Innovative Extraction and Identification of Rutin Flavonoid from *Moringa oleifera* Leaves Using UHPLC-qTOF-MS and Computational Metabolomics Tools

By

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Declaration

I, **Dakalo Lorraine Ndou (19023430)**, declare that this thesis entitled 'Innovative Extraction and Identification of Rutin Flavonoid from *Moringa oleifera* Leaves Using UHPLC-qTOF-MS and Computational Metabolomics Tools' is my own original work. This work is being submitted for the Doctor of Philosophy degree in Chemistry at the University of Venda and has not been submitted for any degree at any other university or institution. The thesis does not contain other persons' writing unless specifically acknowledged and referenced accordingly.

Candidate signature: 

February 2024

Preface

In this thesis, Chapter 1 outlines the introduction to the study by giving the background of the study as well as the problem statement, aim and objectives of the study. In Chapter 2 the literature review relating to the study is outlined. Chapter 3 gives insight on the experimental procedures conducted in this study. Experimental chapters (Chapters 4-7) were written in the journal format to which they were submitted (more detail is given below). Chapter 8 gives the concluding remarks pertaining to the results of the study, and as well as the future work and recommendations. The outline of this thesis is shown below:

Experimental chapter 4. Dakalo Lorraine Ndou, Ntakadzeni Edwin Madala, Ashwell Rungano Ndhlala, Nikita Tawanda Tavengwa. **Distribution of rutin metabolite from domesticated *Moringa oleifera* plants in Vhembe District of Limpopo Province using UHPLC-qTOF-MS** (to be submitted)

Experimental chapter 5. Dakalo Lorraine Ndou, Ntakadzeni Edwin Madala, Ashwell Ndhlala, Nikita Tawanda Tavengwa. **Ultrasonic-assisted aqueous two-phase extraction for the extraction of rutin from *M. oleifera* leaves by response surface methodology** (to be submitted to Results in Chemistry)

Experimental chapter 6. Dakalo Lorraine Ndou, Ntakadzeni Edwin Madala, Ashwell Ndhlala, Nikita Tawanda Tavengwa. **Extraction of rutin from *M. oleifera* leaves by PT-µSPE using hollow carbon nanospheres as sorbent** (submitted to International Journal of Analytical Chemistry)

Experimental chapter 7. Dakalo Lorraine Ndou, Ntakadzeni Edwin Madala, Ashwell Ndhlala, Nikita Tawanda Tavengwa. **A relook into the flavonoid chemical space of *Moringa oleifera* Lam. leaves through a combination of LC-MS and molecular networking.** *Journal of Analytical Methods in Chemistry*, 2023:1-15.

Abstract

Moringa oleifera is a tree that has been studied extensively and it has been found to host a variety of medicinal and nutritional properties. Owing to these properties, it has often been referred to as a 'miracle tree'. *M. oleifera* contains a variety of metabolites such as flavonoids, glucosinolates, phenolic acids, tannins, and carotenoids that are responsible for the pharmacological properties of this plant. In this study, the metabolite of interest in *M. oleifera* was the rutin flavonoid. Rutin has various pharmacological properties and its presence in *M. oleifera* makes the plant more bioavailable. In this study, the presence of rutin was evaluated in *M. oleifera* plants in households from different villages within the Vhembe District. The Molecular Networking (MN) approach was utilized to revisit the chemical space of flavonoids in *M. oleifera*. The aim was to establish the biochemical modifications responsible for the chemical diversity of these compounds, which have been reported to be associated with the purported pharmacological properties of this plant. Modern extraction methods such as ultrasonic-assisted aqueous two-phase extraction (UA-ATPE) and pipette tip micro-solid phase extraction (PT-μSPE) were explored in the extraction of the most bio-available and most sought-after flavonoid, rutin, in *M. oleifera* leaf extracts. This work has been divided into four experimental chapters.

In **Experimental Chapter 4**, the presence of rutin was evaluated in the leaves of 135 *M. oleifera* plants from households in different villages within the Vhembe District of Limpopo Province of South Africa. The metabolite extraction was carried out using the conventional liquid extraction method using 80% MeOH and the resulting extracts were analyzed using UHPLC-qTOF-MS. The results from the UHPLC-qTOF-MS showed that only 15 plants produced rutin. This was confirmed using tandem mass spectrometry (MS2) and an authentic standard, which further validated the detected ion as a true representation of rutin. It was concluded that different cultivars of this plant are being grown in various households within the Vhembe District. These differences are expected to result in a negative perception towards Moringa plants, and as such, knowledge of the cultivar-chemical relationship should be made public with the intention to encourage the cultivation of proper plant species. The extraction of rutin from *M. oleifera* leaves using UA-ATPE was reviewed in **Experimental Chapter 5**. An ethanol/salt ATPE was formed for the extraction of rutin. Ultrasonication was used to assist in the extraction of rutin from the leaves. Central composite design (CCD) was used to design experiments and two factors which were optimized

are ultrasonic time and ultrasonic temperature. The ammonium sulphate ($(NH_4)_2SO_4$), sodium chloride (NaCl), and magnesium sulphate ($MgSO_4$) salts were used to form the ethanol/salt ATPE two phase system. The resulting Response Surface Model (RSM) was observed to be a linear fit for the ethanol/$(NH_4)_2SO_4$ and the ethanol/$MgSO_4$ ATPE systems, with R^2 values of 0.7339 and 0.5782, respectively, as obtained from the analysis of variance (ANOVA). The ethanol/NaCl ATPE system yielded a quadratic fit with $R^2 = 0.7865$ and was observed to be the best performing in the extraction of rutin from the *M. oleifera* leaves with optimum extraction at a temperature of 25 ºC and time of 22.5 minutes. Based on multiple reaction monitoring (MRM) through the UHPLC-qTOF-MS technique, the concentration of rutin extracted by the ethanol/NaCl ATPE system was 240 µg L^{-1}. According to ANOVA, temperature (the B-term) was found to be the significant term with a $p < 0.0500$ that the extraction of rutin through UA-ATPE is temperature-dependent. Therefore, it was concluded that the extraction of rutin from *M. oleifera* leaves favors low temperatures. The results of the current study further demonstrate the usefulness of simple extraction techniques, such as heated water with additives like salts, as a feasible method to enrich pharmacologically relevant metabolites from plants. This reaffirms traditional protocols that are currently used by communities which include boiling plants in water to extract useful chemical compounds for the treatment of common ailments such as colds and headaches. In **Experimental Chapter 6**, PT-µSPE was applied in the extraction of rutin from *M. oleifera* leaves using activated hollow carbon nanospheres (HCNSs) as the sorbent. The activated HCNSs were characterized using FTIR, which confirmed the presence of the functional groups of interest such as OH stretch, $-COO^-$ vibration, and C=O stretch. TGA thermogram showed a difference in the thermal stability of the raw and activated HCNSs, proving that the raw HCNSs are more stable than the activated HCNSs, and SEM displayed the difference in the morphology of the raw and activated HCNSs by observing the difference in the coalescence of the material. Parameters such as loading cycles, elution solvent, concentration of rutin, pH, loading volume, and mass of sorbent were optimized. The analysis of the extracts was conducted with a UV-Vis spectrophotometer to ascertain the recovery of rutin. The optimal conditions for rutin recovery using PT-µSPE were determined to be 15 loading cycles, i-PrOH as the elution solvent, ~~a~~ 2 ppm as standard concentration of rutin, a pH of 2, 500 µL as loading volume, and 1.5 mg of sorbent. The LOD, LOQ, and RSD values were found to be 0.604 mg L^{-1}, 1.830 mg L^{-1}, and 3.26%; respectively. It was thus confirmed that the PT-µSPE method is effective in the extraction of rutin even at trace levels based on the low LOD

value obtained and the RSD value obtained proved that this method is a reliable pre-concentration technique and is thus repeatable for the analysis of complex samples. Therefore, in cases where some plants produce these compounds in minute concentrations, methods such as this one presented herein can be used to estimate and concentrate the pharmacologically relevant compounds.

Molecular networking (MN) was used to study the global metabolic profile of *M. oleifera* and is outlined in **Experimental Chapter 7**. Herein, Global Natural Products Social (GNPS) platform was used to generate the MN from the LC-MS data obtained from the methanolic leaf extracts of *M. oleifera*. The MN was viewed and analyzed using Cytoscape. Through MN, it was observed that *M. oleifera* contains a variety of metabolites. Other GNPS tools such as network annotation propagation (NAP), DEREPLICATOR, MS2LDA, and MolNetEhancer were further used to compliment the classical MN model. To this end, MS2LDA was used to annotate the flavonoids found within *M. oleifera*. Kaempferol, quercetin, and isorhamnetin flavonoids were successfully annotated by MS2LDA. Additionally, Chrysin-6,8-C-diglucoside was also annotated and reported for the first time in *M. oleifera* leaves. The results of this study further suggest MN models as useful tools for chemical exploration, enabling the discovery of new metabolites by leveraging existing knowledge as "chemical charms" to unearth hidden metabolites.

Dedications

This thesis is dedicated to:

- My beautiful children, Ishe and Renda; this one is for you my babies!
- My parents for the support and encouragement and for just believing in me even when it did not make sense. You were my pillar. I couldn't have asked for better parents.
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List of abbreviations

ATPE/S	Aqueous Two-Phase Extraction/System
CID	Collision Induced Dissociation
CNTs	Carbon Nanotubes
DES	Deep Eutectic Solvent
DI-SDME	Direct Immersion-Single Drop Micro-extraction
DI-SPME	Direct Immersion-Solid Phase Micro-extraction
DLLME	Dispersive Liquid-Liquid Micro-extraction
DVB/CAR/PDMS	divinylbenzene/carboxen/polydimethylsiloxane
EtOH	Ethanol
GC-MS	Gas Chromatography – Mass Spectrometry
GNPS	Global Natural Product Social
GO	Graphene Oxide
HF-LPME	Hollow Fiber-Liquid Phase Micro-extraction
HHPE	High Hydrostatic Pressure Extraction
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High Performance Liquid Chromatography-Diode Array Detector
HPLC-ESI-qTOF-MS	High Performance Liquid Chromatography coupled to Electrospray Ionization Quadrupole Time of Flight Mass Spectrometry
HPLC-PDA	High Performance Liquid Chromatography-Photo Diode Array
HS-SDME	Headspace-Single Drop Micro-extraction
HS-SPME	Headspace-Solid Phase Micro-extraction
IM-QTOF-MS	Ion Mobility Quadrupole Time of Flight Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LPME	Liquid Phase Micro-extraction
LOD	Limit of Detection

LOQ	Limit of Quantification
MAE	Microwave Assisted Extraction
MANPs	Magnetic Agarose Nanoparticles
M-D-µSPE	Magnetic Dispersive micro Solid Phase Extraction
MeOH	Methanol
MF	Molecular Family
MGF	Mascott Generic Format
MGO/MHNTs@MIPs	Magnetic Graphene Oxide/Magnetic Halloysite Nanotubes Molecularly Imprinted Polymers
MHz	Megahertz
MIPS	Molecularly Imprinted Polymers
MN	Molecular Networking
MS	Mass Spectrometry
MSPD	Matrix Solid-Phase Dispersion
NADES	Natural Deep Eutectic Solvents
NMR	Nuclear Magnetic Resonance
PCSS-DLPME	Phosphatidylcholine Supramolecular Solvent Dispersive Liquid Phase Micro-extraction
PEI	Polyethyleneimine
PHWE	Pressurized Hot Water Extraction
PLE	Pressurized Liquid Extraction
PS-DVB	Poly (styrene divinyl-benzene)
PT-µSPE	Pipette Tip Micro Solid Phase Extraction
Pt@r-GO@MWCNTs)	Platinum nanoparticle, reduced Graphene Oxide, Multi-walled Carbon nanotubes
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SBME-DES	Solvent Bar Micro-extraction based on a Deep Eutectic Solvents

SDME	Single Drop Micro-extraction
SFE	Supercritical Fluid Extraction
SLM	Supported Liquid Membrane
SPE	Solid Phase Extraction
SPME	Solid Phase Micro-extraction
TLL	Tie Line Length
UA-ATPE	Ultrasound Assisted-Aqueous Two-phase Extraction
UAE	Ultrasonic Assisted Extraction
UHPLC-qTOF-MS	Ultra-High Performance Liquid Chromatography – quadrupole Time of Flight Mass Spectrometry
UV-Vis	Ultraviolet Visible Spectrometry
VA-DLLME	Vortex-Assisted Dispersive Liquid-Liquid Micro-extraction
WHO	World Health Organization

Chapter 1

Introduction and background of study

The introduction and background of the study are outlined in this chapter. The problem statement, aim and objectives are also detailed.

1.1. Background

Chronic diseases pose a significant challenge to the wellbeing of human beings as well as the healthcare system, globally. The WHO has reported that each year, approximately 71% of deaths are due to chronic diseases (Cardoso *et al*., 2018; Li *et al*., 2019; Reiners *et al*., 2019). In this modern day and age, these diseases are known to affect both young people and the elderly. The most common chronic diseases include cancer, chronic respiratory diseases, cardiovascular diseases, and diabetes. These diseases are usually a result of behavioral patterns such as tobacco use, unhealthy diet, lack of physical activity, and the use of alcohol. It is, therefore, believed that promoting healthy lifestyles through following a healthy nutritious diet, smoking cessation, and participating in physical activities can help prevent these diseases (Raghupathi and Raghupathi, 2018; Zhou *et al*., 2018; Anderson and Durstine, 2019; Wilkins *et al*., 2019). The use of natural herbal medicines is an age-old tradition and the recent progress in modern therapeutics has stimulated the use of natural products for the treatment of diseases (Eddouks *et al*., 2012; Altemimi *et al*., 2017; Brilhante *et al*., 2017; Jamshidi-Kia *et al*., 2018). Plants have shown great promise in the treatment of diseases as they contain secondary metabolites that are sources of pharmaceutical compounds (Hemalswarya and Doble, 2006).

Nutraceuticals, found in plants, are natural bioactive or chemical compounds that have both nutritional value and provide health-promoting, disease curing or prevention properties. Nutraceuticals have been reported to treat certain conditions such as cardiovascular diseases, diabetes, cancer, and neurological disorders (Dima *et al*., 2020; Durazzo *et al*., 2020; Makkar *et al*., 2020; Sachdeva *et al*., 2020; Williamson *et al*., 2020; Alagawany *et al*., 2021; Reque and Brandelli, 2021). Numerous plant species have been used for food and medicinal purposes globally. One such source of nutraceuticals is *Moringa oleifera* Lam. This plant is a cruciferous herb and is a member of the Moringaceae family. It is native to India but is now being grown in various parts of the world (Makita *et al*., 2017; Yi *et al*., 2017; Dhakad *et al*., 2019; Islam *et al*., 2021; Milla *et al*., 2021; Sreeja *et al*., 2021). *M. oleifera* is cultivated in six provinces in South Africa, namely Limpopo, Gauteng, Mpumalanga, Kwazulu Natal, Free State, and North West. *M. oleifera* is primarily cultivated in Limpopo by farmers and households among the mentioned provinces. (Mashamaite *et al*., 2021). *M. oleifera* has previously been reported to have therapeutic benefits such as anti-diabetes, antioxidant, anti-inflammatory, anti-fertility, pain relief, anti-

depression, and diuretic and thyroid control (Kou *et al*., 2018; Padayachee and Baijnath, 2019; Chhikara *et al*., 2020; Meireles *et al*., 2020; Sreeja *et al*., 2021; Abdel-Latif *et al*., 2022).

The biological activities of *M. oleifera* are attributed to its phytochemicals such as sterols, terpenoids, flavonoids, saponins, anthraquinones, alkaloids, glucosinolates, isothiocyanates, glycosides, and polyphenolic compounds (Omotoso *et al*., 2018; Singh *et al*., 2019; Fernandes *et al*., 2020; Liu *et al*., 2020; Suresh *et al*., 2020; Zainab *et al*., 2020). Flavonoids are one of the major groups of phytochemicals that have been studied extensively for their biological properties (Gorniak *et al*., 2019; Karak, 2019; Maleki *et al*., 2019, Ullah *et al*., 2020). Flavonoids have played significant roles in successful medical treatments in ancient and modern times. It has been reported that *M. oleifera* leaves are rich in flavonoids with high antioxidant and anti-inflammatory activities (Xu *et al*., 2019; Lin *et al*., 2021; Ati *et al*., 2022; Silva *et al*., 2022; Wang *et al*., 2022). The most common flavonoids in *M. oleifera* are kaempferol, isorhamnetin, quercetin, and apigenin. These flavonoids exist abundantly as glycosides attached to a wide range of sugar moieties such as acetyl dihexose, hexose and rutinoside (Lin *et al*., 2018; Pollini *et al*., 2020; Khan *et al*., 2021; Mohanty *et al*., 2021; Wang *et al*., 2022), thereby contributing towards a structurally diverse flavonoid chemical space.

Quercetin is a major flavonoid in the leaves of *M. oleifera*. It exists as a glycoside linked to one or more sugar molecules via a glycosidic bond. Glycosides of quercetin include hyperoside, isoquercetin, quercitrin, rutin and reinutrin (Shervington *et al*., 2018; Sulastri *et al*., 2018). Rutin exhibits various pharmacological properties such as antioxidant, anti-inflammatory, antiallergic, cardiovascular, neuroprotective, antidiabetic, and anticancer activities. These properties make this flavonoid important in the pharmaceutical industry (Enogieru *et al*., 2018; Peng *et al*., 2018; Kim and Lim, 2019; Tursynbolat *et al*., 2019; Rahman *et al*., 2021). This compound has been reported in over 70 plant species and, due to its diverse health benefits, it is frequently extracted as researchers seek optimal extraction conditions (Kim and Lim, 2019).

Due to the importance of rutin, as highlighted above, many extraction methods that have been applied for its extraction including Soxhlet extraction (Al-Majmaie *et al*., 2019; Vaidehi *et al*., 2020; Nuralin and Guru, 2021), ultrasound-assisted extraction (UAE) (Moreira and Dias, 2018; Papoutsis *et al*., 2018; Banozic *et al*., 2019; Fan *et al*., 2020), microwave-assisted extraction

(MAE) (Zhao *et al*., 2018; Ayouaz *et al*., 2020; Weremfo *et al*., 2020), pressurized liquid extraction (PLE) (Corazza *et al*., 2018; Tripodo *et al*., 2018; Barbosa *et al*., 2019; de Aguiar *et al*., 2020), supercritical fluids extraction (de Souza *et al*., 2019; Nuralin and Guru, 2021), and the application of deep eutectic solvents (Molnar *et al*., 2018; Peng *et al*., 2018; Ali *et al*., 2019; Jakovljevic *et al*., 2020). In this study, two modern extraction methods were applied for the extraction of rutin from *M. oleifera* leaves. The methods are ultrasound-assisted aqueous two-phase extraction (UA-ATPE) (**Experimental Chapter 5**) and miniaturized pipette tip solid-phase extraction (PT-µSPE) (**Experimental Chapter 6**). PT-µSPE is the miniaturized form of conventional SPE in which a microscale amount of sorbent is placed in a pipette tip. This method overcomes some of the practical problems of conventional SPE disks and cartridges (Hashemi *et al*., 2019; Kahkha *et al*., 2019; Seidi *et al*., 2019; Sun *et al*., 2019; Amini *et al*., 2020; He *et al*., 2021). Nanomaterials such as functionalized magnetic nanoparticles (MNPs), graphene-based sorbents, and carbon nanotubes have been successfully applied in miniaturized pipette tip SPE (Fresco-Cala *et al*., 2018; Esrafili *et al*., 2020; Sun *et al*., 2021; Tsai *et al*., 2021). In this study, hollow carbon nanospheres (HCNSs) were used as sorbents for the extraction of rutin from *M. oleifera* leaves. PT-µSPE is discussed at length in Chapter 2.

UA-ATPE combines UAE and ATPE with the field-enhanced effect and two-phase separation and it completes the extraction and purification of bioactive components in one step. UA-ATPE is advantageous because it shortens the extraction time and improves the extraction yield and purity of the target compounds (Ji *et al*., 2018; Luo *et al*., 2018; Yan *et al*., 2021; Zhou *et al*., 2021; Tan *et al*., 2022). In this study, three different ethanol/salt ATP systems were developed for the extraction of rutin from *M. oleifera* leaves with ultrasound assistance as discussed in **Experimental Chapter 5**.

Quantification studies of rutin from the *M. oleifera* leaves were performed on the ultra-high performance liquid chromatography time of flight mass spectrometry (UHPLC-qTOF-MS) using the multiple reaction monitoring (MRM) approach. UHPLC-qTOF-MS has emerged as an effective analytical tool for the rapid screening of plant metabolites. This analytical tool is highly sensitive, selective, specific, and has shorter analysis time when compared to other conventional methods (Want, 2018; Nadeem *et al*., 2019; Beccaria and Cabooter, 2020; Bian *et al*., 2020). Low

resolution MS coupled with MRM transitions is well suited for analyzing targeted metabolites but is restricted in its metabolite coverage. The combination of UHPLC-qTOF-MS and MRM helps to achieve the requirements of high resolution and excellent quantification (Zhao and Li, 2020; Tan *et al*., 2021).

In molecular networking, untargeted tandem mass spectrometry (MS/MS) data is organized and represented in a graphical form where each node represents an ion and its associated fragmentation pattern. The links between the nodes indicate similarities in the fragmentation spectra (Beniddir *et al*., 2020; Le Dare *et al*., 2020). Molecular networking can be used to map the chemical space of complex samples to facilitate the discovery of new molecules (Quinn *et al*., 2016; Le Dare *et al*., 2020; Schmid *et al*., 2021). Molecular networking has led to the development of Global Natural Products Social (GNPS) and has been widely used for the annotation of MS-based chemical signatures (Quinn *et al*., 2016; Xu *et al*., 2019; Rawlinson *et al*., 2020; Silva *et al*., 2020). GNPS molecular networking provides other information including insight on regularities for many structurally related metabolites detected in experiments and is therefore becoming a powerful tool for the development of metabolomics (Xu *et al*., 2019; Aron *et al*., 2020). More information on molecular networking can be found in Chapter 2 and **Experimental Chapter 7**.

1.2. Problem statement

Moringa oleifera plant leaves, roots, flowers, and pods have been reported to exhibit various medicinal and nutritional properties. This plant can also be used as a source of nutrition to combat malnutrition in Third World countries. The seeds of this plant have been used to purify water and the leaves of this tree have been used to treat a variety of diseases and that is why this tree is also referred to as the 'miracle tree'.

The leaves of *M. oleifera* have been reported to have a range of biological properties owing to the flavonoids present (Lin *et al*., 2021; Wang *et al*., 2022). One flavonoid that is most popular for its pharmacological properties is quercetin rutinoside, also known as rutin. Rutin is a glycoside of the quercetin aglycone which is attached to the rutinoside sugar. The presence of rutin in *M. oleifera* enhances its ability in the treatment of different diseases such as inflammatory diseases, diabetes, cancers, and neuro-dysfunctional diseases. However, there has been a controversy centered around

the presence of rutin in *M. oleifera*. In a study by Habtemarium and Varghese (2015), *M. oleifera* was studied with its closely related species *M. stenopelata* and it was found that rutin was present only in *M. stenopelata*. It was concluded that *M. oleifera* is incapable of producing rutin. A similar study was, however, conducted by Makita *et al*. (2016) where *M. oleifera* was studied alongside *M. ovalifolia* and it was also found that rutin was present in *M. ovalifolia* but not in *M. oleifera*. Again, it was then concluded that *M. oleifera* is incapable of producing rutin. However, Makita *et al*. (2017) conducted another study on twelve different cultivars of *M. oleifera* and found that only three of those cultivars were rutin producing and this led to the conclusion that *M. oleifera* can produce rutin, however, its production in this tree is cultivar specific. These findings further propagate an interest to determine the cultivar that is most commonly being grown, not only on commercial farms but also in different households.

Another challenge poses to the extraction of rutin from natural products. Rutin is believed to exist in low concentrations which makes it difficult to extract from plants (Sampaio *et al*., 2018). This poses the need to investigate robust extraction methods that will extract this compound from *M. oleifera* plants that produce rutin even at lower concentrations. This study, therefore, investigates the use of pre-concentration techniques such as UA-ATPE and PT-µSPE to extract rutin from household rutin-producing *M. oleifera* plants. A sensitive analytical instrument such as the UHPLC-qTOF-MS is also necessary to detect the low concentration of rutin in the extracts.

There have been inconsistent reports regarding the presence of rutin in *Moringa oleifera*. This study reaffirms that rutin accumulation is specific to certain cultivars, meaning not all *M. oleifera* plants produce rutin. Among the 135 *M. oleifera* plants investigated, only 15 (11%) were found to produce rutin, and in varying amounts. Environmentally friendly methods such as UA-ATPE and PT-µSPE were demonstrated to be effective in preconcentrating rutin from plants with lower concentrations of the compound. The results of this study effectively show that not all Moringa plants are the same, emphasizing the importance of specimen authentication to achieve standardized pharmacological outcomes. Additionally, these findings should encourage the cultivation of rutin-producing *M. oleifera* cultivars to enhance public perception and utilization of this plant.

1.3.Aim and objectives

1.3.1 Aim

To investigate the presence of rutin in household *M. oleifera* plants within the Vhembe District in Limpopo Province of South Africa using UHPLC-qTOF-MS, the use of modern extraction methods in extracting rutin, and to study the flavonoid chemical space of this plant using computational metabolomics tools such as molecular networking.

1.3.2 Objectives

- Sampling of *M. oleifera* leaves from different households within the Vhembe District in Limpopo Province of South Aftica.
- Extraction of metabolites using 80% MeOH and analyzing the extracted metabolites using UHPLC-qTOF-MS.
- Determination of the distribution of rutin from the leaf extracts and quantification of rutin using UHPLC-qTOF-MS.
- Extraction of rutin from the rutin-producing plants using modern extraction techniques such as UA-ATPE and PT-µSPE and analyzing using UHPLC-qTOF-MS, UV-Vis spectrophotometry, and HPLC.
    - Optimization of ultrasonic time and ultrasonic temperature using three different ethanol/salt UA-ATPE systems for the extraction of rutin.
    - Optimization of mass of sorbent, loading cycles, aspirating/dispersion cycles, pH, concentration, and solvent for the extraction of rutin using PT-µSPE.
- To study the chemical space of the flavonoids within *M. oleifera* using molecular networking and to annotate the flavonoids through MS2LDA and MolNetEnhancer.

Chapter 2

Literature review

This chapter gives a literature survey on *Moringa oleifera* and the flavonoids it contains. Particular interest is also given to the rutin flavonoid. Modern extraction techniques have also been highlighted in this chapter. The use of computational tools to study the chemical space of flavonoids has also been elaborated.

2.1. The importance of natural products

Natural products such as plants provide new opportunities for the discovery of drugs because of their unmatched availability of chemical diversity (Sasidharan *et al*., 2011; Anand *et al*., 2019; Suntar, 2020). Plants have been and are still used in medicine owing to their therapeutic properties (Salmeron-Manzano *et al*., 2020). They have the ability to produce a variety of diverse bioactive compounds and thus play a significant role in the discovery of drugs (Muhammad *et al.*, 2016; Altemimi *et al.*, 2017; Chopra and Dingra, 2020; Atanasov *et al*., 2021). The number of plant metabolites have been estimated to be more than 100 000 with new analogues still being discovered (Etalo *et al*., 2018). These are secondary metabolites like alkaloids, terpenoids, and phenolic compounds, which are synthesized by the plants as a defense against herbivores and pathogens (Muhammad *et al*., 2016; Etalo *et al*., 2018; Thomford *et al*., 2018). The different parts of the plants such as the roots, leaves, seeds, fruit, bark, stem, or sometimes even the whole plant are used because of the active compounds that are stored therein (Jamshidi-Kia *et al*., 2018).

The World Health Organisation (WHO) has reported that more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Sasidharan *et al*., 2011; Awuchi, 2019). It has also been reported that up to 20% of known plants have been used in pharmaceutical studies and they impact the healthcare system in positive ways such as treating many harmful diseases (Altemimi *et al*., 2017; Jain *et al*., 2019; Adhikari *et al*., 2021). These medicinal plants have a large impact on the health of individuals and the community at large in treating and preventing various diseases (Edeoga *et al*., 2005; Brilhante *et al.*, 2017; Jamshidi-Kia *et al*., 2018). One such medicinal plant is the *Moringa oleifera* Lam. tree which has been reported to have active compounds against several diseases such as diabetes, inflammation, heart diseases, and ulcers (Brilhante *et al*., 2017; Matic *et al*., 2018; Prabu *et al*., 2019; Mohanty *et al*., 2021).

2.2. Introduction to *Moringa oleifera*

The Moringaceae family is known to be monogeneric, with thirteen species of the genus *Moringa* (Leone *et al*., 2015). Amongst these species, eleven species are indigenous to Africa (i.e. *M. arborea*, *M. rivae, M. ruspoliana*, *M. borziana*, *M. pygmaea, M. longituba*, *M. stenopetala*, *M. ovalifolia, M. drouhardii*, *M. hildebrandtii* and *M. peregrine*) while the remaining two species are indigenous to Asia (i.e. *M. concanensis* and *M. oleifera*) (Boopathi and Abubakar, 2021). These

Moringa species are used for different purposes such as sources of medicine, food, cosmetics, and the production of oil (Padayachee and Baijnath, 2012). Table 2.1 lists the different Moringa species with their characteristics, i.e. their place of origin, their growth form, and their uses. This table also highlights the similarities/differences between the different Moringa species. However, the most widely known and studied species is *M. oleifera* (Stadtlander and Becker, 2017).

Moringa oleifera is cultivated in tropical and sub-tropical areas (Fahey, 2005; Wadhwa *et al.*, 2012; Bhattacharya *et al*., 2014; Ratshilivha *et al.*, 2014; Gopalakrishnan *et al*, 2016; Metwally *et al.*, 2017). It is native to India, Pakistan, Bangladesh, and Afghanistan and it is a perennial tropical evergreen tree. It is currently cultivated in West, East and Southern Africa, tropical Asia, Latin America, Florida, and in Pacific Islands (Fahey, 2005; Wadhwa *et al.*, 2012; Ratshilivha *et al.*, 2014; Muhammad *et al.*, 2016; Brilhante *et al.*, 2017; Makita, 2017; Yi *et al*., 2017). African countries recently started cultivating this plant because of its medicinal uses for both humans and animals, and countries such as Ghana and Senegal have been reported to have the highest cultivation of this tree (Ratshilivha *et al*., 2014).

Moringa is often referred to as a miracle tree because of the positive impact it has on people's livelihoods (Islam *et al*., 2021). It contains a variety of essential phytochemicals that have various medicinal and nutritional values that are present in the leaves, pods, roots, bark, gum, flowers, seed, and seed oil (Brilhante *et al*., 2017; Metwally *et al*., 2017; Tshabalala *et al*., 2019; Boumenjel *et al*., 2021; Vaknin *et al*., 2021). The nutritional composition of this plant differs at different locations. For example, the tree that is grown in India slightly differs in nutrient composition to the tree grown in Nigeria (Gopalakrishnan *et al*., 2016). Soil is thus an important factor that determines the nutritional content and the strength of the plant (Gopalakrishnan *et al*., 2016). Almost every part of the *M. oleifera* possesses a variety of important nutrients such as calcium, potassium, zinc, magnesium, iron, copper, vitamins A – E, proteins, lipids, carbohydrates, and dietary fibres (Brilhante *et al*., 2017; Yi *et al*., 2017; Do *et al*., 2021; Bania *et al*., 2023). It is also used as a food source to overcome malnutrition because the leaves, flowers, and seeds of the Moringa tree can be consumed by humans (Gopalakrishnan *et al*., 2016; Brilhante *et al*., 2017; Islam *et al*., 2021).

Table 2.1: List of the 13 Moringa species with their respective place of origin, growth form, and their uses (Padayachee and Baijnath, 2012).

***Moringa* species**	**Place of origin**	**Growth form**	**Use(s)**
M. arborea	Kenya	Shrub or tree	Medicinal
M. rivae	Kenya and Ethiopia	Shrub or tree	Medicinal
M. ruspoliana	Ethiopia and Somalia	Tree	Medicinal
M. borziana	Somalia and Kenya	Herb or small shrub	Medicinal
M. pygmaea	Somalia	Herb or small shrub	Medicinal
M. longituba	Somalia, Kenya and Ethiopia	Tree or shrub	Water coagulant, medicinal
M. stenopetala	Kenya and Ethiopia	Tree	Vegetable, spice, medicinal, water coagulant, seed oil, lubricant, perfume and soap production, ornamental
M. ovalifolia	Angola and Namibia	Tree	Vegetable, oil (cooking and cosmetics), ornamental
M. drouhardii	Madagascar	Tree	Oil (cooking and cosmetics), water coagulant, medicinal, ornamental
M. hildebrandtii	Madagascar	Tree	Medicinal, ornamental

M. peregrina	Arabia	Shrub or small tree	Oil (cooking and cosmetics), medicinal, water coagulant, ornamental, building material
M. concanensis	Pakistan, India and Arabia	Tree	Cooking oil, medicinal
M. oleifera	India	Tree	Food source, water coagulant, oil (cooking and cosmetics), honey clarifier, medicinal, ornamental, firewood

Moringa oleifera has been reported to have a broad range of pharmacological activities such as antimicrobial, anti-inflammatory, hypotensive, antidepression, antioxidant, antidiabetic, hypoglycemic and immunomodulatory properties (Padayachee and Baijnath, 2019; Meireles *et al*., 2020; Sreeja *et al*., 2021). The chemical constituents of the stems, leaves, flowers, pods, and seeds have been analyzed to determine the presence of bioactive compounds, and they were found to contain predominant secondary metabolites such as phenolic acids, gallic acid, polyphenolic compounds, sterols, terpenoids, flavonoids, alkaloids, sugars, anti-cancerous agents such as gluconisolates, isothiocyanates, glycoside compounds, and glycerol-1-9-octadecanoate which have nutritional, pharmaceutical, and anti-microbial properties (Bhattacharya *et al*., 2014; Gopalakrishnan *et al*., 2016; Makita *et al*., 2016; Brilhante *et al*., 2017; Yi *et al*., 2017).

Moringa provides a rich and rare combination of zeatin, quercetin, kaempferol, and other phytochemicals. Faizi *et al.* (1998) performed a bioassay-guided analysis of the ethanolic extract of the leaves and it revealed the presence of 10 compounds. The different extracts of its roots, barks, leaves, flowers, pods, and fruits have been reported to possess cardiac and circulatory stimulant, antifertility, antitumor, antioxidant, antifungal, anti-inflammatory, hypoglycemic, antiulcer, hepaprotective and therapeutic potential. In another study by Sinaga *et al*. (2021), 96% ethanol was used to extract the phytochemicals from *M. oleifera* leaves by maceration and 14 compounds were successfully extracted.

2.3. Extraction of metabolites in *Moringa oleifera*

The amount of the metabolites in the *M. oleifera* plant extracts can vary according to the geographic location, soil, sun exposure, and climatic conditions (Brilhante *et al*., 2017). However, the method and solvents used to extract can also have an influence on the content of the compounds obtained from the plant, mainly phenols and flavonoids (Brilhante *et al*., 2017). There are several methods that can be used for the extraction of plant metabolites. The extraction yield does not only depend on the extraction method but also on the solvent used for extraction and the physico-chemical properties of the metabolites (Tugizimana *et al.*, 2013; Do *et al.*, 2014; Złotek *et al.*, 2016).

Conventional methods of treatment have proven to make use of large amounts of reagents and solvents which generate large amounts of waste and have long analysis times. This has risen human

and environmental concerns (Gbashi *et al.*, 2016; Masike *et al.*, 2017). The various steps that are integrated in the treatment of the samples can also result in analyte loss and/or degradation. The steps involved with these conventional methods include sampling, sample preparation, and management of sample preparation being the most time consuming stage of this process (Escobar-Arnanz and Ramos, 2015; Moliner-Martinez *et al*, 2015). In a study by Okechukwu *et al*. (2021), the phytochemical constituents in *M. oleifera* were extracted from the leaves using the conventional MeOH extraction (600 mL), 80 g of leaves, and the extraction was performed for 72 h. In another study by Akintelu *et al*. (2021), a mass of 400 g of powdered seeds was extracted using 800 mL of methanol and the extraction time was 72 h. The use of the large amounts of reagents, solvent volume, and long extraction times have resulted in the need for the development of green sample preparation techniques and therefore replacing conventional methods by simpler and environmentally friendly techniques that can minimize these short comings (Escobar-Arnanz and Ramos, 2015; Moliner-Martinez *et al*, 2015; Wen *et al*., 2020; Mohapatra *et al*., 2021).

Green technology aims to replace toxic reagents, miniaturization, and automation of analytical methodologies with the intention of minimizing environmental hazards. The use of green techniques results in less time, energy, and solvents being used (Pena-pereira *et al*., 2010; Ivanovs and Blumberga, 2017; Ak *et al*., 2020; Sajid and Plotka-Wasylka, 2022). Extraction methods such as microwave-assisted extraction (MAE), ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) have been used for the extraction of bioactive compounds (Ardiles *et al*., 2020; Chavez-Gonzalez *et al*., 2020). These technologies eliminate or reduce the use of toxic solvents and extraction time; they enhance the extraction efficiency as well as the yield and quality of extract obtained (Fomo *et al*., 2020; Ummat *et al*., 2021). In a study by Fan and Gao (2022), the use of subcritical water was investigated in the extraction of bioactive compounds from licorice. Maximum contents of total flavonoids, phenolics, and glycyrrhizic acid were extracted at 140 °C for 20 min. Environmentally friendly solvents such as ionic liquids can be used to eliminate the use of the toxic traditional solvents (Fomo *et al*., 2020).

