LC-MS based metabolomic investigation of the effects of phytohormones on cultured cells of *Moringa oleifera*

By

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TO THE LORD, MY GOD, ALL THE GLORY…!!!

I can do all things through Christ who strengthens me.

~Philippians 4: 13

Dedication

I dedicate this work to the following:

My late grand-parents **Josue Tchokonte**, **Madelaine Tchuathi** and **Job Tchoualack** and great grand-parents **Sarah Djadeu** and **Anne Tcheukoua** for being my role models and inspiration

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Preface

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"Not everything that counts can be counted and not everything that's counted truly counts"

~Albert Einstein

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List of Abbreviations

ABA	Abscisic acid
ACX1	Acyl-CoA oxidase 1
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
ATP	Aqueous two phase
BA	Benzyl adenine
BAP	Benzylaminopurine
BPI	Base peak intensity
CE-MS	Capillary electrophoresis-mass spectrometry
CGA	Chlorogenic acid
CL	4-Coumarate-CoA ligase
CV-ANOVA	Cross-validation analysis of variation
CYP94	Cytochrome P450 (CYP) 94 family proteins
DES	Deep eutectic solvents
dnOPDA	Dinor-oxo-photodienoic
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ESI	Electron spray ionization
ET	Ethylene
ET	Electron transfer
F-C	Folin-Ciocalteu
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HAT	Hydrogen transfer
HCA	Hierarchical clustering analysis
H_2JA	Dihydrojasmonic acid
H_2JA-Ile	Dihydrojasmonoyl-isoleucine
JA	Jasmonate

JA-Ile/Leu	Jasmonoylisoleucine/leucine
JA-Phe	Jasmonoylphenylalanine
JA-Val	Jasmonoylvaline
KAT	L-3-Ketoacyl CoA-thiolase
Kin	Kinetin
LOX	Lipoxygenase
LC-EC-MS	Liquid chromatography-electrochemistry-mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-NMR	Liquid chromatography-Nuclear magnetic resonance
MeJA	Methyl jasmonate
MGDG	Monogalactosyldiacylglycerol
NAA	Naphthalene acetic acid
MFP	Multifunctional protein
NMR	Nuclear magnetic resonance
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MVDA	Multivariate data analysis
NADES	Natural deep eutectic solvent
NaOCl	Sodium hypochlorite
OPDA	12-Oxo-phytodienoicacid
OPLS-DA	Orthogonal projection to latent structures-discriminant
OPR3	OPDA reductase 3
PCA	Principal component(s) analysis
PDA	Photodiode array
PGRs	Plant growth regulators
PHW	Pressurised hot water
PPP	Pentose phosphate pathway

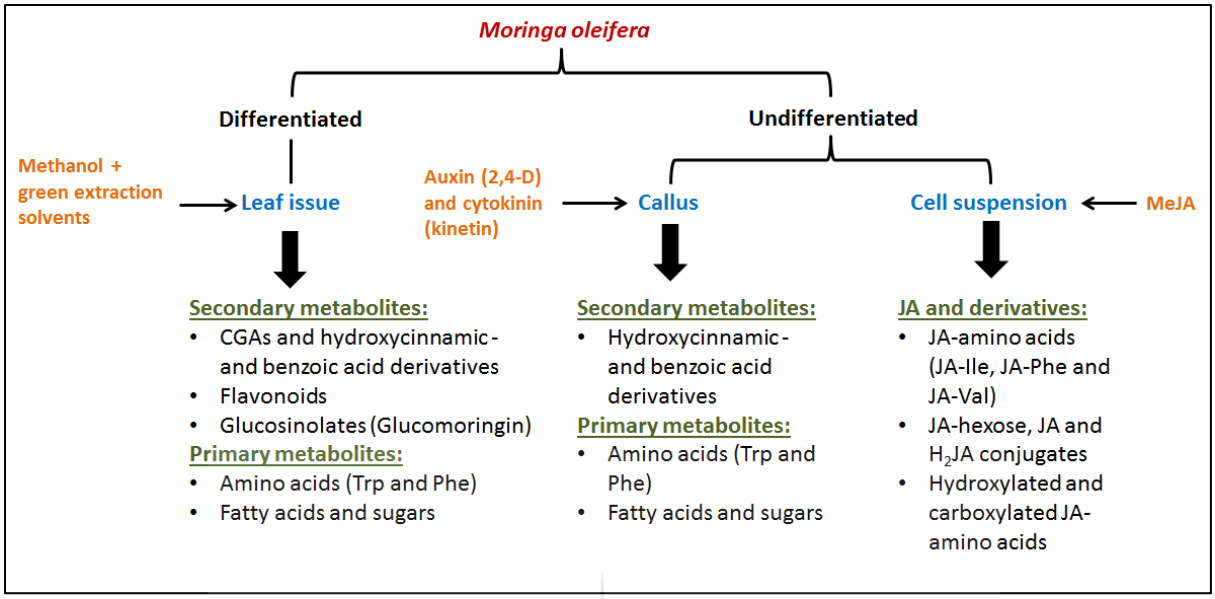
QqQ	Triple quadrupole
RNS	Reactive nitrogen species
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
SA	Salicylic acid
TOF	Time-of-flight
TPC	Total phenolic content
UHPLC-HDMS	Ultra-high performance liquid chromatography - high definition mass spectrometry
VIP	Variable importance in projection
12COOH-JA-Ile/Leu	12-Carboxyjasmonoyl-isoleucine/leucine
2,4-D	2,4-Dichlorophenoxyacetic acid
11-HPHT	11 (S)-Hydroxyperoxy linoleic acid
13-HPOT	13 (S)-Hydroxyperoxy linolenic acid
12OH-JA-Leu/Ile	Hydroxyjasmonoyl-leucine/Isoleucine
12OH-JA-Phe	Hydroxyjasmonoyl-phenylalanine
12OH-JA-Val	Hydroxyjasmonoyl-valine

