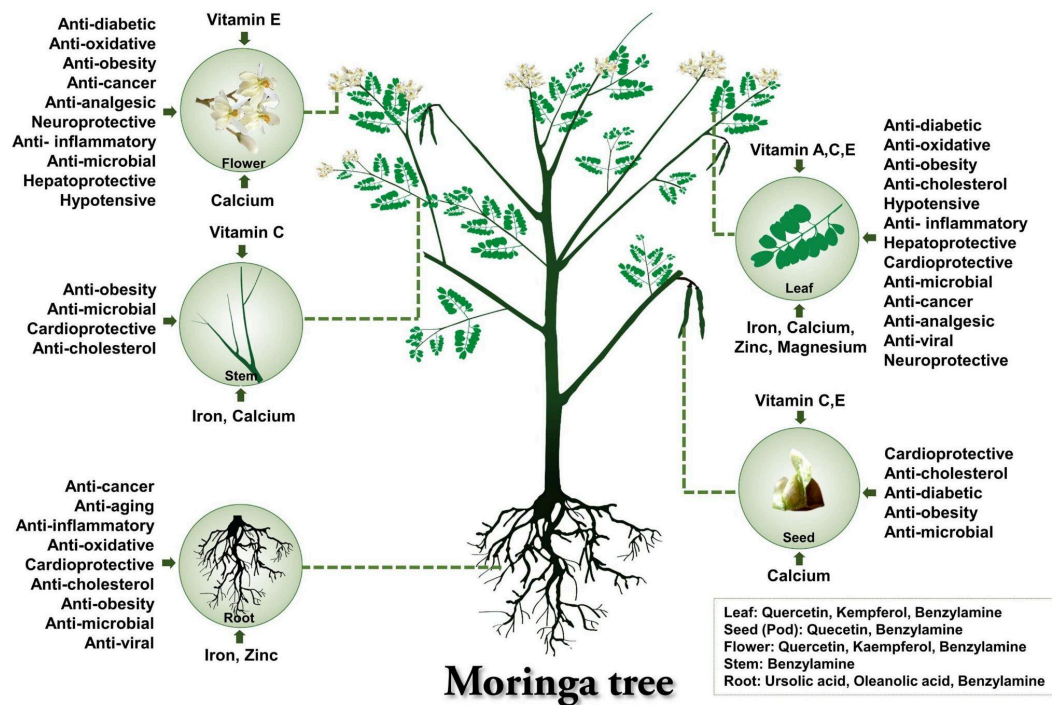


Figure 1.4.1: *Moringa Oleifera* tree

1.5: Nutritional and medicinal importance of *Moringa Oleifera*

M. oleifera is important for both nutrition and medicine since it has a very high concentration of phytochemicals and bioactive compounds. Each cup of fresh moringa leaves has 6.7g of protein, 1.7g of fat, 12.5g of carbs, and 19% of the daily value (RDA) of Vitamin B6, 12% of (RDA) of Vitamin C, and 1 (RDA) of iron. The dried leaves have an even higher concentration of nutrients, with 29.4g of protein per 100g. But Moringa leaves have different kinds of amino acids in them. Moringa leaves include amino acids like Thr, Met, Ile, Lys, and Val, which are similar to proteins found in meat (Islam et al., 2021). *M. oleifera* has more than 90 bioactive plant components that have different pharmacological effects (Pareek et al., 2023). Moringa leaves can help treat such as antioxidants, anti-inflammatory, antibacterial, anti-diabetic, anti-Obesity activity, wound healing effect, fertility and anti-fertility activity, asthma, hyperglycemia, Dyslipidemia, flu, heart burn, syphilis, malaria, pneumonia, diarrhea, headaches, scurvy, skin diseases, bronchitis, eye and ear infections, also reduces cholesterol and anticancer. Moringa seeds can help treat hyperthyroidism, Crohn's disease, antiherpes-simplex virus arthritis, rheumatism, gout, cramps, epilepsy, and sexually transmitted infections. They can also kill bacteria and reduce inflammation. Root bark is a cardiac stimulant, an anti-ulcer, and an anti-inflammatory compound. Moringa flowers are hypocholesterolemic and anti-arthritic agents that can treat urinary tract problems and colds. Moringa pods can help with diarrhea, liver and spleen disorders, and joint pain (Gopalakrishna et al., 2016). Due to its high nutritional value, the plant is a useful strategy for treating malnutrition, especially in developing countries where it provides children and breastfeeding mothers with important nutrients (Pareek et al., 2023b)

1.6: The Rationale for Phytochemical Diversity and the Selection of *Moringa Oleifera* Leaf

Moringra oleifera were selected for research because of its medicinal properties and high nutritional value, which makes it an effective natural source for therapeutic uses. They are full of nutrients that are beneficial for our health and nutrition, such as proteins, vitamins (including vitamin C and vitamin B6), and minerals (Islam et al., 2021). Moringa leaves contain a variety of phytochemicals. It includes phenolic acids, alkaloids, tannins, saponins, and flavonoids (quercetin and kaempferol). These compounds have been shown to be highly effective

antioxidants, anti-inflammatory agents, and antimicrobials (Pareek et al., 2023b). *M. oleifera* have shown that these bioactive compounds are effective in inhibiting the proliferation of a variety of bacteria, including multidrug-resistant strains that are frequently associated with hospital infections, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Kalpana et al., 2013) . Their capacity to resist free radicals and reduce oxidative stress may facilitate the rapid recovery of individuals from diseases (Kim et al., 2023). Additionally, Moringa leaves are easily accessible. These were harvested in a way that minimizes environmental impact and have been used in traditional medicine for a prolonged time. This gives them an excellent option for the development of plant-based antibacterial medications that can be substituted for conventional antibiotics as resistance to them increases. We selected these subjects for the study due to their bioactivity and beneficial effects. It has also shown that it is capable of synthesizing over 90 unique bioactive chemicals with substantial therapeutic potential (Pareek et al. 2023b) . The plant's phytochemicals include bioactive compounds from different categories, and each one makes it more effective against pathogens. Alkaloids are a very significant group of organic chemicals. They are nitrogen-containing substances that stop the growth of bacterial cell walls, proteins, and nucleic acids. These compounds go through the peptidoglycan barrier and mess up important biological processes, which is why they work against Gram-positive bacteria. Advanced analytical techniques identified quercetin, kaempferol, rutin, isoquercitrin, and vitexin as the principal flavonoids. Flavonoids alter bacterial cell membranes by lipid peroxidation, chelate essential metal ions for metabolic processes, and interfere with enzymatic systems. Quercetin, the most common flavonoid in moringa extracts, stops pathogenic bacteria's quorum sensing systems, which lowers the creation of protective biofilm matrices that make bacteria more resistant to antibiotics (Othman et al., 2019). Phytochemical screening experiments consistently indicated that the majority of these bioactive compounds were present in the ethanol and aqueous extracts of *M. oleifera* leaves. Ethanolic extracts were also better at reducing infections than other solvent systems. It's more effective since ethanol has the best polarity. This means that you may easily separate polar and moderately polar molecules without losing their biological activity. It has been proven that using ethanol in levels between 50% and 70% is the best way to remove phenolic compounds out of plants. For example, extracts prepared with 70% ethanol have the greatest quercetin, kaempferol, and other essential flavonoids. Ethanol can break down the walls of plant cells, which allows bioactive chemicals to go inside cells while

keeping their chemical structure intact. This improves the extraction procedure (Tambe & Gadhave, 2024).

1.7: Global Antibiotic resistance crisis

Antibiotic resistance is now one of the most serious medical issues in the world in the 21st century. The World Health Organization (WHO) says that antimicrobial resistance (AMR) is the 5th major threat to world health. A huge number of people are getting multidrug-resistant (MDR) bacterial infections, therefore it's crucial to find new ways to treat them. New research shows that antimicrobial resistance from pathogens (AMR) caused 1.27 million deaths worldwide in 2019. Also added to 4.95 million more deaths (World Health Organization: WHO, 2023). AMR is estimated to kill more than 39 million people between 2025 to 2050 (Naghavi et al., 2024). Because of this concerning condition, scientists are increasingly seeking novel antimicrobials. They are looking at natural plant-based chemicals that can be used instead of standard antibiotics and have the same effect.

1.8: Antioxidant potential and antimicrobial Assessment of *M. oleifera*

Moringa oleifera shows antibacterial properties that are effective against both Gram-positive and Gram-negative microorganisms, as it is shown by several types of scientific trials. Moringa leaf extracts showed significant antibacterial efficacy against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, with MIC values between 200mg/ml and 800mg/ml. For microorganisms, *M. oleifera* can break down their cell walls and membranes, letting their cytoplasm leak out and damaging the structure. Scientists used transmission electron microscopy to see these processes clearly by showing that the shapes of the treated microbe cells changed in significant ways. Flavonoids, phenols, alkaloids, tannins, and saponins are only a few of the bioactive elements that interact together to make a wide spectrum of antibacterial effects. Different solvent extracts have different levels of activity (Kalpana et al., 2013). When an antioxidant reduces it, it turns pale yellow (Abd El-Wahab et al., 2023). A spectrophotometer is used to evaluate the color change at 517 nm, providing a direct measurement of the effectiveness of radical scavenging. Higher levels of decolorization are linked to higher levels of antioxidant activity. This test is very sensitive and quick, making it an effective means to see how well plant extracts can combat oxidative stress, which is linked to a number of diseases, such as infections

and inflammation. phenolic and flavonoid compounds with antioxidant properties can be extracted by preparing *Moringa oleifera* leaf extracts using solvents such as ethanol, methanol, chloroform, or ethyl acetate. Research shows that ethanolic extracts usually have higher DPPH scavenging capacity, due to the polarity of ethanol, which effectively isolates components such as quercetin, kaempferol, and other phenolics (Panwar et al., 2016). The experiment's outcomes are typically expressed as IC 50. 50, the quantity of extract required to inhibit 50% of the DPPH radicals. IC50 values for ethanolic extracts of Moringa leaves have been reported as low as around 0.3 mg/mL, indicating substantial free radical neutralization comparable to standards like ascorbic acid and butylated hydroxytoluene (BHT) (Abd El-Wahab et al., 2023).

1.9: Thin-Layer Chromatography (TLC) to find the phytochemicals in *Moringa oleifera*

Thin-layer chromatography (TLC) is a widely used and effective technique for the isolation and identification of multiple bioactive phytochemical compounds in plant extracts, including those derived from *Moringa oleifera*. This study will utilize thin-layer chromatography (TLC) on extracts obtained from multiple solvents, such as ethanol, chloroform, and ethyl acetate, to generate detailed phytochemical profiles. The stationary phase is made up of plates covered in silica gel. The mobile phases, like chloroform:methanol: ethyl acetate in precise ratios, help separate things based on their polarity and affinity (Ghavate et al., 2023). Ultraviolet light and chemicals such as iodine spray reveal distinct colored bands corresponding to compounds including flavonoids, alkaloids, tannins, and phenols. These chemicals are recognized for their medicinal properties. Previous study indicates that ethanol and ethyl acetate extracts yield the most distinctive bands, signifying a broader spectrum and greater concentration of bioactive compounds compared to other solvents. These bands typically exhibit retention factor (Rf) values below 1 (International Journal of Creative Research Thoughts, 2023). This profile not only confirms the presence of these phytochemicals but also enables the subsequent isolation and quantification of compounds believed to be responsible for antibacterial and antioxidant properties.

Chapter 2

Literature Review

2.1.1 Role of Natural Extracts in Antibiofilm and Antioxidant Research

Natural plant extracts are proving to be of promise as an alternative to synthetic antimicrobials as a result of their wide range of bioactive phytochemicals and antioxidant ability. These substances neutralize reactive oxygen species (ROS) and at the same time interfere with the action of bacterial quorum sensing (QS) and biofilm formation pathways. *Moringa oleifera* is one of these botanicals that occurs as one of the potent sources of multifunctional phytochemicals with proven antibiofilm, antioxidant, and anti-quorum-sensing properties (Haris and Ahmad, 2024; El-Sherbiny et al., 2024).

2.1.1.1 *Moringa oleifera* Leaf Extracts – Phytochemical Constituents

Moringa oleifera has a wide range of secondary metabolites such as flavonoids, tannins, alkaloids, saponins, and steroids in its leaves (Syahputra et al., 2021). All of these compounds contribute to the antioxidative and antimicrobial activity of the plant. The quality characterization of *M. oleifera* leaves by various localities (e.g., Sidikalang, North Sumatra; Sirsa, Haryana) showed that it met the requirements of pharmacognostic criteria, which confirms their uniformity as a high-quality herbal crude material (Syahputra et al., 2021; Deepali et al., 2025). Specifically, methanolic and ethanolic extracts have been shown to have a high free-radical scavenging potential (IC₅₀ 17.5 mg/mL) and a high ability to affect foodborne and clinical pathogens (MIC = 14-26 mg/mL) (El-Sherbiny et al., 2024; Deepali et al., 2025). The results establish *M. oleifera* as an excellent source of therapeutically useful bioactives in dealing with biofilm-related infection.