Moringa oleifera leaves have been reported to exhibit antioxidant activity both *in vitro* and *in vivo* due to the abundance of phenolic compounds and flavonoids (Rocchetti *et al*., 2020; Hassan *et al*., 2021). Phenolic compounds are also known to interact with other plant components which makes

extraction the most important step in sample pre-treatment. The extraction of the phenolic acids and flavonoids depends largely on the solvent's polarity, the method, and extraction time which determine the qualitative and quantitative composition of the compounds. The total phenolic compound that is determined from the same plant and its antioxidant activity may vary because of the extraction conditions applied (Osorio-Tobon *et al*., 2020; Saifullah *et al*., 2020; Alara *et al*., 2021). Rodriguez-Perez *et al.* (2015) studied ultrasound assisted extraction (UAE) and maceration and found that UAE produced an extract of *M. oleifera* leaves with the largest number of phenolic compounds. This could be because ultrasounds can disrupt the plant cell walls and thus increase solvent penetration which helps to obtain a higher extraction yield. In this study, high performance liquid chromatography-electrospray interface-quadrupole time-of-flight-mass spectrometry (HPLC–ESI–QTOF–MS) was used to determine that the *M. oleifera* leaves are a good source of bioactive compounds, such as flavonoids and phenolic acid derivatives. Table 2.2 gives a summary of the extraction of different metabolites from *M. oleifera* and their extraction methods and extraction conditions. This table highlights the effects that extraction methods as well as the conditions have on the metabolites extracted. For example, Hamany Djande *et al*. (2018) and Khoza *et al*. (2014) extracted metabolites from *M. oleifera* leaves using the PHWE method. At a temperature of 100 °C and a flow rate of 3 mL min^{-1}, Hamany Djande *et al*. (2018) extracted glucosinolates, chlorogenic acids, and flavonoids, whereas only flavonoids and chlorogenic acids were extracted when the temperature was increased to 150 °C in the study by Khoza *et al*. (2014). This goes to suggest the effect that extraction conditions have on extraction of metabolites.

Table 2.2: A summary of the different metabolites extracted from *M. oleifera* using different extraction methods and extraction conditions.

Extraction method	**Metabolites extracted**	**Extraction conditions**	***M. oleifera* tissue**	**Analysis method**	**Reference**
PHWE, ATPE, NADES	Glucosinolates Chlorogenic acids Flavonoids	NADES Solvent: choline chloride/citric acid (20 mL) ATPE 20% ammonium sulfate salt/ethanol PHWE Temperature: 100 ºC Pressure: 1000 ± 200 psi Flow rate: 3 mL s^{-1}	Leaves	UHPLC-qTOF-MS	Hamany-Djande *et al.* (2018)
UAE	Amino acids Nucleosides Organic acids Phenolic acids and derivatives	25 mL EtOH:H_2O (50:50, v/v) Time: 15 min Temperature: room temperature	Leaves	HPLC-ESI-QTOF-MS	Rodriguez-Perez *et al.* (2015)

	Glucosinolates Lignans Flavonoids				
MAE PLE	Amino acids Glucosinolates Organic acids Phenolic acids and derivatives Flavonoids	MAE Maximum power: 850 W Frequency: 2455 MHz PLE Time: 20 min Pressure: 10 MPa (1500 psi) Heat-up time: 5 min Static time: 5 min Flush volume: 60% Purge: N_2, 60 s No. of cycles: 1	Leaves	HPLC-ESI-QTOF-MS	Rodriguez-Perez *et al*. (2016)
MeOH	Alkaloids Saponins Phenols	800 mL MeOH Extraction time: 72 h	Seeds	Bacterial analysis	Akintelu *et al*. (2021)

	Flavonoids				
MeOH	Terpenoids Phenols Alkaloids Saponins Glycosides	600 mL MeOH Extraction time: 72 h	Leaves	GC-MS FTIR	Okechukwu *et al*. (2021)
PHWE	Chlorogenic acids Flavonoids	Temperature: 50 and 150 ºC Pressure: 1000 ± 200 psi Flow rate: 2 mL min^{-1}	Leaves	UPLC-qTOF-MS	Khoza *et al*. (2014)

2.4. Properties of flavonoids

Flavonoids are naturally occurring polyphenols that accumulate in the different parts of plants, more particularly in fruits and vegetables (Kopustinskiene *et al*., 2020; Shen *et al*., 2022). Flavonoids are divided into several subgroups which have different biological and chemical properties (Prabhu *et al*., 2021; Koop *et al*., 2022). The carbon atoms in the flavonoid molecules are organized into two benzene rings, denoted as A and B, and are connected by an oxygen containing pyrene ring (Figure 2.1) (Brodowska, 2017; Iriti *et al*., 2017; Dias *et al*., 2021).

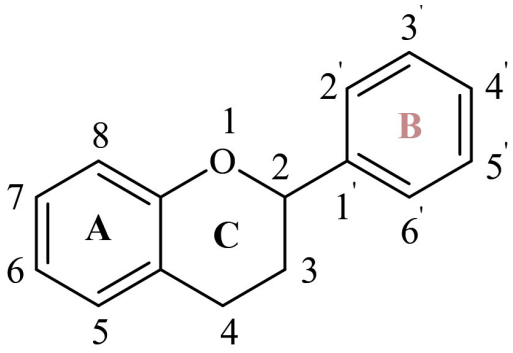

Figure 2.1: Basic skeleton structure of a flavonoid wherein the aromatic rings A and B are connected by an oxygen-containing pyran ring.

The subdivision of flavonoids into different subgroups depends on the C ring on which the B ring is attached and the degree of unsaturation and oxidation of the C ring. Flavonoids in which the B ring is linked in position 3 of the C ring are called isoflavones. Those in which the B ring is linked in position 4 are called neoflavonoids while those in which the B ring is linked in position 2 can be subdivided into several subgroups based on the structural features of the C ring. These sub-groups include flavones, flavanols, flavanones, flavonols, flavanonols, isoflavones and anthocyanins (Ekalu and Habila, 2020; Addi *et al*., 2022). The structures of the subgroups of flavonoids are represented in Figure 2.2. Flavonoids with open C ring are called chalcones. Flavonols are represented by quercetin and kaempferol, among others, and are one of the most analysed sub-groups of flavonoids owing to their antioxidant properties and other biological properties (Xiao, 2017). Quercetin is the major representative of this sub-group, and it is a powerful antioxidant (Brodowska, 2017; Iriti *et al*., 2017).

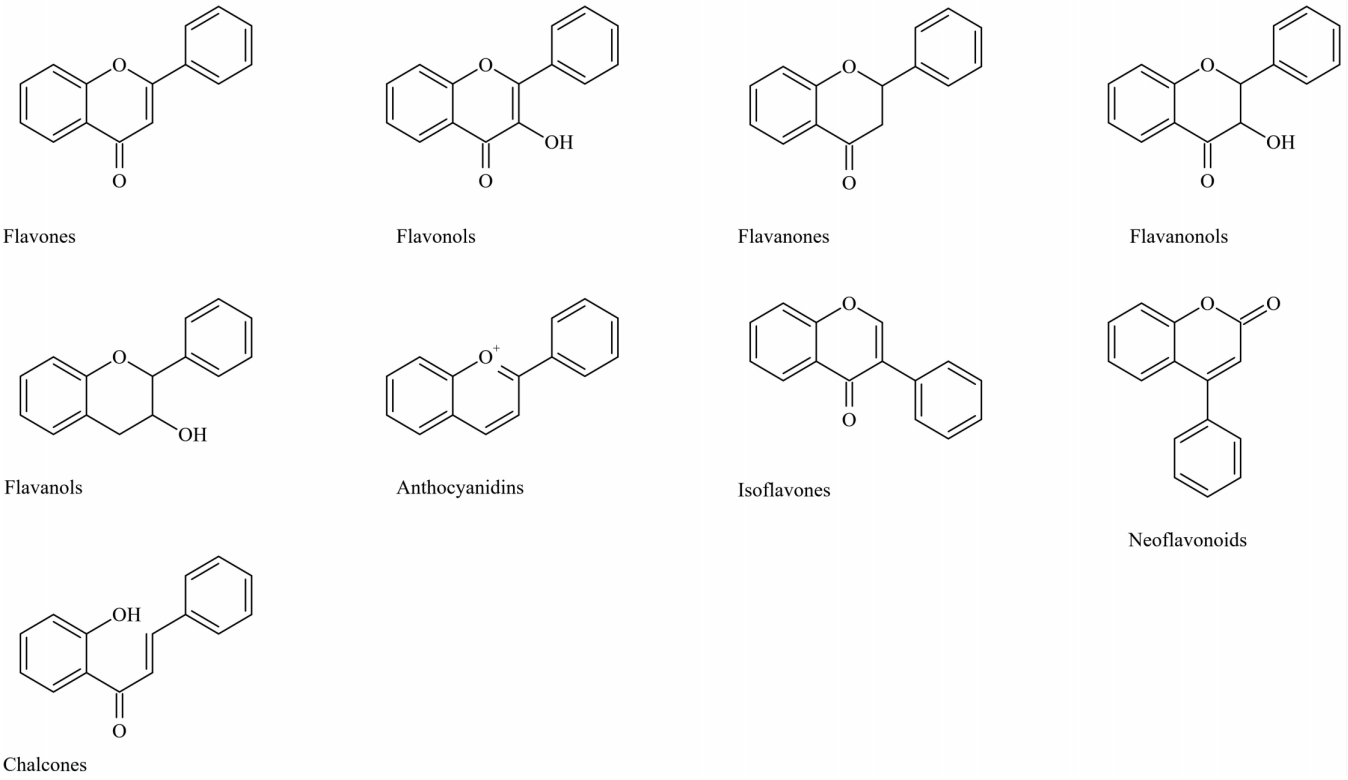

Figure 2.2: An illustration of the sub-groups of flavonoids which arises due to the differences in the structures.

Structurally, flavonoids are polyphenolic compounds with a nuclear structure base of C6-C3-C6. They exist as either free aglycones or glycosylated with different sugar moieties. The sugar attachment on the flavonoid aglycone moiety can be at different positions (Madala *et al*., 2016; Iriti *et al*., 2017; Addi *et al*., 2022; Song *et al*., 2022). The chemical diversity of flavonoids in *M. oleifera* make it an interesting plant. The reason for this being that the flavonoids that are found in *M. oleifera* undergo a type of glycosylation which is not found in any other plant species (Rodriguez-Perez *et al*., 2015; Makita *et al*., 2016). This plant diversifies its flavonoids through glycosylation patterns. Upon comparison with *M. ovalifolia*, *M. oleifera* produces similar aglycone flavonoids such as quercetin, kaempferol and isorhamnetin. Differences arise in the glycosides attached to the aglycones. *Moringa oleifera* attaches different types of sugars to its flavonoid aglycones. The attachment of different groups on the sugar moiety of the flavonoid changes the polarity as well as the bioavailability. The process of glycosylation in *M. oleifera* indicates that

this plant can be a useful source of bioavailable flavonoid compounds which can be used in physiological environments at different polarities (Tshabalala *et al*., 2019).

The four types of flavonoids that are mainly found in *M. oleifera* include quercetin, kaempferol, isorhamnetin, and apigenin and they exist abundantly as glycosides attached to sugar moieties such as acetyl dihexose, hexose, and rutinoside (Matshediso *et al*., 2015; Lin *et al*., 2018). Figure 2.3 represents the common flavonoids found in *M. oleifera*. The multiple hydroxyl groups in the flavonoid skeleton are targets for glycosylation with sugars such as glucose, mannose, or galactose, and their 6-deoxyderivatives (fucose and rhamnose) and pentoses (arabinose, apiose, and xylose). They accumulate in the form of mono-, di-, or triglycosides (Alseekh *et al*., 2020; Ushasree and Lee, 2020). Natural dietary flavonoids accumulate in the stems, leaves, flowers, and fruits of plants and they exist as glycosides, such as glucoside, galactoside, rhamnoside, arabinoside, and rutinoside. Flavone *O/C*-glycosides and flavonol *O*-glycosides are the most abundant flavonoid glycosides in plants (Xiao, 2017; Ushasree and Lee, 2020). These flavonoid glycosides exist mainly as their 3 or 7 *O*-glycosides, although 5, 8, and 4 *O*-glycosides have also been reported. Fruits such as apples and berries contain anthocyanidin, flavonol, and flavone *O*-glycosides that exist in the *C*-3 position (Xiao, 2017). Flavonols such as kaempferol, quercetin, and isorhamnetin are an important class of bioactive flavonoids, which are most abundant as their 3-*O*-glycosides (i.e. glucoside, glucuronoside, galactoside, and rutinoside). The glycosylation of flavonols enhances their water solubility such that high concentrations of flavonols can accumulate in plant cells (Ono *et al*., 2010). Flavonoid glycosides are of interest owing to their pharmacokinetic properties such as antibacterial, estrogenic, endocrinological, anti-inflammatory, antiviral, and anticancer properties (Alseekh *et al*., 2020).

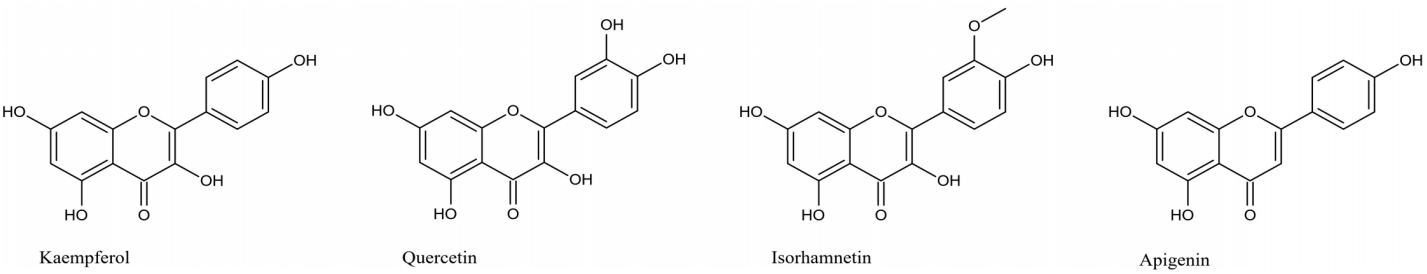

Figure 2.3: The types of flavonoids commonly found in *M. oleifera*.

2.4.1. Biological importance of flavonoids

Flavonoids have several health benefits in humans and a diet rich in these compounds helps prevent some chronic diseases. Most flavonoids exist as glycosides and the number, and the position of the sugar attachment affects the antioxidant properties of the flavonoid (Karak, 2019). Although flavonoids are recognized for their antioxidant activity, flavonoids are also described to have anti-inflammatory, anticancer, antidiabetic, cardioprotective, antimicrobial, neuroprotective, and antiviral properties (Wang *et al*., 2018; Juca *et al*., 2020; Rufino *et al*., 2021). The structural diversity of flavonoids contributes to differences in their ability to modulate specific molecular pathways. Differences in absorption, distribution, metabolism, and elimination after consumption modify their bioavailability, site of action, and formation of bioactive metabolites. Some flavonoids are well absorbed and distributed to tissues, and some have limited absorption. However, such flavonoids could still have systematic effects via interaction with the microbiota (Mozaffarian and Wu, 2018).

2.4.1.1. Antioxidant activity

Flavonoids act as antioxidants and are oxidized by radicals to form less reactive species via four mechanisms, i.e. (1) the inhibition of nitric-oxide synthase activity, (2) inhibition of xanthine oxidise activity, (3) modulation of channel pathways, or by (4) interacting with other enzyme systems (Ullah *et al*., 2020). The antioxidant capacity within the flavonoid classes depends on the functional group and its arrangement around the nuclear structure (Karak, 2019; Dias *et al*., 2021). The number and position of hydroxy groups in the B-ring and on the C-ring influence the free radical scavenging ability (Ullah *et al*., 2020; Dias *et al*., 2021). The functional hydroxy group of the structure can donate an electron and hydrogen to a radical through resonance, stabilize them, and originate a relatively stable flavonoid. The antioxidant action mechanisms of flavonoids can be by (a) direct scavenging of reactive oxygen species (ROS), (b) inhibition of ROS formation through the chelation of trace elements or inhibition of the enzymes that participate in the generation of free radicals and (c) activation of antioxidant defenses. Figure 2.4 represents the free radical scavenging mechanism of ROS by flavonoids. A combination of some of these mechanisms, for example, radical scavenging action with suppression of some enzyme functions, may also occur (Dias *et al*., 2021).

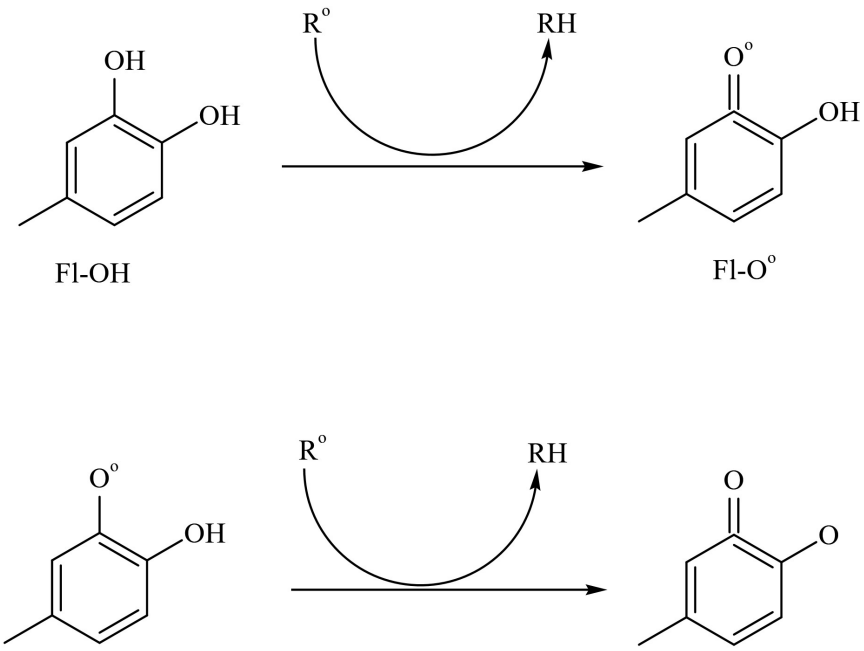

Figure 2.4: Free radical scavenging mechanism of ROS (R^o) by flavonoids (Fl-OH) whereby the hydroxy group on the flavonoid donates an electron and a hydrogen to a radical.

Besides the radical scavenging activity of flavonoids, another antioxidant mechanism may result from the interactions between transition metal ions, such as iron and copper, and flavonoids to produce metal complexes that prevent the participation of these metal ions in free radical generating reactions. Iron and copper play a major role in the production of the very reactive $HO^{\cdot}$. Chelates are formed through three metal-binding sites within a flavonoid molecule containing hydroxyl groups at 3, 5, 3', and 4' positions (Gomes *et al*., 2008; Kejik *et al*., 2021; Simunkova *et al*., 2021) (Figure 2.5).

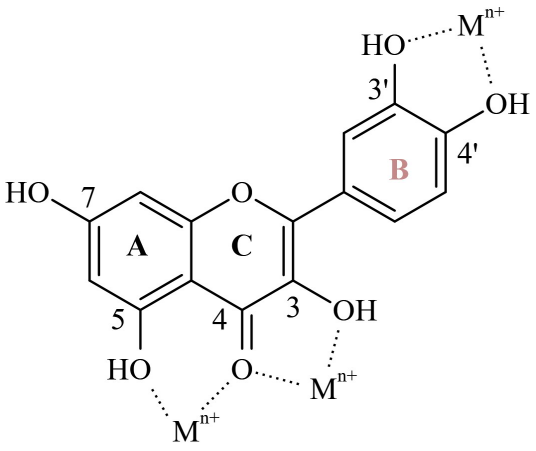

Figure 2.5: The possible binding sites for trace metal ions (M^{n+}) to flavonoids.

These sights are between the 3-OH group and the 4-oxo group, the 5-OH group and the 4-oxo group, and between the *o*-dihydroxyl groups in the B-ring. It was reported in literature that UV-Vis and ESI-MS showed that only flavones and flavonols (e.g. quercetin, myricetin, luteolin, kaempferol, rutin, and apigenin) and the flavon-3-ol catechin interacted with metal ions when chelation studies were performed with iron and copper ions at pH 7.4 and pH 5.5 (Gomes *et al*., 2008). Flavanones (taxifolin, naringenin, and naringin) and isoflavones (daidzein and genistein) do not chelate metal ions. This is because in flavones and flavonols, the presence of the 2,3-double bond increases the planarity of the molecule and confers higher rigidity to the ring and holds the A and C rings in a more coplanar position and thus allowing the 3-OH/4-oxo groups and the 5-OH/4-oxo groups to be closer. The metal chelating properties of flavonoids suggest that they may play a role in metal-overload diseases and in all oxidative stress conditions involving a transition metal ion (Gomes *et al*., 2008).

2.5. Quercetin rutinoside (Rutin)

Rutin is a flavonol glycoside that has been reported to present clinically relevant functions, beneficial in preventing diseases and protecting genome stability (Gullon *et al*., 2017). It is also called sophorin, rutoside, quercetin-3-rutinoside, and vitamin P. It is a polyphenolic bioflavonoid that is extracted from natural sources such as fruits and vegetables and plant derived beverages (Chua, 2013; Frutos *et al*., 2019; Kim and Lim, 2019). The basic structure of rutin is illustrated in Figure 2.6. Rutin is a glycoside with the flavonol aglycone quercetin attached to disaccharide

rutinose (Enogieru *et al*., 2018; Rahman *et al*., 2021). It was first reported in *Ruta graveolens* L., which gave its name to the compound (Frutos *et al*., 2019). Seventy plant species have been reported to contain rutin and Buckwheat is reported as the major source of natural rutin (Chua, 2013; Kim and Lim, 2019).

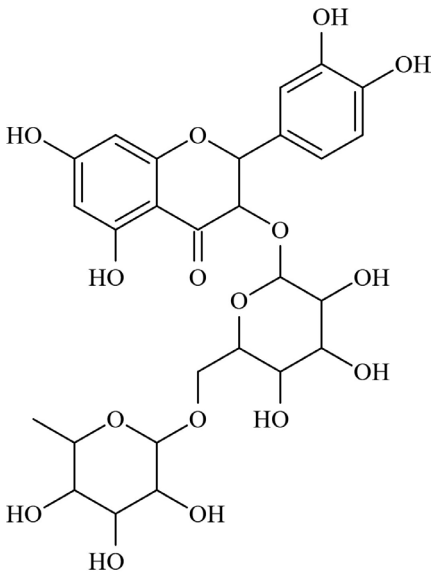

Figure 2.6: Structure of quercetin rutinoside (rutin).

Rutin is an attractive phytochemical because of its pharmacological properties and is thus an important flavonoid in the pharmaceutical industry (Chua, 2013; Peng *et al*., 2018; Tursynbolat *et al*., 2019). The flavonoid exhibits properties such as antioxidant, anti-inflammatory, antiallergic, cardiovascular, neuroprotective, antidiabetic, and anticancer activities (Enogieru *et al*., 2018; Peng *et al*., 2018; Kim and Lim, 2019; Tursynbolat *et al*., 2019; Rahman *et al*., 2021). More than 130 medicinal preparations in the market contain rutin; either alone or in combination with other active ingredients (Chua, 2013; Gullon *et al*., 2017; Molnar *et al*., 2018). The major challenge of rutin is the poor bioavailability caused by its low aqueous solubility, poor stability, and limited membrane permeability (Gullon *et al*., 2017). The phenolic part of the molecule is linked to a sugar and thus makes it hydrophilic and increases its solubility in water. The hydrolysis of this molecule catalyzed by glucosidase results in quercetin and rutinoside (Chua, 2013; Frutos *et al*., 2019). Rutin has

antioxidant activity which is associated with the presence of the hydroxyl groups bound to the aromatic rings (Frutos *et al*., 2019).

2.5.1. Extraction of rutin from natural products

Various extraction methods (conventional and innovative) have been reported for the extraction of rutin and other flavonoids from natural sources. An accurate comparison between the different procedures is not straightforward because of the difference in the plant materials (Liu *et al*., 2022; Chew *et al*., 2023). However, current trends on the discovery and design of green and sustainable extraction techniques to optimize the extraction of rutin are being investigated (Chua, 2013; Milescu *et al*., 2020). Rutin is generally extracted using organic solvents such as ethanol and methanol (Milescu *et al*., 2020). The problem with conventional extraction methods is that they require large quantities of organic solvents and long extraction times. They are also not suitable for use in food, biotechnological, and pharmaceutical industries due to the solvent toxicity. This can be limited by introducing green techniques such as infrared, ultrasonic, microwave, and supercritical fluids methods (Chavez *et al*., 2020; Zhang *et al*., 2020; Alara *et al*., 2021). The advantages of these methods are that they increase the extraction yield, they use small volumes of organic solvents, and they have short processing times and some of these methods have been used successfully in the food industry (Singla and Sit, 2021). However, as much as these methods are advantageous, the downfall is that scaling-up these methods can be difficult because of the costs of the equipments and installations (Frosi *et al*., 2021; Kumar *et al*., 2021; Ummat *et al*., 2021).

In a study by Chahyadi *et al*. (2020), extraction methods such as UAE and MAE were investigated for the extraction of rutin in cassava leaves. These extraction methods showed a higher yield for rutin when compared to the conventional extraction methods. Table 2.3 further summarizes the extraction of rutin from different plant sources using different extraction methods and their respective yields. From the results presented in this table, it can be deduced that factors such as temperature and extraction method have a huge impact on the amount of rutin extracted from natural products.

Table 2.3: Extraction of rutin from different plant sources using different extraction methods and their respective rutin yields.

Name of plant	Method of extraction	Method of detection	Extraction temperature	Yield of rutin	Reference
Satureja montana L.	DES (choline chloride-based)	HPLC-DAD	70 ºC	1.40 – 17.29 µg mg^{-1}	Jakovljevic *et al*. (2020)
Saphora japonica bud	DES (choline chloride/triethlene glycol)	Spectrometer	70 ºC	279.8 mg g^{-1}	Peng *et al*. (2018)
Buckwheat	Subcritical water extraction	HPLC	120 ºC	91.0%	Kim and Lim (2019)
Lemon by-products	Aqueous UAE	HPLC-PDA	48 ºC	3.20 ± 0.12 mg g^{-1}	Papoutsis *et al*. (2018)
Morinda citrifolia L.	High hydrostatic pressure extraction (HHPE)	HPLC	RT	82.2%	Jamaludin *et al*. (2020)
Solanum lycopersicum L.	MeOH	HPLC	RT	95 ± 1%	Junker-Frohn *et al*. (2019)
Ilex paraguariensis	Solid-liquid extraction	HPLC	80 ºC	48.31 mg L^{-1}	Gerke *et al*. (2018)

Citrus sinensis L.	Solid-liquid extraction	LC-MS/MS	90 ºC	4.70 mg g^{-1}	Gomez-Mejia *et al*. (2019)
Citrus lemon L.			90 ºC	3.30 mg g^{-1}	
Citrus clementina			90 ºC	3.88 mg g^{-1}	
Lycium babarum L.	DES-UAE	HPLC	25 ºC	9.1 mg g^{-1}	Ali *et al*. (2019)
Saphora japonica (in grape juice)	MGO/MHNTs@MIPs	HPLC	RT	132 mg g^{-1}	Wang *et al*. (2022)
Orange juice	Pt@r-GO@MWCNTs)	Electrochemical cyclic voltammetry (CV)	25 ºC	108.46%	Tursynbolat *et al*. (2019)
Physalis angulate L.	UAE	HPLC-DAD	30 ºC	75.6 – 88.2 µg g^{-1}	Moreira and Dias (2018)
Ruta graveolens L.	DES (choline chloride/citric acid)	HPLC	70 ºC	1.88 g/100 g	Molnar *et al*. (2018)

RT = Room temperature

2.6. Modern methods of extraction

2.6.1. Aqueous two-phase extraction (ATPE)

Aqueous two-phase extraction (ATPE) is a powerful method that is popular for the separation and purification of biological materials such as proteins, enzymes, polysaccharides, phenolic compounds, lipids, and carotenoids via a single-step procedure (Ji *et al*., 2017; Khoo *et al*., 2020; Jiang *et al*., 2021; Wang *et al*., 2021). It is composed of two different immiscible liquids that are separated by the interface layer (Phong *et al*., 2018; Jiang *et al*., 2021). ATPE can be formed using polymer-polymer (Chairez-Cantu *et al*., 2023), polymer-inorganic salt (Gonzalez-Amado *et al*., 2021), low molecular weight alcohol-inorganic salt (Caldeira *et al*., 2022), or ionic liquid-inorganic salt aqueous solutions (Zheng *et al*., 2022). The phase-forming components produce physiochemical interactions which distribute the target compound to either the top or bottom phase; depending on the selectivity (Figure 2.7) (Khoo *et al*., 2020; Jiang *et al*., 2021). Factors that contribute to the distribution of the target compound are: (i) the characteristics of the system such as the type and concentration of the liquid and (ii) the characteristics of the target compound such as the charge, size, and hydrophobicity (Jiang *et al*., 2021). ATPE systems have been extremely used for the isolation/extraction of biomolecules owing to their attractive advantages such as high yield, ease of separation, mild conditions, environmental friendliness, easy scale-up, rapidness, and low cost (Chikari *et al*., 2019; Tan *et al*., 2022). It is also regarded as an efficient technique for the recovery and purification of biomolecules (Chikari *et al*., 2019; Khoo *et al*., 2020). ATPE is favourable over traditional organic solvents extraction because of its phase-forming components which contain a large amount of water while maintaining a low interfacial layer that separates both phases (Khoo *et al*., 2020).

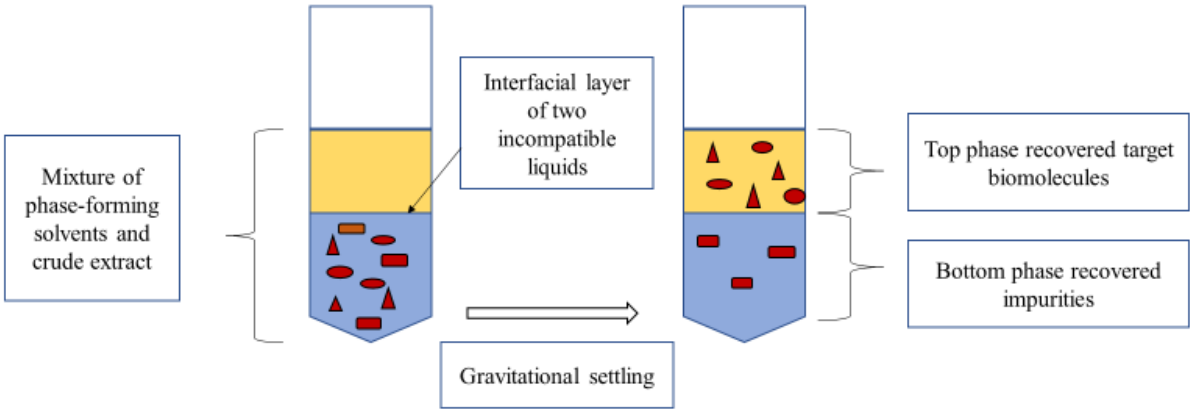

Figure 2.7: Schematic diagram of the principle in aqueous two-phase extraction (ATPE) system (Khoo *et al*., 2020).

The combined method of UAE and ATPE, which is known as ultrasound assisted aqueous two-phase extraction (UA-ATPE), has been used to extract some effective components from herbal materials. In this method, the extraction and purification are achieved in one step (Tan *et al*., 2022). UA-ATPE possesses a variety of advantages such as low viscosity and quick phase separation of the system, fast mass transfer, high extraction efficiency, and gentle biocompatible environment (Luo *et al*., 2018; Zhou *et al*., 2021). This technique has been widely used for the green extraction of natural products (Yuan *et al*., 2022; Liu *et al*., 2023; Yue *et al*., 2024). UA-ATPE has been acknowledged to improve polysaccharides yield. The synergistic effect of UA-ATPE through ultrasonic cell wall disintegration and diffusion of the solvent into the cell wall has a positive impact on the polysaccharide extract, thus significantly increasing mass transfers of the active nutrients. However, this technique degrades polysaccharides due to prolonged ultrasonic treatment (Chikari *et al*., 2019). UA-ATPE is mainly affected by parameters such as the ATPE composition, ultrasonic power and frequency, ultrasonic time, ultrasonic temperature, particle size, and solvent to material ratio. It is therefore important to optimize the extraction process to obtain target flavonoids with high yields (Zhang *et al*., 2018; Zhou *et al*., 2021).

A study by Wang *et al*. (2020) focused on the extraction of flavonoids from *Moringa* leaves using an ATPE system. The single factors they selected to optimize were the solid-liquid ratio, ethanol

concentration, ultrasonic extraction time, and ultrasonic extraction temperature. The extraction process was optimized through single factor experiments and orthogonal experiments. The optimal process observed was: 1:110 of solid-liquid ratio, 45% concentration of ethanol, 15 minutes ultrasonic extraction time, and 70 ºC ultrasonic extraction temperature. Under these conditions, the extraction of *Moringa* leaf flavonoids reached 8.37%. Hamany Djande *et al*. (2018) reported on the use of an ammonium sulphate and ethanol ATPE system for the extraction of metabolites from *M. oleifera* leaf powder. The conditions used were: 20% ammonium sulphate in an ultrasonic bath at 50 ºC for 1 h. They found that at these conditions the metabolites extracted by this process were chlorogenic acids and flavonoids. Glucosinolates were not detected in the extracts from using ATPE. Table 2.4 continues to give a summary of the extraction of rutin from different plant sources using ATPE, the conditions of ATPE, and their rutin yields. It is noted that different ATPE conditions have an effect on the amount of rutin extracted as was observed by Chong *et al*. (2020a) and Chong *et al*. (2020b).

Table 2.4: Extraction of rutin from different plant species using ATPE and the yield of rutin obtained.

Plant species	ATPE conditions	Optimum extraction conditions	Method of detection	Yield of rutin	Reference
Lonicera caerulea	Ammonium sulphate/ethanol	High TLL, 87.5 min, 0.19 wt% leaves	HPLC NMR	~2.5 µg rutin mg^{-1} leaves	Chong *et al*. (2020a)
	Sodium dihydrogen phosphate/ethanol	Low TLL, 5 min, 0.5 wt% leaves		~1.6 µg rutin mg^{-1} leaves	
	Glucose/1-propanol	High TLL, 5 min, 0.1 wt% leaves		11.52 µg rutin mg^{-1} leaves	
	Maltose/1-propanol	Medium TLL, 120 min, 0.427 wt% leaves		~1.9 µg rutin mg^{-1} leaves	
Lonicera caerulea	Ammonium sulfate/ethanol	5 mg leaves, 120 min flotation time, 28.6 mL/min air flow rate	HPLC UV-Vis	0.027 mg rutin mg^{-1} leaves	Chong *et al*. (2020b)
	Sodium phosphate/ethanol	5 mg leaves, 120 min flotation time, 11.4 mL/min air flow rate		0.016 mg rutin mg^{-1} leaves	

Cyclocarya paliurus	Ammonium sulphate/ethanol	33% EtOH concentration, 15% salt concentration, 52 min, 33:1 liquid-solid ratio	UPLC-TQ-MS	3.045 µg mL^{-1}	Yang *et al*. (2020)
Ribes nigrum L.	13.72% ammonium sulphate/31.40% ethanol	70:1 mg/L (*v/w*) liquid-to-material ratio, 65 ºC extraction temperature, 10 min extraction time, 600 W microwave power	HPLC-MS/ESI	-	Zhao *et al*. (2021)
Ziziphus Jujuba Mill.	35% (w/w) K_2HPO_4/20% (w/w) ethanol	Solid:liquid ratio 1:30 g/mL (w/v), ultrasonic power 200 W, extraction time 50 min	UPLC-MS/MS	0.28 mg g^{-1}	Zhu *et al*. (2022)

"-" = Data not available

2.6.2. Miniaturized extraction techniques

Micro-extraction techniques are environmentally friendly because they eliminate the use of a large volume of organic solvent and thus minimizing the generation of waste. These techniques make use of a small volume of the extracting phase relative to the volume of the sample and thus implying high enrichment factors (Oliveira *et al*., 2021; Elia *et al*., 2024). Miniaturization techniques are additionally advantageous because they are easy to operate, are miniaturized, cost effective, and are adaptable to a wide range of samples and analytes (Moreda-piñeiro and Moreda-piñeiro, 2015; Fusari *et al*., 2019; Gutierrez-Serpa *et al*., 2021). Miniaturized techniques are of advantage because they provide a straightforward procedure, faster analysis, higher extraction performance, and reduced amount of sample required. These techniques also use recent advances in the synthesis of new sorbent materials and the use of greener extraction solvents (da Silva Burato *et al*., 2019; Agrawal *et al*., 2021). Micro-extraction techniques can be divided into three broad groups depending on the type of the extracting phase, i.e. solid-phase micro-extraction (SPME) (Jalili *et al*., 2020a), liquid-phase micro-extraction (LPME) (Chormey *et al*., 2020) and membrane-based micro-extraction (Shishov *et al*., 2020).