Units

Da	Dalton
eV	Electron Volt
kV	Kilo Volt
g	Gram
mg	Milligram
µg	Microgram
mL	Milliliter
µL	Microliter
mg/L	Milligram per liter

mM	Millimolar
µM	Micromolar
°C	Celsius
h	Hour
min	Minute
R_t	Retention time
%	Percentage
nm	Nanometer

Summary



Graphical abstract

Moringa oleifera is the most widely studied species in the family of Moringaceae. The plant is well-known for the medicinal properties owed to the wide range of phytochemicals therein. The latter includes polyphenolics, glucosinolates, terpenoids and alkaloids. In order to investigate these compounds, plant hormones such as auxins, cytokinin, salicylic acid, ethylene and jasmonic acid are usually added to cell cultures that are good systems employed for the study of cellular activities in plants since they represent plant responses to both internal and external environments at a cellular level.

The present study mainly aimed to explore changes in metabolites following application of hormones to *M. oleifera in vitro* cells. In order to achieve this, additional aims included firstly to establish and compare the metabolite profiles of *M. oleifera* leaves. This was achieved with three different green extraction solvents: aqueous two phase (ATP) extraction solvent, pressurized hot water extraction (PHWE) and natural deep eutectic solvent (NADES). Secondly, to identify and characterize metabolites from the callus treated with different ratios of auxin and cytokinin; and finally to identify and characterize metabolites present in *M. oleifera* cell suspensions after exogenous application of methyl jasmonate (MeJA).

M. oleifera leaf tissue was extracted with PHW, ATP (20% ammonium sulfate and ethanol), NADES (choline chloride and citric acid) and methanol. For the callus, different ratios of auxin (2,4-dichlorophenoxyacetic acid, 2,4-D) and cytokinin (kinetin) were added to the culture medium in order to induce organogenesis and subsequent production of secondary metabolites. In the case of cell suspension cultures, the hormone MeJA (100, 200 and 300 µM) was added and incubated for 24 h. All methanolic extracts resulting from these experiments as well as green extracts were analyzed using a reverse phase ultra-high performance liquid chromatography high definition quadrupole time-of-flight mass spectrometry (UHPLC-qTOF-MS) instrument. The multidimensional data generated were further analysed using multivariate models.

A differential extraction of phenolics, flavonoids and glucosinolates was observed with the green solvents, yielding in some cases (*e.g.* vicenin 2, quercetin hexose) higher concentrations than methanolic extracts. In *M*. *oleifera* callus, no organogenesis was observed under any of the 25 auxin/cytokinin conditions. However, in addition to primary metabolites such as tryptophan and phenylalanine, secondary metabolites including derivatives of hydroxycinnamic acids and hydroxybenzoic acids were differentially observed in the extracts and putatively annotated. Anti-oxidant activity assays revealed the scavenging ability of extracts from *M*. *oleifera* callus in all conditions. Following exogenous application of MeJA to cell suspension cultures, jasmonic acid (JA) and derivatives (JA-amino acids and JA-hexose) as well as hydroxylated and carboxylated amino acid derivatives of JA were identified.