2.1.1.2 Comparative Solvent Extraction (Ethanol, Methanol, n-Hexane, Ethyl Acetate)

The yield and bioactivity of phytochemicals are hugely affected by the polarity of extraction solvents. The relative analysis with ethanol, methanol and n-hexane and ethyl acetate showed that polar solvents (ethanol and methanol) yield higher levels of phenolics and flavonoids, which

are associated with better antioxidant and antibiofilm activity (Haris and Ahmad, 2024; Famurewa, 2023). MOEF fractions in particular were active and exhibited better anti-QS and anti-biofilm effects- inhibiting the expression of virulence factors including violacein and pyocyanin by more than 70% (Haris and Ahmad, 2024). Conversely, the n-hexane extractions produced low antioxidant and antibacterial properties (Segwatibe et al., 2023), implying that non-polar solvents are not efficient in the extraction of active phenolics. In addition, more sophisticated methods, including ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), have increased the effectiveness of recovery and at the same time preserved thermolabile compounds (Liga et al., 2025; Ihsanpuro et al., 2022).

2.1.1.3 Bioactive Compounds: Quercetin, Rutin, Kaempferol, Chlorogenic Acid

The HPLC and LC-qTOF-MS methods revealed that several major flavonoid and phenolic acids of *M. oleifera* extracts include quercetin, rutin, kaempferol derivatives, and chlorogenic acid (Haris and Ahmad, 2024; El-Sherbiny et al., 2024). Quercetin, which may take up to 38% of the total phytochemical profile, is capable of high antioxidant, QS-interfering activity, that inhibits bacterial virulence and biofilm (El-Sherbiny et al., 2024). Rutin and kaempferol also add to the disruption of membranes and the decrease in the production of EPS. Less abundant, chlorogenic acid supplements the redox modulation effect and hydroxyl radicle scavenging capacity, therefore, reinforcing the antibiofilm effect by inhibiting oxidative stress. These compounds do not act alone, they act synergistically, which represents the need to maintain phytochemical complexity during extraction and formulation.

2.1.1.4 Correlation Between Antioxidant and Antibiofilm Activities

It has been shown that there is a consistent positive relationship between antioxidant and antibiofilm activities in a variety of studies (Haris and Ahmad, 2024; Madhvi et al., 2020; Asif et al., 2021). As an example, a model of *P. aeruginosa* and *C. violaceum* that showed significant decrease in biofilm biomass and pigment production in Ethyl acetate and methanolic extracts with an IC₅₀ of less than 60 mg/mL (Haris and Ahmad, 2024). The implication of this redox-QS interaction is that antioxidant phytochemicals interfere with intracellular communications by altering the oxidative equilibrium and thus inhibiting bacterial communication and adhesion.

Table 2.1.1.4.1: Comparative Analysis of Extraction Solvents and Identified Bioactives

Solvent / Fraction	Major Compounds Identified	Antioxidant Activity (IC ₅₀ , µg/mL)	Antibiofilm / Anti-QS Effect	Key References
Ethanol	Flavonoids, tannins, alkaloids, quercetin	56.5 (moderate)	Moderate antibiofilm inhibition	Syahputra et al. (2021)
Methanol	Quercetin (38.23%), rutin, kaempferol	17.5 (strong)	Potent antibacterial and antibiofilm (MIC 14–26 µg/mL)	El-Sherbiny et al. (2024); Deepali et al. (2025)
Ethyl acetate (MOEF)	Rutin, kaempferol, chlorogenic acid	159.4 (moderate)	>70% inhibition of violacein, pyocyanin, and motility	Haris & Ahmad (2024)
n-Hexane	Non-polar triterpenoids, sterols	Low activity	Negligible antimicrobial effect	Segwatibe et al. (2023)
Advanced (MAE, UAE)	Similar to methanol + enhanced purity	—	Increased yield and stability; reduced degradation	Liga et al. (2025); Ihsanpuro et al. (2022)

2.1.2 Analytical and Characterization Techniques in Literature

In this subsection, the technical principles, strengths, and limitations of the key tools of analysis employed in the extracted studies to profile phytochemicals, quantify antioxidant activity, and visualize biofilm disruption are synthesized. The objective will be functional: demonstrate what each approach can add to the goals (identification, quantification, functional attribution), acknowledge the sources of bias or blind sides cited in the literature and suggest how the techniques should be used to achieve robust and cross-validated outcomes.

2.1.2.1 TLC, HPLC, and LC-qTOF-MS for Compound Profiling

Thin-Layer Chromatography (TLC), is a fast and inexpensive screening method where components present in the sample are fractionated on a stationary phase (silica gel) by a difference in their solubility in a mobile solvent system. TLC densitometric scanning is still a relevant procedure in terms of routine quality control due to the identification of major flavonoids/phenolic acids (e.g., quercetin, rutin, caffeic acid) with hardly any instruments or consumables (Skorek et al., 2016). Nevertheless, the reduced sensitivity and the inability to separate co-eluting low-abundance analytes are mentioned time and again; TLC presents

qualitative or semi-quantitative prints as opposed to actual molecular identification (Skorek et al., 2016).

High Performance Liquid Chromatography (HPLC) enhances the quantitative abilities of TLC through the separation of the analytes on packed columns (mostly reversed-phase C18) and the subsequent identification of the analytes using UV/diode-array observators. HPLC could be used to precisely measure major phytochemicals - in this case, quercetin was measured with high accuracy as a key constituent (~38.23) of methanolic extracts of *M. oleifera* (El-Sherbiny et al., 2024). However, HPLC is insensitive to trace-level compounds and structural isomers without sensitive detectors or mass spectrometers; a number of authors warn that HPLC data can be incompatible with more sensitive techniques (Skorek et al., 2016).

LC-qTOF-MS is a combination of liquid chromatography and high-resolution accurate mass determination and MS/MS fragmentation, which allows identifying low-abundance compounds with confidence and determining the structure (Haris and Ahmad, 2024). Lc-qtof-ms was utilized to profile an ethyl-acetate fraction (MOEF) by Harris and Ahmad (2024) and found rutin and derivatives of kaempferols and chlorogenic acid that were associated with anti-QS and antibiofilm activity. Therefore, a tiered approach (TLC screening - HPLC quantification - LC-qTOF-MS identification) is the most reasonable methodology to cover both high and low bioactives.

2.1.2.2 DPPH, ABTS, and IC₅₀-Based Antioxidant Quantification

DPPH (2,2-diphenyl-1-picrylhydrazyl) & ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) are conventional colorimetric techniques that determine levels of free-radical scavenging by a compound/extract through a reduction in absorbance at specific wavelengths (DPPH $\approx$ 517 nm; ABTS $\approx$ 734 nm). Findings are usually presented in the form of IC₅₀ (concentration that results in 50% radical inhibition) or in the normalized form of Trolox Equivalent Antioxidant Capacity (TEAC) to enable inter-study comparability (Kedare & Singh, 2011). The use of DPPH has been characterized by simplicity and cost-effectiveness, although it has also been reported to be limited by the following: sensitivity to sample colour and turbidity, radical instability in the presence of light, variable dependence on pH, and reaction kinetics that make its use complex in

situations where stoichiometry is not a factor (Kedare & Singh, 2011). ABTS is a more universal method of antioxidants (both lipophilic and hydrophilic), but must also be standardized rather cautiously.

Hyphenated and advanced formats have also been developed and used in the literature to overcome the shortcomings of single assays: HPLC-DPPH online antioxidant output onto chromatography analysis to attribute activity to individual peaks; in Sequential Injection Analysis (SIA) and HSCCC-DPPH, preparative level pre-assay fractionation is followed by activity testing (Kedare & Singh, 2011). It is emphasized in the literature that providing an IC50 out of context and without kinetics (in terms of standardization e.g., Trolox standard curves, defined incubation times, etc.) compromises cross-study comparability, something replicated in comparative reviews (Kedare and Singh, 2011) and extraction-method analysis (Liga et al., 2025).

Table 2.1.2.2.1: Practical Comparison of Key Analytical Techniques

Technique	Principle / Output	Strengths	Limitations	Recommended role
TLC (+ densitometry)	Plate separation; visual/peak-scan fingerprinting	Cheap, fast, useful for QC screening	Low sensitivity; semi-quantitative; co-elution risk	First-line screening and batch QC (Skorek et al., 2016)
HPLC (UV/DAD)	Liquid chromatographic separation with UV detection	Robust quantification of major compounds (e.g., quercetin)	May miss trace compounds; needs standards	Quantification of known marker compounds (El-Sherbiny et al., 2024)
LC-qTOF-MS	LC separation + high-res accurate mass and MS/MS	High sensitivity, structural ID, low-level detection	Requires expertise; higher cost	Comprehensive profiling and identification (Haris & Ahmad, 2024)
DPPH / ABTS (IC ₅₀ , TEAC)	Radical scavenging colorimetry	Simple, comparable with standards	Interference by pigments; kinetic/stoichiometry issues (Kedare & Singh, 2011)	Bulk antioxidant screening; use with Trolox standardization
HPLC-DPPH, SIA, HSCCC-DPPH	Hyphenated antioxidant assays	Assigns activity to chromatographic peaks; higher throughput	More complex instrumentation	Link chemical identity to antioxidant function (Kedare & Singh, 2011)

2.1.3 Comparative Observations Across Plant Sources

Comparative studies show that the phytochemical richness and bioactivity of *Moringa oleifera* differ among ecotypes and geographical locations. Phenolic content and consequently antioxidant and antibiofilm efficacy are affected by regional differences in climate as well as soil. Cross-species comparison will reveal, for example, that *Moringa peregrina*, distributed in the arid Middle East, has an even more marked radical-scavenging capacity under some (other) circumstances among other medicinal plants, including *Rhododendron arboreum* [7.89% (p<0.05)], *Mimusops elengi* [5.47% (p<0.001)], and *Syzygium aqueum* as C-range alkaloid antioxidants or against microbes for antiseptic benchmarking of the efficacy of Moringa trees' drug applications made to man.

Table 2.1.3.1: Comparative Bioactivity of Plant Sources Reported in Literature

Plant Source / Region	Major Active Compounds	Antioxidant Activity (IC ₅₀ , µg/mL)	Antibacterial / Antibiofilm Findings	Key Observations	Reference
<i>M. oleifera</i> (India)	Quercetin, kaempferol	17.5	MIC 14–26 µg/mL; strong antibacterial and safe profile	High bioactivity; solvent-dependent yield	El-Sherbiny et al., 2024; Deepali et al., 2025
<i>M. oleifera</i> (South Africa)	Novel phenanthrene and friedelane derivatives	—	Weak antibacterial at 1 mg/mL	Ecotype variation affects potency	Segwatibe et al., 2023
<i>M. oleifera</i> (Indonesia)	Alkaloids, flavonoids, saponins	—	Good extract quality, standard moisture/ash levels	Suitable for herbal standardization	Syahputra et al., 2021
<i>M. peregrina</i> (Middle East)	Rutin, phenolic acids	8.06	Strong antioxidant; limited antibacterial data	Superior antioxidant but underexplored antibacterial potential	Dehshahri et al., 2012
<i>Rhododendron arboreum</i>	Phenolics, flavonoids	—	High antioxidant potential; correlation with phenolic content	Requires in vivo validation	Madhvi et al., 2020
<i>Mimusops elengi</i>	Phenolic & flavonoid-rich methanol extract	—	Potent antioxidant; strong DPPH scavenging	Supports phenolic–antioxidant link	Asif et al., 2021
<i>Syzygium aqueum</i>	Phenolics, triterpenes	—	Strong antioxidant; part-dependent variation (leaf vs. bark)	First report on stem bark bioactivity	Itam et al., 2021

2.1.4 Summary of Standard Laboratory Assays Referenced (DPPH, Kirby-Bauer)

In most of these studies, antioxidant power is either assayed by the DPPH radical scavenging test or antimicrobial activity is evaluated using the Kirby-Bauer disk diffusion assay. The DPPH technique provides an easy and fast analysis of free-radical scavenging activity expressed as IC₅₀, or in Trolox equivalents; however, attention should be paid to light and pH sensitivity (Kedare & Singh, 2011). The American Society for Microbiology approved Kirby–Bauer technique employs Mueller–Hinton agar and a 0.5 McFarland inoculum to promote consistency (Hudzicki, 2009). Together, these assays offer supporting quantitative and functional evidence in the antioxidant as well as antibacterial aspects of Moringa extracts.

2.1.5 Integration of Visual Data

Recent reports focus on visualization tools that can combine chemical and functional information to aid cross-comparison as well as mechanistic understanding. Tables compile extraction-solvent efficiency and bioactivity relationships (e.g., Table 2.1.2.2) and flow charts depict the cascade in which antioxidant activity leads to quorum-sensing inhibition with subsequent biofilm dispersion—the manner by which redox modulation weakens EPS formation, enhances cell wall permeability or inhibits virulence-factor synthesis is also outlined. Such visual integration enables clear interpretation of multi-parameter datasets and a mechanistic underpinning to the synergistic effect from combining Moringa phytochemicals, Vitamin C, and Fe₃O₄ nanoparticles in targeted antibiofilm formulations.