2.6.2.1. Solid phase micro-extraction (SPME)

Solid phase micro-extraction (SPME) is a modern, non-exhaustive sample preparation technique, which integrates sampling, pre-concentration, and extraction in a single step, and the analytes can be directly introduced to analytical instruments like chromatographic systems (Jalili *et al*., 2020a; Feng *et al*., 2021). This technique is advantageous because it is simple, rapid, has improved sample clean-up, accurate analysis, high pre-concentration factor, and low organic solvent consumption or solvent-free (Jalili *et al*., 2020b; Feng *et al*., 2021; Zhou *et al*., 2023). SPME is used in fields ranging from agriculture to medicine (Huang *et al*., 2019; Jagirani and Soylak, 2020; Khan *et al*., 2020). SPME can be used as an efficient and sensitive method for the extraction and pre-concentration of a wide range of analytes (Rahimi *et al*., 2019). SPME can be performed in three modes, viz headspace, direct immersion, and membrane protected. Figure 2.8 illustrates the different modes of SPME and Table 2.5 gives a summary of the application of different SPME techniques for the extraction of flavonoids. Different sorbent materials were used as reported in

the table. The number of metabolites extracted by each sorbent material highlights the specificity and selectivity of the particular sorbent.

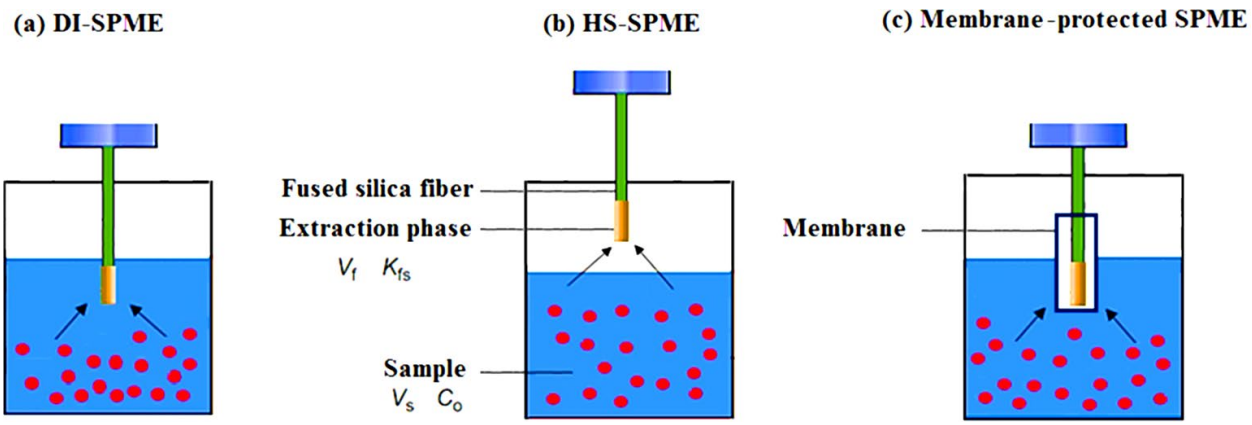

Figure 2.8: Illustration of the different modes of SPME: (a) DI-SPME, (b) HS-SPME, and (c) membrane-protected SPME (Jalili *et al*., 2020a).

In direct immersion SPME (DI-SPME), the fiber coated with an adsorbent is inserted into the sample matrix and the analytes are transmitted directly from the sample to the extracting phase as shown in Figure 2.8(a) (Jalili *et al*., 2020a). The fiber coating for this technique should have matrix compatibility, robustness, and good affinity towards the analytes of interest (Zhang *et al*., 2018). DI-SPME is preferred for the extraction of analytes for both targeted and untargeted studies owing to its rapid and sensitive analysis abilities. A disadvantage of this technique is that it can coextract undesirable species and matrix components. The presence of the undesirable compounds can reduce the extraction efficiency or introduce matrix effects in the electrospray ionization source of the mass spectrometric detection system (Alam and Pawliszyn, 2018). This disadvantage has, however, been eliminated using the highly specific molecularly imprinted polymers (MIPs) (Arabi *et al*., 2020).

Headspace SPME (HS-SPME) is preferred for volatile compounds and complex samples such as biological samples (Jalili *et al*., 2020a; Shokrollahi *et al*., 2020). In HS-SPME, a fused-silica fiber coated with an adsorbent is exposed in the headspace above the sample. Volatile or semi-volatile compounds are distributed among a three-phase system, i.e. the sample, headspace, and sorbent as

shown in Figure 2.8(b) (Mascrez *et al*., 2020; Asadi and Maddah, 2022). The volatile and semi-volatile compounds that equilibrate the sample and the headspace are trapped by the fiber coating. HS-SPME is advantageous because it leads to purer extracts and greater selectivity and the lifespan of the sorbent is increased because the sample matrix is not in direct contact with the coating (Zhu *et al*., 2021). The fiber in the headspace over the sample is not contaminated by non-volatile compounds, which extends its lifespan, increases the extraction selectivity, and improves reliability of GC analysis (Zhakupbekova *et al*., 2019).

However, DI-SPME and HS-SPME are limited to non-volatile and high-molecular-interfering compounds such as proteins and fatty acids. Therefore, a membrane protected SPME can be used for sampling biological fluids, soil samples, and food samples (Jalili *et al*., 2020a). Membrane protected SPME uses a very small amount of solid that is packed inside a membrane bag (Figure 2.8(c)). The extraction material is protected inside the membrane bag, and it is suitable for extraction from complex liquid samples because matrix components and macromolecules cannot penetrate the membrane bag enclosed sorbent. The membrane bag can be dipped into a suitable solvent, with the provision of sonication, to desorb the analytes. Membrane protected SPME is advantageous because of its ease of fabrication, low cost, simplicity of operation, and protection of the sorbent against complex matrix. A major drawback of this technique is its weak enrichment capacity, especially when low water sample volumes are to be extracted.

Table 2.5: Application of different SPME techniques in the extraction of flavonoids.

SPME technique	Sorbent	Flavonoids extracted	Method of analysis	LOD	LOQ	Recovery (%)	Reference
SPME	MIPS	Quercetin	HPLC	9.94 ng mL^{-1}	33.1 ng mL^{-1}	94.92-98.50	Rahimi *et al*. (2019)
HS-SPME	DVB/CAR/PDMS	Quercetin Quercetin glycosides Isorhamnetin glycosides	HPLC-DAD	-	-	-	Cecchi *et al*. (2020)
HS-SPME		Kaempferol-*O*-glycosides Quercetin-*O*-glycosides	UPLC-ESI–qTOF-MS	-	-	-	Mohsen *et al*. (2020)
M-D-µSPE	MANPs	Quercetin Morin Kaempferol	HPLC	0.2-1.1 µg L^{-1}	0.66-3.63 µg L^{-1}	91	Majidi and Hadjmohammadi (2021)
MSPD micro-extraction	C_{18}	Narirutin Naringin Hesperidin Neohesperidin	IM-QTOF-MS	3.70-6.52 ng mL^{-1}	-	96.78-104.67	Zheng *et al*. (2021)

"-" = Data not available

2.6.2.2. Liquid phase micro-extraction (LPME)

Liquid-phase micro-extraction (LPME) is an important research and development tool that is available in analytical, forensic, pharmaceutical, food, clinical, and industrial analysis laboratories for the extraction, purification, and concentration of trace amounts of chemicals present in aqueous, solid, gaseous, and oil-based samples (Kokosa, 2019). This technique has been developed to overcome the drawbacks of conventional liquid-liquid extraction (LLE) such as the use of toxic organic solvents, large amounts of solvents and samples, the extraction time, and their tedious operations; and thus, it is considered a green technique (Aguirre *et al*., 2018; Kokosa, 2019; Santos *et al*., 2022). Pre-concentration is a major advantage of LPME. Other advantages of this technique include simplicity, low cost, low energy consumption, negligible carry-over, high enrichment factor, and reduced waste generation (Aguirre *et al*., 2018; Carasek *et al*., 2018; Hansen *et al*., 2020; Pacheco-Fernandez *et al*., 2020). In the most basic form of LPME, a drop of acceptor phase is held at the top of a microsyringe needle and is either immersed directly into the sample or suspended over its surface for headspace extraction (Rutkowska *et al*., 2019). LPME can be divided into three groups: viz. (a) single-drop micro-extraction (SDME) (Liu and Dasgupta, 1996; Kailasa *et al*., 2021), (b) hollow fiber liquid-phase micro-extraction (HF-LPME) (Shen and Lee, 2002; Moret *et al*., 2023), and (c) dispersive liquid-liquid micro-extraction (DLLME) (Garcia-Lopez *et al*., 2007; Salim *et al*., 2021). The main difference is in how the solvent comes in contact with the aqueous phase. In Table 2.6, the extraction of various flavonoids using different LPME techniques is summarized. The LODs and LOQs obtained from each detection are also indicated. These values (LODs and LOQs) were different for each method and they also give insight into the sensitivity of the detection methods.

SDME is the oldest LPME technique and is based on the use of a drop of acceptor phase. SDME is a facile and rapid miniaturized sample preparation technique for the selective extraction and pre-concentration of target molecules from complex samples. The target analytes are isolated from the sample solution by optimizing conditions such as temperature, pH, solvent, time, and stirring rate (Kailasa *et al*., 2021). In this technique, the drop may be immersed directly into the sample solution in direct-immersion single-drop micro-extraction (DI-SDME) or suspended over the sample in headspace single-drop micro-extraction (HS-SDME) (Figure 2.9). After extraction, the drop is

withdrawn and injected into an analytical instrument (Mafra *et al*., 2019; Neri *et al*., 2019; Abolghasemi *et al*., 2020). Unlike DI-SDME, HS-SDME avoids interferences from the sample matrix and has been used in the extraction of volatile compounds (Tang *et al*., 2018). In DI-SDME, a solvent drop that is suspended from the tip of a syringe needle is directly immersed into an aqueous phase. This technique combines extraction, pre-concentration, and sample introduction in one step. The analyte is isolated from the aqueous phase to the organic drop and therefore this technique is considered a two-phase SDME (Tegladza *et al*., 2020; Kailasa *et al*., 2021). Major advantages of this technique are its simplicity and cost effectiveness (Tegladza *et al*., 2020). A major disadvantage is the instability of the drop under high stirring rates, elevated temperatures, and long extraction times (Mafra *et al*., 2019). In HS-SDME, volatile compounds are extracted from the sample headspace without contact between the solvent droplet and the sample. Matrix interferences are eliminated in this technique. The droplet is usually analysed by gas chromatography (GC) or liquid chromatography (LC) (Tang *et al*., 2019; Qi *et al*., 2020). This method is fast, simple, inexpensive, and requires only microliters of solvents. The choice of solvent is also an important factor. HS-SDME suffers from volatility and lack of drop stability (Mehravar *et al*., 2020; Triaux *et al*., 2020). SDME is advantageous because it is simple, efficient, cost effective, and no special equipment is needed to create the drop.

In a study by Li *et al*. (2019), methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and isopropyl methanesulfonate (IPMS) were tested in active pharmaceutical ingredients (APIs) using HS-SDME with room temperature ionic liquid (RTIL) as the extractant was employed to preconcentrate the analytes and eliminate the drug matrix simultaneously. Sodium iodide (NaI) was used to derivatize the methanesulfonates to the corresponding iodoalkanes. *N, N*-diethyldithiocarbamate (DDTC) was then used to derivate the iodoalkanes in the extract after HS-SDME. The derivatized iodoalkanes were separated and detected with high-performance liquid chromatography with ultraviolet detection (HPLC-UV).

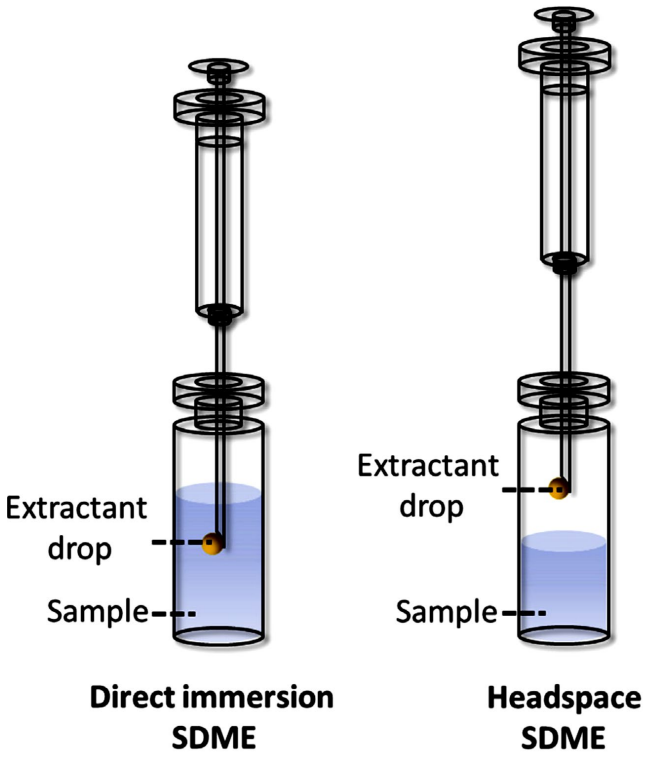

Figure 2.9: Illustration of the modes of SDME where the drop is immersed in the sample in DI-SDME, or the drop is suspended over the sample in HS-SDME (Tegladza *et al*., 2020).

HF-LPME makes use of disposable propylene porous hollow fibers that are filled with a small amount of extracting solution, called the acceptor phase, immersed in an aqueous solution, known as the donor phase. In this method, the organic phase is immobilized and sustained in the pores of a porous hollow fiber (Esrafili *et al*., 2018). The extractive phase is protected by the porous hollow fiber that is attached to the needle of the microsyringe, which is used to fill its lumen with an extractant phase and to maintain it during the extraction. To eliminate contamination, a new piece of hollow fiber is used in each analysis (Salvatierra-Stamp *et al*., 2018). This method is divided into two major types, i.e. two-phase and three-phase HF-LPME. Figure 2.10 illustrates the two types of HF-LPME. The three-phase method is described as an aqueous-organic-aqueous system in which the immobilized organic solvent or supported liquid membrane (SLM) is exposed to two aqueous phases of sample solution and the aqueous acceptor phase located inside the hollow fiber. In the two-phase HF-LPME, on the other hand, the acceptor phase solution is the same as the organic solvent of SLM and both are immiscible in water. Two-phase HF-LPME can extract

uncharged hydrophobic analytes while in the three-phase mode, the pH gradient between the sample solution and the aqueous phase can be used to tune the extraction conditions and it provides better cleanup than the two-phase mode (Esrafili *et al*., 2018). The benefits of HF-LPME are the use of microliters of solvent, highly selective, cost effectiveness, efficient cleaning, and high pre-concentration factor (Rutkowska *et al*., 2019; Venson *et al*., 2019). The disadvantages of this technique, however, are the ability to absorb hydrophobic substances (typically found in biological samples) or the formation of air bubbles on the surface of the fibers during extraction, which can affect the reproducibility of the results (Rutkowska *et al*., 2019).

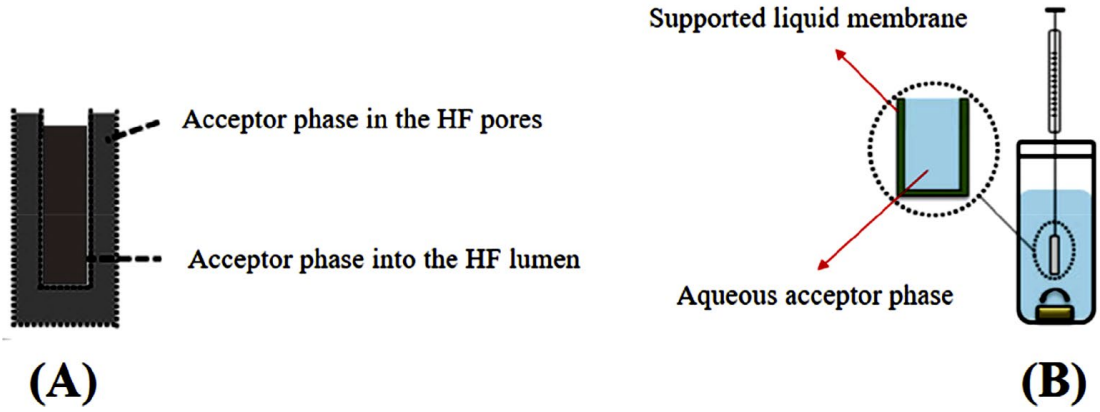

Figure 2.10: A schematic diagram illustrating (A) the two-phase HF-LPME, and (B) three-phase HF-LPME (Esrafili *et al*., 2018).

DLLME is established through the formation of tiny droplets and organic extracting solvent into the sample using a disperser solvent that is fully miscible in both the aqueous and organic phases. Figure 2.11 illustrates the DLLME procedure. The high contact surface of the sample and the extracting phase causes the rapid and efficient extraction of the target analytes (Yamini *et al*., 2019). Three phases are used in this technique: the extraction solvent, the dispersive solvent, and the aqueous phase. The analyte is in the aqueous phase (Mousavi *et al*., 2018; Musarurwa and Tavengwa, 2021). DLLME is simple, inexpensive, offers high pre-concentration factors, requires minimal sample, and offers high enrichment across a variety of donor and acceptor phases. The selection of extraction conditions and the choice of dispersive solvents for the extraction of

analytes are important parameters of this technique (Rykowska *et al*., 2018; Li and Row, 2019). However, the main disadvantages of this technique are the centrifugation of the dispersed solvent for phase separation and the extractant solvent which is usually a highly toxic halogenated solvent (Yamini *et al*., 2019; Zahiri *et al*., 2020). These drawbacks are overcome by using the solidification of organic drop-DLLME (SFO-DLLME) wherein a few microlitres of an organic solvent that is lighter than water is used as an extraction solvent (Zahiri *et al*., 2020).

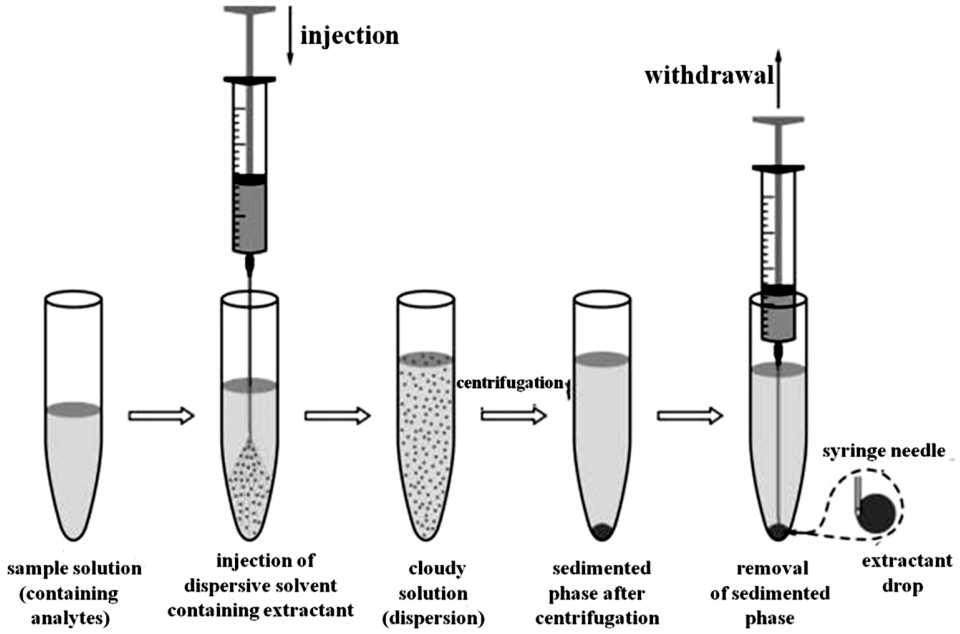

Figure 2.11: Schematic diagram illustrating the dispersive liquid-liquid micro-extraction (DLLME) procedure (Quigley *et al*., 2016).

Table 2.6: Extraction of flavonoids using different LPME techniques.

Type of technique	Flavonoids extracted	Method of detection	LOD	LOQ	Recoveries (%)	References
PCSS-DLPME	Myricetin Quercetin Isorhamnetin Baicalein Wogonin Kaempferide	HPLC/UV-vis	0.15-0.5 ng mL^{-1}	0.45-1.5 ng mL^{-1}	87.6-104.4	Tie *et al*. (2021)
SBME-DES	Quercetin Morin	HPLC/UV-vis	0.2-2.6 ng mL^{-1}	0.6-8.8 ng mL^{-1}	90.3-94.4	Nia and Hadjmohammadi (2019)
Amine-based LPME	Quercetin	UV-vis	0.07 µg mL^{-1}	0.24 µg mL^{-1}	97-107	Soylak *et al*. (2020)
HF-LPME	Morin Naringenin Quercetin Luteolin	HPLC/UV	0.5-7 ng mL^{-1}	-	40-85	Hadjmohmmadi *et al*. (2013)

	Kaempferol Apigenin					
VA-DLLME	Eriocitrin Rutin Hesperidin Diosmin Narirutin	UPLC-TOF/MS	0.70-0.81 ng mL^{-1}	2.01-2.44 ng mL^{-1}	88.5-102	Xue *et al*. (2019)
DLLME	Apigenin Chrysin Galangin Hesperitin Kaempferol Luteolin Myricetin Pinobanksin Pinocembrin Quercetin	HPLC/UV	1.4-6.9 ng mL^{-1}	4.7-23.0 ng mL^{-1}	57.4-104.9	Campone *et al*. (2014)

DSA-HST-LPME	Quercetin Isorhamnetin Chrysin Kaempferide	HPLC/UV	2.5-150 ng mL^{-1}	4.0-200 ng mL^{-1}	90.3-111.2	Chen *et al*. (2019)
DLLME	Hesperitin Fistenin Naringenin Chrysin Myricetin Quercetin Kaempferol	LC-DAD	0.4-4.1 ng mL^{-1}	-	80-111	Campillo *et al*. (2015)

'-' = Data not available

2.6.2.3. Pipette tip micro solid phase extraction (PT-µSPE)

Pipette tip micro solid phase extraction (PT-µSPE) is a new type of miniaturized SPE that is simple, potable, and a rapid sample pre-treatment method for proteins, peptides, and drugs (Hashemi *et al*., 2019; Sun *et al*., 2019). In this technique, a microscale amount of a sorbent is packed inside a pipette tip with the aim of reducing volume of solvents and samples (Amini *et al*., 2020). The chosen sorbent is placed between two filters made of degreasing cotton or frits. The extraction process begins by drawing the sample solution into the tip and then dispensing it back into the sample tube. Figure 2.12 illustrates the extraction procedure for PT-µSPE. These two steps are defined as one aspirating/dispensing cycle. By replicating the aspirating/dispensing cycles, the extraction procedure reaches equilibrium, and this makes the procedure controllable, stable, and smooth (Seidi *et al*., 2019; Sun *et al*., 2019; Tsai *et al*., 2021). An appropriate solvent is then used to desorb the analytes. To achieve quantitative desorption, this step may be performed in several cycles (Seidi *et al*., 2019).

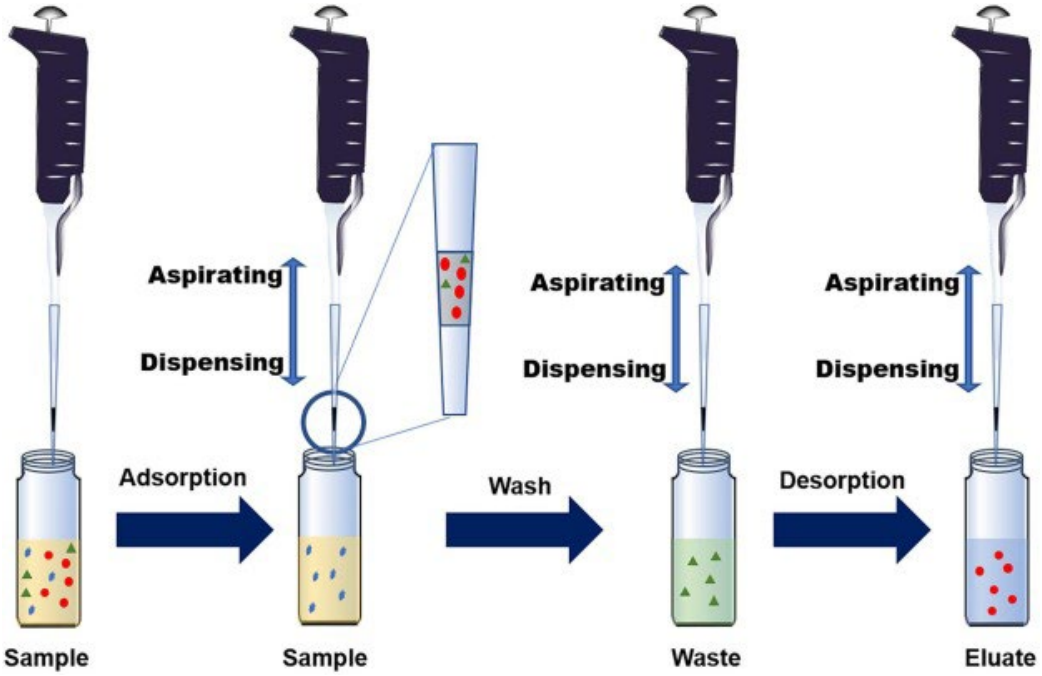

Figure 2.12: Schematic illustration of the PT-µSPE procedure (He *et al*., 2021).

PT-µSPE is advantageous over traditional SPE because the pipette tip facilitates labor-intensive steps, it makes on-site sampling possible, especially in the case of low sample amounts, and it reduces the sample pre-treatment time. It also reduces the amount of sorbent and solvent required, it is inexpensive, and it comprises extraction, concentration, and purification in one step (Seidi *et al*., 2019; Tsai *et al*., 2021). The downside of this technique is that it is not efficient for the quantitative analysis of high volumes of target samples and that the pipette tips may clog during the extraction process when biological specimen such as blood or plasma are used (Seidi *et al*., 2019). It has been used in fields like environmental analysis, food analysis, bioanalysis, and proteomics (Tsai *et al*., 2021). It has been used for the determination and extraction of analytes such as pharmaceuticals, bioanalytical molecules, proteins, and metals in various matrices (Kahkha *et al*., 2019).

The choice of extraction sorbent in the PT-µSPE is important for sample pre-treatment process and to achieve maximum extraction capacity of the target analyte (He *et al*., 2021; Tsai *et al*., 2021). Sorbents are normally loaded in cartridges such as in SPE. However, PT-µSPE is used when there are small amounts of sorbents available (Tavengwa *et al*., 2016b). Inorganic adsorbents such as silica, graphene, graphene oxide (GO), carbon nanotubes (CNTs), and metal oxides have been used in analytical chemistry (Sun *et al*., 2021). Nanomaterials can be used as SPE sorbents because of their high superficial area and variety of interactions. Carbon nanotubes have been used as SPE sorbents for the extraction of drugs, pesticides, or natural compounds in different media such as biological fluids, drug preparations, environment, plants, or animal organs. These materials exhibit interesting chemical properties and a high surface area when used as a sorbent. To benefit from their sorbent capacity, these materials must be embedded into a material or immobilized on a surface/support (Fresco-Cala *et al*., 2018).

Feng *et al*. (2019) extracted two flavonoids, quercetin and myricetin, from *Epipremnum aureum* rhizome using pipette tip micro solid phase extraction (PT-µSPE). In this study, three polymers were synthesized and applied as sorbents in the extraction of myricetin and quercetin. Poly (styrene-divinylbenzene) (PS-DVB) in combination with nano-TiO_2, PS-DVB-TiO_2, and modified with polyethyleneimine (PEI) was chosen as the sorbent owing to its high adsorption ability, high mechanical strength, and special selectivity to the target flavonoids. HPLC was used to detect the

quercetin and myricetin from the extracted solution. The limit of detection (LOD) for myricetin and quercetin were found to be 0.009 and 0.004 µg mL^{-1}, respectively, and the limit of quantification (LOQ) were observed to be 0.03 and 0.01 µg mL^{-1} for the target flavonoids, respectively. The method recoveries of myricetin were in the range of 83.15-97.52% with the relative standard deviation (RSD) less than 2.11% while the method recoveries of quercetin were in the range of 81.25-93.16% with RSD less than 3.91%. These recoveries were reported to be satisfactory for the detection of the two flavonoids. It was concluded that the PT-µSPE method with PEI modified PS-DVB-TiO_2 as the adsorbent showed good performance for the purification of the two flavonoids.

2.7. Metabolomics

Metabolomics is a field of study that gives a comprehensive view of the molecular contents of organisms. Metabolomics increasingly generates amounts of metabolome profiles of complex metabolite mixtures that aim to provide biochemical insights. A metabolomics study includes sample collection, sample extraction, analytical measurement, data processing and analysis, and biochemical interpretation (Ernst *et al*., 2019; Ivanisevic and Want, 2019; Beniddir *et al*., 2021). Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are reported to be the analytical workhorses of metabolomics (Caudy *et al*., 2017; Liu *et al*., 2018; LeVatte *et al*., 2022). Metabolite annotation and identification are important in metabolomics, i.e. assignment of structures to spectral data (Beniddir *et al*., 2021). Untargeted experiments result in qualitative and semi-quantitative information on thousands of molecular ions across many samples (Ernst *et al*., 2019). In biological samples, many metabolites share molecular substructures and form structurally related molecular families (MFs) of different chemical classes. Based on the assumption that structurally similar molecules generate similar MS fragmentation spectra, they can be grouped by comparing their fragmentation spectra in the construction of MFs. To do this on a larger scale, computational tools have been developed such as molecular networking (MN) (Watrous *et al*., 2012; Yang *et al*., 2013; Ernst *et al*., 2019; Vincenti *et al*., 2020).

The annotation of structural information needs additional sources such as library matches, candidate structures from libraries or chemical class annotations. Since the introduction of MN in numerous metabolome mining workflows, annotation and classification tools have been

introduced including SIRIUS (Duhrkop *et al*., 2019), CSI:Finger ID (Duhrkop *et al*., 2015), Met Fusion (Gerlich and Neumann, 2013), and MetFamily (Treutler *et al*., 2016). Metabolome mining tools take MS/MS spectra as input in the Mascott Generic Format (MGF), mzML, or mzXML formats and generate tables where a fragmented mass feature is linked to other fragmented mass features or substructure patterns. On average, only 2 – 5% of MS/MS spectra acquired in a typical LC-MS/MS experiment can be matched to known molecules (Spicer *et al*., 2017; Ernst *et al*., 2019; Li *et al*., 2019).

Molecular networking is a popular tool in the analysis of MS/MS-based metabolomics data. MN aids the elucidation of the structure of many compounds of untargeted MS (Quinn *et al*., 2016; Aron *et al*., 2020). MN is fundamentally based on the observation that two structurally related molecules share fragment ion patterns when subjected to MS/MS fragmentation methods such as collision induced dissociation (CID) (Aron *et al*., 2020). The fragmentation spectra acquired in MS/MS analyses are hypothesized to be related to their original chemical structures and thus molecules that have similar structures are likely to exhibit similar MS/MS spectra. If we can calculate the spectral similarities between all spectra within a complex mixture, the spectral similarities can be extrapolated to the structural similarities between molecules in the mixture (Kang *et al*., 2019). The matrix of spectral similarity can be visualized as a MN where each node is a MS/MS spectrum and the edges between the nodes indicate spectral similarity. The mass spectral similarity is calculated with a modified cosine score (Le Dare *et al.,* 2020; Beniddir *et al*., 2021). Figure 2.13 shows an example of how a MN is created from a complex sample. MN goes beyond spectral matching against reference spectra, by aligning experimental spectra against one another and connecting related molecules by their spectral similarity. In MN, related molecules are referred to as a molecular family and differ only by transformations such as glycosylation, alkylation, and oxidation/reduction (Nothias *et al*., 2020).

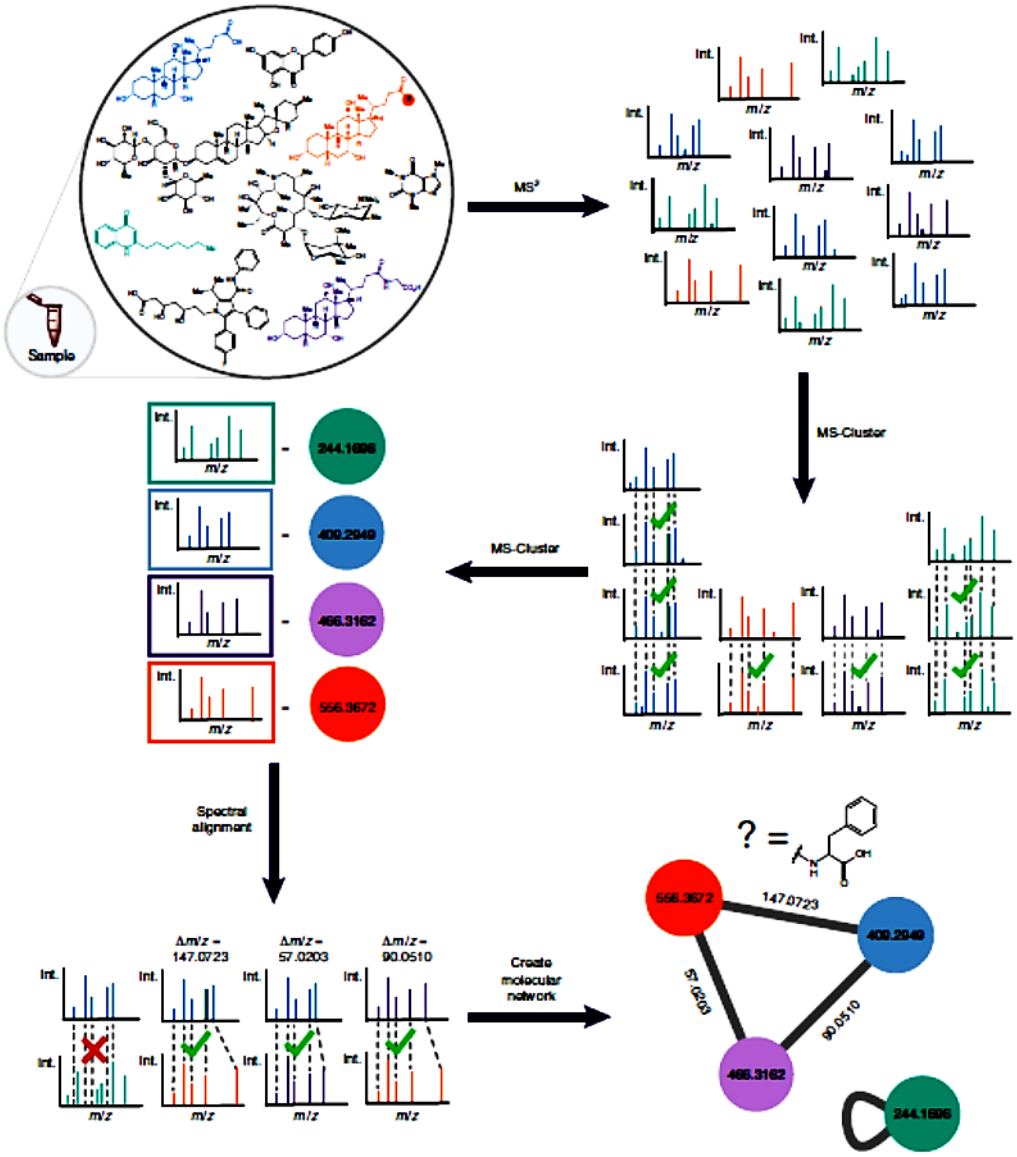

Figure 2.13: Schematic representation of how a molecular network is created from tandem mass spectra for metabolites in a complex sample (Aron *et al*., 2020).

MN has a great potential to aid both MS-based disease diagnostics and drug development. Untargeted MS is a key metabolite discovery and annotation strategy in metabolomics. MN provides a visual overview of all the ions of molecules that were detected and fragmented during an MS experiment and the chemical relationships between them. Since MN was introduced into the field of metabolomics, it has been applied for drug discovery, for the identification of novel virulence factors and natural products, and in drug metabolism studies (Quinn *et al*., 2016; Le Dare

et al., 2020). MN has led to the development of Global Natural Products Social (GNPS) which is a molecular networking and data-sharing web-based platform (Xu *et al*., 2019; Rawlinson *et al*., 2020). MN analyses the chemical relationships between every MS/MS spectrum and thus visualizes the entire metabolome detected in a sample (Quinn *et al*., 2016).