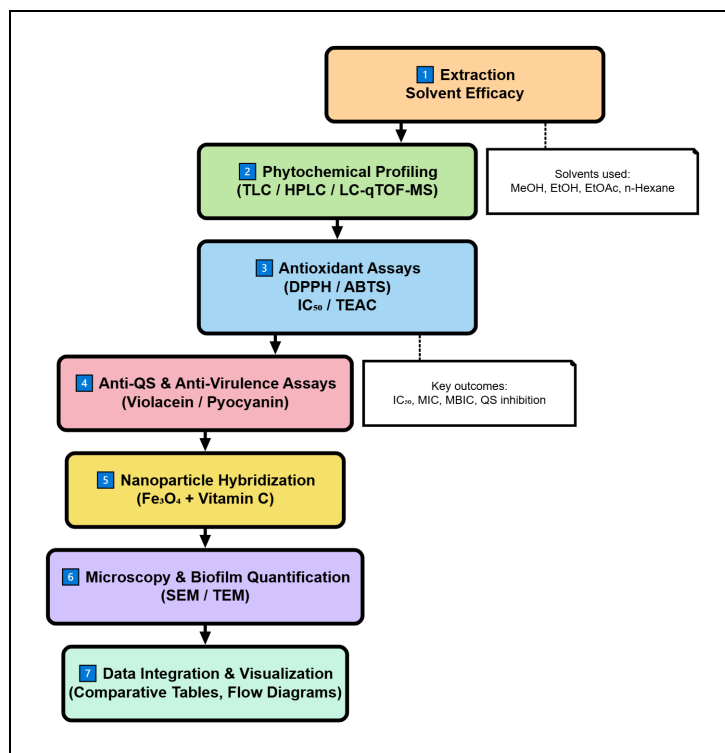


Figure 2.1.5.1: Integrated Analytical and Bioactivity Workflow for *Moringa oleifera* Extracts, Vitamin C, and Fe₃O₄ Nanoparticles

The flowchart is designed based on the compiled sequential data relationships in published analytical and biological investigations of *Moringa oleifera* (Haris & Ahmad, 2024; El-Sherbiny et al., 2024). There is a detailed AD workflow following the workflow of phytochemical research provided in the data extraction table, which includes steps from solvent (ethanol, methanol and ethyl acetate) extraction for compound recovery to chromatographic profiling [TLC, HPLC and LC-qTOF-MS], antioxidant quantification [DPPH/ABTS assays and IC₅₀] estimation through bioactivity based on anti-QS and antibiofilm assays against *C. violaceum* and *P. aeruginosa*. This is followed by an assay of functional efficacy by hybridization to nanoparticles and subsequent microscopic observation (SEM/TEM). The flow is ended with data synchronization and comparison by means of tables as well as pathway diagrams, which connect the antioxidant performances to biofilm formation.

2.2 Novel Contributions and Emerging Technologies

Literature survey Recent literature reveals that there is a movement from traditional extraction and testing to eco-efficient, hybridized, and translation technology for natural antibiofilm studies. The improvements converge on reduction of solvent consumption, accuracy of analysis as well phytochemical and nanomaterial integration for a dual antioxidant–antimicrobial effect are introduced.

2.2.1 Green and Advanced Extraction Technologies

2.2.1.1 Ultrasound- and Microwave-Assisted Extraction (MAE):

UAE and MAE enhance solute diffusion and cell rupture leading to higher phenolic recovery with low solvent consumption and energy power (Liga et al., 2025; Ihsanpuro et al., 2022). The closed-vessel heating of MAE protects the labile compounds and is better than in sensitive flavonoids such as quercetin and rutin.

2.2.1.2 Two-Phase Solvent Systems (EtOAc:EtOH:Water):

Selectivity of polarity partitioning is achieved in two-phase EtOAc–EtOH–H₂O systems, which increase flavonoids and phenolic fractions by decreasing unwanted impurities (Ihsanpuro et al., 2022). It is thought that such biphasic extraction ensures the reproducibility and reasonable efficiency in the subsequent analyses.

2.2.1.3 Standardization of Extract Quality:

Moisture ($\leq 5\%$), total ash ($\leq 10\%$) and solvent-dependent extractives—pharmacognostic quality parameters of Moringa preparations enforce consistency and uniformity (Syahputra et al., 2021). A common platform also ensures that products are: so you can compare between labs, just like with your other lab tests.

Table 2.2.1.3.1: Comparison of Conventional vs. Green Extraction Approaches

Parameter	Conventional Soxhlet	UAE / MAE	Two-Phase EtOAc:EtOH:H ₂ O
Solvent volume	High	Low	Moderate
Energy/time	Long, high energy	Rapid, low energy	Moderate
Preservation of actives	Moderate loss	High retention	High retention
Environmental impact	High	Low	Low
Yield consistency	Variable	Reproducible	Reproducible
Key References	Famurewa (2023)	Liga et al. (2025); Ihsanpuro et al. (2022)	Ihsanpuro et al. (2022)

2.2.2 Analytical Innovations

2.2.2.1 DPPH-HPLC and Sequential Injection Analysis (SIA):

Attachment of DPPH radical detection to HPLC allows for peak-by-peak determination of antioxidant activity, enhancing specificity over bulk DPPH assays (Kedare & Singh, 2011). Sequential Injection Analysis (SIA) provides automation of this step, thus increasing throughput and diminishing reagent consumption.

2.2.2.2 ADMET and In Ovo (HET-CAM) Bioassays:

In ovo, Hep's Egg Chorioallantoic Membrane (HET-CAM) tests and in-silico ADMET modeling examine extract cytocompatibility and absorption–distribution–metabolism excretion–toxicity profiles (El-Sherbiny et al., 2024). They complement in-vitro assays with predictive safety validation.

2.3 Research Gap Analysis

Although the intensive studies on *M. oleifera* or other bioactive systems, several unexplored and partially exploited categories identified through literature review prevent translation of knowledge into practical reality are presented herein. These gaps are collated into two primary thematic clusters which represent experimental and analytical limitations, and mechanistic and environmental gaps.

(A) Experimental and Analytical Limitations

In vivo validation studies of the potent phytochemical–nanoparticle formulations are still not available as an impact of these synergistic properties combined with uncertainty regarding pharmacokinetics, toxicity, bioavailability (El-Sherbiny et al., 2024) and biological activity potency. Research on tri-agent synergy of Moringa extracts, Vitamin C and Fe₃O₄ nanoparticles is also scant—the vast majority of studies focus on elements in isolation without providing quantification. Moreover, solvent optimization and standardization are also incoherent among studies; yields and bioactivities differ tremendously as a result of unregulated extraction parameters and reporting discrepancy (Famurewa, 2023). The analysis of these methods by analytical cross-validation is less frequently attempted, which leads to piecemeal interpretation of data (Skorek et al., 2016).

(B) Mechanistic and Environmental Gaps

At the mechanistic level, the molecular mechanism of quorum sensing (QS) inhibition is not well understood. Auto-inducer interruption, oxidative tension changes, direct signaling disturbances Rare research work has been performed around the mechanism of action where compounds from Moringa can act either by an autoinducer interference or through promoting oxidative stress or disruption in signaling (Haris & Ahmad, 2024). Ecotype and regional phytochemical variability (India vs. Africa vs Indonesia) have also received less attention, although there is some strong evidence of bioactive profile being affected by the environment (Segwatibe et al., 2023). Environmental biofilm sources such as fecally contaminated urban water (for instance, Hatirjheel Lake of Dhaka) have been poorly studied, thereby presenting a large void in ecologically applicable and sustainable aspects (Deepali et al., 2025). Future research will need to combine

standardized analyses, in vivo validation and multi-pathogen testing, in order to develop robust and applicable antibiofilm strategies.

Table 2.3.1: Summary of Key Research Gaps and Their Implications

Gap Area	Description / Missing Aspect	Implication for Current Study
In vivo validation	Lack of biological system testing	Validate tri-agent safety and efficacy experimentally
Tri-agent synergy	No quantified combined effect of Extract + Vit C + Fe ₃ O ₄	Evaluate synergistic inhibition and redox correlation
Solvent inconsistency	Unstandardized extraction protocols	Use optimized, reproducible green solvent ratios
Analytical cross-validation	Poor TLC–HPLC–LC-MS comparability	Employ multi-technique verification
Ecotype variability	Regional phytochemical variation unaccounted	Source-localized <i>Moringa</i> and compare profiles
Pathogen diversity	Limited strain coverage	Include UTI, burn, diarrheal, and water isolates
Environmental focus	Rare exploration of water biofilms	Integrate Hatirjheel water samples for

2.4 Challenges to Address Research Gaps

(A) Extraction and Yield Optimization Challenges

Solvent polarity and the efficiency of extraction are among the major causes of inter-study variation. Polar solvents (methanol, ethanol) extract relatively higher quantities of phenolics while non-polar solvent (n-hexane) imparts least activity; however, the ratio used is seldom provided by standard methods (Haris & Ahmad, 2024; Famurewa, 2023). Also the long-lasting heat extraction may destroy sensitive to temperature antioxidants such as quercetin and rutin (Liga et al., 2025). Collectively these problems demand green, low energy assistance extraction techniques such as UAE or MAE that permit higher production yields along with compound integrity.

(B) Analytical and Characterization Constraints

Reproducibility of the analyses remains an important inherent issue. The differences in sensitivity of TLC, HPLC and LC-qTOF-MS detection often result in contradictory compound identification and quantification (Skorek et al., 2016). There are no also defined criteria of radical stability, pH, inoculum density of the antioxidant (DPPH/ABTS) and antimicrobial (Kirby-Bauer) tests so that their outcomes cannot be compared across studies (Kedare and Singh, 2011). The standardization is highly reliant on the IC₅₀ and MIC, such as Trolox and McFarland standard application.

(C) Data Standardization and Integration Challenges

The range of IC₅₀, MIC and inhibition assay values in recent literature is very diverse as experimental procedures and endpoints vary (Deepali et al., 2025). Irregularity in the depiction of data and inter-technique calibration is detrimental to reliability. These discrepancies could be overcome with the assistance of the incorporation of visual data, both comparative tables and flow diagrams, and validation matrices by which the relationships between technique, outcome, and performance metric would be illustrated. This is meant to be integrated so as to report sufficient reproducible and transparent experimental efficacy.

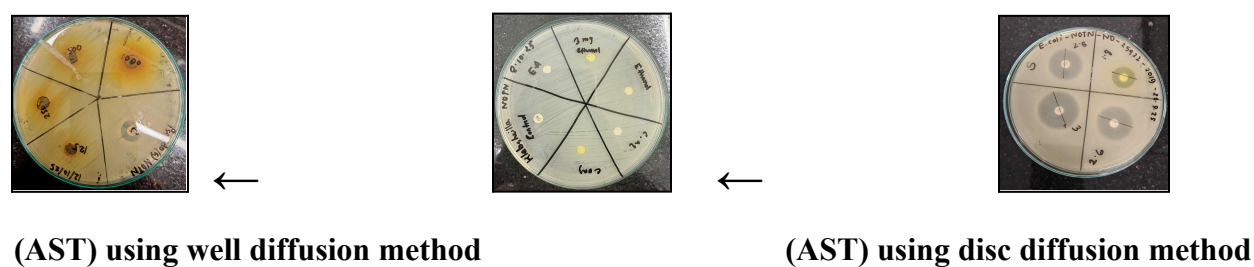
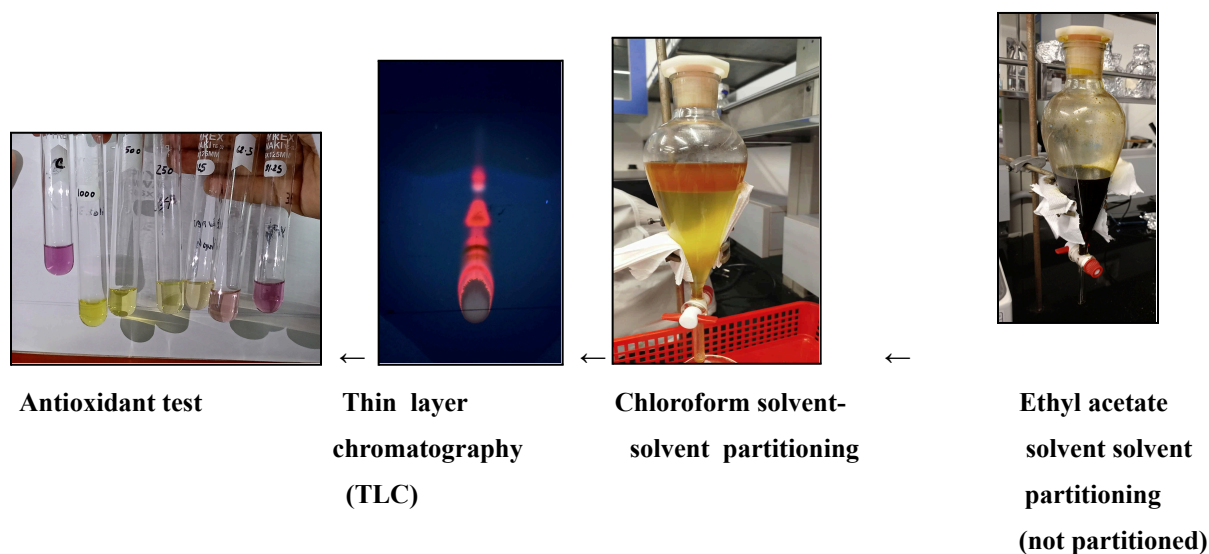
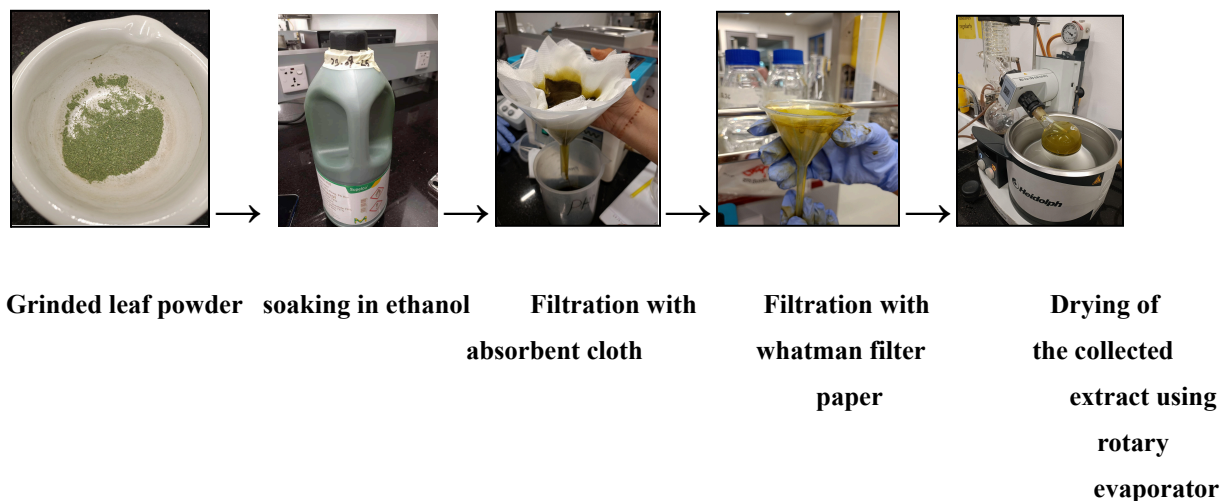
Table 2.4.1: Challenges and Mitigation Strategies

Challenge Category	Identified Issues	Suggested Mitigation Strategy
Extraction	Solvent polarity inconsistency; loss of thermolabile compounds	Adopt UAE/MAE; optimize solvent ratios; maintain low-temperature conditions
Analytical	Poor inter-method reproducibility; unstandardized assay conditions	Apply method calibration; use Trolox/McFarland standards; cross-validate TLC–HPLC–LC-MS
Data & Interpretation	Non-uniform reporting (IC ₅₀ , MIC); weak data visualization	Use comparative tables and flow-based summaries for cross-study validation

Chapter 3

Materials & Methods

The flow chart shows the step by step process of production of antioxidant and antimicrobial properties of *Moringa oleifera* leaf extracts through the extraction, partitioning and testing process.



3.1 Methods

The chemical analysis of a plant can be broken down into several steps:

1. Collection and Identification of Plant Material
2. Preparation of Plant Sample
3. Extraction Process
4. Solvent-Solvent Partitioning
5. Thin Layer Chromatography (TLC)

3.1.1 Collection and Identification of Plant Material

Initially, a thorough literature review was conducted to select an appropriate plant for investigation. The *Moringa oleifera* (drumstick tree) leaf was collected from an authentic source in November 2024. The leaves were verified to ensure authenticity.

3.1.2 Preparation of Plant Sample

The collected *Moringa oleifera* leaves were initially fresh. They were air-dried over a period of 20 days to facilitate grinding. The dried leaves were then processed into a coarse powder using a grinding machine, yielding approximately 600 g of powder. The powder was stored in an air-tight container, properly labeled, and kept in a cool, dark, and dry environment to preserve its integrity for future use.

3.1.3 Extraction Process

The leaf powder (600 g) was placed in a flask and soaked in a mixture of 1110 ml ethanol and 130 ml distilled water (8.5:1 ratio). The flask was wrapped in aluminum foil and left undisturbed for 14 days, with occasional shaking and stirring to facilitate extraction. Following the soaking period, the mixture underwent sequential filtration steps:

- a) **Initial filtration:** The mixture was first filtered through a clean, white, 100% cotton cloth. The filtrate was collected, and the residue on the cloth was squeezed to extract additional liquid.
- b) **Secondary filtration:** The previous step was repeated using Whatman filter paper.
- c) **Final filtration:** The filtrate was passed through a double layer of Whatman filter paper. The final filtrate was run in a rotary evaporator set at 45°C and 50 rpm.

Following these steps, the plant's chemical constituents were systematically extracted by removing ethanol and distilled water (Pereira et al., 2024).

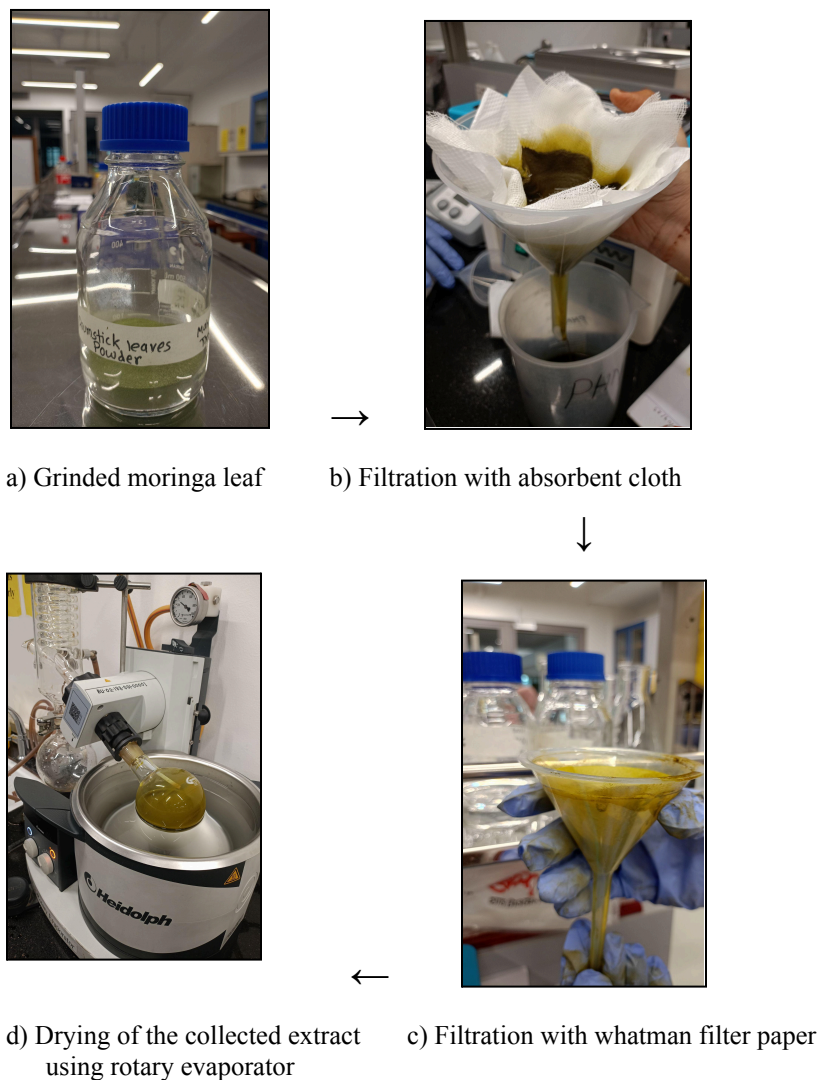


Figure 3.1.3: Extraction of leaf of *Moringa oleifera*

3.1.4 Solvent-solvent partitioning

Solvent-solvent partitioning was performed on the crude extract using chloroform and ethyl acetate to separate compounds based on polarity. For chloroform partitioning, 4.6 g of the crude extract was mixed with 150 ml chloroform and 100 ml distilled water in the first run, followed by separation of the organic and aqueous layers. The process was repeated with 150 ml chloroform (without additional water) in the second and third runs to ensure maximum sample extraction. For ethyl acetate partitioning, 4.6 g of crude extract was mixed with 150 ml ethyl acetate and 100 ml distilled water in the first run; however, no partitioning occurred, and further runs were not pursued. Finally, the entire fraction, including both the organic and aqueous phases, and non partitioned crude were collected and evaporated to dryness separately. (Azwanida, 2020).



Figure 3.1.4.1: Chloroform solvent-solvent partitioning of *Moringa oleifera* leaf
(upper part: organic phase,
lower part: aqueous phase)



Fig 3.1.4.2: Ethyl acetate solvent
solvent (non partitioned) of
Moringa oleifera leaf

3.1.5 Thin layer chromatography (TLC)

Thin-layer chromatography was conducted to detect mid-polar or less-polar chemical compounds in the ethanol soaked crude extract, chloroform partitioned organic crude, chloroform partitioned aqueous crude, and ethyl acetate non partitioned crude. Commercially available silica gel plates were cut to 6.5 cm in length and 4 cm in width. The analysis was based on the polarity, size, and affinity of the compounds.

Specific mobile phases were prepared as follows:

- For **flavonoids** (mid-polar compounds): chloroform:methanol (9:1 ratio).
- For **alkaloids** (less-polar compounds): toluene:ethyl acetate (9:1 ratio).
- For **amino acids and proteins** (polar compounds): n-butanol:acetic acid:distilled water (4:1:5 ratio).
- For **anthraquinones** (mid-polar compounds): toluene:ethyl acetate:formic acid (5:4:1 ratio).
- For **phenolic acids** (mid-polar compounds): chloroform:methanol (8:2 ratio).

A small amount of each extract was dissolved in a suitable solvent to form a solution. Using a clean pipette tip, a small spot of the solution was applied near the baseline (drawn with a pencil) on the TLC plate. A TLC tank with an airtight lid was prepared by pouring the selected mobile phase to a sufficient depth and allowing it to equilibrate for a few minutes to saturate the atmosphere with solvent vapor. The TLC plate was introduced into the tank using forceps, ensuring the spots remained above the solvent level. The solvent migrated up the plate via capillary action, separating the components based on their interactions with the stationary (silica) and mobile phases. The plate was removed when the solvent front reached a specified distance.

Separated compounds were visualized under UV light at 365 nm, revealing distinct spots indicative of various components. This method is essential for initial screening and purity assessment of plant extracts (Cieřla, 2012; Matysik et al., 2016).

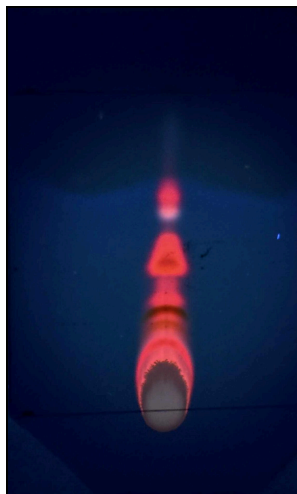


Figure 3.1.5.1: Visual detection of alkaloid chemical compounds (midpolar) in ethanol soaked crude extract of *Moringa oleifera* leaf

3.2 Antibacterial Test Method

The antibacterial activity of *Moringa oleifera* extracts was evaluated using a modified Kirby-Bauer disk diffusion method against various bacterial strains, focusing on potential inhibition relevant to biofilm-forming pathogens. This approach addresses the rising challenge of antibiotic-resistant bacteria by screening plant extracts for novel antibacterial compounds (Hudzicki, 2009).

3.2.1 Test Organisms

A total of eight bacterial strains were used, sourced from hospital samples. The strains included both Gram-positive and Gram-negative bacteria. These include- *Salmonella typhi*, *Escherichia coli* ATCC: 25922, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Vibrio cholera*, *Shigella flexneri*, *Enterococcus faecalis*.

Table 3.2.1: Gram-positive and Gram-negative bacteria selected for the antibacterial activity test

Gram positive	Gram negative
<i>Staphylococcus aureus</i>	<i>Salmonella typhi</i>
<i>Enterococcus faecalis</i>	<i>Escherichia coli ATCC: 25922</i>
	<i>Pseudomonas aeruginosa</i>
	<i>Klebsiella pneumoniae</i>
	<i>Vibrio cholerae</i>
	<i>Shigella flexneri</i>

These organisms were selected to represent pathogens commonly associated with the targeted samples.

3.2.2 Preparation of Culture Media

Mueller-Hinton Agar (MHA) and Nutrient Agar (NA) were used as culture media. Appropriate amounts of agar were dissolved in distilled water and boiled until clear. The media were sterilized by autoclaving at 121°C for 1.5 hours. Fresh bacterial cultures were prepared by streaking the test organisms onto NA plates and incubating at 37°C for 18-24 hours. (Kaur et al., 2023).

3.2.3 Sterilization Techniques

All procedures were conducted in a laminar flow hood to minimize contamination. Glassware and Petri dishes were sterilized using an autoclave at 121°C for 15 minutes.

3.2.4 Preparation of Discs

Three types of discs were prepared:

- i) Standard discs:** Ciprofloxacin (30 µg/disc) served as positive controls.
- ii) Blank discs:** Treated with solvents (without extract) as negative controls to exclude solvent or filter paper effects.
- iii) Sample discs:** Discs were prepared by soaking them in solutions of *Moringa oleifera* extracts (ethanol soaked crude, chloroform aqueous, chloroform organic, ethyl acetate) at a concentration of 10 mg/disc and 30 mg/disc. The discs were then dried and placed on MHA plates pre-inoculated with bacterial cultures.