GNPS is widely used by scientists in industry, academia, and government in the fields of biomedical research, environmental science, ecology, forensics, microbiology, chemistry and more. GNPS facilitates data, stores knowledge, and enables knowledge capture, sharing, dissemination and data-driven social networking while promoting reproducible data analysis (Aron *et al*., 2020). GNPS analyses data and compares it to all data that is available publicly. It provides public data set deposition and/or retrieval through the Mass Spectrometry Interactive Virtual Environment (MassIVE) data repository. GNPS spectral libraries enable dereplication, variable dereplication and the identification of spectra in molecular networks. GNPS can be used for molecular networking and is currently the only public infrastructure that enables molecular networking (Xu *et al*., 2019). The related molecules in a MN can be viewed online at GNPS or on Cytoscape for analysis (Wang *et al*., 2016; Beniddir *et al*., 2021). GNPS has the largest collection of publicly accessible natural products and metabolomics MS/MS data sets and is the only infrastructure where public data sets can be reanalysed together and be compared with each other. In GNPS, natural products data can be shared, analysed and annotated by researchers worldwide (Wang *et al*., 2016).

Other tools in GNPS include network annotation propagation (NAP) and DEREPLICATOR, and an unsupervised substructure identification tool called MS2LDA. These tools are used to complement classic MN output and integration using MolNetEnhancer within GNPS (Ramabulana *et al*., 2021). In GNPS, a MN is created when peaks from one MS/MS are aligned with peaks from another MS/MS and a cosine score is assigned to each combination to describe their similarity (Aron *et al*., 2020; Beniddir *et al*., 2021). This is done in their original *m/z* position or with their *m/z* shifted according to the difference in the precursor *m/z* of the two molecules. The beginning of a network analysis approach means that the relationships between the spectra started to be considered while each spectrum was being analysed independently. The grouping of spectra based upon their similarity allows molecular networking to identify clusters in network theory (Beniddir *et al*., 2021).

The primary focus of this study was to investigate the distribution of rutin in the leaves of *M. oleifera*. The study utilized plants from different households in the Vhembe District of Limpopo Province, South Africa. The amount of rutin was estimated using the optimized UHPLC-qTOF-MS technique. For the first time, modern extraction methods such as PT-µSPE and UA-ATPE were explored in the extraction of rutin from the leaves of *M. oleifera*. Computational metabolomics tools, including molecular networking, MolNet Enhancer, and MS2LDA, were employed to reevaluate the flavonoid chemical space of *M. oleifera* leaves with the intention of establishing the chemical diversity brought about by various biochemical modifications with anticipated pharmacological attributes.

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Chapter 3

Materials and methods

This chapter summarizes the experimental procedures conducted for the extraction of rutin from *M. oleifera* using aqueous methanol and modern extraction methods such as UA-ATPE and PT-µSPE. The creation of a molecular network on the GNPS system is also summarized in this chapter.

3.1. Chemicals and reagents

The chemicals and reagents used to extract rutin from *M. oleifera* using the different extraction methods are outlined in **Experimental Chapters 4 – 7**. The procedure on how a molecular network was created is outlined in **Experimental Chapter 7**.

3.2. Methods

Sample collection and preparation of the samples are outlined in **Experimental Chapter 4**. Figure 3.1 summarizes the extraction methods applied in this study for the extraction of rutin from *M. oleifera* leaves. In **Experimental Chapter 4**, 80% aqueous MeOH was used as the extraction method and the methanolic extracts were analyzed quantitatively and qualitatively on the UHPLC-qTOF-MS. In **Experimental Chapter 5**, which involved the UA-ATPE method, the experimental design was conducted using the central composite design (CCD) software which included 2 factorial inputs. The quantification of rutin was done based on the multiple reaction monitoring (MRM). A response surface model (RSM) was also generated. The extraction of rutin using PT-μSPE is outlined in **Experimental Chapter 6** wherein a carbon nanospheres sorbent is used and is placed inside the pipette tip. Optimization studies were carried out and the extracts were analyzed using a UV-spectrophotometer. **Experimental Chapter 7** outlines the creation of a molecular network (MN) within the global natural product social (GNPS) system. The molecular network created was analyzed using Cytoscape.

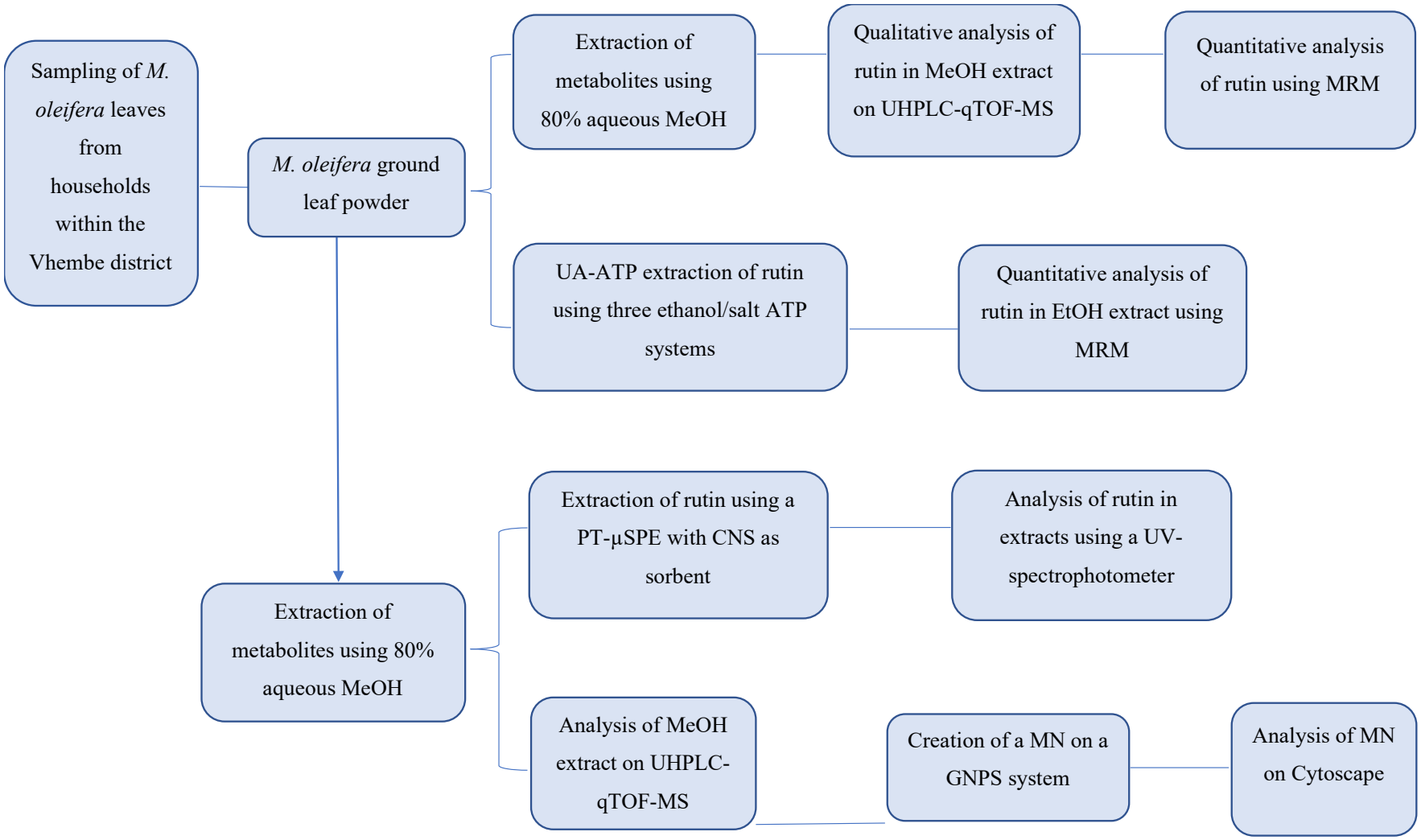

Figure 3.1: Workflow of the extraction methods used in this study for the extraction of rutin from *Moringa oleifera* leaves and analysis using UHPLC-qTOF-MS.

Experimental Chapter 4

This chapter focuses on the work titled 'Distribution of rutin metabolite from domesticated *Moringa oleifera* plants in Vhembe District of Limpopo Province using UHPLC-qTOF-MS'.

4.1. Abstract.

Moringa oleifera, recognized for its diverse nutritional and medicinal advantages, is categorized as a functional crop. Various phenolic compounds, particularly flavonoids metabolites from this plant are identified as potential nutraceutical agents. The chemical composition of these metabolites, especially flavonoids, exhibit unique glycosylation patterns (sugar attachment), thereby enabling them to cover a wider pharmacophoric space. Due to these compelling nutraceutical properties, many individuals have chosen to cultivate this species in their backyard for immediate use. Among the numerous glycosides present in *M. oleifera*, flavonoids attached to a rutinoside disaccharide have also been reported, although conflicting reports exist regarding their levels. Rutinoside-bearing flavonoids are also highly bioavailable for humans and are consequently the most sought-after nutraceutical flavonoids in plants. The current study focused on analyzing Moringa plants from various households in the Vhembe District of Limpopo Province, South Africa, specifically for the presence of the rutin flavonoid molecule (Quercetin rutinoside) using UHPLC-qTOF-MS. The study results reveal that out of the 135 samples analyzed, only 15 household plants can produce rutin and at different concentrations. The highest concentrations were found to be 4088, 4908, 8444, and 14701 mg kg^{-1}. This indicates the existence of different cultivars in different households, potentially impacting the perception of this species. Therefore, it is crucial to communicate these findings to institutions responsible for cultivating medicinal plants, urging the cultivation of the relevant cultivar.

Keywords: flavonoids, LC-MS/MS, *Moringa oleifera*, phytochemicals, rutin, Vhembe District

4.2. Introduction

The Moringacea family is known to be a monogeneric one, with thirteen species of the genus *Moringa* (Leone *et al*., 2015). These *Moringa* species are used for different purposes such as source of medicine, food, cosmetics, and the production of oil (Padayachee and Baijnath, 2012). However, the widely known and studied species is *M. oleifera* (Stadtlander and Becker, 2017). Often dubbed a "miracle tree," this species holds essential phytochemicals with medicinal and nutritional value in its leaves, pods, roots, bark, gum, flowers, seeds, and seed oil. (Wadhwa *et al*., 2013; Gopalakrishnan *et al*., 2016; Brilhante *et al*., 2017; Metwally *et al*., 2017).

This species has been studied for its various biological activities including immune boosting (Mehwish *et al*., 2017), anti-cardiovascular diseases (Dhakad *et al*., 2019), anti-viral (Biswas *et al*., 2019), anti-microbial (Singh *et al*., 2013; Al-Obaidi *et al*., 2021a), anti-oxidant (Shih *et al*., 2011; Lin *et al*., 2018), and anti-inflammatory (Falowo *et al*., 2018; Mahdi *et al*., 2018; Al-Obaidi *et al*., 2021b) properties.

The above activities are due to the presence of a wide spectrum of metabolites (Kim, 2005; Beran *et al*., 2019), including polyphenols, carotenoids, alkaloids, terpenes, and sulfur containing compounds (Maldini *et al*., 2014).

Flavonoids are highly active and major metabolites that are produced by *M. oleifera* (Tshabalala *et al*., 2019). This class of compounds has various metabolic functions owing to their structural configuration since they can exist as either aglycones (free forms) or glycosides (linked to a sugar moiety) (Kim *et al*., 2012). Quercetin, kaempferol, isorhamnetin, and apigenin are among the reported flavonoids in *M. oleifera*. The chemical complexity, due to diverse chemical modifications (i.e. glycosylation patterns), of flavonoids in this species makes its chemistry very interesting (Makita *et al*., 2016). *M. oleifera* diversifies its flavonoids by attaching different types of sugar derivatives on the flavonoid aglycones (Makita *et al*., 2016; Tshabalala *et al*., 2019). The same sugars help for bioavailability of these compounds during consumption by humans and other animals (Makita *et al*., 2016). *In planta,* flavonoids are generally glycosylated for storage purposes (Ogo *et al*., 2016). Rutinoside-bearing flavonoids such as kaempferol rutinoside, quercetin rutinoside (rutin) and isorhamnetin rutinoside are pharmacologically potent and are responsible for most of the bioactive properties (Tshabalala *et al*., 2021). Yet, among these three rutinoside-bearing flavanoids, rutin stands out as the most abundant (Makita *et al*., 2017). Rutin is highly

sought-after in the pharmaceutical industry for its pharmacological properties, making it a key flavonoid (Peng *et al*., 2018; Tursynbolat *et al*., 2019).

The presence of rutin in *M. oleifera* has previously been reported with mixed outcomes. A study conducted by (Habtemariam and Vargehese, 2015) reported the presence of rutin in *M. stenopetala* but not in *M. oleifera* and, as such, they concluded the former species to be more pharmacological superior. Furthermore, (Makita *et al*., 2016) reported the presence of rutin in *M. ovalifolia* and again not in *M. oleifera*; which led to the conclusion that *M. oleifera* is incapable of producing rutinoside-bearing flavanoids. However, in another follow-up study by (Makita *et al*., 2017), rutin was reported in three of twelve different cultivars of *M. oleifera*. This led to the conclusion that the presence of rutin in *M. oleifera* is cultivar specific and, as such, differences in the pharmacological potency of these plants should be expected and which may lead tonegative perception of this plant. This is particularly concerning as households cultivate this plant without knowing the specific cultivar they have.

This study focused on evaluating various *M. oleifera* cultivars cultivated in households across the Vhembe District of Limpopo Province, South Africa. This involved assessing rutin presence in each sampled plant from households using UHPLC-qTOF-MS analyses. Additionally, the study quantified rutin levels in these plants, providing insights into metabolite accumulation differences among cultivars.

4.3. Materials and Methods

4.3.1. Chemicals and reagents

Methanol (99% CP) was purchased from Associated Chemical Enterprises (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) obtained from a Direct-Q 5UV distiller (Massachusetts, United States of America) was used for the preparation of the 80% methanol solution. The extraction was performed on a DIAB MX-RL-Pro dragon shaker. Chromatographic separation of the metabolites in the extracts was done using a reverse phase Shim-pack Velox C18, 2.1 x 100 mm, 2.7 µm with a serial number 227-32009-03 (Columbia, USA). The UPLC was connected to a Shimadzu 9030 LC, qTOF-MS detector (Shimadzu, Kyoto). The solvents used for

the chromatographic runs were methanol and formic acid, which were purchased from Romil Pure Chemistry (Cambridge, UK).

4.3.2. Sampling

Leaves of domesticated *Moringa oleifera* were collected from different households in different villages in the Vhembe District of Limpopo Province in South Africa (Maniini, Tshisaulu, Dzwerani, Mvelaphanda, Gombameni, Thohoyandou, Muledane, Makhado, Shakadza, Mafukani, Tshidimbini, Makonde, Maungani, and Itsani) (Table 4.1). A total of 130 household trees was sampled, and an additional 5 cultivars were obtained from the Agricultural Research Council (ARC) in Pretoria. This brought the total number of samples to 135. Vhembe District is located in a semi-arid area that is frequently troubled by dry spells, which can escalate to severe droughts (Mpandeni and Maponya, 2013) and Pretoria is reported to have warm and free sunny days and winter days that are clear and crisp. Pretoria gets its rainfall during summer (Maluta and Mulaudzi, 2018). The sampled household trees were air dried and ground to a fine powder.

4.3.3. Extraction of metabolites

A modified method presented by Makita *et al*. (2016) was used with further modification. Briefly, ground leaf powder (1 g) for each cultivar was extracted with 10 mL of 80% aqueous methanol (MeOH) with the aid of a dragon shaker overnight. The mixture was then centrifuged for 20 min at 25 °C at a high speed of 5000 rpm. Thereafter, the liquid supernatant was transferred to an Eppendorf tube, filtered through 0.22 µm filters into a vial and analyzed on the UHPLC-qTOF-MS. The remaining supernatant solutions were stored in a refrigerator.

4.3.4. Analysis on the UHPLC-qTOF-MS

Chromatographic separation was conducted on an Acquity HSS T3 C18 column (150 mm × 2.1 mm with particle size of 1.7 µm) using a mobile phase which consisted of formic acid (0.1%) in deionised water (solvent A) and acetonitrile with 0.1% formic acid (solvent B) at a column temperature of 40 °C. A 30 min gradient elution method wherein the initial conditions were 5% solvent B and a flow rate of 0.4 mL min^{-1} was used to achieve chromatographic separation. The conditions were increased to 40% solvent B in 5 min and again to 95% solvent B in 15 min and

kept constant at 95% solvent B for 2 min. The conditions were returned to the initial condition at 5% solvent B in 2 min and the column was allowed to re-equilibrate at 5% solvent B for 3 min. Elution was monitored using a photodiode-array detector (PDA) collecting 20 spectra per second between the 200 and 500 nm range.

For mass spectrometry, the acquisition parameters discussed by Ramabulana *et al*. (2015) were followed. Briefly, MS data were acquired using the negative electron spray ionization (ESI) mode. The MS was configured to scan the range of 100–1000 Da with a scan time of 0.2 s. After a series of optimization, the following settings were found to be optimal: capillary voltage of 2.5 kV, sample cone potential of 30 V, source temperature of 120 °C, desolvation temperature of 450 °C, cone gas flow of 50 L h^{-1}, desolvation gas flow of 550 L h^{-1}, and multichannel plate detector potential of 1600 V. In order to achieve efficient fragmentation to aid during identification, the mass spectrometry data were collected using a collision energy ramp of 10–30 eV. Structural elucidation was done using Knapsack online metabolite database.

Table 4.1: A list of the areas where *M. oleifera* leaves were sampled in the Vhembe District and the ARC.

Area	**No. of plants collected**	**Sample codes**	**Coordinates**
ARC	5	ARC131 – ARC135	25°59”S, 28°35”E
Dzwerani	7	DZWE4 – DZWE5, DZWE11, DZWE15, DZWE18 – DZWE20	23.0655° S, 30.4214° E
Gombameni	2	GOMB7, GOMB9	23.254038° S, 30.108484° E
Itsani	67	ITSA64 – ITSA130	23.0059° S, 30.4189° E
Mafukani	3	MAFU26 – MAFU28	22.6715° S, 30.5553° E
Makhado	1	MAKH24	23.0462° S, 29.9047° E
Makonde	11	MAKO53 – MAKO63	22.8064° S, 30.5826° E
Maniini	2	MANI1, MANI8	22.9870° S, 30.4814° E

Maungani	1	MAUN2	22.9637º S, 30.4214º E
Muledane	1	MULE23	22.9895º S, 30.5020º E
Mvelaphanda	5	MVEL3, MVEL6, MVEL16 – MVEL17, MVEL22	26.0050º S, 28.2216º E
Shakadza	11	SHAK25, SHAK29 – SHAK30, SHAK33 – SHAK36, SHAK38 – SHAK39, SHAK46, SHAK49	22.6160º S, 30.5711º E
Thohoyandou	1	THOH14	22.8785º S, 30.4818º E
Tshidimbini	14	TSHID31 – TSHID32, TSHID37, TSHID40 – TSHID45, TSHID47 – TSHID48, TSHID50 – TSHID52	22.8685º S, 30.5423º E
Tshisaulu	4	TSHIS2, TSHIS10, TSHIS13, TSHIS21	23.0061º S, 30.3983º E

4.4. Results and discussion

4.4.1. Metabolite profile

Metabolites from 135 samples of domesticated *Moringa oleifera* trees from the Vhembe District in Limpopo Province of South Africa and ARC-VIMP were extracted using 80% methanol. These samples were all analyzed for the presence of rutin on the UHPLC-qTOF-MS analytical platform in ESI negative mode. The base peak (BP) chromatograms are illustrated in Figure 4.1. Through visualization of the chromatograms, differences were noted in the intensities of peaks and the presence and absence of different metabolites, which clearly indicates the differences in the metabolite accumulation in this species. For instance, taking the phenolic compounds such as chlorogenic acids and flavonoids (1-20 minutes), there were more peaks that showed variability across different chromatograms.

Flavonoids have been reported to be the dominant metabolite group identified in *M. oleifera*. In this study, quercetin and kaempferol derivatives (glycosylated with different sugars) appeared to be the most abundant of the flavonoid group. Lin *et al*. (2019) also reported on flavonoids such as quercetin (*m/z* 301) and kaempferol (*m/z* 285) moieties attached to different sugars as the most abundant flavonoids. Makita *et al*. (2017) further reported that kaempferol rutinoside, quercetin rutinoside (rutin), and isorhamnetin rutinoside are three flavonoids that exhibit an interesting distribution among different cultivars. Rutin, however, appeared to be the most abundant flavonoid when compared to the other rutinoside-bearing flavonoids. The presence of these metabolites can be seen in Figure 4.1, wherein rutin (when present) is the most abundant of the three rutinoside-bearing flavonoids.

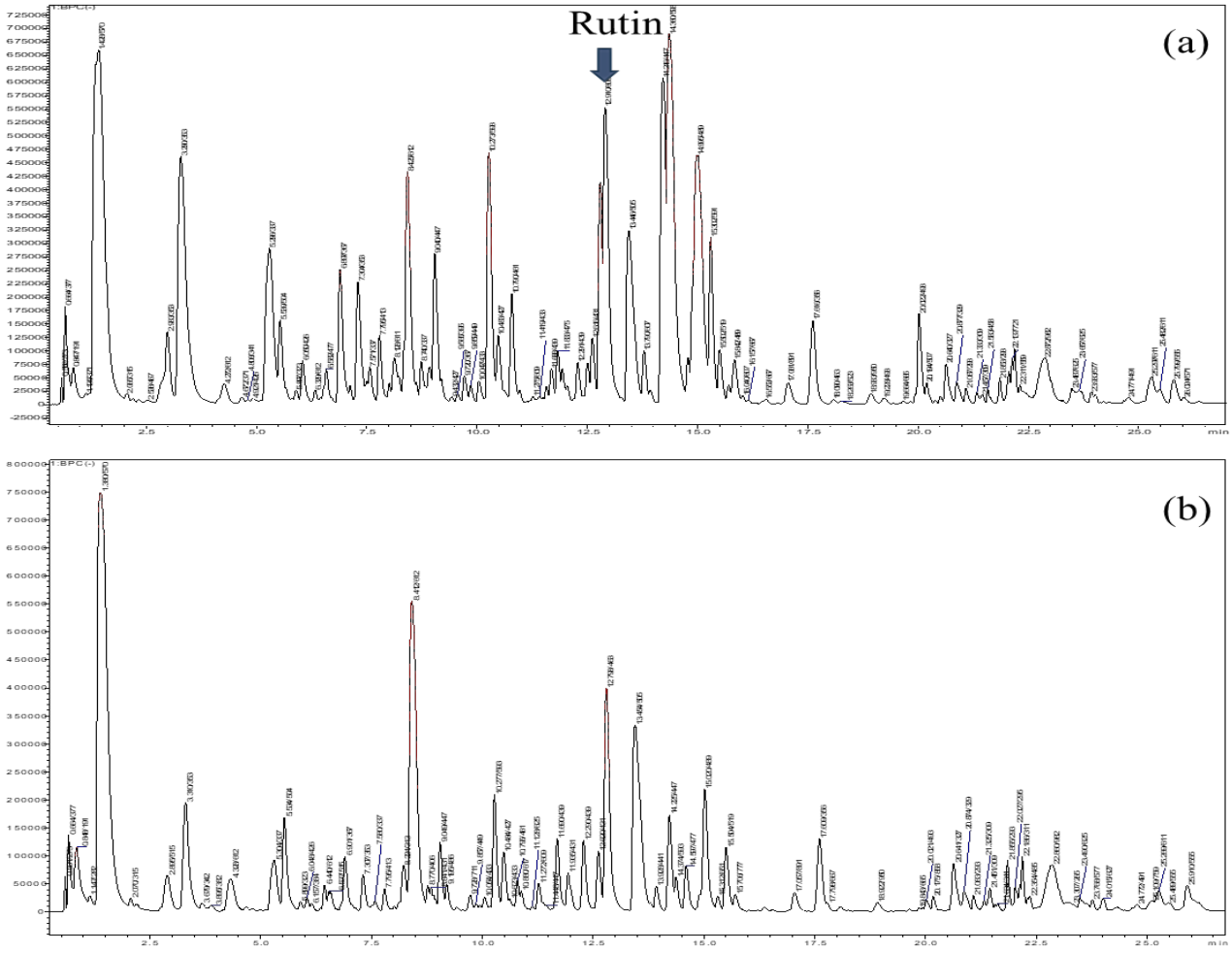

Figure 4.1: UHPLC-qTOF-MS chromatogram of *M. oleifera* leaf extracts from Makonde that (a) produces rutin (MAKO53), and (b) does not produce rutin (MAKO59).

4.4.2. Analysis of rutin

Rutin (Figure 4.2) was identified in some of the domesticated plants that were analyzed. Figure 4.1 shows the chromatograms of two trees from Makonde village wherein (a) is the chromatogram of a *M. oleifera* tree that produces rutin and (b) is the chromatogram of a tree that does not produce rutin. Rutin has a precursor ion at *m/z* 609.147 $[M - H]^-$. According to Figure 4.1 (a), rutin was eluted at a retention time of 12.91 min. Figure 4.3 is the LC-MS BP chromatogram of rutin obtained from sample MAKO53. Deprotonated rutin undergoes homolytic cleavage of the 3-O glycosidic bond to produce the quercetin aglycone ion observed at *m/z* 300 $[M - H]^-$ [37-38]. Figure 4.4 illustrates the fragmentation pattern of rutin. It is noted that rutin fragments to the quercetin aglycone molecule at *m/z* 301.0325 $[M - H]^-$ showing the

loss of the rhamnose-glucose unit (Rodriguez *et al*., 2015; De Graaf *et al*., 2014; Wang *et al*., 2020). From the 135 trees sampled; 15 of these trees were found to produce rutin. The presence of rutin from these 15 domesticated trees was confirmed through the observation of the fragmentation pattern of the precursor ion at *m/z* 609.

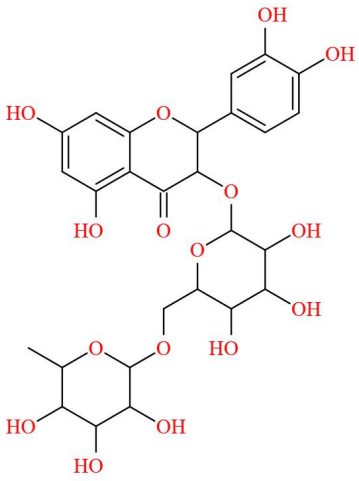

Figure 4.2: Structure of quercetin rutinoside (rutin).

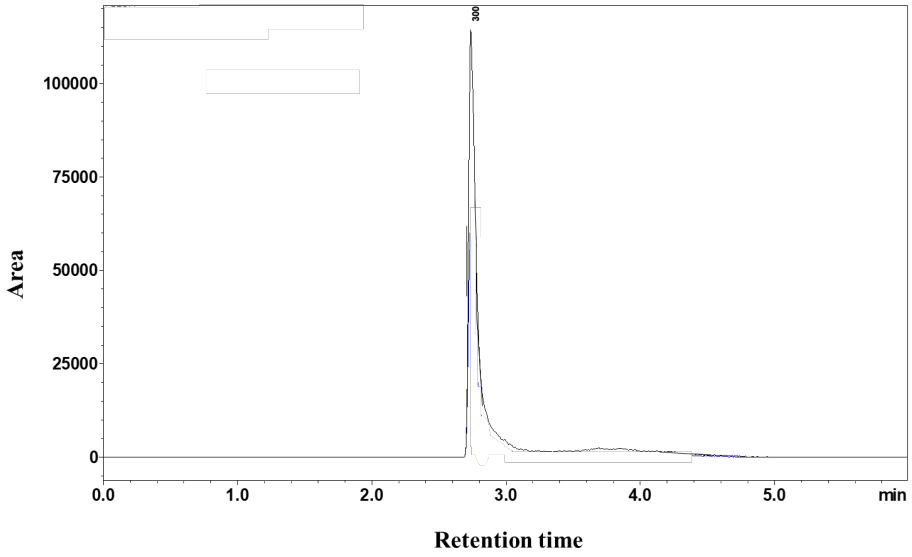

Figure 4.3: Single ion monitoring LC-MS chromatogram of rutin showing that the extract does not produce any isobaric peak which might lead to misidentification.

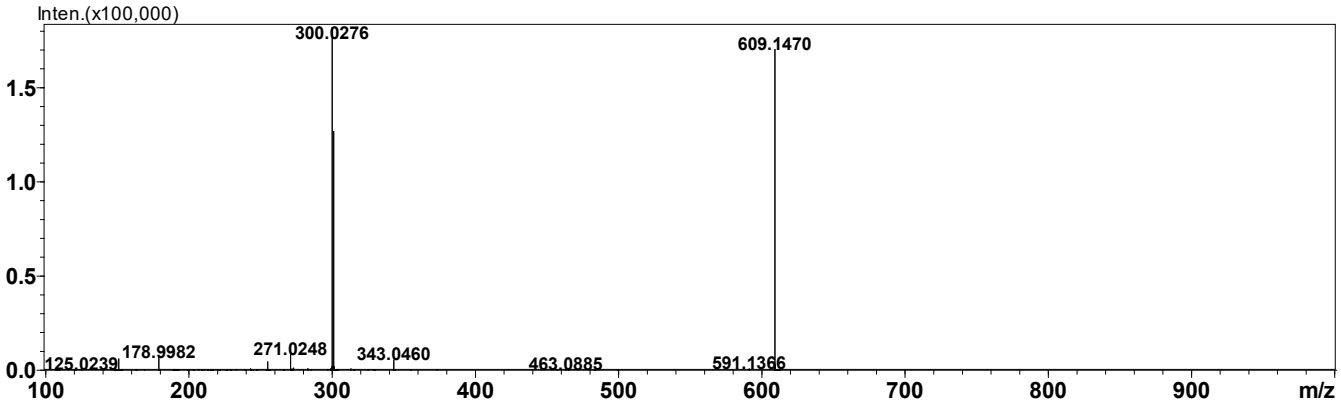

Figure 4.4: Mass spectrum of the fragmentation pattern of rutin showing that rutin fragments to the quercetin aglycone.

Figure 4.5 is a representation of the domesticated trees that produced rutin and highlights the differences in the metabolite accumulation of *M. oleifera* and further confirms that indeed different cultivars of this tree are being grown in the different households. The rutin-producing trees are from the villages Gombameni (GOMB7 and GOMB9), Itsani (ITSA77, ITSA81, ITSA82, ITSA99 and ITSA119), ARC (131), Makonde (MAKO53, MAKO57 and MAKO61), Dzwerani (DZWE18 and DZWE19) and Shakadza (SHAK36 and SHAK39). However, these trees produced rutin at varying concentrations (Table 4.2). Some produced rutin at very low concentrations while others produced rutin at high concentrations. The trees that produced high concentrations of rutin were notably from Itsani (ITSA 82 and ITSA99), Makonde (MAKO61), and Shakadza (SHAK39) and the highest concentration (14701.887 $mg.kg^{-1}$) was observed from an Itsani tree. A view of the trees that produce rutin is also shown in Figure 4.5.

Table 4.2: A summary of the rutin-producing *M. oleifera* leaf samples and their respective concentrations.

Sample code	Concentration (mg kg^{-1})
ARC131	45.198
GOMB7	26.141
GOMB9	73.994
DZWE18	32.433
DZWE19	44.653
SHAK36	31.578
SHAK39	8444.679
MAKO53	206.139
MAKO57	62.596
MAKO61	4908.468
ITSA77	99.347
ITSA81	43.984
ITSA82	14701.887
ITSA99	4088.063
ITSA119	495.147

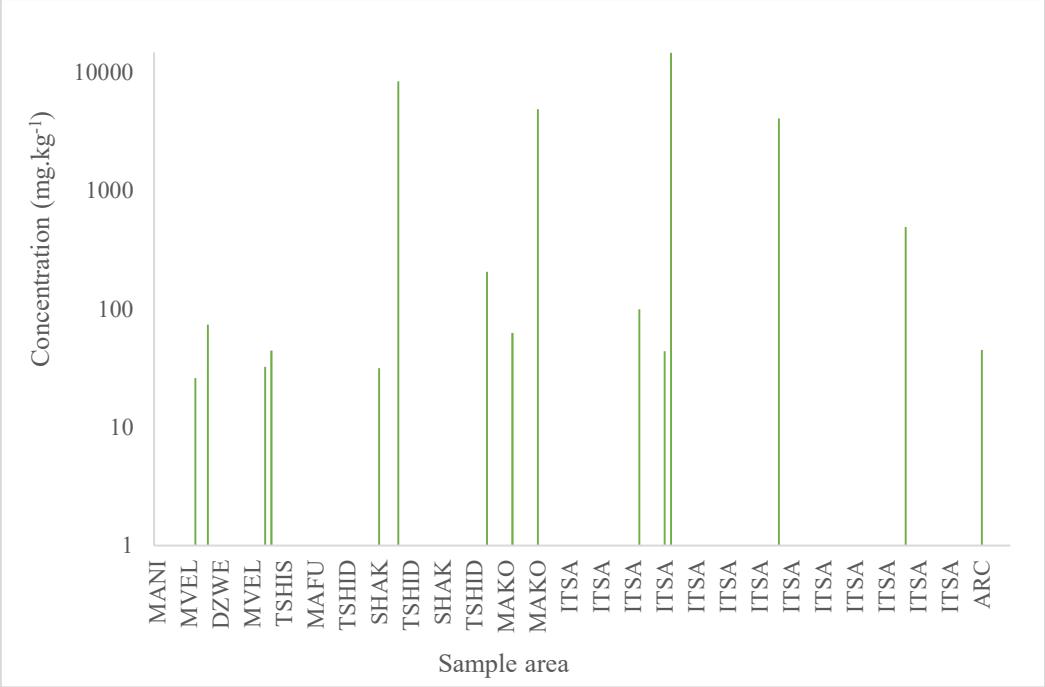

Figure 4.5: Typical bar graph showing the varying concentrations of rutin in *M. oleifera* leaf extracts.

In the study by Habtemaraim and Vargehese (2015), it was concluded the *M. oleifera* does not accumulate rutin. According to Makita *et al*. (2016), this is because *M. oleifera* is incapable of glycosylating its flavonoids with the rutinoside sugar. There are, however, some factors that possibly contribute to the inability of the plant to glycosylate its flavonoids with the rutinoside sugar. The difference in genetic make-up, the maturity of the leaves, and the different environmental conditions of these trees could be possible contributors to the accumulation or non-accumulation of rutin in these trees and the varying concentrations of rutin (De Graaf *et al*., 2014; Makita *et al*., 2017). Environmental factors such as soil, climate, topography, and biotic factors play a role in the accumulation of secondary metabolites in medicinal plants. These factors affect the plant growth and development and thus having an effect on the content of active components in such plants (Li *et al*., 2020; Li *et al*., 2020). Medicinal plants grown in different environments have been reported to produce different contents of secondary metabolites and thus resulting in differences in their medicinal qualities (Liu *et al*., 2015).

However, Makita *et al*. (2017) concluded in another study that *M. oleifera* does infact produce rutin, but the production of rutin in this species is cultivar specific. The results obtained from this study show that most households from the different villages are growing the non-rutin

producing cultivar. The overwhelming absence of rutin in various households could be due to sharing of explants used to grow the plants, thereby propagating the non-rutin producing plant. This results in the non-rutin producing plant being distributed throughout most of the households in the entire village. This then means that most households miss out on the full pharmacological potency of this plant. This could also mean that although *M. oleifera* is capable of producing rutin, the rutin-producing cultivar is a rare one.

4.5. Conclusions

The flavonoids in *Moringa oleifera* are known to undergo unique glycosylation patterns by attaching to different sugars and thus diversifying the flavonoid pool. In this study, the distribution of rutin in 135 *M. oleifera* plants from different households was determined and only 15 plants were found to produce rutin at varying concentrations. This study further reaffirms that the accumulation of rutin in *M. oleifera* is cultivar specific and not all Moringa plants have the same metabolite distribution patterns and, as such, differences in pharmacological properties should be expected. Based on the results of this study, it is observed that most of the households within the villages are growing the less pharmacologically potent cultivar in their backyards. This then raises a concern regarding the accessibility of the rutin-producing cultivar; considering that the rutin-producing cultivar might be a rare one. The results of the current study can, therefore, act as a catalyst to encourage selection of cultivar to propagate in order to have only the fully pharmacologically potent plants utilized by communities. As such, future studies should focus on the genetic make-up of *M. oleifera* in order to unearth the genes responsible for rutin biosynthesis. Other techniques, such as those involving remote sensing, can also be used for cultivar selection too.

4.6. References

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Experimental Chapter 5

This chapter focuses on the work titled ‘Ultrasonic-assisted aqueous two-phase extraction for the extraction of rutin from *M. oleifera* leaves by response surface methodology’.