3.2.5 Preparation of Extract Solutions

Initially 10 µg and 30 µg crude concentration of each test material (ethanol-soaked crude, chloroform-partitioned aqueous crude, chloroform-partitioned organic crude, and ethyl acetate non-partitioned crude) were dissolved in 700 µl DMSO and 300 µl distilled water in sterile vials. The solutions were vortexed for complete mixing. The extract solution was prepared for the disc diffusion method.

Subsequently, only the ethanol-soaked crude extract was used at concentrations of 125 mg, 250 mg, 500 mg, and 1000 mg. Each concentration was dissolved in 100 µL of DMSO and 900 µL of distilled water in sterile vials, followed by vortexing to achieve homogeneous solutions. The extract solution was prepared for the well diffusion method.

3.2.6 Disk Preparation

Autoclaved Whatman filter paper was punched into 6 mm diameter discs using a sterile punch-hole tool under laminar flow. Discs were prepared to match the size and thickness of standard antibiotic discs. Using a micropipette, 25 µl of each extract solution was applied to the discs, which were allowed to dry for even distribution.

3.2.7 McFarland Standard

Bacterial suspensions were standardized to 0.5 McFarland turbidity (approximately 1.5×10^8 CFU/ml). A few isolated colonies were transferred to sterile 0.85% NaCl solution and vortexed until uniform turbidity was achieved (Acharya, 2023).

3.2.8 Antimicrobial Susceptibility Test (AST):

AST has been performed to observe which antibiotics (commercial discs) are effective against each specific bacterial strain, measuring the zone of inhibition (ZOI). Larger zones of inhibition were considered for each bacterial strain as it means bacteria are more susceptible towards that specific antibiotic. Then incubated at 37°C for 18-24 hours.

3.2.9 Disk Diffusion Method

MHA plates were inoculated with bacterial suspensions using sterile cotton swabs after matching turbidity to the 0.5 McFarland standard. Discs were placed on the agar surface with sterile forceps, ensuring even spacing. Plates were pre-chilled at 4°C for 2 hours to allow diffusion, then incubated at 37°C for 18-24 hours.

3.2.10 Well Diffusion Method

MHA plates were inoculated with bacterial suspensions using sterile cotton swabs after matching turbidity to the 0.5 McFarland standard. Then, wells were created in the agar media by cutting media using sterile pipette tips keeping proper size and spacing similar to the disc size. Test samples were added to the wells using a micro pipette and kept in an incubator for 18-24 hours at 37°C .

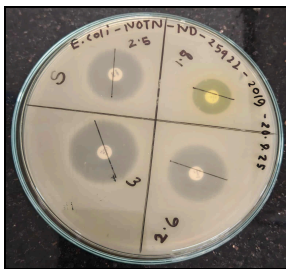


Figure 3.2.9.1:

AST using commercial disc

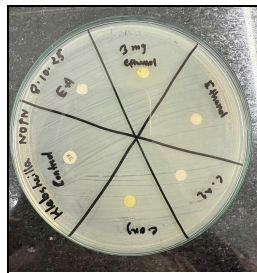


Figure 3.2.9.2:

Disc diffusion method

using crude disc

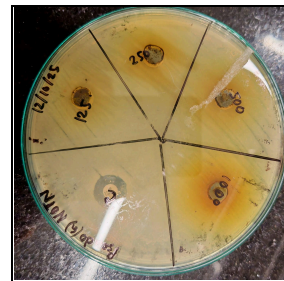


Figure 3.2.9.3:

Well diffusion method

using crude solution

3.2.11 Measurement of Inhibition Zones

Post-incubation, inhibition zones were measured in millimeters using a ruler. Measurements were taken three times per zone, and averages were recorded

3.3 Antioxidant Test: DPPH assay

3.3.1 DPPH solution preparation

0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared by dissolving 1.57 mg of DPPH powder in 40 mL of methanol in an amber-colored volumetric flask, protected from light to prevent photodegradation (Kedare and Singh, 2011).

3.3.2 Preparation of sample and standard stock solutions

Dissolve 10 mg of each test crude sample in 10 ml methanol.

3.3.3 Preparation of working concentrations (serial dilutions)

From the stock solution, prepared the following concentrations using methanol: 500, 250, 125, 100, 65 mg/ml. Used micropipettes for accurate measurement.

3.3.4 Preparation of reaction mixtures

1 ml of each sample or standard solution and mix it with 1 ml of that 0.1 mM DPPH solution that was prepared earlier. A gentle swirl was done to mix it all up nicely.

3.3.5 Preparation of control and blank

For the blank, 2 ml of methanol was used. Then, for the control, 1 ml of methanol was combined with 1 ml of the 0.1 mM DPPH solution.

3.3.6 Incubation

All tubes were kept in the dark for 30 minutes at room temperature.

3.3.7 Measurement of absorbance

Absorbance was measured at 517 nm using a UV-visible spectrophotometer and the blank was used as reference.

3.3.8 Radical scavenging activity

Radical scavenging activity is calculated using the following formula (Mahdi-Pour et al., 2012),

$$\%RSA = ((\text{Absorbance of control} - \text{Absorbance of Sample}) / \text{Absorbance of Control}) * 100\%$$

Then, IC₅₀ was calculated from a line chart equation.



Fig 3.3.8.1: Change of purple color of DPPH to yellow indicating antioxidant property

Chapter 4

Results

This section shows the results of the current study on *Moringa oleifera* leaf extracts. Various tests were conducted to assess the antibacterial and antioxidant properties of the plant. There were also TLC tests done. The results are structured as follows:

4.1 TLC Result & Analysis

Flavonoids

For flavonoid Mobile Phase

(E.A 6.76, FORMIC ACID 0.74, ACETIC ACID 0.74, DH20 1.76) - Ethyl acetate:Formic acid:Acetic acid:Water (100:11:11:26 v/v/v/v) — ideal for polar

For mid polar

Chloroform:Methanol (7:3)

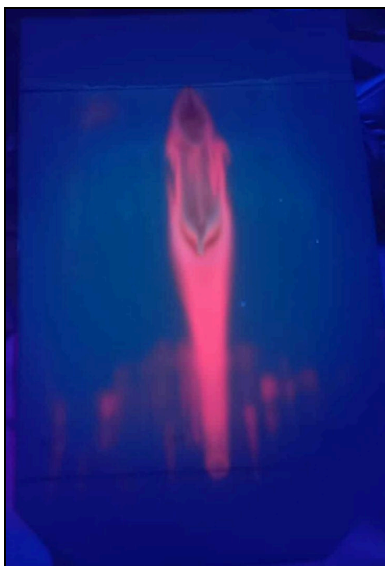


Figure 4.1.1: Ethanol

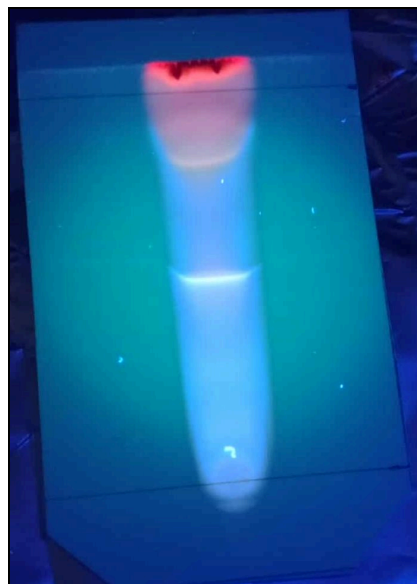


Figure 4.1.2: Ethanol

Mid polar
Chloroform:Methanol (9:1)

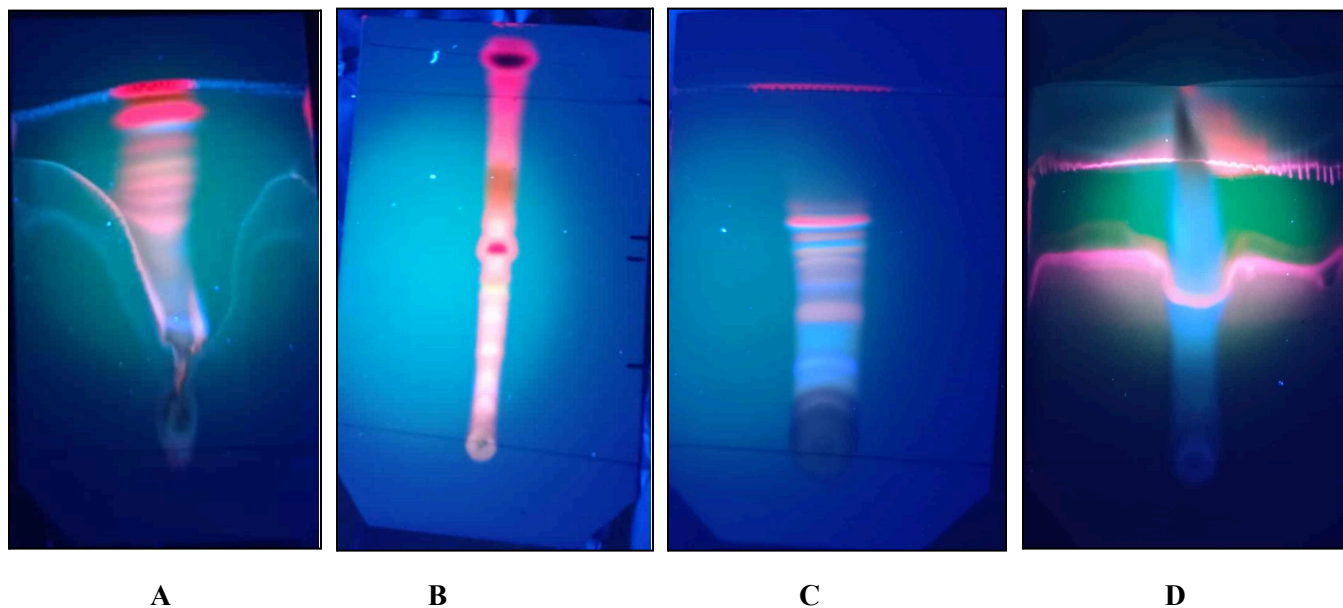


Figure 4.1.3: Ethanol Crude (A), Chloroform organic (B), Chloroform aqueous (C), Ethyl acetate (D)

Alkaloids

Mid polar
Toluene:Ethyl acetate (7:3)

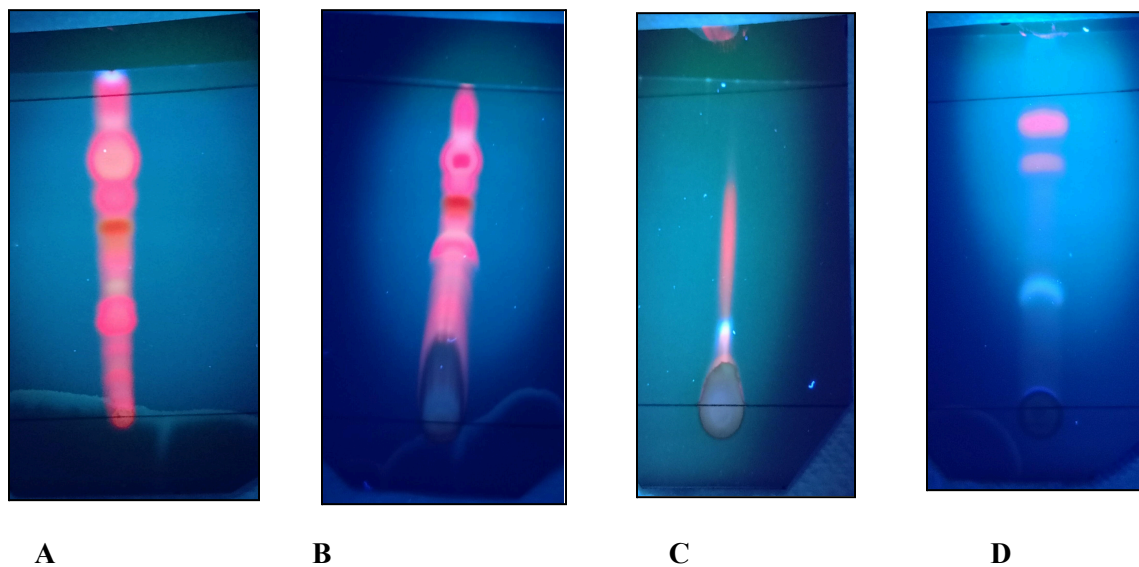
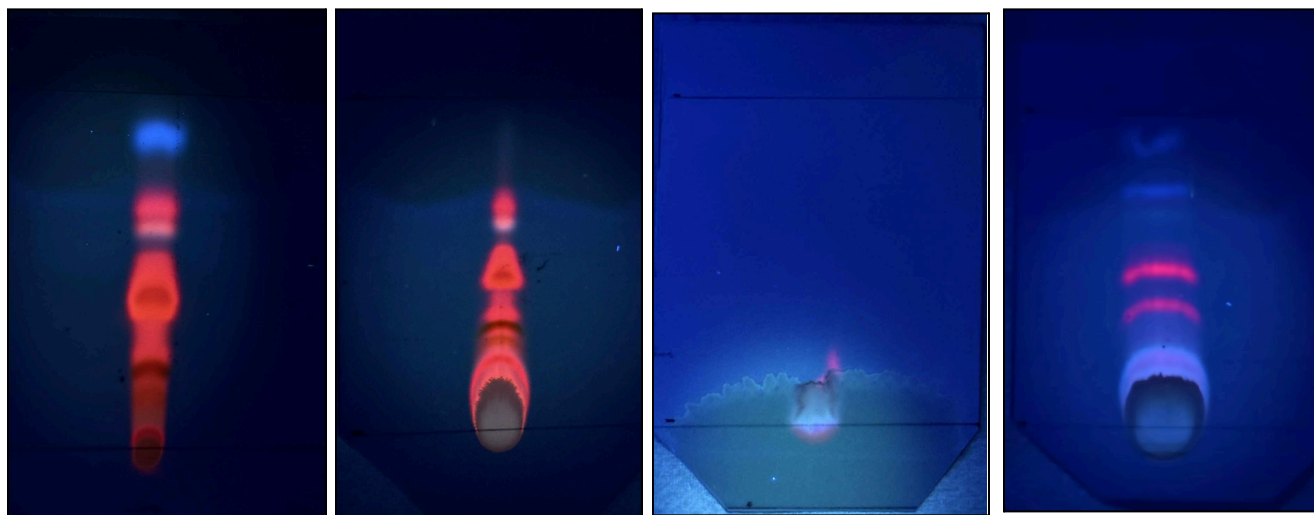


Figure 4.1.4: Chloroform organic (A), Ethanol (B), Chloroform aqueous (C), Ethyl acetate (D)

Mid polar
Toluene:Ethyl acetate (9:1)
9:1 ratio, more concentration



A

B

C

D

Figure 4.1.5: Chloroform organic (A), Ethanol (B), Chloroform aqueous (C), Ethyl acetate (D)

****Red marks are alkaloids**

****white parts are other compounds**

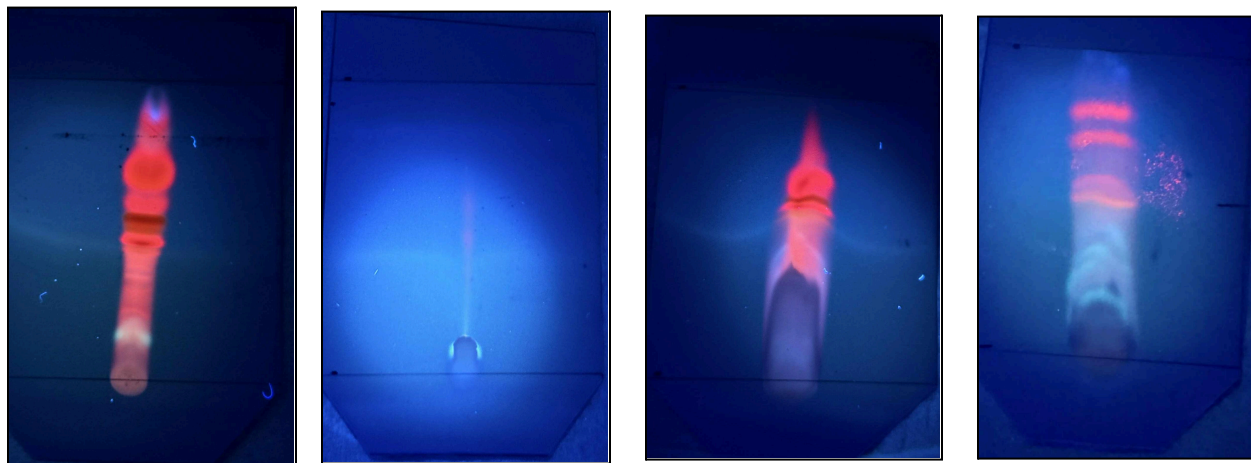
****Chloroform aqueous (c) - No band.**

Anthraquinone

Mid polar

Toluene:Ethyl acetate:Formic acid (5:4:1)

5:4:1 ratio



A

B

C

D

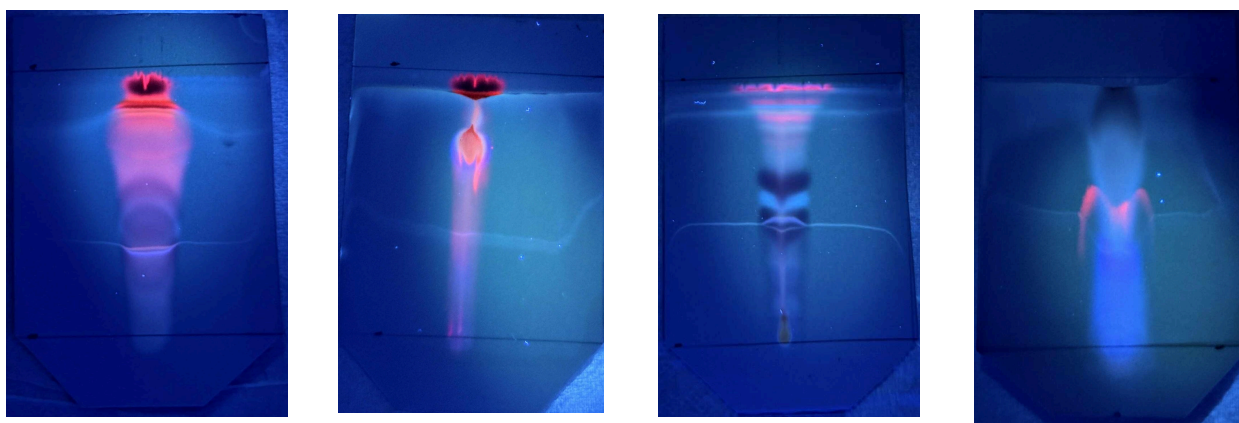
Figure 4.1.6: Chloroform organic (A), Chloroform aqueous (B), Ethanol (C), Ethyl acetate (D)

Phenolic compound

Mid-polar

Chloroform:Methanol (8:2)

8:2 ratio



A

B

C

D

Figure 4.1.7: Ethanol (A), Chloroform organic (B), Ethyl acetate (C), Chloroform aqueous (D)

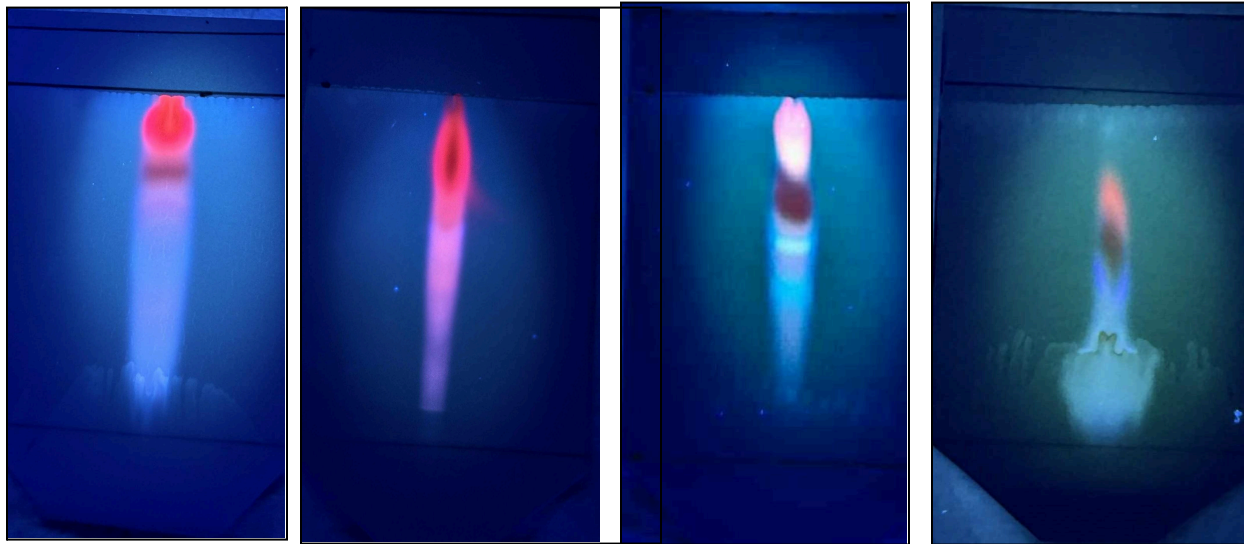
****Chloroform organic- 1 Band.**

Protein / amino acid

Polar amino acids

n-Butanol:Acetic acid:Water (4:1:5)

4:1:5 ratio



A

B

C

D

Figure 4.1.8: Ethanol (A), Chloroform organic (B), Ethyl acetate (C), Chloroform aqueous (D)

Table 4.1.1: Bioactive Compounds of TLC Data

Bioactive Compounds	Mobile Phase	Ratio	Crude Extract	Rf Value
Flavonoids	Ethyl acetate:Formic acid:Acetic acid:Water (Polar)	(100:11:11:26 v/v/v/v)	Ethanol	0.55 0.89 0.93
	Chloroform: Methano	7:3	Ethanol	0.51 0.93
		9:1	Ethyl acetate	No Band
			Chloroform organic	0.27 0.56 0.62 1
			Chloroform aqueous	0.24 0.53 0.58 0.84 0.87 1
			Ethanol	0.67 0.73 0.84 0.91 1
Alkaloids	Toluene: Ethyl Acetate	7:3	Ethanol	0.51 0.64 0.71 0.88
		9:1	Ethyl acetate	0.37 0.75 0.88

			Chloroform organic	0.26 0.37 0.57 0.66 0.75
			Chloroform aqueous	No Band
			Ethanol	0.22 0.36 0.48 0.6
			Ethyl acetate	0.4 0.51 0.68 (White) 0.73 0.77 (White)
			Chloroform organic	0.22 0.6
			Chloroform aqueous	No Band
Anthraquinone	Toluene: Ethyl Acetate: Formic Acid	5:4:1	Ethanol	0.57 0.62 0.64
			Ethyl Acetate	0.48 0.55 0.68 0.78
			Chloroform Organic	0.31 0.44 0.51 0.57 0.64
			Chloroform aqueous	No Band

Phenolic Compound	Chloroform: Methanol	8:2	Ethanol	0.28 0.8 0.82 0.88
			Ethyl Acetate	0.78 0.84 0.86 0.88 0.93
			Chloroform organic	0.88
			Chloroform aqueous	No Band
Amino Acid and Protein(Polar)	n-butanol: Acetic acid:Water	4:1:5	Ethanol	0.66 0.78 0.84
			Ethyl Acetate	0.58(White)
			Chloroform organic	0.53
			Chloroform aqueous	No Band

4.2 Antioxidant Test Result

$$IC_{50} = X_1 + (50 - Y_1) * \frac{X_2 - X_1}{Y_2 - Y_1}$$

$$\%RSA = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} * 100\%$$

Table 4.2.1: IC₅₀ value of Ethanol extract of the leaf of *Moringa Oleifera*

Concentration (n µg/mL)	Absorbance of Control	Absorbance of Sample	%RSA	IC ₅₀
1000	0.647	0.108	83.307	56.535
500	0.647	0.073	88.717	
250	0.647	0.055	91.499	
125	0.647	0.106	83.616	
62.5	0.647	0.288	55.486	
31.25	0.647	0.474	26.738	

Based on the table above, we could conclude that the antioxidant activity of the Ethanol extract is **strong** since the value is slightly above the cutoff of very strong which lies at **50µg/ml** (Itam et al., 2021).

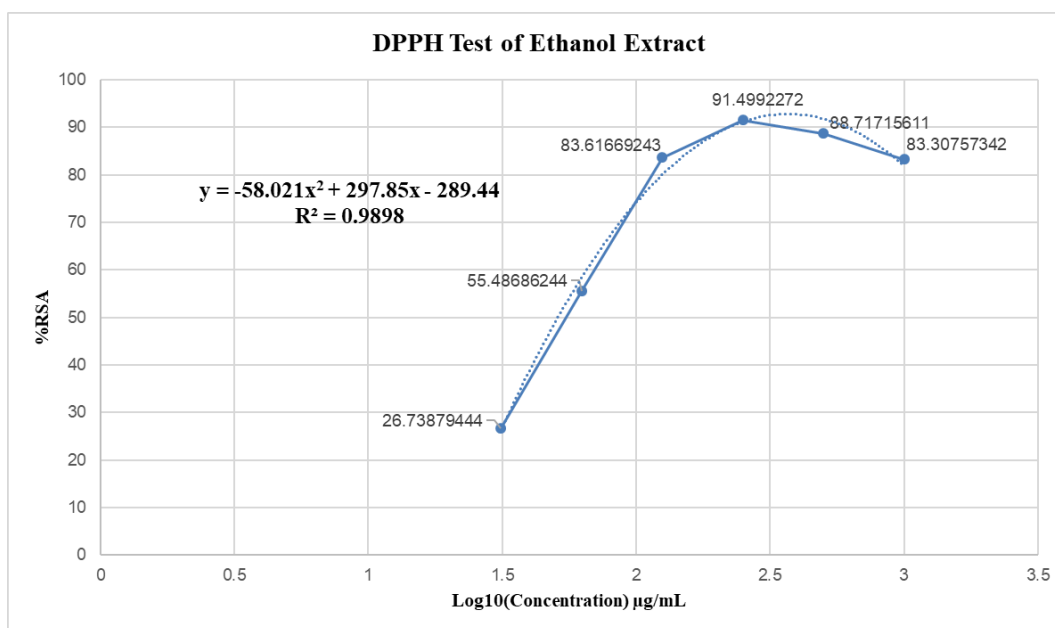


Figure 4.2.1: Antioxidant activity of Ethanol extract of *Moringa Oleifera* leaf

Table 4.2.2: IC50 value of Ethyl Acetate extract of the leaf of *Moringa Oleifera*

Concentration (n µ-gm/mL)	Absorbance of Control	Absorbance of Sample	%RSA	IC50
1000	0.578	0.043	92.560	159.395
500	0.578	0.046	92.041	
250	0.578	0.073	87.370	
125	0.578	0.371	35.813	
62.5	0.578	0.538	6.920	
31.25	0.578	0.605	-4.671	

Based on the table above, we can conclude that the Ethyl Acetate extract has **moderate** antioxidant activity since it falls between the range of **above 150 µg/ml and below 250µg/ml** (Itam et al., 2021).