5.1. Abstract

Rutin, a natural flavonol glycoside prevalent in various fruits and vegetables, is known for its purported health benefits. This flavonoid possesses diverse pharmacological properties, and its extraction from plant materials has been explored through various methods, yielding different outcomes. In the present investigation, ultrasonic-assisted aqueous two-phase extraction (UA-ATPE) was employed to extract rutin from *Moringa oleifera* leaves, utilizing a 25% saturated salt solution to create an aqueous two-phase. Different salts, including $MgSO_4$, $(NH_4)_2SO_4$, and NaCl, were used, and the optimization focused on ultrasonic time and ultrasonic temperature. A Central Composite Design (CCD) approach was implemented to determine the optimal experimental conditions. The various ATPE systems resulted in distinct response surface models (RSMs), displaying a linear fit for ethanol/$MgSO_4$ and ethanol/$(NH_4)_2SO_4$ systems, and a quadratic fit for ethanol/NaCl system. The optimal conditions were identified as 25 °C and 22.5 minutes for the ethanol/$(NH_4)_2SO_4$ and ethanol/NaCl systems. Based on multiple reaction monitoring (MRM) using UHPLC-qTOF-MS, rutin concentrations at these conditions were determined to be 170.30 µg L^{-1} for the ethanol/$(NH_4)_2SO_4$ system and 240.00 µg L^{-1} for the ethanol/NaCl system. The study highlighted the significant impact of temperature on rutin extraction and concluded that low temperatures favor the extraction of rutin from *M. oleifera* leaves. Among the UA-ATPE systems tested, the ethanol/NaCl system exhibited the best performance in rutin extraction.

Keywords: aqueous two-phase extraction, rutin, ultrasonic, central composite design, response surface model

5.2. Introduction

Moringa oleifera is known for its nutritious pods and edible leaves and is used as food and medicine. It is also known to contain a great number of bioactive compounds. The leaves are the most used parts of the plant as they are rich in vitamins, carotenoids, polyphenols, phenolic acids, flavonoids, alkaloids, glucosinolates, isothiocyanates, tannins and saponins (Paikra *et al*., 2017; Vergara-Jimenez *et al*., 2017). These bioactive compounds are responsible for the pharmaceutical properties of *M. oleifera* (Falowo *et al*., 2018; Lin *et al*., 2018). Rutinoside-bearing flavonoids such as kaempferol rutinoside, quercetin rutinoside (rutin), and isorhamnetin rutinoside are an important class of bioactive flavonoids. The disaccharide, i.e. rutinoside, enables these flavonoids to be highly bio-available. Rutin is an attractive phytochemical because of its pharmacological properties and is thus an important flavonoid in the pharmaceutical industry (Chua *et al*., 2013; Peng *et al*., 2018; Tursynbolat *et al*., 2019).

Rutin is a flavonol glycoside that has been reported to present clinically relevant functions, beneficial in preventing diseases and protecting genome stability (Gullon *et al*., 2017). This flavonoid exhibit other favourable properties such as antioxidant (Enogieru *et al*., 2018), anti-inflammatory (Peng *et al*., 2018), antiallergic (Rahman *et al*., 2021), cardioprotective (Fei *et al*., 2019), neuroprotective (Yang *et al*., 2019), antidiabetic (Tursynbolat *et al*., 2019) and anticancer activities (Molnar *et al*., 2018; Negahdari *et al*., 2021). More than 130 medicinal products in the market contain rutin; either alone or in combination with other active ingredients (Chua *et al*., 2013; Gullon *et al*., 2017; Molnar *et al*., 2018).

Rutin is generally extracted using various organic solvents such as methanol, ethanol, acetone, isopropanol, ethyl acetate, diethyl ether and water-solvent mixtures (Huang *et al*., 2016; Gullon *et al*., 2017; Vetrova *et al*., 2017; Kim and Lim, 2019). Conventional extraction methods are simple to use but they present drawbacks such as poor efficiency, high solvent consumption, and long extraction times (Izza *et al*., 2018; Chou *et al*., 2020). Non-conventional extraction methods, on the other hand, use dedicated processing aids/energy inputs to improve the extraction efficiency and/or selectivity. Some of these methods employed in the extraction of rutin include deep eutectic solvents-based ultrasound-assisted extraction (DES-UAE) (Ali *et al*., 2019), ultrasound-assisted extraction (UAE) (Banozic *et al*., 2019) and microwave assisted extraction (MAE) (Dobrincic *et al*., 2020). These methods offer superior extraction efficiency in terms of cost, yield, extraction time and/or selectivity. The drawback with these methods, however, is that scaling-up can be difficult because of the costs of the equipment and

installations (Huang *et al*., 2013; Tatke *et al*., 2014; Barba *et al*., 2016; Kim and Lim, 2019). One method of extraction to consider is aqueous two-phase extraction (ATPE).

ATPE is a non-conventional extraction method that is formed from two or more phase-forming substances in water (e.g. two polymers, a polymer and a salt/sugar) (Zhou *et al*., 2018). It is a liquid-liquid fractionation technique that has been used in separation, extraction, purification and enrichment of some biomolecules (Jiang *et al*., 2019). ATPE allows the use of environmentally friendly phase-forming components such as alcohol, salts and sugars and can achieve rapid separation at room temperature. This contradicts conventional extraction that often uses toxic solvents, long extraction times and high temperatures (Chong *et al*., 2021). The alcohol/salt ATPE system is advantageous because of the easy recovery, low environmental impact, high selectivity, low viscosity, fast separation effect, and low cost (Toledo *et al*., 2019). The most common alcohols used are short-chain alcohols such as methanol, ethanol, and 2-propanol and the most common salts are those with cations such as Na^+, K^+, Ca^{2+}, and NH_4^+ and with anions such as Cl^-, SO_4^{2-}, CO_3^{2-}, and NO_3^{2-} (Gomis *et al*., 2021). Ethanol has been reported to extract weakly polar compounds such as flavonoids, saponins, and alkaloids while 2-propanol has been reported to extract lipid-soluble compounds (Xi *et al*., 2023). The combination of ATPE with another method such as the ultrasonic method enhances the extraction efficiency (Xi *et al*., 2023). ATPE-based ultrasonic-assisted extraction reduces the consumption of solvents, labor and energy, and also destructs the structure of plant cell walls by acoustic cavitation that passes through the solvent and thereby enhancing the recovery of bioactive compounds (Toledo *et al*., 2019; Gomis *et al*., 2021; Xi *et al*., 2023).

Hence, the primary objective of this research is to enhance the efficiency of rutin extraction from *Moringa oleifera* leaves by employing ultrasonic-assisted aqueous two-phase extraction (UA-ATPE). Notably, this is the first study to attempt to concentrate rutin extraction from *M. oleifera* leaves using an ethanol-salt UA-ATPE configuration. The salts examined herein for ATPE include NaCl, $MgSO_4$, and $(NH_4)_2SO_4$. Two key extraction variables, namely ultrasonic time and ultrasonic temperature were systematically investigated. To achieve optimization, a central composite design (CCD) approach was implemented. The outcomes were analyzed using response surface methodology (RSM) using LC-MS data; chosen for its advantages of shorter processing time, predictive response capabilities, and cost-effectiveness. This study thus contributes novel insights into the targeted rutin extraction process from *M. oleifera* leaves using the ethanol-salt UA-ATPE method.

5.3. Materials and Methods

5.3.1. Chemicals and reagents

Ammonium sulphate ($(NH_4)_2SO_4$), magnesium sulphate ($MgSO_4$), sodium chloride (NaCl), and rutin hydrate were purchased from Sigma-Aldrich (Johannesburg, South Africa). Methanol (99% CP) and ethanol (99% CP) were purchased from Associated Chemical Enterprises (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) obtained from a Direct-Q 5UV distiller (Massachusetts, United States of America) was used for the preparation of the salt solutions. The extractions were performed on Scientec Ultrasonic Cleaner and a DIAB MX-RL-Pro dragon shaker. A reverse phase Shim-pack Velox C18, 2.1 x 100 mm, 2.7 µm with a serial number 227-32009-03 (Columbia, USA) was used to achieve chromatographic separation of the metabolites in the extracts. The UPLC was connected to a Shimadzu 9030 LC-qTOF-MS detector (Shimadzu, Kyoto). Methanol and formic acid, which were purchased from Romil Pure Chemistry (Cambridge, UK), are the solvents that were used for the chromatographic runs.

5.3.2. Chromatographic and mass spectrometry conditions

Rutin was separated using a Shimpack C18, 2.1 x 100 mm, 2.7 µm column from Shimadzu (Honeydew, South Africa). The column was maintained at 40 ºC at a flow rate of 0.4 $mL.min^{-1}$ and the injection volume was 3 µL was used. Mobile phase A was 0.1% formic acid in ultrahigh purity water (v/v) and mobile phase B was 0.1% (v/v) formic acid in methanol.

An UPLC-QTOF-MS 9030 mass spectrometer (Shimadzu, Japan) was used for all mass spectral measurements. The mass spectrometer was equipped with an electrospray interface (ESI) operating in positive mode. ESI parameters were optimized for rutin by direct infusion of standard solutions into the mass spectrometer. The mass spectrometer was operated in the multi reaction monitoring (MRM) mode to confirm the identity of rutin. This was achieved by selecting specific precursor to product ion transitions for each rutin based on MRM transitions. High-purity nitrogen (N_2) was used as the nebulizing and drying gas. The optimum parameters were as follows: drying gas temperature, 250 °C; drying gas flow, 10 L min^{-1} and collision energy, 30 – 60 eV. For the chromatographic separation a Shimadzu 9030 LC instrument (Shimadzu, Japan) was used. The instrument consisted of an autosampler, thermostated column

compartment and a binary pump. Labsolutions software was used to control the LC-MS/MS instrument and for data acquisition and the mass range was *m/z* 100-1000.

5.3.3. Preparation of ATPE

A salt (ammonium sulphate, magnesium sulphate, and sodium chloride) (8 g) was dissolved in water (32 mL) to form a 25% saturated solution. The saturated salt solution (20 mL) was mixed with *M. oleifera* powder (1 g). The resulting mixture was placed in an ultrasonic bath and the reaction was performed at various temperatures and times. After the elapsed time, ethanol (20 mL) was added to the mixture resulting in an aqueous two-phase system. The ethanol phase was filtered and analysed using UHPLC-qTOF-MS. The extractions were performed in triplicates.

5.3.4. Preparation of rutin standards

The stock standard solution of rutin was prepared in methanol at a concentration of 500 mg L^{-1}. Seven standard working solutions (5 - 3120 µg L^{-1}) were prepared from the stock solution through serial dilutions. The rutin standards were quantified based on scheduled multiple reaction monitoring (MRM). The regression equation for the calibration curve was y = 192.78x + 12795 and an R^2 of 0.996.

5.3.5. Statistical analysis

The central composite design response surface model (CCD RSM) was fitted to experimental data to obtain the relationship between factors and optimize the response of Z (rutin concentration) in relation to A (time), B (temperature) using Design expert 13. A Two-level full factorial CCD was designed, a total of 9 experimental runs (including 3 repetitions) were designed for all the salts. This included numerical factors such as time (10, 20, 22.5, 25 and 35 min) and temperature (25, 39, 42.5 and 46 ºC). Model parameters and model significance were determined at $p < 0.05$. The fitness of the models were determined by evaluating the coefficient of regression (R^2) obtained from the analysis of variance (ANOVA). The model fit generated the response surface that defined the behavior of the response variable (i.e., concentration of rutin).

5.4. Results and discussion

CCD was performed to determine the optimum ATPE conditions using an ultrasonic bath for the extraction of rutin from *M. oleifera* leaves. The results for the ethanol/$MgSO_4$, ethanol/$(NH_4)_2SO_4$, and ethanol/NaCl ATPE systems are shown in Table 5.1. The highest concentration of rutin extracted was 240 µg L^{-1} with conditions 25 ºC for 22.5 min using the ethanol/NaCl ATPE system. According to Table 5.1, in the ethanol/$MgSO_4$ system the amount of rutin extracted increased with an increase in temperature and decreased with an increase in time. However, increasing the temperature to 46 ºC resulted in a decrease in the amount of rutin extracted. In the ethanol/$(NH_4)_2SO_4$ system, it was observed that an increase in temperature and time resulted in a decrease in the amount of rutin extracted. Therefore, the highest concentration of rutin was extracted at 25 ºC with a 22.5 minutes extraction time.

The use of the salts in the UA-ATPE is to allow partitioning of the ethanol from water, wherein the ethanol phase is enriched with rutin. The addition of salts in this extraction method minimizes the water solubility of ethanol. This is then referred to as the salting-out effect. Salting-out is a pre-concentration method and is dependent on the type of salt involved (Mokgehle *et al*., 2021). Introducing a salt in the aqueous phase resulted in the salting out effect wherein the bond between the rutin and water was disturbed. This reduced the solubility of rutin in water and thus causing its precipitation from the aqueous phase. The precipitation of rutin from the aqueous phase is dependent on the type of salt used. Upon introduction of ethanol, the precipitated rutin moved from the aqueous phase to the ethanol phase (Mokgehle *et al*., 2021; Xi *et al*., 2023). According to Table 5.1, it was observed that the ethanol/NaCl UA-ATPE system was the best performing system because it extracted the highest concentration of rutin from the *M. oleifera* leaves extract. Such an observation could be attributed to this salting-out effect. It can thus be concluded that NaCl formed stronger bonds with the water molecules than $MgSO_4$ and $(NH_4)_2SO_4$ which resulted in a higher recovery of rutin from the aqueous phase. NaCl has also been reported to be the most common salt added to an ATPE system (Ng *et al*., 2021).

Furthermore, in a study by Shiran *et al*. (2020), the effect of cations in the salting-out effect was studied. In their study, a polymer-salt ATPE system was formed with polyethylene glycol (PEG) as the polymer and Na_2SO_4 and $MgSO_4$ as the salts. It was observed that Na_2SO_4 had a more salting-out effect than $MgSO_4$, and this observation was attributed to the cation effect. It was observed that the salt with multiple valence cations (Mg^{2+}) interacted with the ether oxygen of PEG which resulted in salting-in. This led to the conclusion that the more

valence of the cations, the more the salting-in ability. Therefore, the Na^+ cation led to a more salting-out effect than the Mg^{2+} cation.

However, according to the Hofmeister series, the salting-out ability is dependent on the cations and anions. For anions, the salting out ability of $SO_4^{2-} > Cl^-$, while for cations it is $NH_4^+ > Na^+ > Mg^{2+}$ (Hyde *et al*., 2017). Based on these trends, it would therefore be expected that the ethanol/$(NH_4)_2SO_4$ ATPE system be the best performing of the three, which was not the case herein. It should be noted that $(NH_4)_2SO_4$ is more acidic than $MgSO_4$ and NaCl. Rutin, on the other hand, is more stable in acidic medium (Ran *et al*., 2017). Therefore, during the salting-out process, less rutin is partitioned to the ethanol phase because of its high affinity for the acidic $(NH_4)_2SO_4$ aqueous phase. This then results in a low concentration of rutin in the ethanol phase. Another possible explanation is the hydration energy of the cations. Hydration energy increases with an increase in the cation's charge, meaning Mg^{2+} would have the highest hydration energy among the three cations. Based on this study's observations, it can be concluded that higher hydration energy corresponds to lower extraction efficiency. However, NH_4^+ and Na^+ have the same charge, so the ion size must be considered. NH_4^+ is larger than Na^+, and hydration energy decreases with increasing ionic size. According to a study by Parsons et al. (2011), the Hofmeister series can be reversed when ionic size is taken into account. Therefore, it is possible that the Hofmeister series was reversed in this study, resulting in the order $Na^+ > NH_4^+$ and consequently the high extraction efficiency of the NaCl/ethanol system.

Table 5.1: List of CCD experiments for ATPE optimization and concentration of rutin extracted using different salts.

	Factors		Rutin concentration extracted (µg L^{-1})			
Run order	Time (min)	Temperature (°C)	$MgSO_4$	$(NH_4)_2SO_4$	NaCl	Standard order
1	25	46	169.09	157.25	204.41	3
2	25	39	165.17	154.43	240.99	2
3	22.5	42.5	168.68	154.94	233.77	9
4	10.0	42.5	188.74	162.79	230.86	4
5	22.5	25.0	145.63	170.30	240.00	6
6	22.5	42.5	173.37	152.29	234.26	8
7	35	42.5	150.16	152.48	216.60	5
8	22.5	42.5	141.45	159.36	220.32	7
9	20	39.0	173.09	157.37	239.95	1

5.4.1. Statistical data

The regression models were obtained by fitting the model obtained from the different salts to the experimental data. The adequacy of the models was assessed by model statistics and the results are shown in Tables 5.2, 5.3, and 5.4 for the ethanol/$MgSO_4$, ethanol/$(NH_4)_2SO_4$, and ethanol/NaCl systems, respectively.

The model significance was evaluated by probability values (p-values), while the evidence of goodness of fit was assessed by R^2 values for the experimental values. From the results, a higher Fisher's F-test value and lower p-values indicate the relative significance of each term. The suitability of the models was assessed through the lack of fit. If the lack of fit values are non-significant ($p > 0.05$), then it implies that the model is valid, reliable and precise. The lack of fit values for the ethanol/$MgSO_4$, ethanol/$(NH_4)_2SO_4$, and ethanol/NaCl systems were determined as 0.9392, 0.5596, and 0.7091, respectively. This indicates the validity, reliability, and precision of the models. The fitting of the models revealed that the independent factors, namely ultrasonic temperature and ultrasonic time, exerted an influence on the rutin content in the response.

A model is considered insignificant if it has a p-value greater than 0.500. In this study, the model fitted to the data according to ANOVA for the ethanol/$MgSO_4$ ATPE system was observed to be a linear fit with a p-value of 0.0751, which thus implied that the fit is not significant. According to Table 5.2, both temperature and time were regarded as insignificant terms with p-values of 0.1505 and 0.0518, respectively. These values ($p > 0.05$) imply that the model is not significant and is thus deemed not valid (Shokrollahi *et al*., 2020). The lack of fit F-value was 4.11 which is not significant relative to the pure error (Uzoejinwa *et al*., 2019). The non-significant lack of fit is desirable because the intention is for the model to fit. The goodness of fit, R^2, was found to be 0.5782.

For the ethanol/$(NH_4)_2SO_4$ ATPE system, the model fitted to the data according to ANOVA was observed to be a linear fit (Table 5.3). The p-value was observed to be 0.0188 proving that the model fit is significant. Time was observed to be an insignificant term with a p-value of 0.0848 while temperature had a p-value of 0.0144. This indicated that temperature was a significant term in this model. This model showed a goodness of fit of $R^2 = 0.7339$.

For the ethanol/NaCl ATPE system, the model fitted to the data according to ANOVA was observed to be a quadratic fit (Table 5.4). The p-value was found to be 0.0097 which shows

that the model fit is significant. Temperature appeared to be the significant fit in this model with a p-value of 0.0047 and the B^2 term had a p-value of 0.0124. The goodness of fit for this model was observed to be 0.7865.

Table 5.2: Fit statistics of a linear model based on ANOVA for the ethanol/$MgSO_4$ system.

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	1080.36	2	540.18	4.11	0.0751	not significant
A-Time	770.37	1	770.37	5.86	0.0518	-
B-Temperature	356.67	1	356.67	2.71	0.1505	-
Residual	788.24	6	131.37	-	-	-
Lack of Fit	194.40	4	48.60	0.1637	0.9392	not significant
Pure Error	593.84	2	296.92	-	-	-
Cor Total	1868.60	8	-	-	-	-

Table 5.3: Fit statistics of a linear model based on ANOVA for ethanol/$(NH_4)_2SO_4$ system.

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	192.47	2	96.23	8.28	0.0188	significant
A-Time	49.46	1	49.46	4.25	0.0848	-
B-Temperature	134.94	1	134.94	11.60	0.0144	-
Residual	69.77	6	11.63	-	-	-
Lack of Fit	44.15	4	11.04	0.8617	0.5996	not significant
Pure Error	25.62	2	12.81	-	-	-
Cor Total	262.24	8	-	-	-	-

Table 5.4: Fit statistics of a reduced quadratic model based on ANOVA for ethanol/NaCl system.

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	1000.78	2	500.39	11.05	0.0097	significant
B-Temperature	863.10	1	863.10	19.07	0.0047	-
B^2	563.58	1	563.58	12.45	0.0124	-
Residual	271.62	6	45.27	-	-	-
Lack of Fit	146.48	4	36.62	0.5853	0.7091	not significant
Pure Error	125.13	2	62.57	-	-	-
Cor Total	1272.40	8	-	-	-	-

5.4.2. Identification and quantification of rutin using UHPLC-qTOF-MS

The presence of rutin was confirmed using the UHPLC-qTOF-MS. The confirmation of rutin's presence was reinforced through the analysis of its fragmentation pattern in tandem MS experiments (MS/MS) (Figure 5.1). In this process, rutin undergoes fragmentation, resulting in its flavonol aglycone, quercetin, at *m/z* 301, attributed to the loss of the rutinoside sugar. These results are consistent with those achieved elsewhere (Wojdylo and Nowicka, 2019; Fu *et al*., 2020).

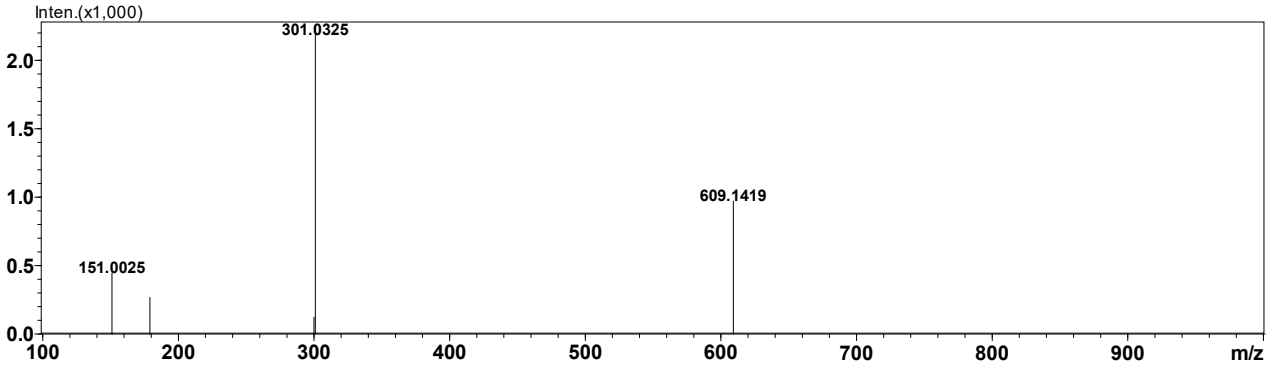

Figure 5.1: Tandem mass spectrum generated from a single ion monitoring of rutin at *m/z* 609.1419 showing the fragmentation pattern characterized by loss of a rutinoside sugar thereby resulting in formation of the quercetin aglycone at *m/z* 301.0325.

5.4.3. Parameter effects and response surface model

Figure 5.2 is the bar chart which shows the influence of ultrasonic temperature and ultrasonic time on the extraction of rutin from *M. oleifera* in comparison to the three ethanol/salt systems. It was noted that an increase in the ultrasonic temperature resulted in a decrease in the concentration of rutin extracted whereas ultrasonic time did not have a significant effect in the concentration of rutin extracted. The decrease in the amount of rutin extracted from the *M. oleifera* leaves extracts with an increase in temperature is rather in contrast with several previous results on the extraction of rutin from different plant materials such as *Satureja montana L*. (Jakovlijevic *et al*., 2020), *Ruta graveolens L.* (Molnar *et al*., 2018), buckwheat (Kim and Lim, 2019), and lemon by-products (Papoutsis *et al*., 2018) using DES, subcritical water (SW), and aqueous ultrasound-assisted extraction (AUAE) methods, respectively. This could be because each plant requires specific extraction conditions for the

optimum recovery of its compounds (Cardona *et al*., 2017; Shafi *et al*., 2019). It is therefore necessary to validate the extraction method for phenolic compounds to avoid enzymatic oxidation which results in loss of phenol function and antioxidant potential (Shafi *et al*., 2019).

In this study, the highest concentration of rutin extracted was at 25 ºC. However, a similar trend was observed by Ali *et al*. (2019) wherein a decrease in the amount of rutin extracted with increasing temperature has been reported in *Lycium barbarum* L. fruits where a temperature of 25 ºC was used as the optimum temperature for the extraction of rutin (Ali *et al*., 2019). The differences in optimal temperatures could be because different polyphenols show different sensitivity to heat treatment depending on their structures. Flavonoids, for example, have been reported to be more sensitive to thermal degradation than phenolic acids. Elevated temperatures usually improve the extraction yield and shortens the extraction time. However, intense temperatures or prolonged exposure to elevated temperatures can cause degradation of the thermally sensitive compounds and thus result in poor extraction yields (Dobrincic *et al*., 2020). The extraction yield of rutin from different plant materials is also dependent on the method of extraction. In this study, the ultrasonic power combined with the increase in temperature are most likely the reason for the observed results. In a review by Dzah *et al*. (2020), it was stated that the effects of ultrasound-assisted extractions and temperature on the extraction yield of polyphenolic compounds were also dependent on the plant material.

In this study it was observed that ultrasonic temperature had a significant effect on the extraction of rutin when using the ethanol/NaCl and ethanol/$(NH_4)_2SO_4$ systems. However, when the ethanol/$MgSO_4$ system was used, time was observed to be a more significant term than temperature. These observations agree with the fit statistics detailed above in Tables 5.2, 5.3, and 5.4.

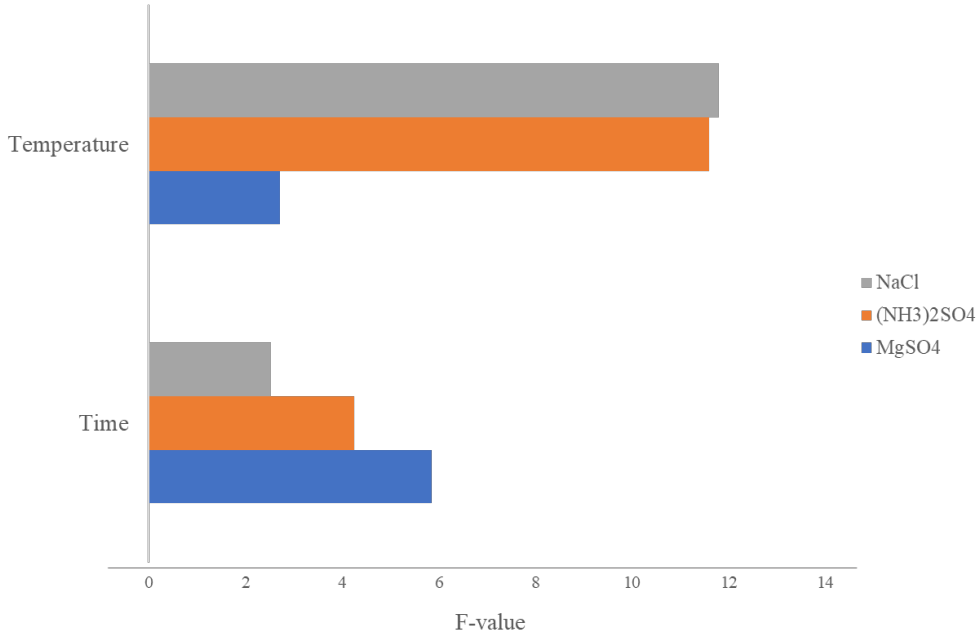

Figure 5.2: Bar chart showing the effects of ultrasonic time and ultrasonic temperature on the extraction of rutin from *M. oleifera* leaves in an ATPE.

The response surface plots in Figure 5.3 show the effect of ultrasonic time and ultrasonic temperature in the extraction of rutin from *M. oleifera* leaves in the ethanol-salt ATPE-systems. Figure 5.3 (a) represents the response surface plot for the ethanol/$MgSO_4$ UA-ATPE system. This plot shows that the concentration of rutin extracted increases with an increase in temperature and decreases with a decrease in time. The ethanol/$(NH_4)_2SO_4$ UA-ATPE system is represented in Figure 5.3 (b) wherein it was observed that the concentration of rutin extracted decreased with an increase in time and temperature. Figure 5.3 (c) represents the ethanol/NaCl UA-ATPE system wherein it was observed that the concentration of rutin decreased with an increase in temperature. It was also observed that time did not have a significant effect on the concentration of rutin extracted. The observations from the response surface plots for the three ethanol/salt UA-ATPE systems represent the trend observed in Table 5.1.

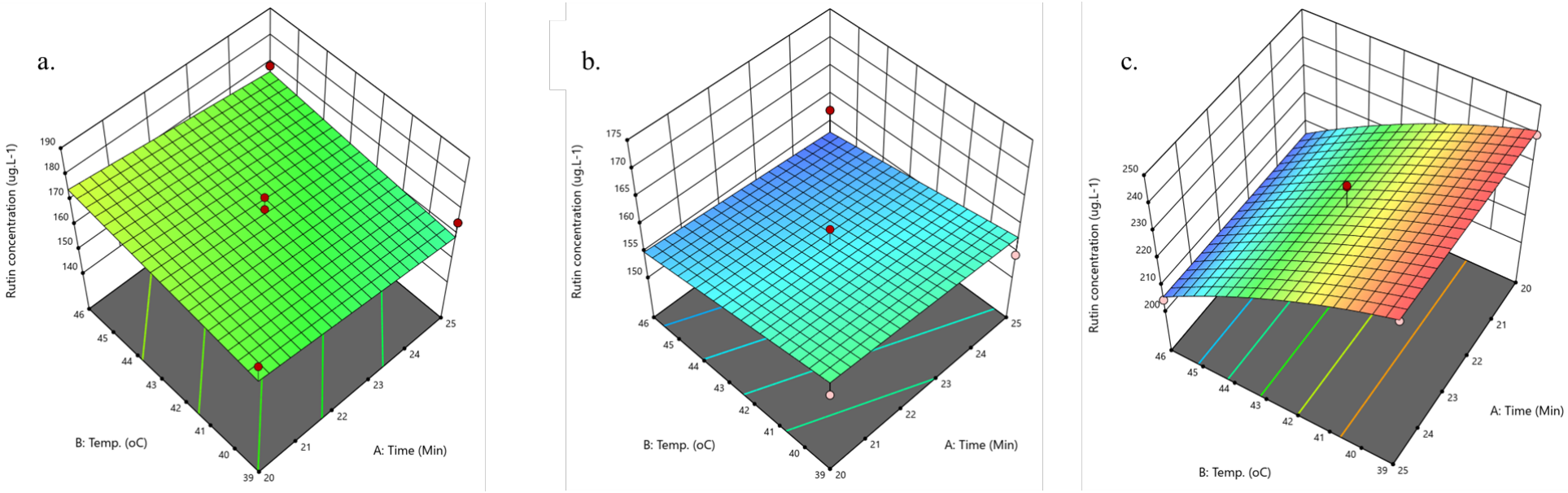

Figure 5.3: Response surface plots on the effect of ultrasonic time and ultrasonic temperature on the extraction of rutin from *M. oleifera* leaves for the a) ethanol/$MgSO_4$, b) ethanol/$(NH_4)_2SO_4$, and c) ethanol/NaCl ATPE systems

5.5. Conclusions

The extraction of rutin from *Moringa oleifera* leaves extracts through three ethanol-salt UA-ATPE systems was performed. The salts investigated in the ethanol/salt ATPE systems were NaCl, $MgSO_4$, and $(NH_4)_2SO_4$. Ultrasonic temperature and ultrasonic time were optimized and the maximum concentration of rutin was extracted through the ethanol/NaCl UA-ATPE system at 25 ºC. It was observed in this study that an increase in ultrasonic temperature and ultrasonic time resulted in a decrease in the extraction of rutin from the *M. oleifera* leaves extracts. However, based on the ANOVA statistics it was noted that the ultrasonic temperature was a significant factor ($p < 0.05$) and the ultrasonic time was an insignificant factor ($p > 0.05$) for the ethanol/NaCl and ethanol/$(NH_4)_2SO_4$ UA-ATPE systems. However, for the ethanol/$MgSO_4$ UA-ATPE system, time had a more significant effect than temperature on the extraction of rutin. The response surface models confirmed the effect of temperature and time on the extraction of rutin using the three ethanol/salt UA-ATPE systems. From this study, it was observed that the extraction of rutin from *M. oleifera* leaves is favourable at low temperatures. This could be because rutin is sensitive to high temperatures and therefore degrades at these elevated temperatures resulting in low extraction yield. Owing to its multiple bio-activites, the extraction of rutin is therefore imperative and because of its presence in many herbal medicines, the findings of the current study can therefore form a basis on which traditional healers can use to optimize its extraction during their preparation, instead of using just pure water, as is the case currently. Furthermore, it was also noted that the settings of the central composite experimental design were very narrow, thus resulting in narrow RSM models. It is therefore imperative that, in future studies, other axial and central points be included in the design to maximize the prediction.

5.6. References

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Experimental Chapter 6

This chapter is focused on the work titled ‘Extraction of rutin from *M. oleifera* by pipette-tip micro-solid phase extraction using activated hollow carbon nanospheres as sorbents’.

6.1. Abstract

Herein, a micro-solid phase extraction (µSPE) method was developed using a pipette tip for rutin extraction, employing activated hollow carbon nanospheres (HCNSs) as the sorbent. Characterization of the activated carbon nanospheres through TGA, FTIR, and SEM analysis confirmed the success of the activation process. Our study demonstrated the efficacy of PT-µSPE in rutin extraction under pH 2 conditions with a standard concentration of 2 mg L^{-1}. The optimal mass of HCNSs was found to be 2 mg, and a loading volume of 500 µL resulted in the maximum recovery of rutin, as indicated by the study results. Propan-2-ol was the best elution solvent with 15 aspirating/dispensing cycles. The linear regression (R^2) for the calibration curve was found to be 0.9991 and the LOD and LOQ values were 0.604 and 1.830 mg L^{-1}, respectively. The applicability of the method was demonstrated by extracting rutin from a complex *Moringa oleifera* leaf extract with the relative standard deviation (RSD) of 3.26%, thereby validating this method as feasible for extraction of useful bioactive compounds from complex plant samples.

Keywords: pipette tip micro-solid phase extraction (PT-µSPE); hollow carbon nanospheres; rutin; *Moringa oleifera*; sorbents

6.2. Introduction

Conventional methods of extraction have proven to make use of large amounts of reagents and solvents which generate large amount of waste and have long analysis times, resulting in undesirable consequences to both humans and their environment (Gbashi *et al*., 2016; Belwal *et al*., 2020; Fomo *et al*., 2020). These drawbacks have resulted in the need for the development of green sample preparation techniques to replace the conventional methods with the simpler and environmentally friendly techniques ((Escobar-Ananz and Ramos, 2015; Moliner-Martinez *et al*., 2015; Pollini *et al*., 2021). Environmentally friendly extraction methods such as microwave-assisted extraction (MAE) (Bagade *et al*., 2021), ultrasound assisted extraction (UAE) (Kumat *et al*., 2021), supercritical fluid extraction (SFE) (Uwineza and Waskiewicz, 2020), and pressurized liquid extraction (PLE) (Garcia *et al*., 2021) have been used for the extraction of bioactive compounds. The extraction methods facilitated by these advancements eliminate or minimize the use of harmful solvents and reduce extraction time. They improve the efficiency of extraction, resulting in higher yields and enhanced extract quality (Fomo *et al*., 2020; Ummat *et al*., 2021).

Micro-extraction techniques make use of a small volume of the extracting solvents relative to the volume of the sample and thus implying high enrichment factors (Fusari *et al*., 2019; Gutierrez-Serpa *et al*., 2021). Miniaturized techniques are of advantage because they provide a straightforward procedure, faster analysis, higher extraction performance, and reduced amount of sample required (Soares da Silva Burato *et al*., 2019; Bouvarel *et al*., 2020). These techniques also use recent advances in the synthesis of new sorbent materials and the use of greener extraction solvents (Soares da Silva Burato *et al*., 2019; Agrawal *et al*., 2021). Micro-extraction techniques can be divided into three broad groups depending on the type of the extracting phase, i.e. solid-phase micro-extraction (SPME) (Jalili *et al*., 2020), liquid-phase micro-extraction (LPME) (Yamini *et al*., 2019), and membrane micro-extraction (Moreda-pinero and Moreda-pinero, 2015). SPME is a modern and non-exhaustive sample preparation technique which integrates sampling, pre-concentration, and extraction in a single step and the analytes can be directly introduced to analytical instruments like chromatographic systems (Jalili *et al*., 2020; Feng *et al*., 2021).

Pipette tip micro solid phase extraction (PT-µSPE) represents a novel form of miniaturized SPE. It serves as a straightforward, portable, and swift sample pre-treatment method applicable to proteins, peptides, and drugs (Sun *et al*., 2019; Hashemi *et al*., 2019a; Rezaie *et al*., 2022). In this

technique, a microscale amount of a sorbent is packed inside a pipette tip with the aim of reducing volume of solvents and samples (Amini *et al*., 2020; Sun *et al*., 2020; Kandeh *et al*., 2021). The extraction process begins by drawing the sample solution into the tip and then dispensing it back into the sample tube. By replicating the aspirating/dispensing cycles, the extraction procedure reaches equilibrium, and this makes the procedure controllable, stable, and smooth (Seidi *et al*., 2019; Sun *et al*., 2019; Dugheri *et al*., 2020; Tsai *et al*., 2021). An appropriate solvent is then used to desorb the analytes. To achieve quantitative desorption, this step may be repeated several times (Seidi *et al*., 2019). It has been used for the determination and extraction of analytes such as pharmaceuticals, bioanalytical molecules, proteins, and metals in various matrices (Kahkha *et al*., 2019). Sorbents such as multi-walled carbon nanotubes (MWCNTs), silica, molecularly imprinted polymers (MIPs), graphene oxide (GO), and carbonaceous materials have been used as sorbents (Du *et al*., 2019a; Wang *et al*., 2019; Sun *et al*., 2021). Carbon nanotubes have been used as SPE sorbents for the extraction of drugs (Othman *et al*., 2020), pesticides or natural compounds in different media such as biological fluids (Fresco-Cala *et al*., 2018), environment (Aguinaga Martinez *et al*., 2020), plants or animal organs (Azzouz *et al*., 2018; Hashemi *et al*., 2019b). Carbonaceous materials such as graphene, carbon nanotubes and porous carbons have been thoroughly investigated. These materials are advantageous because of their low density, high porosity, and large surface area and they have thus attracted many scientific and technological interests. They have thus been applied in various areas such as catalyst supports, adsorption, separation systems, and nanoreactors (Peng *et al*., 2019).