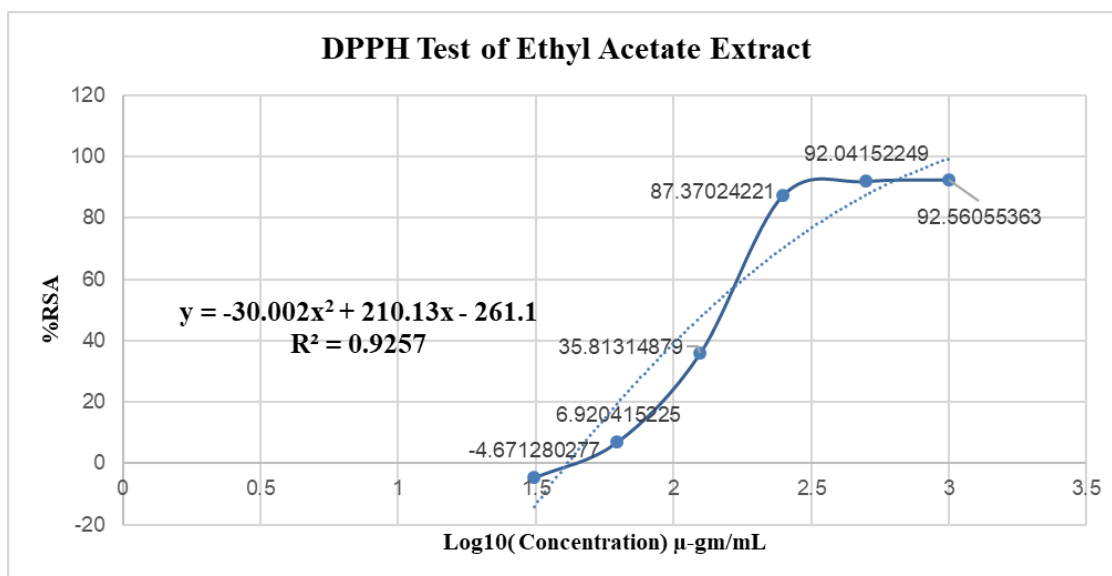


Figure 4.2.2: Antioxidant activity of Ethyl Acetate extract of *Moringa Oleifera* leaf

Table 4.2.3: IC₅₀ value of Chloroform aqueous phase partition extract of the leaf of *Moringa Oleifera*

Concentration (n μ -gm/mL)	Absorbance of Control	Absorbance of Sample	%RSA	IC ₅₀
1000	0.578	0.145	74.913	300.518
500	0.578	0.103	82.179	
250	0.578	0.069	88.062	
125	0.578	0.074	87.197	
62.5	0.578	0.338	41.522	
31.25	0.578	0.463	19.896	

Based on the table above, we can conclude that the **Chloroform aqueous phase partition** extract has **weak** antioxidant activity since it falls over the range of above 150 μ g/ml and below 250 μ g/ml (Itam et al., 2021).

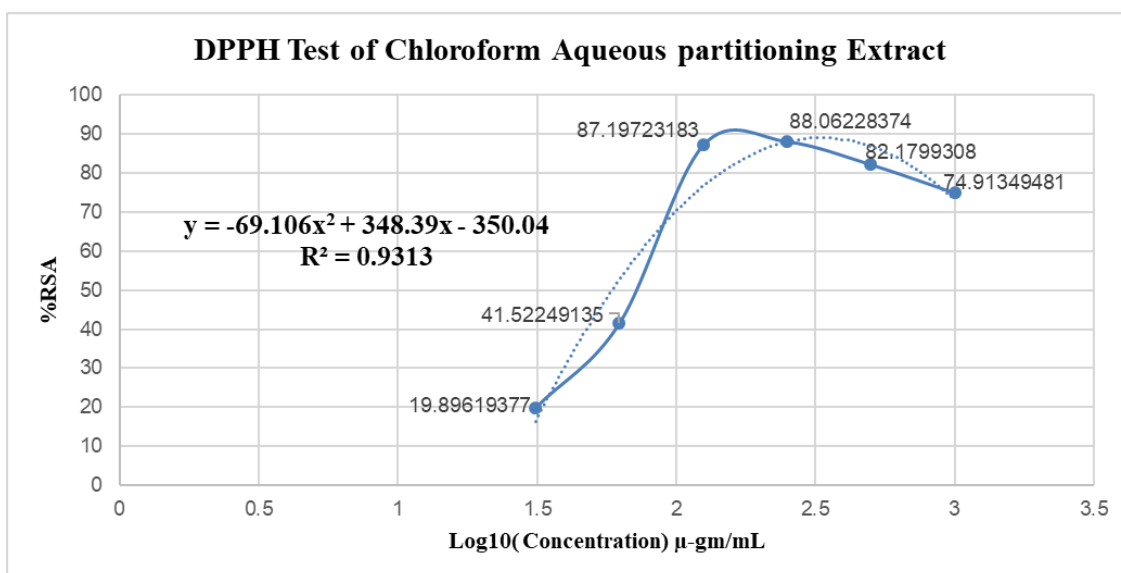


Figure 4.2.3: Antioxidant activity of Chloroform aqueous phase partition extract of *Moringa Oleifera* leaf

Table 4.2.4: IC50 value of Chloroform organic phase partition extract of the leaf of *Moringa Oleifera*

Concentration (n μ -gm/mL)	Absorbance of Control	Absorbance of Sample	%RSA	IC50
1000	0.578	0.425	26.470	580.246
500	0.578	0.263	54.498	
250	0.578	0.129	77.681	
125	0.578	0.098	83.044	
62.5	0.578	0.445	23.010	
31.25	0.578	0.465	19.550	

Based on the table above, we can conclude that the **Chloroform organic phase partition** extract has **the weakest** antioxidant activity since it falls over the range of above 150 μ g/ml and below 250 μ g/ml (Itam et al., 2021).

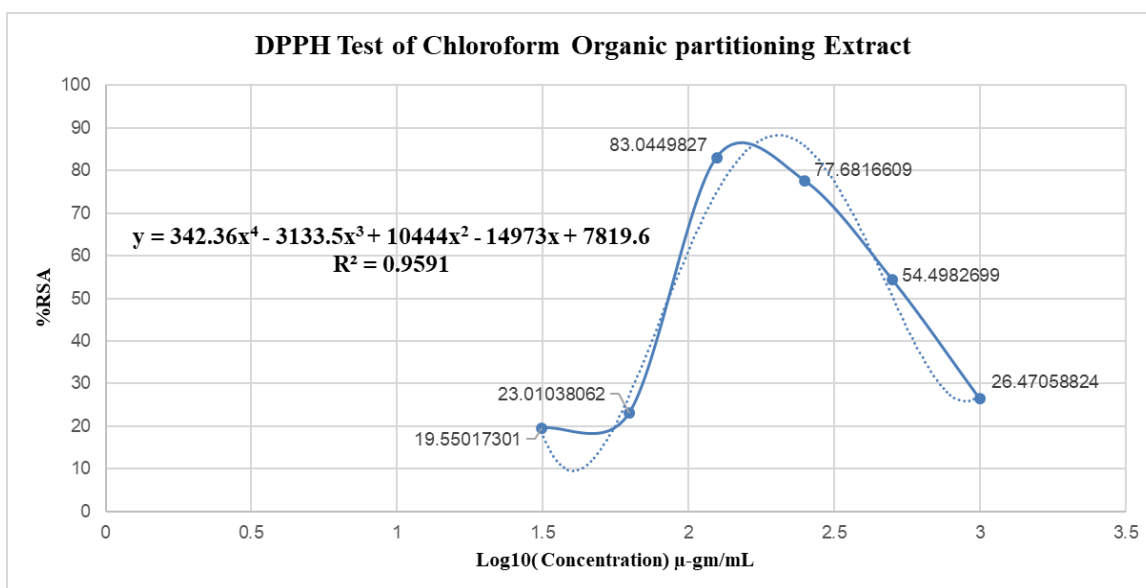


Figure 4.2.4: Antioxidant activity of Chloroform organic phase partition extract of *Moringa Oleifera* leaf

Published ascorbic acid IC_{50} values are variable, due to the DPPH outcome being dependent upon solvent, reaction time, reaction temperature and the concentration of DPPH. The literature values of ascorbic acid IC_{50} in DPPH assay range in the single-digit to low-two-digits μ g/mL.

Published ascorbic acid IC_{50} value (DPPH) values were used to compare results:

- 6.1 μ g/mL (Nariya et al., 2013).
- 7.78 μ g/mL (Asif et al., 2021).
- 10.47 μ g/mL (Ihsanpuro et al., 2022).

Table 4.2.5: Comparison of IC_{50} value

Extract	vs 6.1 μ g/mL	vs 7.78 μ g/mL	vs 10.47 μ g/mL
Ethanol (56.54)	~9.3 $\times$ weaker.	~7.3 $\times$ weaker.	~5.4 $\times$ weaker.
Ethyl-acetate (159.40)	~26.1 $\times$ weaker.	~20.5 $\times$ weaker.	~15.2 $\times$ weaker.
Chl-aq (300.52)	~49.3 $\times$ weaker.	~38.6 $\times$ weaker.	~28.7 $\times$ weaker.
Chl-org (580.25)	~95.1 $\times$ weaker.	~74.6 $\times$ weaker.	~55.4 $\times$ weaker.