In this study, PT-µSPE was used as the extraction method for extracting a highly bio-available flavonoid, rutin, from a multipurpose *Moringa oleifera* plant. Activated hollow carbon nanospheres (HCNSs) were used as sorbent material. This is the first study to report on the extraction of rutin from *M. oleifera* using PT-µSPE.

6.3. Materials and methods

6.3.1. Chemicals and reagents

Rutin (anhydrous ≥ 99% purity) was purchased from Sigma-Aldrich (Modderfontein, South Africa), sodium hydroxide pellets and hydrochloric acid (32%) were purchased from Merck

(Modderfontein, South Africa), potassium chloride and propan-2-ol was purchased from Associated Chemical Enterprises (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) was used for the preparation of the standard solutions. Methanol and acetonitrile were purchased from Fluka (Steinheim, Germany).

A Thermo Orion Star A121 Portable pH Meter was used for pH measurements and a DIAB MX-RL-Pro dragon shaker was also used. A NanoPhotometer was used to measure the concentration of the samples.

6.3.2. Instruments for characterization

FTIR spectroscopy analysis was done using Bruker Tensor 27 Fourier Transform Infrared spectrometer. The absorption measurements of electromagnetic radiation were between 600 to 4000 cm^{-1}.

The surface morphology of the samples was performed using SEM on a ZEISS SIGMA 03-39 FESEM operating at 20 kV. A small amount of the sample was spread on carbon tape and coated with a layer of carbon and gold/palladium alloy before analysis.

Thermal analysis was done using Perkin Elmer TGA 6000 thermogravimetric analyser. A mass of approximately ± 9 mg inside a ceramic pan was placed inside the instrument furnace and heated in the temperature range of 35 to 900 °C at a rate of 10 °C min^{-1} under a flow of air (20 ml min^{-1}) gas while monitoring the change in mass.

6.3.3. Preparation of stock and real solutions

A working stock solution (5 mg L^{-1}) was prepared from a standard stock solution of 500 mg L^{-1} and made to the mark using methanol. Working standard solutions of 0.5, 1, 1.5 and 2 mg L^{-1} were prepared from the stock solution and made to the mark using the same diluent. A series of calibration standard solutions (0.7 – 50 mg L^{-1}) were prepared by appropriate dilutions of the standard stock solution. Extractions from *Moringa oleifera* were performed using 80% MeOH. The supernatant solution was stored in a refrigerator at 4 °C when not in use.

6.3.4. Synthesis of polystyrene spheres (PSs)

The PSs were prepared by adding 0.2 g polyvinylpyrrolidone (PVP), 50 mL water, and 200 mL ethanol in a 500 mL round-bottom flask. After all the PVP had dissolved, styrene (16 mL) was added to the mixture and stirred for 15 min. A solution of potassium persulfate (0.3 g) dissolved in distilled water (20 mL) was added to the flask while stirring. The mixture was then refluxed at 80 °C while stirring for 24 h. The final product was filtered by centrifuge at 18000 rpm for 15 min and washed repeatedly with ethanol. The resulting product was dried at 60 °C for 4 h, crushed into a fine powder, and denoted as PSs.

6.3.5. Synthesis of hollow carbon nanospheres (HCNSs)

The HCSs were synthesized by coating the PSs template with resorcinol-formaldehyde (RF) polymer using the following procedure: PSs (2 g) were dispersed in a mixture of ethanol (100 mL), ammonia solution (25 %, 3 mL), and distilled water (50 mL) in a 500 mL bottle by ultrasonication. A solution containing formaldehyde (37%, 3 mL), resorcinol (1 g), CTAB (1.5 g), and ethanol (75 mL) was added slowly to the latter and the resulting solution was stirred for 12 h at room temperature. This was followed by hydrothermal treatment in an oil bath at 80 °C for 24 h. A layer of RF polymer was formed around the PSs. The resulting product was filtered and washed several times with ethanol/water and dried in the oven at 60 °C for 12 h. The sample was then crushed into a fine powder and denoted as PSs@RF. The dry composite (PSs@RF) was then heated inside a horizontal CVD furnace in a one-step process to remove the template and carbonize the material. This was achieved by heating the composites under a controlled nitrogen flow (80 ml/min) at a heating rate of 5 °C min^{-1} to 350 °C and kept isothermal for 1 h to decompose the PSs followed by carbonization of the RF polymer to form HCSs at 600 °C for 2 h. The resulting product was denoted as HCNSs.

6.3.6. Activation of hollow carbon nanospheres (HCNSs) with NaOH

A modified method by Song *et al*. (2011) was used. To activate the HCNSs for extraction of rutin, 120 mg of HCNSs powder was dispersed in NaOH (0.5 M, 100 mL) aqueous solution. The mixture was stirred on a magnetic stirrer for 1 h at room temperature. The product was filtered and washed

with a large amount of deionized water to remove residual NaOH until the pH of the filtrate was near neutral. The activated HCNSs samples were dried in an oven at 60 °C for 12 h.

6.3.7. Miniaturized pipette tip preparation

A modified method by Tavengwa *et al*. (2016a) was used. A fixed mass of glass wool (5 mg) was pre-loaded into each polypropylene pipette tip (1000 µL). The activated HCNSs (≈ 2 mg) were added into the pre-loaded polypropylene pipette tip. An aliquot rutin standard solution (200 µL) dissolved in methanol was aspirated onto the HCNSs and dispensed back into the same sample tube.

6.3.8. Optimization of the extraction conditions

Parameters capable of influencing the recovery of rutin from the standard methanol solutions were investigated using HCNSs PT-µSPE. The effect of sample pH (adjusted using 0.1 M HCl and 0.1 M NaOH), number of loading cycles, concentration of sample, loading volume, mass of sorbent and choice of eluent were investigated and optimized. All the experiments were done in triplicate.

6.3.9. Point of zero charge (pHpzc)

For the determination of point of zero charge (pHpzc), a 0.01 M KCl solution was prepared and its initial pH (pH_i) was adjusted to between 2 and 11 by the addition of 0.1 M HCl and 0.1 M NaOH in different test tubes. A volume of 10 mL of the pH adjusted KCl solution and 2 mg of HCNSs and HCNS-OH were mixed together at room temperature for 25 h. After the elapsed time, the solution was separated from the CNS and CNS-OH by centrifugation. After decanting, the final pH (pH_f) of the solution was measured.

6.3.10. HPLC Chromatographic method conditions

An HPLC-PDA (LC-2030C, Shimadzu Corporation, Kyoto, Japan) fitted with a Shim-pack GIST column (150 mm x 4.6 mm, particle size 5.0 µm) was used for the analysis. The column oven was set at a constant temperature of 50 °C. A 20 µL injection volume was employed, and the analytes underwent analytical separation over a 16-minute binary gradient at a flow rate of 0.8 mL min^{-1}. The mobile phase was a binary solvent mixture consisting of 0.1% formic acid in water (Eluent

A) and 0.1% formic acid in methanol (Eluent B). The concentration of solvent B was gradually increased between 3 to 16 minutes to achieve optimal separation of rutin in the samples. Briefly, eluent B was held at 10% from 0 to 3 minutes, thereafter, increased gradually from 10 to 60% between 3 and 6 minutes, and further elevated to 90% between 6 and 7 minutes. Eluent B was then maintained isocratically at 90% between 7 and 10 minutes. The concentration of B was returned to 10% in 4 min and the column was allowed to re-equilibrate at 10% until 16 minutes, in preparation for the next run. Chromatography elution was monitored using a PDA detector operating at 190-700 nm and rutin was specifically monitored at its maximum absorption peak at 359 nm.

6.4. Results and discussion

6.4.1. Characterization of the sorbent materials

6.4.1.1.Thermogravimetric analysis

The thermal stability of the raw HCNSs and the activated HCNSs were analysed using thermogravimetric analysis (TGA). Figure 6.1 displays the TGA/DTG profiles of the HCNS samples. The continuous weight loss denotes the breaking of the chemical bonds on the surface of the HCNSs with an increase in temperature. The onset decomposition temperature of the raw HCNSs occurred at *ca.* 443 °C whereas the activated HCNSs began to decompose at *ca.* 423 °C (Fig. 6.1a). This implies that the raw HCNSs were more thermally stable than the activated HCNSs. The lower thermal stability of the activated HCNSs can be associated with the defects in the carbon framework induced by NaOH treatment. The peak observed at *ca.* 208 °C in the derivative profile of the activated HCNSs can be related to the dehydration and decarboxylation of the carboxylic acid functional groups (Fig. 6.1b). A similar observation was reported by Cervantes-Uc *et al*. (2006) in a polymethacrylate polymer containing carboxylic groups wherein the dehydration of the carboxylic acid functional groups was reported to be at 250°C. Another study by Mohamed *et al*. (2020) observed a decomposition at 250 °C in the TGA thermogram of nitrogen-doped microporous carbons from two monomers that contained carboxylic groups and associated it to the partial decarboxylation of the COOH groups. The second peak observed at ca. 353 °C in the DTG profile of the activated HCNSs could be attributed to the removal of the oxygen functional groups. The raw and activated HCNSs exhibited a similar maximum weight loss at 550 °C and 554 °C, respectively, associated with the decomposition of the carbon material. Moreover, complete decomposition of the carbon core was observed at temperatures > 650 °C with no

residues noted at 900 °C, which signified the absence of impurities in the samples. A similar trend was observed by Yan *et al*. (2015) wherein a strong weight loss of the HCNSs was observed at a temperature range of 200 °C to 700 °C. On the other hand, Tavengwa *et al*. (2016) observed a similar trend wherein the oxidized carbon nanofibers (CNFs) were less stable than the raw CNFs. This observation was attributed to the disorder caused by the presence of the hydroxyl functional groups.

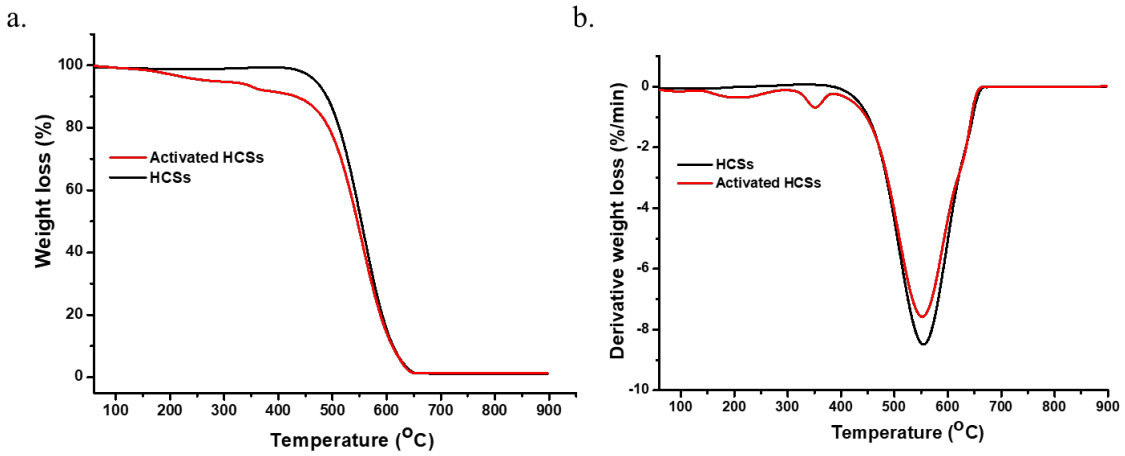

Figure 6.1: The TGA (a.) and DTG (b.) profiles of the raw and activated HCNSs.

6.4.1.2. Functional group analysis

The FTIR spectra of raw HCNS and the activated HCNS are shown in Figure 6.2. The surface of the raw HCNS is observed to not contain any functional groups as it is a pure carbon material. Upon activation of the HCNS, new functional groups were observed in the spectrum as seen in Figure 6.2. The FTIR spectrum for the activated HCNS show a C=O stretching vibration of the carbonyl group at 1610 cm^{-1}, a stretching vibration of a deprotonated carboxyl group, $-COO^-$, at 1324 cm^{-1}, OH bending vibrations in C-OH at 1164 cm^{-1}, and a C-OH stretching at 1052 cm^{-1}. A broad O-H stretching vibration in the range 3000 – 3700 cm^{-1}, which is due to the enrichment of the hydroxyl groups on the surface of HCNS after NaOH activation, is also observed. In the current study, these observations confirmed that the surface of the HCNS was successfully activated by NaOH. A similar observation of the functional groups on the surface of the HCNSs was reported by Song *et al*. (2011) after activating the HCNSs with NaOH.

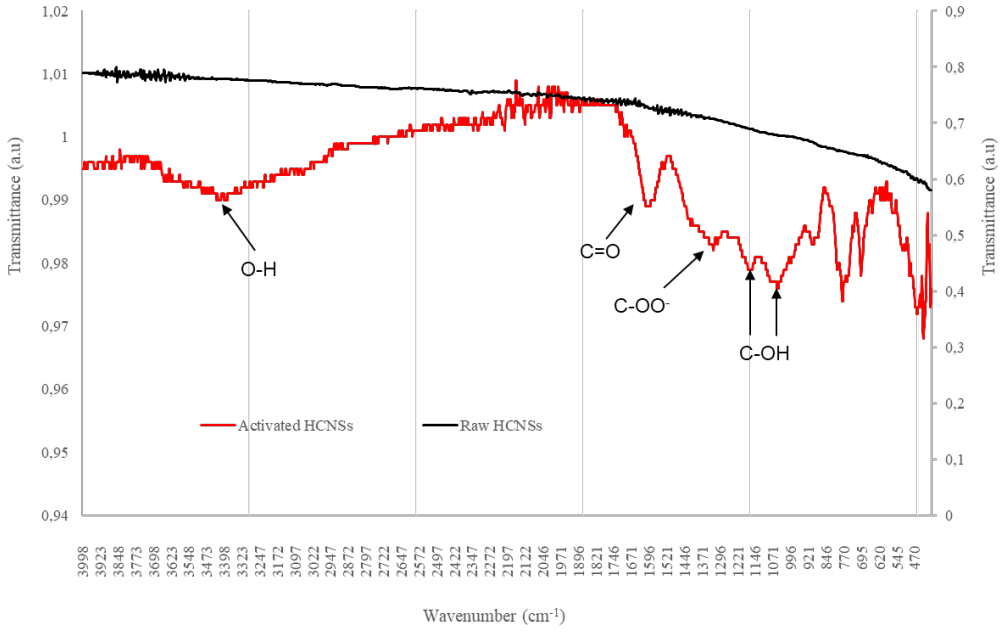

Figure 6.2: FTIR spectra of the raw HCNS and the NaOH activated HCNSs.

6.4.1.3. Scanning electron microscopy

SEM images (Figure 6.3 (a)) shows the raw HCNSs which are spherical in shape and are also hollow. The spheres are grouped together due to the reactive bonds on their surface. This observation is similar to what was observed by Nieto-Marquez *et al*. (2011). In their study they observed that CNSs appeared as a conglomeration of spherical bodies, and this was attributed to the presence of the reactive bonds on their surface which provides them with a high surface reactivity. Figure 6.3 (b) shows the SEM image of the activated HCNSs. The activation of the HCNSs using NaOH increases the presence of the reactive bonds on the surface of the spheres and thereby providing them with a higher surface reactivity. This, therefore, means that the spherical bodies will coalesce to form bigger conglomerate. This is what was observed in this study which confirms the activation of the surface of the HCNSs.

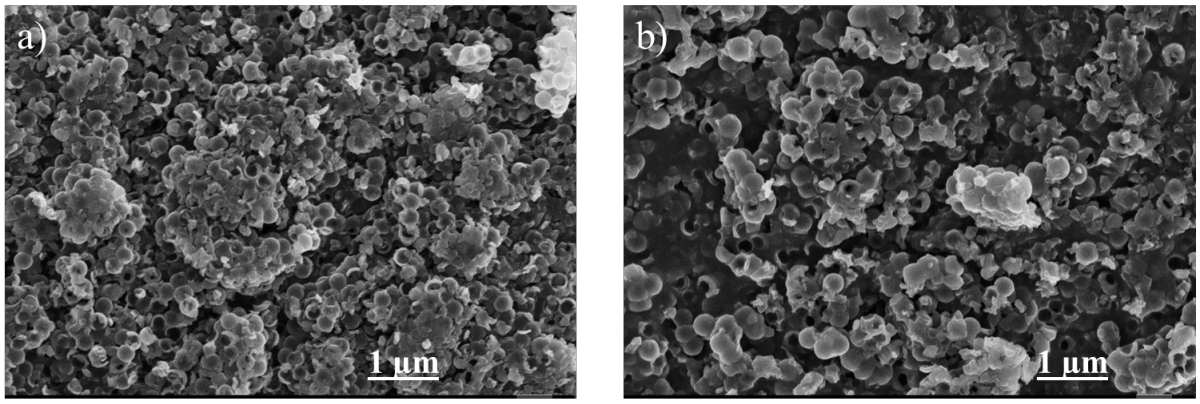

Figure 6.3: SEM images of a) raw HCNSs and b) activated HCNSs.

6.4.2. Effect of aspirating/dispensing cycles

The enrichment of rutin was investigated by optimizing the loading cycles from 5 to 40. The number of aspirating/dispensing cycles was found to be critical for the enrichment of rutin using PT-µSPE, and the maximum amount of rutin extracted was observed after 15 aspirating/dispensing cycles (Figure 6.4). Increasing the number of cycles above 15 resulted in a decrease in the amount of rutin enriched on the sorbent bed. This observation could be because as the number of cycles increase, rutin is desorbed from the sorbent bed and eluted with the loading solvent due to the sheer stress exerted with increasing pipetting cycles. Therefore, throughout the experiment 15 aspirating/dispensing cycles were used. A reduction in the extraction of rutin that was observed after 15 aspirating/dispensing cycles could be probably due to the elution of the analyte during the dispensing action which was undesirable on the loading stage. A similar trend was observed by Tavengwa *et al*. (2016b). Abbaszadehbezi *et al*. (2022) concluded that at a high number of aspirating/dispensing cycles, the back extraction of analytes from the sorbent bed to the sample solution might occur, which causes a decrease in the recovery of analyte.

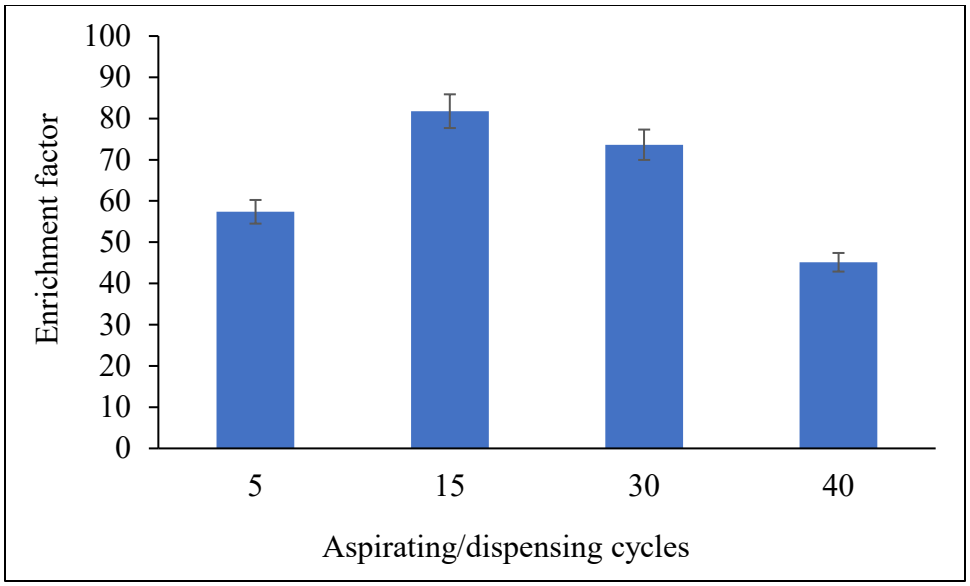

Figure 6.4: Effect of number of aspirating/dispensing cycles on the performance of PT-µSPE. Extraction conditions: initial concentration of rutin = 0.5 mg L^{-1}, loading volume = 1000 µL, elution solvent = MeOH, sample pH = 6, mass of sorbent = 2 mg (n = 3, SD).

6.4.3. Effect of eluting solvent

The elution solvent was also found to have a significant role in the enrichment of rutin in PT-µSPE (Figure 6.5). The choice of the eluting solvent should be able to elute the most amount of rutin from the pipette tip and is hence available for analysis. Herein, three polar organic solvents were investigated; MeOH, ACN and *i*-PrOH. Figure 6.5 shows that propan-2-ol gave the highest desorption ability of rutin and was hence used as the eluting solvent in subsequent experiments. Dramou *et al*. (2019) attributed that the ability of an eluent to desorb an analyte is due to the chemical interactions between the analyte and the sorbent. Rutin is a very polar compound due to the multiple OH groups and the attached disaccharide, and, as such, it could be expected that the more polar solvent (in this case MeOH) should have the highest desorption ability. According to a study by Mukherjee *et al*. (2022), rutin and other flavonoids were detected in methanol, a polar solvent, when compared to other solvents such as n-hexane, chloroform, ethyl acetate, acetone and even water. In the present study, MeOH was employed in the adsorption experiment, suggesting that certain analytes may have displayed a stronger affinity for the solvent than for the sorbent, leading to their elution with the solvent. Consequently, only a limited amount of the analyte

remained on the sorbent bed, resulting in a minimal quantity being desorbed in the desorption studies.

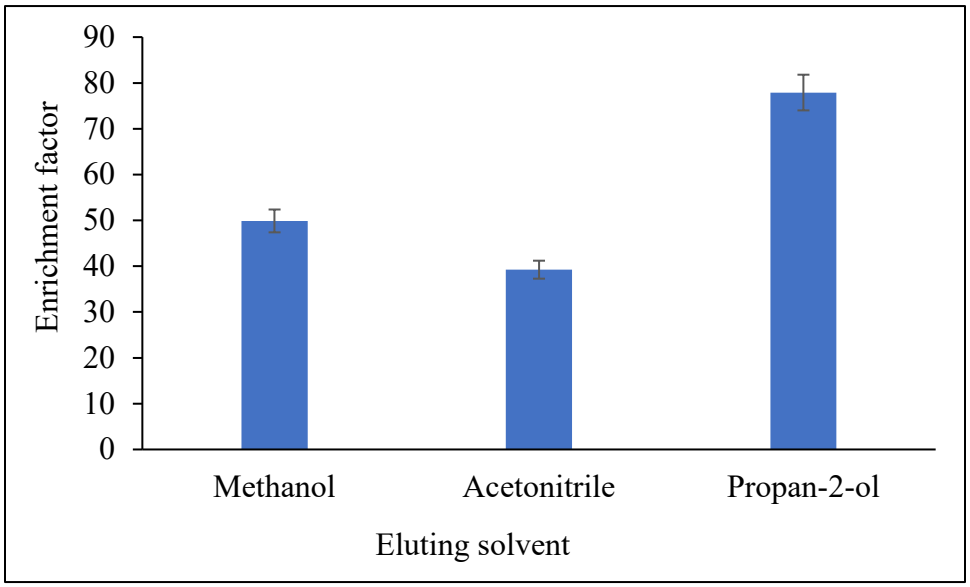

Figure 6.5: Effect of eluting solvent on the performance of PT-µSPE. Extraction conditions: initial concentration of rutin = 0.5 mg L^{-1}, loading volume = 1000 µL, aspirating/dispensing cycles = 15 cycles, sample pH = 6, mass of sorbent = 2 mg (n =3, SD).

6.4.4. Effect of concentration of standard rutin

The effect of the concentration of rutin was also investigated to determine the concentration that aids the highest enrichment. The investigated concentrations were 0.5, 1, 1.5 and 2 mg L^{-1}. As depicted in Figure 6.6, when using a concentration of 2 mg L^{-1}, maximum amount of rutin was enriched. This then implied that a higher concentration of the standard analyte aids a higher enrichment of the analyte because there are more analyte ions to be adsorbed. As per Rais *et al*. (2021), when the analyte ions interact with the sorbent material, an escalation in their concentration leads to the saturation of binding sites. Initially, these binding sites are open for sorption, meaning that elevating the analyte concentration results in the occupation of more binding sites on the sorbent material. However, once all the binding sites are filled, the sorbent material reaches a state of saturation.

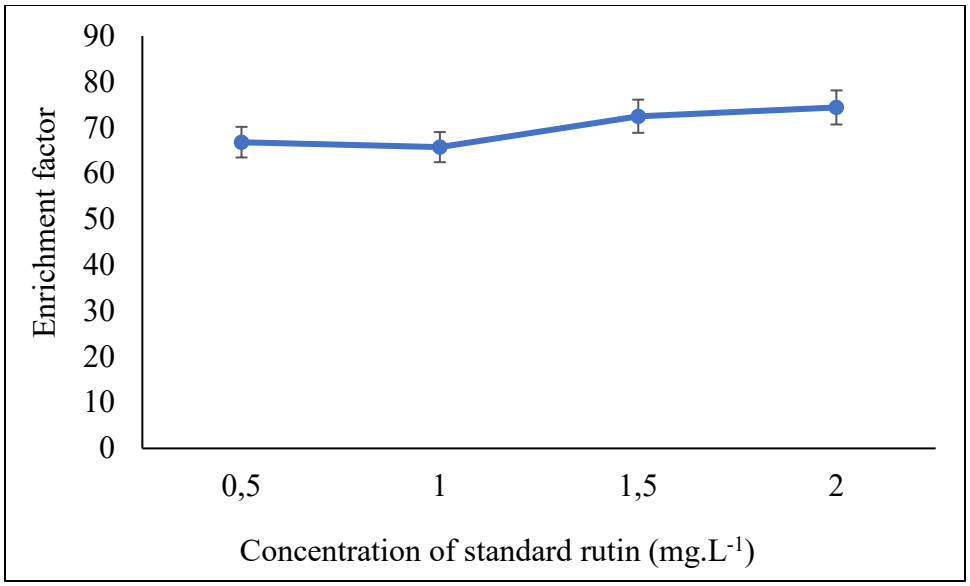

Figure 6.6: Effect of initial concentration of rutin standard on the performance of PT-µSPE. Extraction conditions: aspirating/dispensing cycles = 15 cycles, loading volume = 1000 µL, elution solvent = propan-2-ol, sample pH = 6, mass of sorbent = 2 mg (n = 3, SD).

6.4.5. Effect of pH on the sorbent

The oxidation of HCNS materials induced surface modifications, incorporating more oxygen-containing groups that attract the adsorption of the target analyte. The graph depicting initial pH (pH_i) versus final pH (pH_f) (Figure 6.7 (a)) was generated. The point of zero charge (pHpzc) is identified as the value at which pH_f remains nearly constant. In this experimental setup, the pHpzc was determined to be 6.5. Loading the rutin standard onto the sorbent bed revealed increased adsorption in acidic conditions. However, there was only a slight difference in the adsorption capacity between acidic and basic media, with almost the same amount of rutin being adsorbed. At pH 7, less rutin was observed to be adsorbed onto the sorbent bed. The optimal pH for rutin extraction was determined to be pH 2 (Figure 6.7 (b)), aligning with similar findings by Gholizadeh *et al*. (2019). Their study on multi-walled carbon nanotubes (MWCNTs) showed maximum rutin adsorption at pH 2. The presence of numerous hydroxyl groups in rutin makes pH a crucial factor in the adsorption process. In high pH values, flavonoids dissociate into anions, causing surface functional groups to be either neutral or negatively charged, reducing electrostatic repulsion and subsequently decreasing adsorption capacity. Ran *et al*. (2017) also noted that at pH ≤ 4, analytes exist in a neutral form, facilitating intermolecular interactions and yielding the highest

enrichments. At pH > 4, flavonoid analytes exist in anionic form, resulting in less interaction with the sorbent. This observation stems from the pH-dependent ionization states of flavonoids, influencing the analytes' state in solution and, consequently, extraction efficiency.

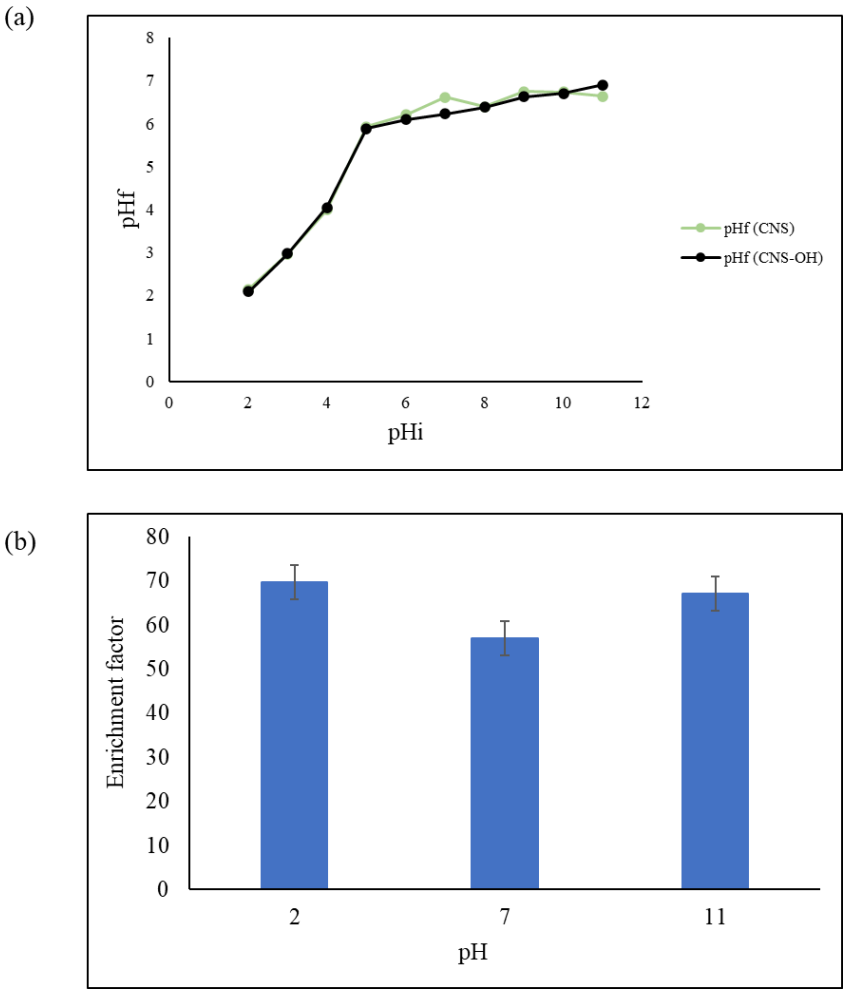

Figure 6.7: (a) pH_i versus pH_f for the determination of point of zero charge of the CNS sorbent (n = 3). (b) Effect of pH on the performance of PT-µSPE. Extraction conditions: initial concentration of rutin = 2 mg L^{-1}, loading volume = 1000 µL, elution solvent = propan-2-ol, aspirating/dispensing cycles = 15 cycles, mass of sorbent = 2 mg (n = 3, SD).

6.4.6. Effect of loading volume

The quantity of standard rutin introduced into PT-µSPE plays a crucial role in rutin enrichment, as the enrichment efficiency hinges on the loading sample solution volume. Therefore, the

investigated loading volumes were 300, 500, and 1000 µL. It was noted that loading PT-µSPE with 500 µL resulted in a higher rutin enrichment compared to the other volumes (Figure 6.8). When the volume of the sample solution was increased to 1000 µL, the amount of rutin adsorbed was observed to decrease because of shear pressure between the solvent and the sorbent material. A study by Bielicka-Daszkiewicz and Voelkel (2009) highlights the effects of sample loading volume on the sorbent bed-analyte relationship. The study states that the sample volume that is loaded onto the sorbent bed can lead to a loss of analyte. This is consistent with the observations in this study that a larger volume could displace the already adsorbed rutin during the aspirating/dispensing cycles. Consequently, 500 µL was deemed the optimal value and was employed in subsequent experiments. Wang *et al*. (2019) observed a parallel trend and attributed it to the partial loss of analytes from the sorbent bed associated with high sample volumes.

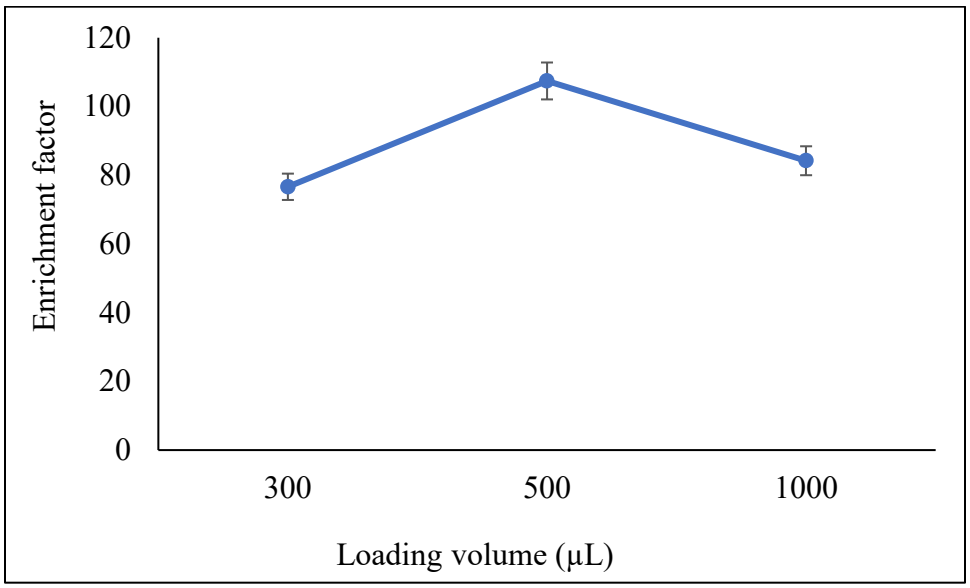

Figure 6.8: Effect of loading volume on the performance of PT-µSPE. Extraction conditions: initial concentration of rutin = 2 mg L^{-1}, aspirating/dispensing cycles = 15 cycles, elution solvent = propan-2-ol, sample pH = 2, mass of sorbent = 2 mg (n = 3, SD).

6.4.7. Effect of mass of sorbent

The sorbent's mass plays a role in rutin enrichment, prompting an investigation at 1, 1.5, 2, and 3 mg. It was noted that at 1.5 mg, the enrichment of rutin was maximized, establishing it as the optimal mass (Figure 6.9). Gomes *et al*. (2021) concluded that an increase in sorbent mass provides more active sites for the removal of desired analytes. Consequently, 1.5 mg was employed in the analysis of real samples. Hu *et al*. (2018) similarly observed a trend where extraction efficiency decreased with an increase in sorbent mass, attributing it to increased sorbent aggregation, reducing the accessible surface for sorbent-analyte interactions. This study witnessed a comparable trend.

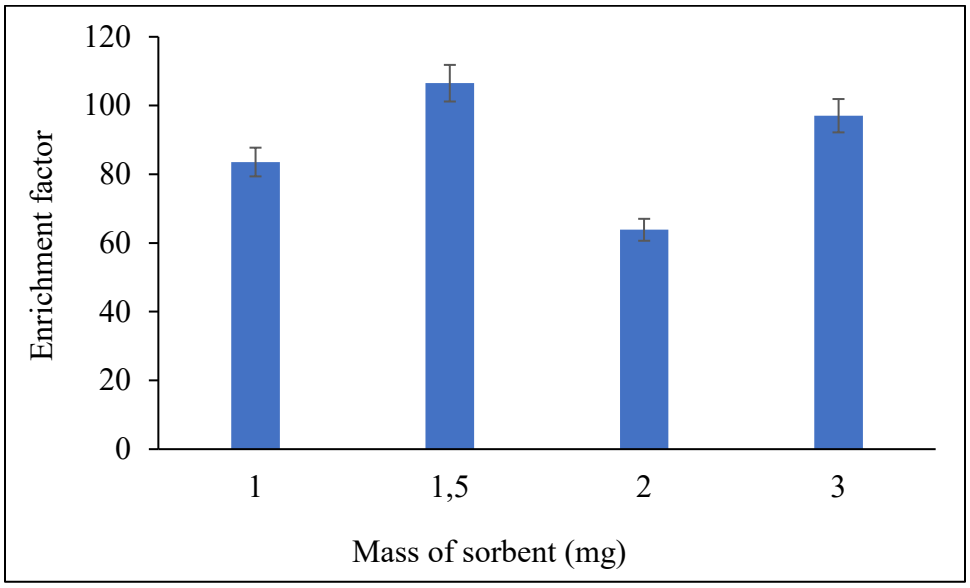

Figure 6.9: Effect of mass of the sorbent on the performance of PT-µSPE. Extraction conditions: initial concentration of rutin = 2 mg L^{-1}, loading volume = 500 µL, elution solvent = propan-2-ol, sample pH = 2, aspirating/dispensing cycles = 15 cycles (n = 3, SD).