4.3 Antimicrobial Activity Results

Well diffusion

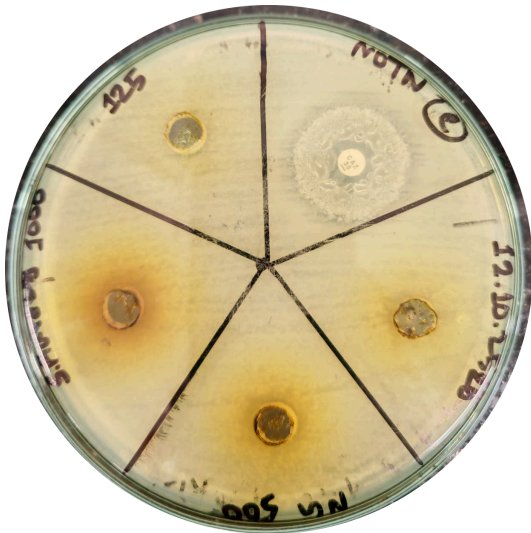


Figure 4.3.1: *Staphylococcus aureus*

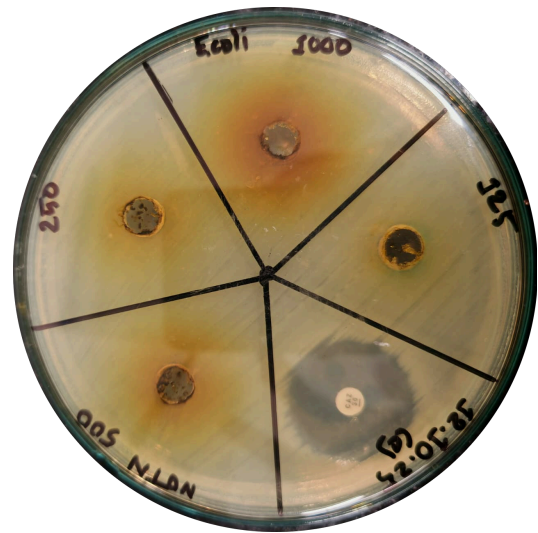


Figure 4.3.2: *Escherichia coli*

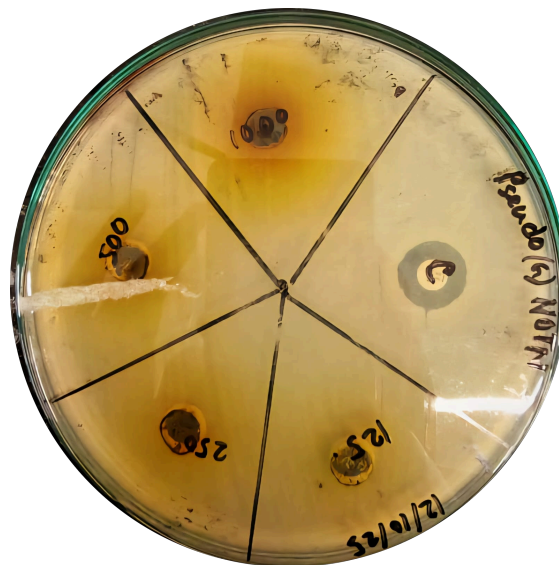


Figure 4.3.3: *Pseudomonas aeruginosa*

Disk Diffusion



Figure 4.3.4: *Klebsiella pneumoniae*

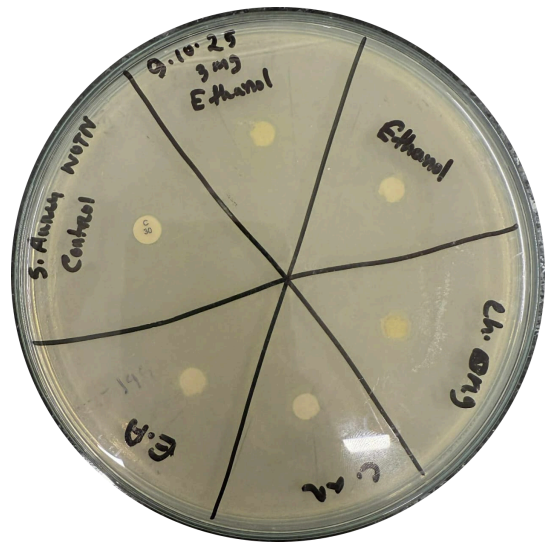


Figure 4.3.5: *Staphylococcus aureus*



Figure 4.3.6: *Shigella flexneri*



Figure 4.3.7: *Pseudomonas aeruginosa*



Figure 4.3.8: *Escherichia Coli*



Figure 4.3.9: *Salmonella typhi*



Figure 4.3.10: *Vibrio Cholerae*



Figure 4.3.11: *Enterococcus faecalis*

Table 4.3.1: Actibacterial activity of *Moringa oleifera* leaf extract

Organisms	Diameter of Zone of Inhibition (mm)										
	Extract Concentrations (mg/disk)						Positive Control		Negative Control	Solvent Control	
	0.010	0.030	125	250	500	1000	LZ	CAZ		DMSO	MeOH
<i>Klebsiella pneumoniae</i>	-	-	-	-	-	-	22	21	-	-	-
<i>Salmonella typhi</i>	-	-	-	-	-	-	18	32	-	-	-
<i>Shigella flexneri</i>	-	-	-	-	-	-	11	27	-	-	-
<i>Vibro cholerae</i>	-	-	-	-	-	-	10	21	-	-	-
<i>Pseudomonas aeruginosa</i> (ATCC: 27853) 2023	-	-	-	-	-	-	10	19	-	-	-
<i>Enterococcus faecalis</i> (ATCC: 29212)	-	-	-	-	-	-	19	-	-	-	-
<i>Escherichia coli</i> (ATCC: 25922)	-	-	-	-	-	-	10	29	-	-	-
<i>Staphylococcus aureus</i> (ATCC: 25923)	-	-	-	-	-	-	25	-	-	-	-
<i>Streptococcus</i>	-	-	-	-	-	-	24	-	-	-	-

“ - “ means susceptibility

Chapter 5

Discussion

The research on the *Moringa oleifera* produced encouraging results and specifically with regards to chemical structure and antioxidants, but the antibacterial effect was less promising.

Qualitative estimation of bioactive compounds - Flavonoids, Alkaloids, Anthraquinones Phenolics, Amino acids and Proteins has been carried out in four leaf extracts of *Moringa oleifera* by Thin Layer Chromatography based on R_f values and chemical polarity which differ with the solvent's solubility. For flavonoids, TLC with Ethyl acetate: Formic acid: Acetic acid: Water (100:11:11:26) revealed several bands in ethanol and chloroform organic extracts of the leaves (Coppin et al., 2013), but distinct zones were visible at R_f - 0.55, 0.89, and 0.93 for the ethanol extract whereas that of chloroform organic extract was between R_f value of 0.24–1.00 indicating multiple fractions for flavonoid groups. Ethyl acetate and chloroform aqueous extracts showed weak or no bands which indicate that the yield of flavonoid fraction might be low and natural version of flavonoids in *Moringa* are more soluble in polar solvents e.g., ethanol and organic chloroform layer. TLC Detection and location of alkaloids: (a) Strong colour bands appeared on spotted plates in the ethanolic, ethyl acetate and chloroform organic extracts with Toluene: Ethyl acetate as mobile phase 7:3 & 9:1 (Faizi et al, 1994) showing R_f value of 0.22–0.88; chloroform organic extract exhibiting several spots (R_f 0.26–0.75); spot red due to different shades found in ethyl acetate extract at R_f 0.37, 0.75, 0.88 while no band seen in aqueous indicating the mid/ non-polar nature of the alkaloids. Anthraquinones identified with Toluene: ethanol acetate: formic acid (5:4:1) were present in all but aqueous extract (Wagner & Bladt, 2009); chloroform organic extract (R_f 0.31-0.64), ethanol (R_f 0.57-0.64), and ethyl acetate (R_f 0.48-0.78) revealed more than one derivative suggesting the solubility of these compounds in mid-polar to organic solvent systems. Phenolic compounds using Chloroform: Methanol (8:2) as revealing agent gave clear band at R_f 0.78–0.93 in ethanol extract followed by ethyl acetate extract, weak in chloroform organic (R_f 0.88), and none with aqueous, thus supporting ethanol and ethyl acetate best solvents for phenolic extraction, being the most likely source of antioxidant activity. For amino acids and proteins, TLC in n-Butanol: Acetic acid: Water (4:1:5) (Faizi et al., 1994) revealed three spots in ethanol (R_f 0.66, 0.78, 0.84), one in ethyl acetate (R_f

0.58), weakly expressed in chloroform organic layer ($R_f=53$) and no spot in aqueous extraction depicting polar protic nature of ethanol supporting its potential for extracting polar biomolecules.

The crude plant ethanol extract ($IC_{50} = 56.536$ ug/mL) displays excellent antioxidant activity characteristics of an ethanol extract, which is extensive compared to published Moringa ethanol/methanol extracts which generally have a polar/alcoholic solvent extract of 20-100 ug/mL; this corroborates reports indicating that polar/alcoholic solvents extract phenols and flavonoids-dominant DPPH-active compounds (Deepali et al., 2025). Phenolic and flavonoid compounds (polar and medium polarity) are specifically soluble in ethanol and are also effective hydrogen/electron donors to DPPH. Consequently, the ethanol fraction exhibits the highest antioxidant capacity, but still less than that of vitamin C in the majority of comparative studies. It remains relevant to food supplements and nutraceuticals, however, as a crude extract, because its various bioactive compounds are synergistic (Matuszewska et al., 2018). The ethyl-acetate extract ($IC_{50}= 159.396$ ug/mL) has an average to weak activity, which is significantly less than the ethanol extract. The moderately polar soluble compounds are extracted by ethyl-acetate, a semi-polar solvent, but can be missed by more polar phenolic glycosides, which are more readily extracted by more polar solvents. Research demonstrates that the ethyl-acetate fractions are usually of mediocre activity, either stronger than nonpolar ones but weaker than ethanol/methanol (Haris and Ahmad, 2024). The data of percent-inhibition corresponding to the highest scavenging rates show that it follows a concentration-dependent mechanism, with high scavenging at 1000 ug/mL (more than 90%), and very low scavenging at 31.25 ug/mL, which confirms that it has a concentration-dependent behavior, with an IC_{50} at the middle range, indicating that larger concentrations are required to produce a 50% reduction in DPPH. The partition (aqueous-phase) of chloroform ($IC_{50} = 300.518$ ug/mL) has low antioxidant activity indicating low radical scavenging ability at low concentrations. This stage still does not have the phenolic richness of the ethanol extract, but it has sugars or tiny nonpolar non-phenolic products with low DPPH scavenging abilities (Deepali et al., 2025). Lastly, the antioxidant activity of chloroform organic-phase ($IC_{50} = 580.247$ ug/mL) is extremely weak, or even non-existent, and has to be used in high concentrations to be minimally scavenging. The concentration of nonpolar and lipophilic compounds, including terpenes and lipids, which are poor hydrogen donors to DPPH, is concentrated in this fraction, which is in agreement with reports that chloroform or n-hexane fractions tend to exhibit the lowest DPPH activity (Madhvi et al., 2020).

Though, the presence of phenolic, flavonoid compounds in *Moringa oleifera* leaf extracts was clearly demonstrated, using thin-layer chromatography and DPPH antioxidant assays, no inhibition spots were detected during the agar diffusion tests against clinical isolates (*Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus*, *Vibrio cholerae*, *Salmonella typhi*). This apparent inconsistency is mostly due to methodological and biochemical shortcomings of the experimental design. To begin with, the antimicrobial activity of compounds that are created through the use of plants greatly varies with the plant part. Although *M. oleifera* leaves have been found to be high in antioxidant flavonoids and phenols, past research has revealed that the bark, seeds, and fruit have even greater concentrations of robust antibacterial alkaloid, isothiocyanates, and tannin that directly interfere with the membranes of the microbes (El-Sherbiny et al., 2024). Therefore, the utilization of leaves must have limited the antimicrobial potential of the extracts.

Second, temperature and treatment at extraction and concentration may play a crucial role in bioactivity. High temperature during rotovaporation or long-lasting contact with air and light may oxidize or volatilize thermosensible molecules, making the antimicrobial activity of the final extract weaker (Angestia et al., 2020). Third, solvent-solvent partitioning might be useful to fractionate compounds according to their polarity; however, it can also divide active compounds or reduce the concentration. The polar fractions (aqueous and ethanol) would contain hydrophilic antioxidants, while certain hydrophobic antimicrobials accumulate in the chloroform or ethyl acetate layers, where they may become insoluble or their diffusion is limited, in an agar-based medium (Eloff, 2019). Lower diffusion of large or nonpolar phytochemicals through agar has been a drawback in disk and well diffusion tests for plant extracts (Balouiri et al., 2015). As a result, even the bioactive compounds can be devoid of measurable inhibition zones if they find it difficult to migrate from the disc or well into abutting agar (Gonzalez-Pastor et al., 2023).

The tested concentrations were also presumably below the minimum inhibitory concentration (MIC) of crude plant extracts, which usually have MICs in the hundreds or thousands of μg per mL for antimicrobial action (Eloff, 2019). Other confounding parameters, such as partial solvent evaporation, pH dependency, or high inoculum density, can at the same time further obscure weak activity. In order to fix these complications, diffusion barrier-free broth microdilution

assays in future studies, the use of concentrated extracts and freshly prepared extracts in place of degraded ones would be necessary. Inclusion of other plant parts, such as the bark and seeds, could also give a more effective antibacterial effect.

Despite such limitations, the findings of the current research have been useful in attracting attention to the critical parameters of the methodology that result in false-negative diffusion using plant extracts. This paper concluded that robust descriptions of antioxidants and phytochemicals do not automatically translate into antimicrobial zones, which further underscores the need of assays of optimization. Such procedural and biological limitations being understood contributes to this study becoming a methodological base in further studies of the research of the plant-derived antimicrobials in *M. oleifera* or other similar plants.

Conclusion

In conclusion, the ethanolic extracts of *Moringa oleifera* leaves as determined by the present research analysis exhibited the most pronounced antioxidant efficacy ($IC_{50} = 56.54 \mu\text{g/mL}$) when compared to partitioned fractions, thus emphasizing the heavy load of phenolic and flavonoid compounds. On the other hand, the absence of significant antimicrobial activity might be due to the constraints of the applied methods and environmental conditions. It is possible that the use of partitioned crude reduced the concentration and combined effect of active compounds, thus resulting in a lower efficiency of the extract. In addition, the use of only leaf extracts while bark, seeds and pods are known to have higher antimicrobial and antioxidant potential limited the overall activity. pH, temperature, crude concentration, inoculum density, solvent ratios (DMSO:DW) and seasonal variations in leaf quality during collection may also have played a role in producing inconsistent results.

To sum up, the present research really pointed out *Moringa oleifera's* great antioxidant potential but also explicitly mentioned the need for more studies on its antimicrobial activity with bark and seeds using optimized extraction techniques. The later incorporation of the water or other solvent extracts, Vitamin C enrichment, and nanoparticle-based formulations may not only improve bioactivity but also widen the therapeutic scope. Therefore, even though the ethanol crude extract showed the greatest antioxidant efficiency, the lack of detectable antimicrobial activity directs the study around the plant's biomedical potential to continue till fully unlocked.

The future studies will prioritize the alteration of extraction and testing conditions so as to increase the biological activity of *Moringa oleifera*. The bark of the plant will be utilized in the upcoming trials as past research has stated that bark and seed extracts are more potent than leaves in terms of antimicrobial and antioxidant properties (Sultan et al., 2023). The research will cover biofilm inhibition by means of raw extracts which will be an evaluation of anti-quorum-sensing and antibiofilm capabilities (Kim et al., 2024). Cytotoxicity tests will be carried out with the aim to find the bioactive compounds with anticancer activity, while thrombolytic tests will be done to isolate the fibrinolytic components that are meant for cardiovascular applications.

Moreover, the use of leaf and bark extracts for the production of bio-nanoparticles will be a main focus area aimed at the United Nations Sustainable Development Goal (SDG) 3 which corresponds to Good Health and Well-being (Perumalsamy et al., 2024). The crude extracts will be combined with Vitamin C, and this combination will be tested to get enhanced antioxidant and antimicrobial efficiency (Penalver et al., 2022). Different solvent-based extractions will be performed using methanol, hexane, or distilled water to check the variation in antimicrobial potential. Furthermore, the crude extracts, which are aqueously soaked, will be assessed in future studies for their relative effectiveness to those based on ethanol.

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