6.5. Method validation

The PT-µSPE method was validated using rutin standards covering a linear range of 0.7 – 50 mg L^{-1}. The calibration curve was constructed by plotting the peak area against the concentration of rutin (Figure 6.10). Linearity, limit of detection (LOD), and limit of quantification (LOQ) are the analytical parameters that were evaluated. The linear regression equation y = 36397x – 9442.2

from the calibration curve of the rutin standards gave a good linearity ($R^2 = 0.9991$). The slope (S) and the intercept (σ) obtained from the linear regression line were 36397 and 9442.2, respectively. The LOD and LOQ were calculated from the standard deviation of the y-intercept and the slope, affording the equations LOD = 3.3 σ/S and LOQ = 10 σ/S, respectively. The LOD was calculated to be 0.604 and the LOQ was calculated to be 1.830. The results of the analysis of variance (ANOVA) test from the calibration curve are shown in Table 6.1. The *p*-value was less than 0.05 which indicates that the linear fit is significant.

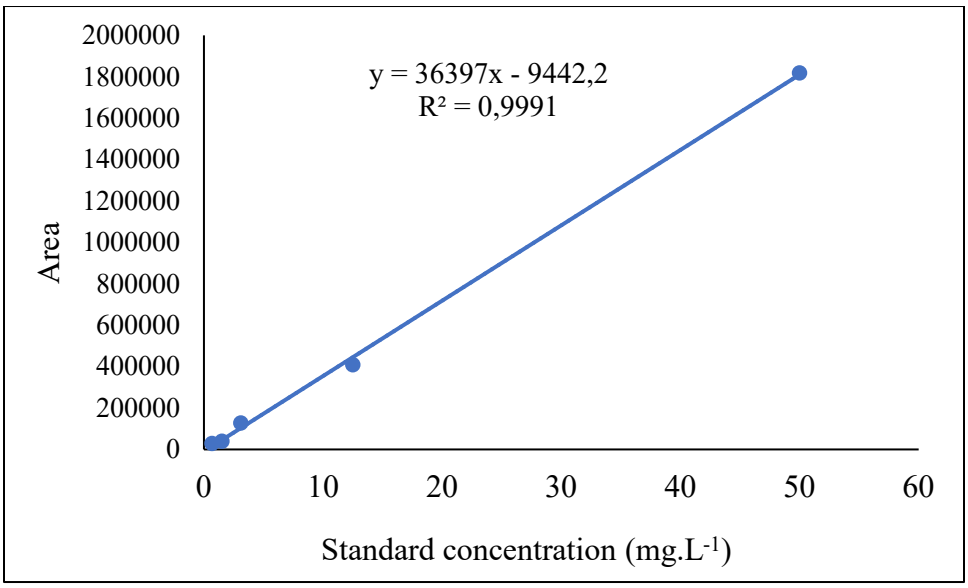

Figure 6.10: Linear fit of the calibration curve of rutin with a linear range of 0.7 – 50 mg L^{-1}.

Table 6.1: Results of the analysis of variance (ANOVA) test for the calibration curve.

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2,32E+12	2,32E+12	3189,072	1,22316E-05
Residual	3	2,18E+09	7,26E+08		
Total	4	2,32E+12			

6.6. Real sample analysis

The applicability and reliability of the PT-μSPE method was investigated by applying it to a *Moringa oleifera* leaf extract sample contained rutin under the optimized conditions as described

above. The investigation was done in triplicate measurements (n = 3). Figure 6.11 shows the chromatograms of (a) a 0.7 mg L^{-1} rutin standard, (b) 50 mg L^{-1} rutin standard, and (c) *Moring oleifera* leaf extract. Rutin was observed to elute at 8.9 min and had a maximum wavelength at 359 nm. Rutin was observed to have strong UV-vis absorption peaks at 255 and 360 nm by Deepika *et al*. (2019). The relative standard deviation (RSD) of the three sample runs was found to be 3.26%. Since RSD < 10%, the method is reliable and repeatable for the analysis of complex samples.

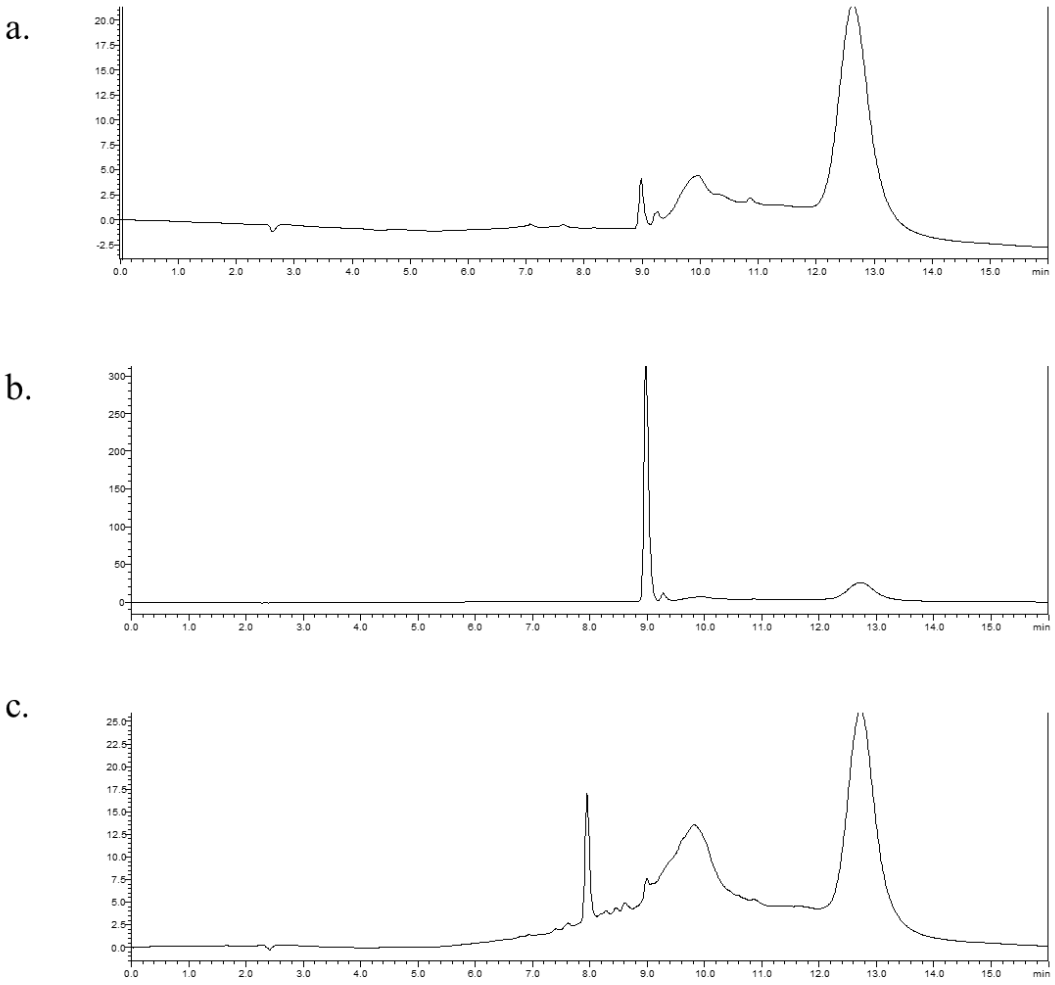

Figure 6.11: Chromatograms of (a) a 0.7 mg L^{-1} rutin standard, (b) a 50 mg L^{-1} rutin standard, and (c) a *M. oleifera* sample.

6.7. Conclusion

The successful extraction of rutin from *M. oleifera* leaves using PT-µSPE with carbon nanospheres as the sorbent was demonstrated. This affirmed the effectiveness of activated HCNSs in rutin extraction. The oxidation of HCNS material introduced more oxygen-containing groups, enhancing the adsorption of rutin on the carbonaceous material's surface. Rutin exhibited optimal adsorption at pH 2 with an initial concentration of 2 mg L^{-1}. Propan-2-ol emerged as the most effective eluting solvent for rutin desorption from the sorbent bed, with an optimal sorbent mass of 1.5 mg. Additionally, employing 15 aspirating/dispensing cycles and a loading volume of 500 µL yielded the optimal extraction of rutin from *M. oleifera*. The achieved low limit of detection (LOD) for rutin attested to the method's suitability for extracting rutin even at trace levels from complex samples. A calculated relative standard deviation (RSD) of 3.26% indicated the reliability and repeatability of the PT-µSPE method for analyzing complex samples.

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Experimental Chapter 7

This chapter focuses on the work titled ‘A relook into the flavonoid chemical space of *M. oleifera* through a combination of LC-MS and molecular networking’.

7.1. Abstract

Moringa oleifera Lam. is a functional tree that is known to produce a variety of metabolites with purported pharmacological activities. It is commonly referred to as the 'miracle tree' because of its application in various nutraceutical and pharmacological applications. This study was aimed at studying the chemical space of *M. oleifera* leaf extracts through molecular networking (MN), a tool that identifies metabolites by classifying them based on their MS-based fragmentation pattern similarities and signals. In this case, a special emphasis was placed on the flavonoid composition. The MN unraveled different molecular families such as flavonoids, carboxylic acids and derivatives, lignin glycosides, fatty acyls, and macrolactams that are found within the plant. In silico annotation tools such as network annotation propagation (NAP) and DEREPLICATOR, an unsupervised substructure identification tool (MS2LDA) and MolNet enhancer were also explored to further compliment the classic molecular networking output within global natural product social (GNPS). In this study, common flavonoids found within *Moringa oleifera* were further annotated using MS2LDA. Utilizing computational tools allowed for the discovery of a wide range of structurally diverse flavonoid molecules within *M. oleifera* leaf extracts. The expansion of the flavonoid chemical repertoire in this plant arises from intricate glycosylation modifications, leading to the creation of structural isomers that manifest as isobaric ions during mass spectrometry (MS) analyses.

Keywords: *M. oleifera*, flavonoids, molecular network, MS2LDA, Mass2Motifs

7.1. Introduction

Moringa oleifera Lam. has been reported to have a broad range of pharmacological activities such as antimicrobial, anti-inflammatory, hypotensive, antidepressant, antioxidant, antidiabetic, hypoglycemic and immunomodulatory properties (Padayachee and Baijnath, 2019; Meireles *et al*., 2020; Sreeja *et al*., 2021). The chemical constituents of the stems, leaves, flowers, pods and seeds of *M. oleifera* have been analyzed to determine the presence of bioactive compounds, and they were found to contain various secondary metabolites such as phenolic acids, sterols, terpenoids, flavonoids, alkaloids, sugars, anti-cancerous agents such as gluconisolates, isothiocyanates, glycoside compounds and glycerol-1-9-octadecanoate which have nutritional, pharmaceutical and anti-microbial properties (Bhattacharya *et al*., 2014; Gopalakrishnan *et al*., 2016; Makita *et al*., 2016; Brilhante *et al*., 2017; Yi *et al*., 2017). However, studies on this plant have shown that the presence of the bioactive compounds is dependent on various factors such as the geographical origin, the harvesting season, and cultivation conditions (Silva *et al*., 2022).

Metabolomics is a field of study that gives a systematic view of the unique chemical fingerprints of metabolites and their small changes in a specific cellular process (Senn *et al*., 2012). A metabolomics study includes sample preparation, analytical measurement, data analyses and interpretation (Ernst *et al*., 2019; Ivanisevic and Want, 2019; Beniddir *et al*., 2021). Mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques are reported to be the analytical workhorses of metabolomics (Caudy *et al*., 2017; Liu *et al*., 2018; LeVatte *et al*., 2022). A molecular family (MF) is constructed by the grouping of structurally related molecules that generate similar fragmentation patterns. To do this on a larger scale, computational tools such as molecular networking (MN) have been developed (Watrous *et al*., 2012; Yang *et al*., 2013; Ernst *et al*., 2019; Vincenti *et al*., 2020). MN is a popular tool in the analysis of tandem MS (MS/MS) based metabolomics data. MN is fundamentally based on the observation that two structurally related molecules share fragment ion patterns when subjected to MS/MS and aids the elucidation of the structure/identity of many compounds of untargeted MS (Quinn *et al*., 2016; Aron *et al*., 2020; Morehouse *et al*., 2023). MN has led to the development of Global Natural Products Social (GNPS) which is a molecular networking and data-sharing web-based platform (Xu *et al*., 2019; Rawlinson *et al*., 2020).

GNPS is widely used by scientists from various platforms in the fields of chemistry, microbiology, forensics and more to perform sample classification with the objective to give identity of the content thereof. GNPS facilitates data, stores knowledge, enables sharing, and promotes reproducible data analysis (Aron *et al*., 2020). GNPS can be used for molecular networking and is currently the only public infrastructure that enables molecular networking (Xu *et al*., 2019). The related molecules as depicted in a MN can be viewed online at GNPS or on Cytoscape for analysis (Wang *et al*., 2016; Beniddir *et al*., 2021). Other tools in GNPS include network annotation propagation (NAP) and DEREPLICATOR, and an unsupervised substructure identification tool called MS2LDA, all of which are meant to strengthen metabolite identification through MN. These tools are used to complement classic MN output and integration using MolNetEnhancer within GNPS (Ramabulana *et al*., 2021).

In this study, the chemical space of *M. oleifera* was studied through computational tools within GNPS. Molecular networking was used to reveal the molecular families of this plant and the unsupervised substructure annotation tool (MS2LDA) was used to annotate the Mass2Motifs of some of the flavonoids that are found within *M. oleifera* by depicting similar fragmentations and neutral losses.

7.2. Materials and methods

7.2.1. Chemicals and reagents

Methanol (99% CP) was purchased from Associated Chemical Enterprises (Johannesburg, South Africa). Ultra-pure water using a Direct-Q 5UV distiller (Massachusetts, United States of America) was used for the preparation of the 80% methanol solution. The extraction was performed on a DIAB MX-RL-Pro dragon shaker. Chromatographic separation of the metabolites in the extracts was done using a reverse phase Shim-pack Velox C18, 2.1 x 100 mm, 2.7 µm (Columbia, USA). The UPLC was connected to a Shimadzu 9030 LC, qTOF-MS detector (Kyoto, Japan). The solvents used for the chromatographic runs were methanol and formic acid, which were purchased from Romil Pure Chemistry (Cambridge, UK).

7.2.2. Plant collection and sampling

Leaves were collected from cultivated *M. oleifera* plants in multiple households across various villages within the Vhembe District of the Limpopo Province of South Africa. After being harvested, these leaves were kept in darkness while being transported to the University of Venda. Subsequently, the leaves were air-dried in the absence of light at room temperature and then finely ground into a powder using a blender. This powdered form was stored in a dark environment until the metabolite extraction process.

7.2.3. Preparation of the extract

A modified version of a previously described extraction method by Makita *et al*. (2017) was utilized. In summary, 1 gram of ground leaf powder from each cultivar was mixed with 10 mL of 80% aqueous methanol (MeOH) and shaken overnight using a dragon shaker. The resulting mixture was then centrifuged at a high speed of 5000 ×g for 20 minutes at a temperature of 25 °C. The supernatant liquid was transferred to an Eppendorf tube, filtered through 0.22 µm filters into a vial, and subjected to UPLC-qTOF-MS analysis. Any remaining supernatant solutions were stored in a refrigerator at 4 °C.

7.2.4. Ultra-High Performance Liquid Chromatography-Quadruple Time-of-Flight Mass Spectrometry (UHPLC-qTOF-MS)

To analyze the extracts, the LCMS-9030 qTOF instrument from Shimadzu Corporation in Kyoto, Japan, was employed, following the method outlined by Ramabulana *et al*. (2021). Liquid chromatography separation took place on a Shim-pack Velox C18 column (100 mm × 2.1 mm, particle size 2.7 µm) housed in a column oven maintained at 55 ℃. A binary mobile phase gradient consisting of solvent A (0.1% formic acid in Milli-Q water) and solvent B (methanol with 0.1% formic acid) was used. An injection volume of 3 µL was applied to all samples. The gradient conditions were as follows: 10% B for 3 minutes, 10-60% B over 3-40 minutes, 60% B from 40-43 minutes, 90% B from 43-45 minutes (maintained for 3 minutes), returned to initial conditions from 48-50 minutes, followed by a 3-minute column re-equilibration time. The chromatographic effluents were analyzed using a qTOF high-definition mass spectrometer in negative electrospray ionization mode. The instrument was calibrated with sodium iodide (NaI), and both MS1 and MS2

data were simultaneously generated (through a data dependent acquisition mode) for all ions within an *m/z* range of 100-1000 and an intensity threshold of 5000, using a data-dependent acquisition (DDA) mode. Fragmentation experiments were conducted using argon as a collision gas, with a collision energy of 30 eV and a spread of 5 eV. The MS settings were as follows: interface voltage of − 4.0 kV, interface temperature of 300 °C, nebulization and dry gas flow rate of 3 L min^{-1}, heat block temperature of 400 °C, DL temperature of 280 °C, and detector voltage of 1.8 kV.

7.2.5. Molecular Networking and Metabolite Annotation

The creation of a molecular network was performed using the GNPS Opensource tool website (http://gnps.ucsd.edu) through an online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/), accessed on August 17, 2021. The data underwent filtering by removing MS/MS fragment ions within +/- 17 Da of the precursor *m/z* and selecting only the top 6 fragment ions in the +/- 50 Da window across the spectrum. The precursor ion mass tolerance was set at 2.0 Da, and a MS/MS fragment ion tolerance of 0.5 Da was applied. The resulting network was filtered to have a cosine score above 0.7 and more than 6 matched peaks for the edges, while nodes were connected if they appeared in each other's respective top 10 most similar nodes. Molecular families were limited to a size of 100, and low-scoring edges were eliminated until the size was below this threshold. The network spectra were then searched against GNPS' spectral libraries using the same filtering criteria. Finally, the visualization of the molecular network was carried out using Cytoscape software. Empirical formulas were generated from accurate mass and fragmentation patterns obtained from the MS2 data to annotate all matched nodes and some unmatched nodes of metabolites. These annotations were compared to dereplication databases such as the KNApSAck chemical database. Substructure annotation was achieved using MS2LDA through the ms2lda.org web interface within GNPS. Structural searches were performed according to the protocol recently outlined by Moyo *et al*. (2023).

7.3. Results and discussion

MS/MS spectra of six (6) methanolic extracts from the *M. oleifera* cultivars were compared to find similarities in the fragmentation patterns (i.e. same fragment ions or similar neutral losses) of the

metabolites. Metabolites that are structurally related and have similar gas phase chemistries were grouped into molecular families based on a cosine score of ≥ 0.7 [26]. Using molecular networking, the MS/MS spectra were organized into 565 nodes, with 338 clustered into 38 different molecular families (with a minimum of two nodes connected by an edge) based on GNPS spectral matching. A total of 227 nodes were not clustered into a molecular family and were represented as individual nodes at the bottom of the network (Figure 7.1).

Previous studies have shown the presence of structurally diverse flavonoid molecules in the plant extracts. However, most of the work conducted in this study was through classical means of chemical identification where obtained mass spectrometry (MS) signals were compared with what is already known in literature. This approach, however, has negative connotation owing to the limitation on information of some uncharacterized metabolites. Molecular network is a computational method aimed at metabolite identification by classifying metabolites based on their MS-based fragmentation pattern similarities and signals.

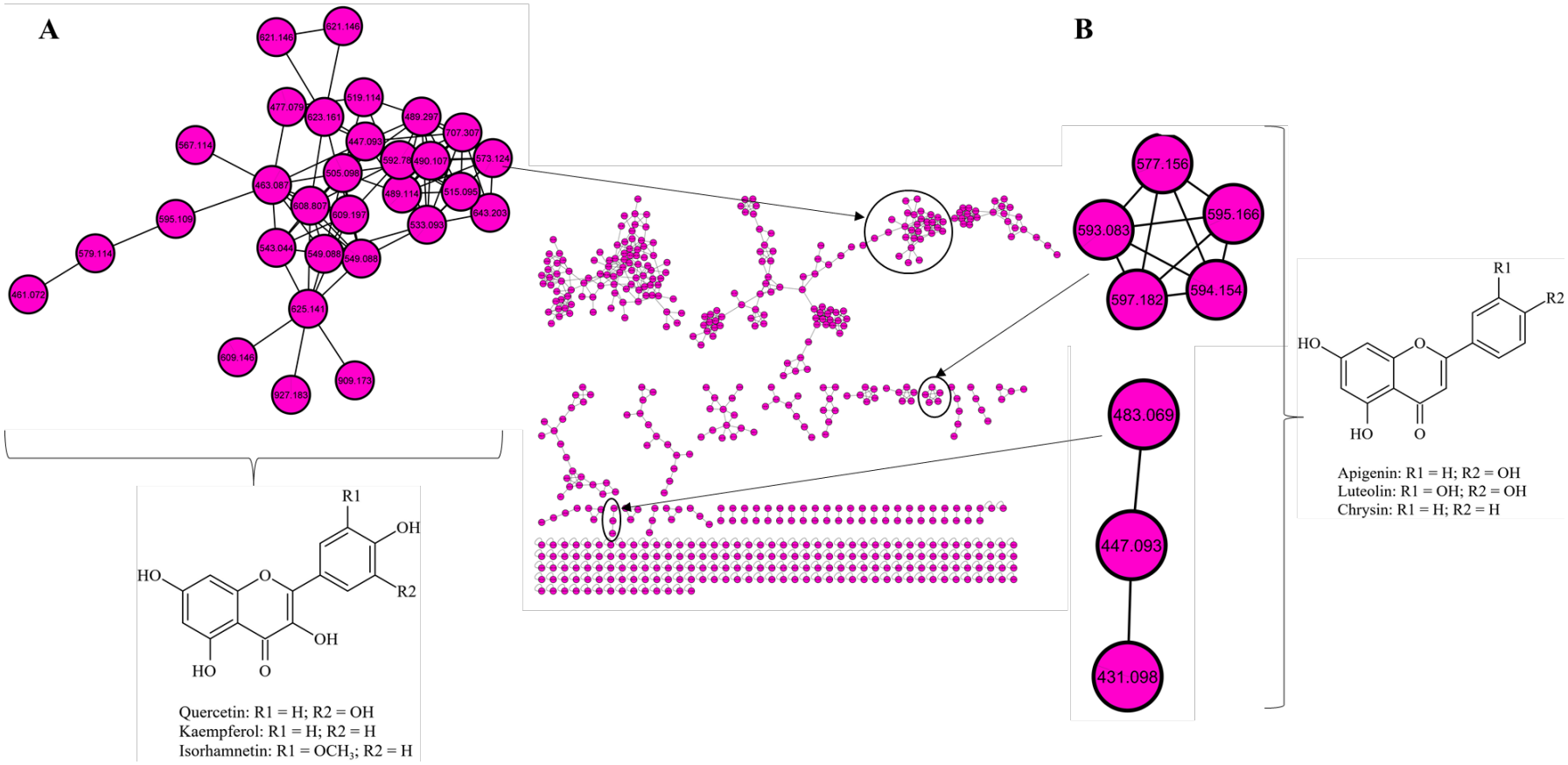

Figure 7.1: Molecular network of *Moringa oleifera* Lam leaf extracts as analyzed by liquid chromatography-tandem mass spectrometry using electrospray ionization in negative mode (center), with two different kinds of flavonoids highlighted: (A) flavonols, and (B) flavones.

7.3.1. Exploration of the chemical space of Moringa oleifera

Moringa oleifera is well known for its nutraceutical and pharmacological metabolic profiles which are characterized by the presence of flavonoids, glucosinolates, and chlorogenic acids. In this study, the metabolic profile of *M. oleifera* was studied with the help of molecular networking from the GNPS website. MolNetEnhancer (Figure 7.2) represents the metabolomes of this plant that were observed in this study. The node annotations of MolNetEnhancer were based on MS2LDA, network annotation propagation (NAP) and DEREPLICATOR output. It was observed that this plant contains 16 different classes of metabolites including carboxylic acids and derivatives, fatty acyls, flavonoids, glycerophospholipids, lignin glycosides, macrolactams, macrolides, napthacenes, organooxygen compounds, prenol lipids, purine nucleotides, and tetrapyrroles and derivatives. A study by Abdel Shakour *et al*. (2022) revealed the presence of hydroxyl fatty acids, phenolic acids, flavonoids, intact glucosinolates, sulfolipids, and phenolic acid derivatives metabolite classes.

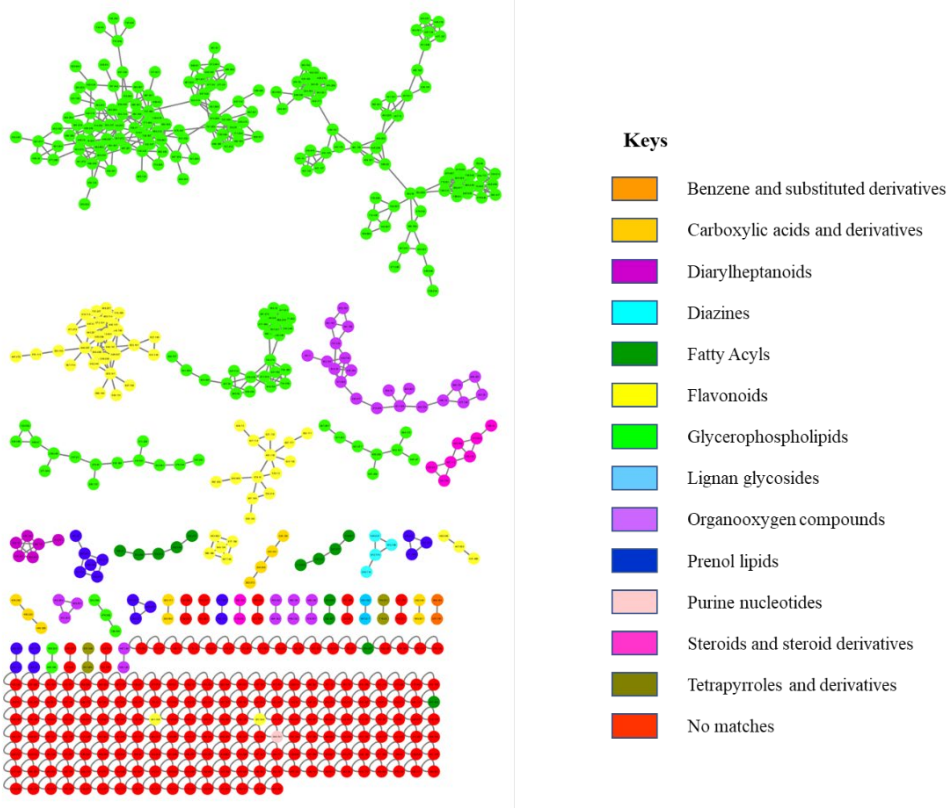

Figure 7.2: An enhanced molecular network in which nodes are highlighted based on their chemical superclass based on MS2LDA, network annotation propagation (NAP) and DEREPLICATOR outputs.

Flavonoids have been reported to be the predominant group of metabolites in *M. oleifera* leaf extracts with kaempferol and quercetin derivatives being the most predominant (Rodriguez-Perez *et al*., 2015). Flavonoids are naturally occurring polyphenols that accumulate in the edible parts of plants, more particular in fruits and vegetables (Iriti *et al*., 2017). Flavonoids can further be subdivided into flavones, flavanols, flavanones, flavonols, flavanonols, isoflavones and anthocyanins. In this study, much attention was given to flavones and flavonols. Flavones and flavonols have antioxidant effects and are essential for protecting plants from UV radiation (Li *et al*., 2020). Quercetin and kaempferol (Figure 7.1A), among others, are abundant dietary flavonols found in fruits and vegetables. Flavonols have various health benefits which include cardiovascular and antioxidant properties. Luteolin and apigenin (Figure 7.1B) are the main flavones that are found in fruits and vegetables and have a wide range of biological effects such as anti-cancer, antioxidant and anti-inflammation properties (Brodowska, 2017; Xiao, 2017; Chagas *et al*., 2022).

In this study, a total of 52 flavonoids was detected. Kaempferol derivatives are known to have a major fragment ion at *m/z* 285 and quercetin derivatives are known to have a major fragment ion at *m/z* 301; both indicating the aglycone moiety thereof. Another common flavonoid in *M. oleifera* leaves is isorhamnetin and derivatives of this flavonoid have a major fragment ion at *m/z* 314; again indicating the aglycone moiety. The detailed mass information of selected flavonoids that were annotated in this study are shown in Table 7.1.

Table 7.1: Identification of flavonoids by UHPLC-qTOF-MS and their Mass2Motifs.

No.	Molecular formula	m/z value	Fragment ions	Compound name
1	$C_{27}H_{30}O_{16}$	609.197	343.048; 301.038; 300.028; 271.098; 255.028; 178.998; 151.003	Quercetin rutinoside
2	$C_{23}H_{21}O_{13}$	505.098	301.038; 300.033; 271.023; 255.028; 178.998; 151.013	Quercetin acetyl hexose
3	$C_{23}H_{22}O_{11}$	549.089	505.103; 463.088; 301.038; 300.033; 271.023; 255.028	Quercetin malonyl hexose
4	$C_{12}H_{20}O_{12}$	463.087	301.038; 300.028; 271.033; 255.028; 178.998; 151.003	Quercetin hexose
5	$C_{24}H_{22}O_{14}$	533.093	489.108; 285.043; 284.033; 257.048; 255.028; 229.053; 227.033	Kaempferol malonyl hexose
6	$C_{26}H_{28}O_{16}$	592.785	489.108; 447.098; 285.043; 284.033; 255.028	Kaempferol rutinoside
7	$C_{27}H_{30}O_{16}$	609.146	489.103; 447.098; 446.088; 327.048; 285.043; 283.023; 255.028	Kaempferol diglucoside

8	$C_{21}H_{20}O_{11}$	447.093	285.043; 284.033; 256.038; 255.028; 227.033	Kaempferol hexose
9	$C_{23}H_{22}O_{11}$	489.114	285.043; 284.033; 255.023; 227.033	Kaempferol acetyl hexose
10	$C_{27}H_{30}O_{16}$	623.161	315.053; 314.043; 300.023	Isorhamnetin rutinoside
11	$C_{28}H_{28}O_{11}$	621.146	559.148; 519.118; 477.103; 315.058; 314.043	Isorhamnetin hydroxy-methylglutaroyl hexose
12	$C_{22}H_{22}O_{11}$	477.079	315.058; 314.043; 299.018; 285.043; 271.023; 257.048; 243.028	Isorhamnetin hexose
13	$C_{24}H_{24}O_{13}$	519.114	357.058; 315.048; 314.043; 299.018; 285.043; 271.023; 257.048; 243.028	Isorhamnetin acetyl hexose
14	$C_{27}H_{30}O_{15}$	593.083	503.118; 473.108; 383.078; 353.068; 311.058	Apigenin-6,8-C-dihexose
15	$C_{27}H_{30}O_{14}$	577.156	487.123; 457.118; 439.103; 397.093; 379.083; 367.083; 337.073; 309.078; 281.083	Chrysin-6,8-C-diglucoside

16	$C_{21}H_{20}O_{10}$	431.098	341.068; 281.048; 269.048	323.053;	311.058;	283.063;	Vitexin
17	$C_{21}H_{20}O_{11}$	447.093	357.063; 297.043; 285.043	339.048;	327.048;	299.053;	Luteolin-8-C-hexose

There are other various tools that are available in GNPS that compliment molecular networking. Such tools are *in silico* metabolite annotation tools such as network annotation propagation (NAP) and dereplication. These tools perform in silico fragmentation of known structures and then search against chemical databases. Within the GNPS, there's another valuable resource known as Mass Spectrometry-Mass Spectrometry Latent Dirichlet allocation (MS2LDA). MS2LDA is an unsupervised computational technique that reveals inherent substructures within compounds by analyzing intricate mass spectrometry (MS) data. This algorithm operates on an unsupervised basis, automatically detecting patterns and substructures within the complex MS data. This capability allows for the identification of shared substructures or fragmentation patterns among compounds. MS2LDA decomposes each molecule into one or more Mass2Motifs which allows for more efficient molecular grouping, searching and exploration (Van der Hooft *et al*., 2016). Mass2Motifs consist of similar fragments and neutral losses (Van der Hooft *et al*., 2017; Wandy *et al*., 2018). The structural annotations of the Mass2Motifs are straightforward and less complex because Mass2Motifs represent smaller substructures (Rogers *et al*., 2019). Figure 7.3 represents the metabolite annotation using MolNetEnhancer and by MS2LDA of flavonoids found in *M. oleifera* leaves. The colored parts are representative of the flavonoids that make up the Mass2Motifs. Quercetin, kaempferol and isorhamnetin are the major flavonols that are represented in Figure 7.3. It is observed that some of these flavonols share the same Mass2Motif owing to their similar fragments and neutral losses. For example, the flavonoids with precursor ion $[M-H]^-$ at *m/z* 533.088 and at *m/z* 592.785 share the same Mass2Motif because they share similar fragments due to the similar aglycone structure.

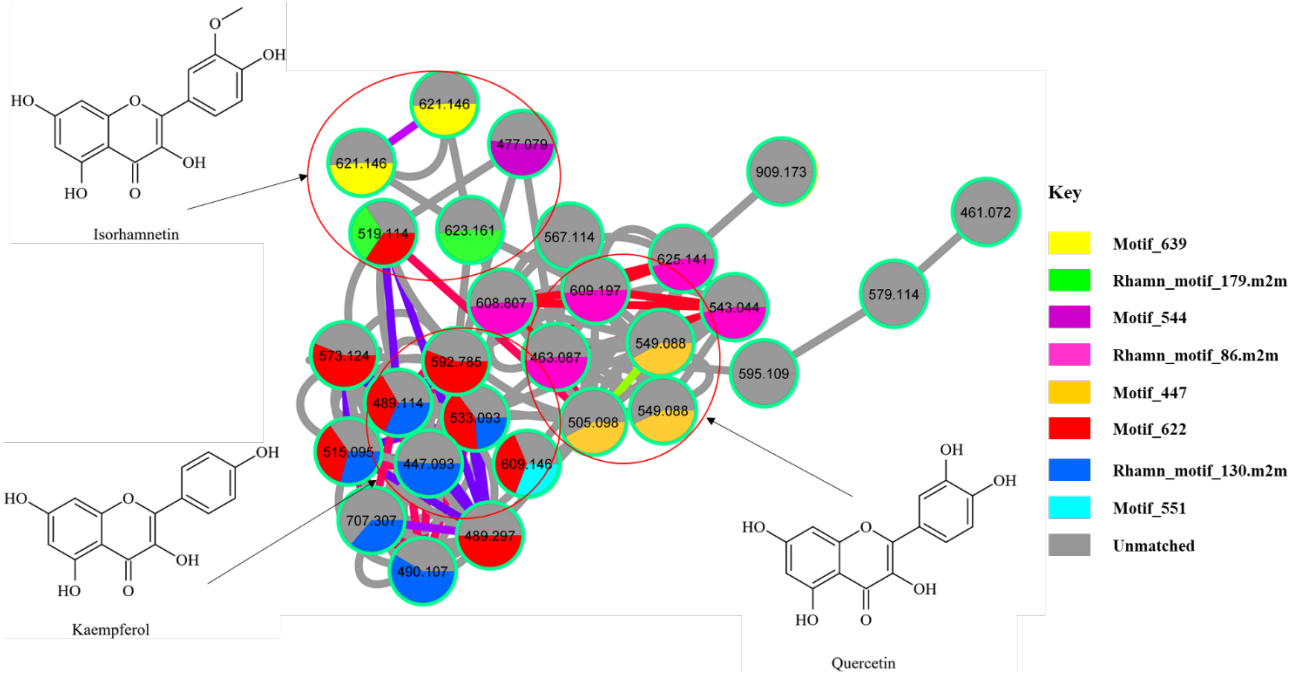

Figure 7.3: Metabolite annotation using MolNetEnhancer by MS2LDA where the colored parts represent the flavonoids that make up the Mass2Motif.

7.3.1.1. Quercetin flavonoids

Quercetin is a flavonoid that is abundantly found in fruits and vegetables and can be used as a nutritional supplement. This compound has been reported to prevent diseases such as tumors, lung and cardiovascular diseases and some forms of cancer (Reyes-Farias and Carrasco-Pozo, 2019; Xu *et al*., 2019; Vafadar *et al*., 2020). Figure 7.4 shows the fragmentation spectra of four quercetin-related flavonoids as annotated by rhamn_motif_86.m2m and motif_447 mass2motifs on MS2LDA approach. Rhamn_motif_86.m2m (a quercetin related motif) indicated the presence of a quercetin aglycone with diagnostic fragments at *m/z* 301, 300, 255 and 179 and a neutral loss of 106 amu. Motif_447 also indicated the presence of a quercetin aglycone with fragments at *m/z* 301, 300, 271 and 255 and neutral loss of 44 amu. Quercetin flavonoids are characterized by a deprotonated quercetin aglycone fragment at *m/z* 300/301 and other characteristic product ions of *m/z* 271, 255, 179 and 151 further confirm the identity of the quercetin aglycone (Li *et al*., 2016). Compound **1** gave a precursor ion $[M-H]^-$ at m/z 609.197 and a fragmentation ion at *m/z* 300.028 due to the loss of the rhamnose and glucose sugars was seen as a base peak. Therefore, this compound was identified as quercetin rutinoside (Latiff *et al*., 2018). Compound **2** gave a precursor ion $[M-H]^-$ at *m/z* 505.098 and showed a fragment ion at *m/z* 445.078 due to the loss of the acetyl moiety (60 amu) and a further loss of

the hexosyl moiety (162 amu) resulting in the fragment at *m/z* 300. This compound was thus identified as quercetin acetyl hexose (Barros *et al*., 2012). Compound **3**, which was identified as quercetin malonyl hexose, gave a precursor ion $[M-H]^-$ at *m/z* 549.089 showed a fragment at *m/z* 505 due to the loss of an acetyl (44 amu) and another fragment at *m/z* 463 due to the loss of the malonyl moiety (86 amu). A further loss of the hexosyl moiety (162 amu) led to the fragment ion at *m/z* 300 (Abu-Reidah *et al*., 2019). Compounds **2** and **3** share the same mass2motif due the similar neutral losses which is the loss of the hexose moiety. Compound **4** gave a precursor ion $[M-H]^-$ at *m/z* 463.087 and a fragmentation ion at *m/z* 300.028 due to the loss of hexose. This compound was identified as quercetin hexose (Pascale *et al*., 2020).

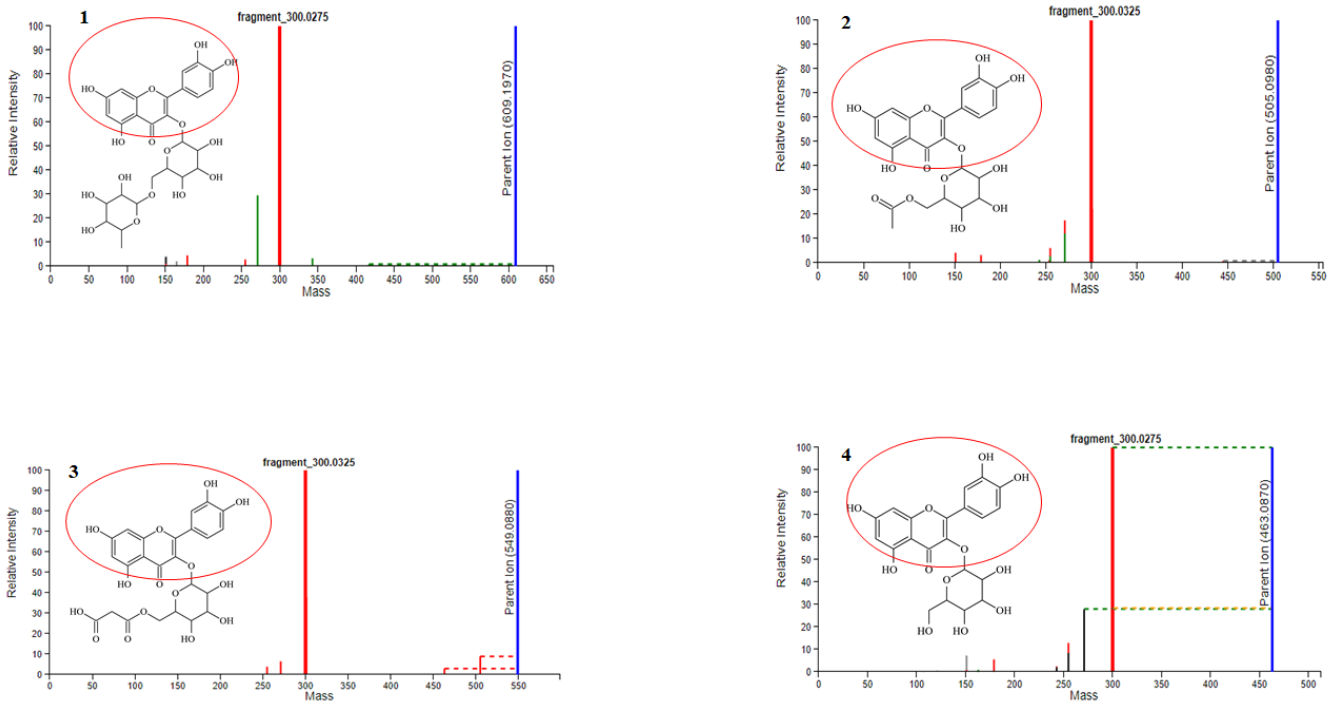

Figure 7.4: Fragmentation spectra of some quercetin-related flavonoids as annotated by MS2LDA.

7.3.1.2. Kaempferol flavonoids

Kaempferol is a flavonoid that is found in various plant parts such as seeds, leaves, fruits, flowers and even vegetables. It has been referred to as a nutraceutical, owing to its medicinal and nutritional benefits (Wong *et al*., 2019). For instance, kaempferol and its glycosides have been reported to have cardioprotective, neuroprotective, anti-inflammatory, antioxidant, and anticancer activities (Du *et al*., 2018; Imran *et al*., 2019). Figure 7.5 shows the fragmentation

spectra of five (5) different kaempferol-related flavonoids as annotated by rhamn_motif_130.m2m (kaempferol related motif), motif_622, and motif_551 mass2motifs on MS2LDA approach. Rhamn_motif_130.m2m is characteristic of kaempferol with diagnostic fragments at *m/z* 285, 284, 255, and 227. Motif_622 also represents kaempferol with diagnostic fragments at *m/z* 285, 284, and 255 and a neutral loss of 68 amu. Motif_551 was characterized by diagnostic fragments at *m/z* 283 and 110 and neutral losses of 162, 167, 182, 193, and 194 amu. Kaempferol flavonoids are characterized by a deprotonated kaempferol aglycone fragment at *m/z* 284/285 and other characteristic product ions at *m/z* 255 and 227 further confirm the identification of the kaempferol aglycone (Li *et al*., 2016). Compound **5** gave a $[M-H]^-$ ion at *m/z* 533.093 while its MS/MS fragmentation gave a base peak at *m/z* 285.043, due to the loss of the malonyl hexose moiety, and was thus identified as kaempferol malonyl hexose (Mosic *et al*., 2019). Compound **6** gave a $[M-H]^-$ ion at *m/z* 592.785 while its MS/MS fragmentation gave a base peak at *m/z* 285.043, due to the loss of the rutinoside sugar, and was identified as kaempferol rutinoside (Aksay *et al*., 2021). Compound **7** was identified as kaempferol diglucoside with a precursor ion at *m/z* 609.146 $[M-H]^-$ with a fragmentation peak at *m/z* 285.043. This compound also has fragments at *m/z* 446.089 and 447.098 due to the loss of the two hexose moieties (162 + 162 amu) (Dusek *et al*., 2021). Compound **8**, which was identified as kaempferol hexose has a precursor ion $[M-H]^-$ at *m/z* 447.093 with a fragmentation ion at m/z 284.033 which is due to the loss of the hexose sugar (162 amu) (Avila *et al*., 2022). Compound **9** gave a precursor ion $[M-H]^-$ at *m/z* 489.114 with a fragmentation ion at *m/z* 284.033 due to the loss of an acetyl hexose moiety. This compound was thus identified as kaempferol acetyl hexose (Rodriguez-Perez *et al*., 2015). Compounds **5** and **6** were annotated by motif_622, compound **7** was annotated by motif_551, and compounds **8** and **9** were annotated by rhamn_motif_130.m2m, as shown in Figure 7.3.

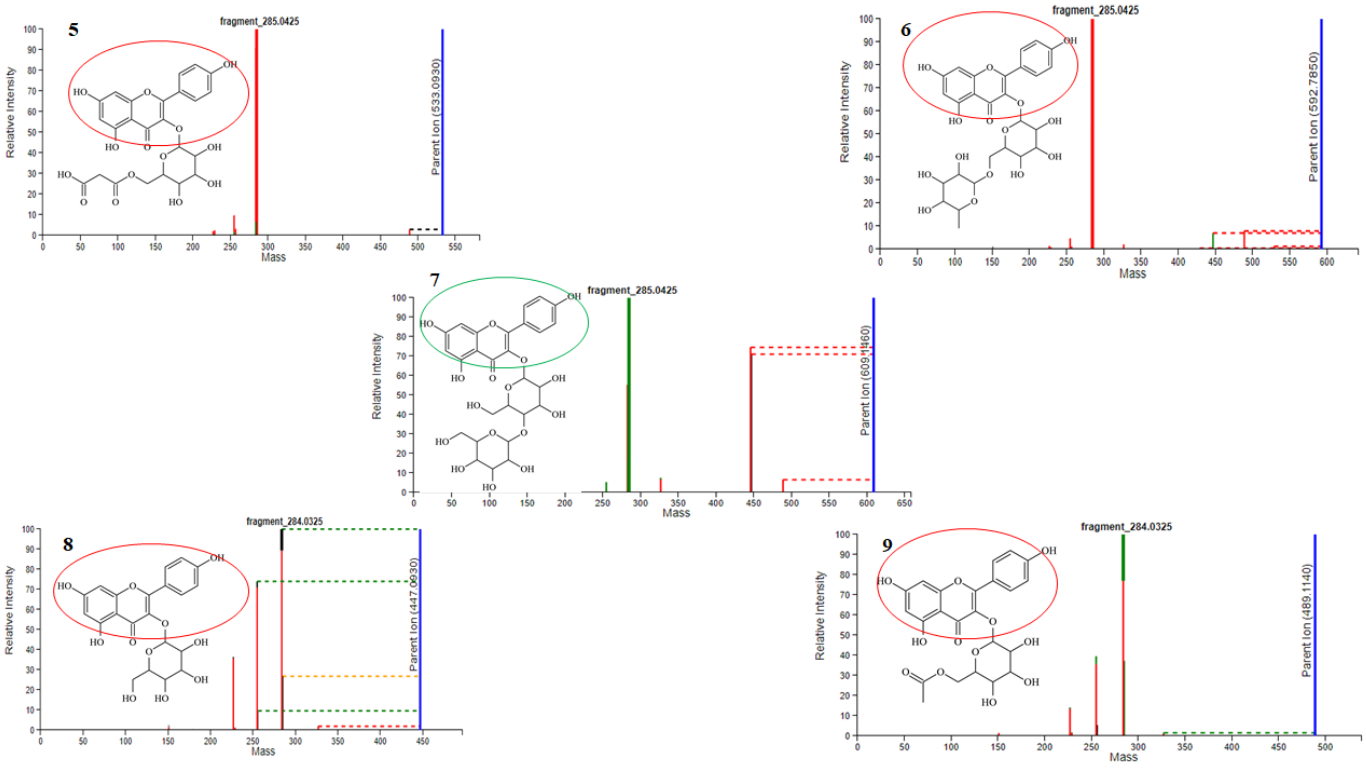

Figure 7.5: Fragmentation spectra of some kaempferol-related flavonoids in *M. oleifera* as annotated by MS2LDA.

7.3.1.3. Isorhamnetin flavonoids

Isorhamnetin is commonly present in the leaves, flowers and fruits of many plants and also forms part of a daily diet. This flavonoid hosts various pharmacological properties such as cardiovascular protection, antibacterial, antiviral, antioxidation, antiinflammation and antitumor properties (Gong *et al*., 2020; Rashed *et al*., 2020; Rodriguez *et al*., 2021). Figure 7.6 shows the fragmentation spectra of 4 isorhamnetin-related flavonoids as annotated by rhamn_motif_179.m2m (rhamnetin (= 7-methylquercetin) motif), motif_639, and motif_544 mass2motifs on MS2LDA approach. Rhamn_motif_179.m2m represents 7-methylquercetin with diagnostic fragments at *m/z* 315, 314, 300, and 299 and a neutral loss of 32 amu. Motif_639 represents isorhamnetin with diagnostic fragments at *m/z* 559, 519, 477, 315, and 314 and neutral losses of 62, 102, and 144 amu. Motif_544 also represents isorhamnetin with diagnostic fragment ions at *m/z* 315, 314, 299, 285, 271, 257, and 243 with neutral losses of 162, 163, 178, and 192 amu. Isorhamnetin flavonoids are characterized by a deprotonated isorhamnetin aglycone fragment at *m/z* 314/315 and other characteristic product ions at *m/z* 300, 271, 255 and 227 further confirm the identification of the isorhamnetin aglycone (Li *et*

al., 2016). Compound **10** gave a precursor ion [M-H]$^-$ at *m/z* 623.161 with a fragmentation ion at *m/z* 315.053 indicating the loss of a rutinoside sugar. This compound was thus identified as isorhamnetin rutinoside (El-Zahar *et al*., 2022). Compound **11**, which was identified as isorhamnetin hydroxy methylglutaroyl hexose, gave a precursor ion [M-H]$^-$ at *m/z* 621.146 and a fragmentation ion at *m/z* 315.053. This compound also gave fragments at *m/z* 559 and *m/z* 519.118 which were due to the loss of hexosy and hydroxy-methylglutaroyl moieties (Makita *et al*., 2016). Another fragment was observed at *m/z* 477.103 which was due to the loss of the hydroxy methylglutaroyl moiety (144 amu). Compound **12** gave a precursor ion [M-H]$^-$ at *m/z* 477.079 with a fragmentation ion at *m/z* 314.043 due to the loss of the hexose sugar (162 amu). This compound was thus identified as isorhamnetin hexose (Soltana *et al*., 2018). Compound **13** gave a precursor ion [M-H]$^-$ at *m/z* 519.114 and a fragmentation ion at *m/z* 314.043 due to the loss of the acetyl hexose moiety. This compound was identified as isorhamnetin acetyl hexose (204 amu) (Nowicka *et al*., 2019).

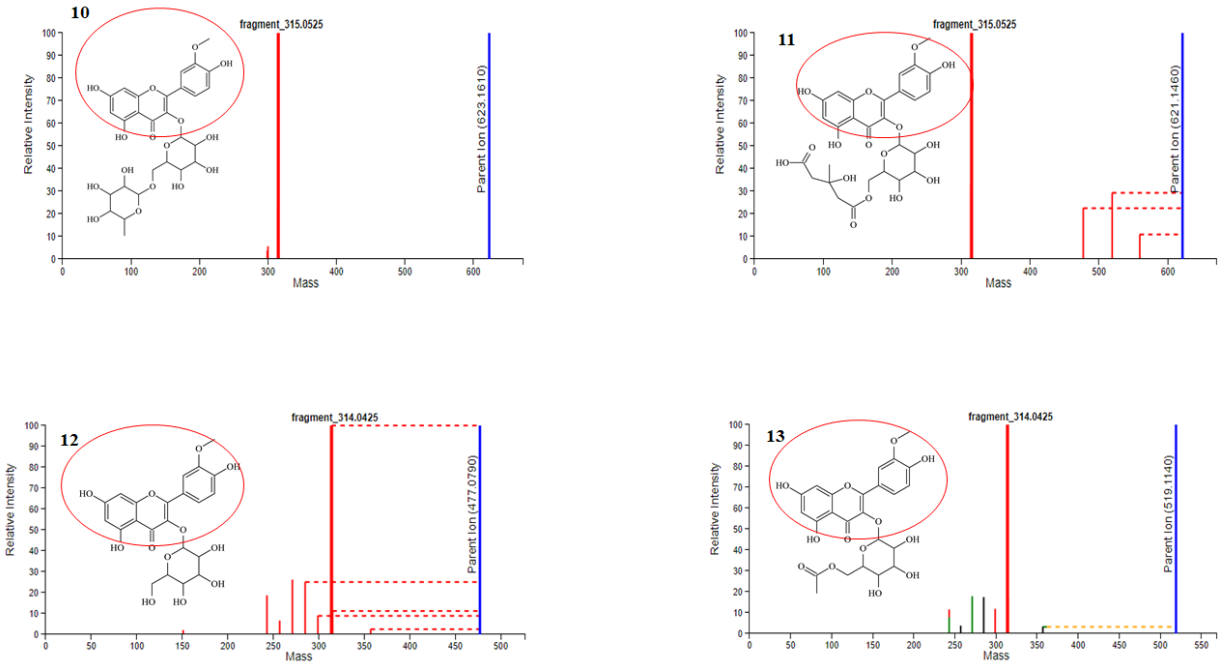

Figure 7.6: Fragmentation spectra of some isorhamnetin-related flavonoids in *M. oleifera* as annotated by MS2LDA.

7.3.1.4. Apigenin, luteolin, and chrysin flavonoids

Apigenin is a natural flavonoid found in a daily diet and has gained attention due to its low toxicity and various nutritional and biological properties. Because of the medicinal and nutritional properties, it is thus termed a nutraceutical. This flavonoid has antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic properties (Salehi *et al*., 2019; Wang *et al*., 2019; Dou *et al*., 2020). Chrysin is also a natural flavonoid that is found in many plants and bee products. This flavonoid has been reported to have a variety of biological properties such as anti-inflammation, anti-oxidation, anticancer, antibacterial, antidiabetic, and neuroprotective effects (Hwang *et al*., 2018; Zhu *et al*., 2019; Liu *et al*., 2021). Luteolin is a flavonoid that is found in medicinal plants, fruits, and vegetables. Plants that are rich in this flavonoid are often used for the treatment of various diseases such as inflammatory disorders, hypertension, and cancer (Aziz *et al*., 2018; Juszczak *et al*., 2019).

Figure 7.7 shows the fragmentation spectra of an apigenin-related flavonoid and a chrysin-related flavonoid as annotated by motif_535 and motif_538 mass2motifs on MS2LDA approach. Motif_538 is characterized by fragment ions 503, 473, 413, 395, 383, and 353 and neutral losses of 90, 120, 180, and 198 amu. Motif_535 is characterized by fragments at *m/z* 337, 367, 379, 457, and 497 and neutral losses of 90, 120, and 198 amu. Compound **14** gave a precursor ion $[M-H]^-$ at *m/z* 593.083. The MS/MS spectrum showed product ions at *m/z* 473.108 $[M-H-120]^-$ and at *m/z* 353 $[M-H-210]^-$ resulting from sugar fragmentations. This compound was identified as apigenin-6,8-C-dihexose (vicenin-2) (Makita *et al*., 2016). Compound **15** gave a precursor ion $[M-H]^-$ at *m/z* 577.156. The product ions observed in the MS/MS spectrum are due to the sugar fragmentations. This compound was thus identified as chrysin-6,8-C-diglucoside (Lin *et al*., 2013; Cherfia *et al*., 2020). This is the first time that this flavonoid is reported in *M. oleifera*.

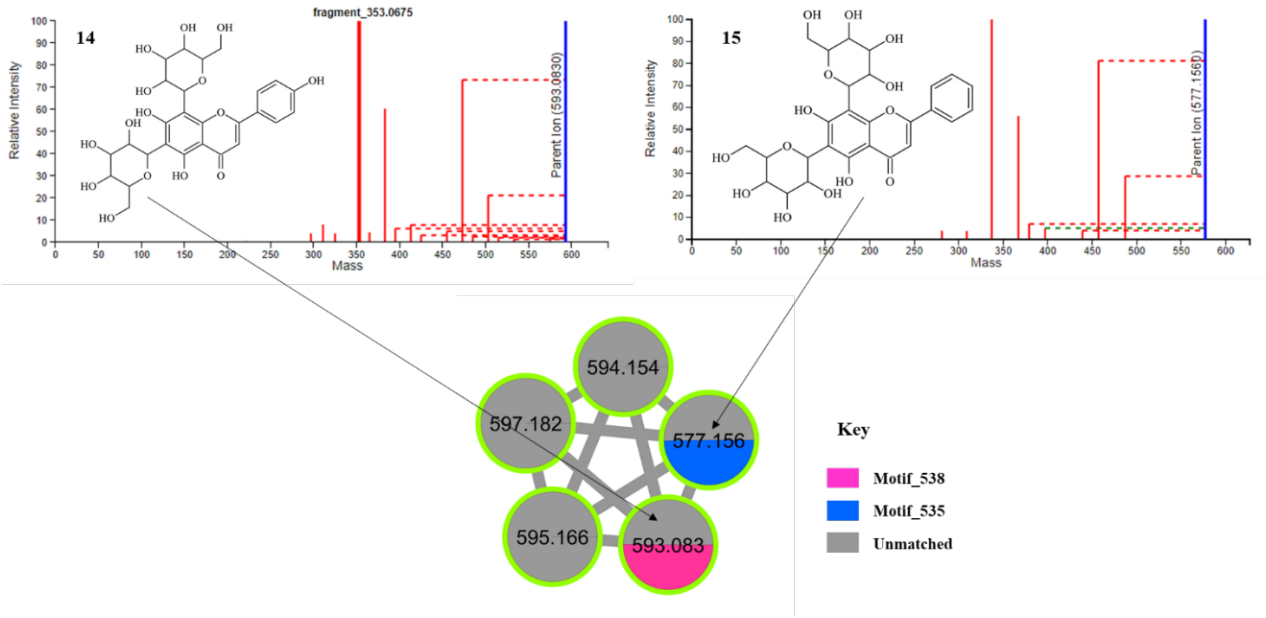

Figure 7.7: Fragmentation spectra of apigenin-6,8-C-dihexose (**14**) and chrysin-6,8-C-diglucoside (**15**) in *M. oleifera* as annotated by MS2LDA.

Figure 7.8 shows the fragmentation spectra of an apigenin-related flavonoid and a luteolin-related flavonoid as annotated by motif_570 mass2motifs on MS2LDA approach. Motif_570 is characterized by fragment ions at *m/z* 575, 357, 341, 339, 327, 323, 311, 299, 283, 215, and 197 and neutral losses of 18, 36, 90, 108, 120, 148, and 162 amu. Compound **16** gave a precursor ion $[M-H]^-$ at *m/z* 431.098. It gave a base peak fragmentation ion at *m/z* 311.058. Further fragments were observed at *m/z* 341.068, at *m/z* 323.053 due to the loss of H_2O and at *m/z* 283.063 due to the loss of a CO moiety. This compound was thus identified as apigenin-8-C-hexose (vitexin) (Wu *et al*., 2013). Compound **17** gave a precursor ion $[M-H]^-$ at *m/z* 447.093. The fragment ion observed at *m/z* 285.043 $[M-H-162]^-$ was due to the fragmentation of the hexose sugar. This compound was thus identified as luteolin-8-C-hexose (orientin) (Tahir *et al*., 2012).

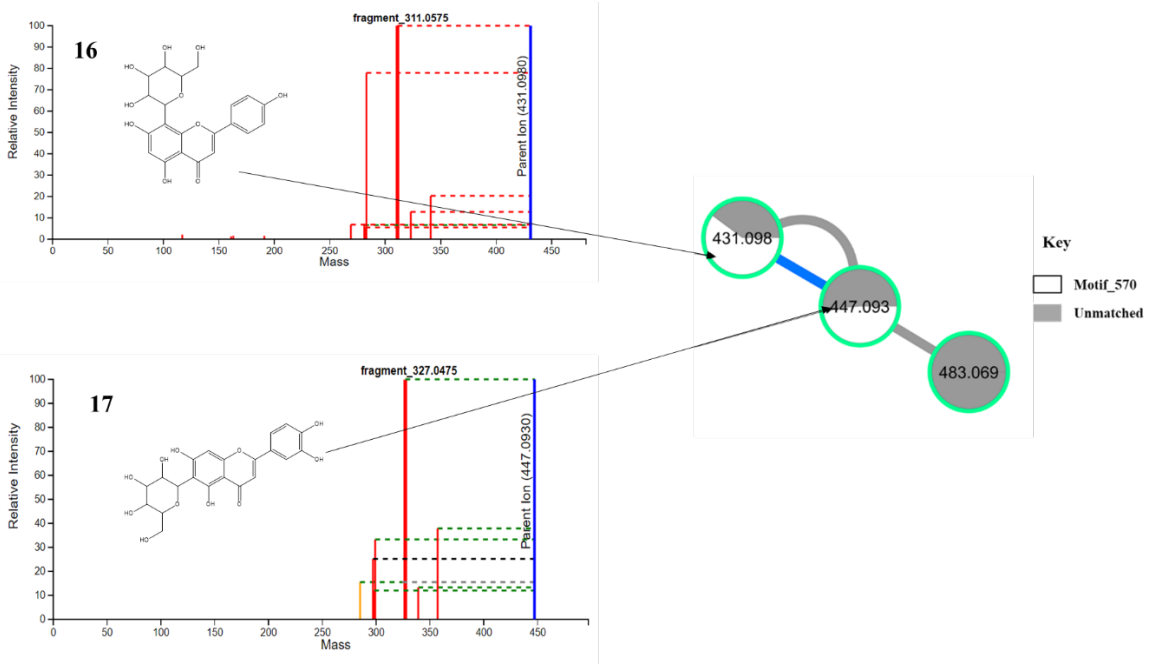

Figure 7.8: Fragmentation spectra of apigenin-8-C-hexose (**16**) and luteolin-8-C-hexose (**17**) in *M. oleifera* as annotated by MS2LDA.

7.3.1.5. Glycoisomerization of flavonoids

Moringa oleifera has been reported to undergo glycosylation patterns in order to diversify its flavonoids. *Moringa oleifera* attaches different types of sugars to its flavonoid aglycones (Tshabalala *et al*., 2019). For example, quercetin is observed to attach different types of sugars to its aglycone structure as observed in Figure 7.3. Furthermore, the glycosylation of flavonoids can undergo further chemical modification such as isomerization, acetylation, malonylation, and acylation. These modifications, however, bring about an analytical challenge because of the isomers are identified as structural artefacts. Some of the flavonoids undergo glycosylation through disaccharide sugar attachments (Nengovhela *et al*., 2021). Co-elution of different flavonoids is often encountered in LC, which makes it difficult to characterize the flavonoid composition. However, MS has a high sensitivity by making use of multiple reaction monitoring (MRM) which helps to improve the selectivity of the flavonoids (Frieden and Sjoberg, 2014).

Compounds that have similar molecular formulae, but different chemical arrangements are considered to be isomeric. For example, compounds kaempferol acetyl hexose (*m/z* 489), quercetin malonyl hexose (*m/z* 549), and isorhamnetin hydroxy methylglutaroyl hexose (*m/z* 621) with molecular formulae $C_{23}H_{22}O_{11}$, $C_{23}H_{22}O_{11}$, and $C_{28}H_{28}O_{11}$; respectively, are considered to be isomeric (Table 7.2). These isomers have similar molecular formula and

molecular mass and are also observed to have similar fragmentation patterns. However, the chemical arrangement of these compounds differs; which could be due to a slight shift in the position of the glycosidic bond between the organic acid and the sugar that is conjugated to the aglycone structure as suggested by Ramabulana *et al*. (2021). It, however, still remains a challenge to distinguish these molecules. There is therefore a need to develop advanced analytical techniques so as to be able to distinguish between molecules of such a nature.

Table 7.2: Isomeric flavonoids identified in *Moringa oleifera* methanolic leaf extracts.

$[M-H]^-$ (*m/z*)	Molecular formula	MS/MS fragmentation	Compound name
489.297	$C_{23}H_{22}O_{11}$	559.148; 519.118; 477.103; 315.053; 314.043	Kaempferol acetyl hexose (isomer 1)
489.114	$C_{23}H_{22}O_{11}$	559.148; 519.118; 477.103; 315.058; 314.043	Kaempferol acetyl hexose (isomer 2)
549.088	$C_{23}H_{22}O_{11}$	505.103; 463.088; 301.038; 300.033; 271.023; 255.028	Quercetin malonyl hexose (isomer 1)
549.088	$C_{23}H_{22}O_{11}$	505.098; 301.038; 300.033; 271.023	Quercetin malonyl hexose (isomer 2)
621.146	$C_{28}H_{28}O_{11}$	559.148; 519.118; 477.103; 315.053; 314.043	Isorhamnetin hydroxy-methylglutaroyl hexose (isomer 1)
621.146	$C_{28}H_{28}O_{11}$	559.148; 519.118; 477.103; 315.053; 314.043	Isorhamnetin hydroxy-methylglutaroyl hexose (isomer 2)

Isobaric molecules were also observed in this study. Isobaric molecules are molecules with the same mass but are different compound composition. In this study, isobaric flavonoids were observed to have similar precursor ion mass at *m/z* 609 and 447 and molecular formula $C_{27}H_{30}O_{16}$ and $C_{21}H_{20}O_{11}$, respectively. However, the compound composition differs. This observation thus makes these compounds isobaric. The flavonoids with molecular formula $C_{27}H_{30}O_{16}$ and precursor ion mass *m/z* 609 were identified as quercetin rutinoside and

kaempferol diglucoside and those with molecular formula $C_{21}H_{20}O_{11}$ and precursor ion mass *m/z* 447 were identified as kaempferol hexose and luteolin-8-C-hexose (orientin). These compounds were difficult to tell them apart using only an LC-MS spectrum. However, upon the untargeted LC-MS/MS approach for metabolite profiling, the difference in the fragmentation spectra were useful in the identification of these flavonoids and was thus easy to distinguish them as can be seen in Table 7.3 (Kachlicki *et al*., 2016).

Table 7.3: Isobaric flavonoids identified in *Moringa oleifera* methanolic leaf extracts.

$[M-H]^-$ (*m/z*)	Molecular formula	MS/MS fragmentation	Flavonoid name
609.197	$C_{27}H_{30}O_{16}$	343.048; 301.038; 300.028; 271.098; 255.028; 178.998; 151.003	Quercetin rutinoside
609.146	$C_{27}H_{30}O_{16}$	489.103; 447.098; 446.088; 327.048; 285.043; 283.023; 255.028	Kaempferol diglucoside
447.093	$C_{21}H_{20}O_{11}$	285.043; 284.033; 256.038; 255.028; 227.033	Kaempferol hexose
447.093	$C_{21}H_{20}O_{11}$	357.063; 339.048; 327.048; 299.053; 297.043; 285.043	Luteolin-8-C-hexose

7.4. Conclusions

The use of computational tools such as molecular networking highlighted the different molecular families that are found within *M. oleifera* and thus bringing insight into the chemical space of the plant. Unsupervised substructure annotation (MS2LDA) was useful in the annotation of Mass2Motifs of some of the flavonoids found within *M. oleifera*. An enhanced molecular network unraveled the different chemical classes found in this plant and thus revealing the metabolome of *M. oleifera*. Seventeen (17) flavonoids (flavonols and flavones) were successfully annotated by MS2LDA in this study and confirms what has been previously reported in literature. MS2LDA was also useful in the annotation of chrysin-6,8-C-diglucoside which is reported in *MO* leaves for the first time through this study.

In the existing literature, it has been documented that flavonoids in *M. oleifera* undergo glycosylation using various sugars as a mechanism to expand their chemical diversity. This glycosylation process has led to the detection of isomeric and isobaric flavonoids in our current study. The untargeted LC-MS/MS approach in combination with computational metabolomics tools such as molecular networking proved valuable in identifying isobaric molecules due to their distinct fragmentation patterns, thereby successfully accomplishing their identification. However, a challenge persists when it comes to identifying isomeric flavonoids, primarily because traditional MS techniques struggle to differentiate them effectively. Consequently, the future application of alternative MS analyzers, such as orbitraps and ion mobility, will become essential in addressing this challenge, especially when hyphenated to other computational metabolomics tools such as a feature based molecular networking.

7.6. References

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Chapter 8

General conclusions, future work, and recommendations

This chapter gives the general conclusions based on the research findings of this project. The future work and recommendations are also outlined in this chapter.

8.1. General conclusions

Moringa oleifera is a plant abundant with diverse metabolites and possesses the potential to contribute to the prevention of various diseases. Notably, the leaf extracts of this tree are particularly rich in flavonoids, with quercetin and kaempferol being the predominant types. The flavonoids in this tree undergo glycosylation with different sugars and thus diversifying the flavonoid chemical pool. This further enhances the bioavailability of the flavonoids from this tree. Rutin, a quercetin flavonoid that is glycosylated by the rutinoside disaccharides (rhamnose-glucose), is an essential flavonoid due to its various pharmacological properties. Currently, there exist mixed reports on the existence of this highly sought after flavonoid in *M. oleifera,* with many reports suggesting that the plant is incapable of producing this compound, with few reports showing the existence of this compound in *M. oleifera*. **Experimental Chapter 4** was aimed at determining the presence of rutin in *M. oleifera* from household plants within the Vhembe District in the Limpopo Province of South Africa. It was found that some plants could produce rutin and some were not capable. This observation led to the conclusion that there are different cultivars of *M. oleifera* and the accumulation of rutin is cultivar specific. This observation is believed to be genetically coded and that a genetic marker associated with production of rutin, and other related flavonoids can be determined for future selection of proper cultivar for propagation.

Furthermore, this study also focused on the extraction of rutin from *M. oleifera* leaf extracts using different extraction methods. Modern extraction methods such as UA-ATPE and PT-µSPE were investigated for the extraction of rutin in this study. UA-ATPE was investigated in **Experimental Chapter 5**, wherein the optimized conditions were ultrasonic time and ultrasonic temperature. Three different ethanol/salt ATPE systems were investigated in the extraction of rutin from *M. oleifera* leaves. The salts investigated were $(NH_4)_2SO_4$, $MgSO_4$, and NaCl. The ethanol/NaCl-UA-ATPE system was found to be the best performing in the extraction of rutin from the *M. oleifera* leaves. The optimal concentration of extracted rutin was found to be 240.00 µg. L^{-1} at a temperature of 25 ºC. It was observed that the rutin concentration decreased as the temperature increased, indicating that higher rutin concentrations are extracted at lower temperatures.

In **Experimental Chapter 6**, PT-µSPE was investigated for the extraction of rutin. Various parameters, including loading volume, dispensing/aspirating samples, elution solvent, concentration of rutin, mass of sorbent, and pH, were explored, providing insights into the optimum conditions for the extraction of this flavonoid. The LOD and RSD values obtained proved that PT-µSPE is a reliable pre-concentration technique that can be used in the analysis of complex samples containing the minute concentration of the intended analyte.

Computational tools were also used to further study the flavonoid chemical space of *M. oleifera* (**Experimental Chapter 7**). Herein, molecular network (MN) approach was applied which revealed the different molecular families within this plant. Other metabolomics computational tools such as network annotation propagation (NAP), DEREPLICATOR, substructure annotation (MS2LDA) and MolNet enhancer were used to complement MN. A combination of MolNetEnhancer and MS2LDA was used to annotate flavonoids in *M. oleifera* on ms2lda.org. The Mass2Motifs helped in the determination and confirmation of the substructures of the flavonoids within this plant. The use of MS2LDA was also useful in the identification of chrysin-6,8-C-diglucoside, reported for the first time in *M. oleifera*, thereby suggesting the robustness and usefulness of this approach to discover new chemical entities in plants. Using LC-MS and MS2LDA, isomeric and isobaric compounds were also identified in the *M. oleiferai* extracts. For instance, two isomers of kaempferol acertyl hexose, quercetin malonyl hexose, and isorhamnetin hydroxy-methylglutaroyl hexose were identified. Quercetin rutinoside and kaempferol diglucoside were also identified as isobaric compounds alongside kaempferol hexose and luteolin-8-C-hexose.

8.2. Future work and recommendations

The extraction of metabolites should be done on fresh leaves of *M. oleifera* and a comparison should be done with the extracted metabolites from matured leaves. A comparison study of the accumulation of the rutin flavonoid in leaves of different physiological states should also be considered. A study of the environment (i.e. soil conditions and climate conditions) should be conducted to evaluate the accumulation of metabolites in the leaf extracts. The genetic make-up of the rutin-producing and the non-rutin producing *M. oleifera* plants is important and should thus be further investigated.

The extraction method used to extract rutin from the leaves of *M. oleifera* is also an important factor. Modern extraction techniques make use of green technology and, therefore, other modern extraction methods such as microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE) should be investigated to get the best method for extracting this compound. It is also important to have an extraction method that is selective towards rutin. Therefore, the use of molecularly imprinted polymers (MIPs) can be used in future to selectively extract rutin from the leaves of *M. oleifera*.

Other rutinoside-bearing flavonoids such as kaempferol rutinoside and isorhamnetin rutinoside are also important since they are amongst the bioavailable components of *M. oleifera*. Further quantification studies of these flavonoids should be established to determine their presence and quantity in *M. oleifera* leaves. Techniques such as preparatory high performance liquid chromatography (prep-HPLC) can be used to isolate the rutinoside flavonoids (quercetin, isorhamnetin, and kaempferol) from the *M. oleifera* leaf extracts. These isolated compounds can then be analyzed using nuclear magnetic resonance (NMR) to ascertain the correct structural configuration of these compounds. Most importantly, other *in silico* methods such as network pharmacology should be used to further understand the nutraceutical outcomes of the flavonoid's composition of *M. oleifera* with or without rutin.

An inclusion of botanists or biochemists to look into the strains of the rutin-producing plants should be considered for a future project. This will assist in determining whether the plants are of the same or different strains.