Although no chlorogenic acids, flavonoids and glucosinolates were observed in the undifferentiated *M. oleifera* callus as it was the case in differentiated leaf tissue, the present study revealed the presence of some novel, phenolic and anti-oxidant compounds in the cultured cells. Furthermore, in the case of *M*. *oleifera* cell suspension cultures, it was demonstrated that addition of MeJA resulted in its metabolism through bioconversion and biotransformation reactions. This study provides a basis for future investigations on the possible pathways employed by undifferentiated *M*. *oleifera* culture systems for the production of secondary metabolites.

Chapter 1: General introduction

1.1 Background

Plants have been utilized for medicinal purposes for centuries. *Moringa oleifera* is a highly valued plant native of India but also cultivated in some parts of Africa such as South Africa, as a multipurpose crop (Goyal *et al*., 2007; Nouman *et al*., 2016; Saini *et al*., 2012). The plant belongs to the Moringaceae family with 12 other species (Paliwal & Sharma, 2011; Sujatha & Patel, 2017). *M. oleifera* is a dicotyledonous plant economically important in Africa because of its multiple usages (*e.g.*: biofuel production, water purification, medicine, cosmetics) (Amaglo *et al*., 2010; Torres-Castillo *et al*., 2013; Rajalakshmi *et al*., 2017), and is also known as horseradish, ben oil, drumstick, miracle tree and 'mother best friend' tree (Vinoth *et al*., 2012).

The name miracle tree comes from the impressive range of medicinal properties of *M. oleifera*. These include anti-microbial (Busani *et al*., 2012), anti-inflammatory (Araújo *et al*., 2013; Minaiyan *et al*, 2014), anti-ulcer (Minaiyan *et al*., 2014), anti-hypertensive, anti-spasmodic (Toma & Deyno, 2014), anti-tumor (Goyal *et al*., 2007), anti-oxidant, anti-pyretic (Sujatha & Patel, 2017), anti-epileptic (Anwar *et al*., 2007), diuretic, cholesterol lowering, and anti-diabetic activities (Sharma & Paliwal, 2013). The pharmaceutical and health beneficial properties are associated with the large spectrum of phytochemicals present in *M. oleifera* plant (Makita *et al*., 2016; Vats & Gupta, 2017).

Phenolic acids and flavonoids are the main group of polyphenols found in *M. oleifera* (Leone *et al*., 2015; Makita *et al*., 2016; Hamany Djande *et al*., 2018). The former include hydroxybenzoic and chlorogenic acids (Amaglo *et al*., 2010; Leone *et al*., 2015; Vongsak *et al*., 2013; Hamany Djande *et al*., 2018), and the latter are quercetin, kaempferol, isorhamnetin and apigenin attached to various sugars molecules (Makita *et al*., 2016; Saini, 2016; Hamany Djande *et al*., 2018). *M. oleifera* is rich in glucosinolates such glucomoringin and derivatives (De Graaf *et al*., 2015; del Carmen Martínez-Ballesta *et al*., 2013;

Ramabulana *et al*., 2016; Hamany Djande *et al*., 2018). Biological activities of these sulphur- and nitrogen-containing compounds can be attributed to the hydrolysis products thereof (Dinkova-Kostova & Kostov, 2012; del Carmen Martínez-Ballesta *et al*., 2013). Terpenes and alkaloids are also found in *M. oleifera*, and contribute to the medicinal properties thereof (Furo & Ambali, 2012).

The investigation of plant secondary metabolites is a vast area of research, in which functions and pharmacological properties are currently being explored (Kabera *et al*., 2014). The use of plant cell and tissue systems for secondary metabolite production date from many years ago and has shown tremendous progress in this regard (Bourgaud *et al*., 2001; Raj *et al*., 2015). Plant cell and tissue culture regroups several approaches employed to grow plant cells, tissue or organs on a medium containing the appropriate nutrients and hormones under sterile conditions (Hellwig *et al*., 2004). It provides a defined production system with a controllable environment, hence ensuring a consistent phytochemical supply.

Plant cell and tissue culture involves dedifferentiation and also differentiation of cells depending on the desired outcome. Both approaches (dedifferentiation and differentiation) are usually achieved with the suitable ratio of plants growth hormones (commonly auxins and cytokinins) added to the medium (Smetanska, 2008). Moreover, plant hormones such as jasmonates (JA) and its methyl ester derivative, methyl jasmonate (MeJA), are naturally produced and released by the plant as part of the defense mechanism against biotic and abiotic stresses, and can be exogenously applied to the cell - and tissue culture medium to elicit the production of secondary metabolites (Mhlongo *et al*., 2016; Schaller & Stintzi, 2009).

Systems biology is the quantitative study of the complex network of interacting and interchanging molecular entities (DNA, mRNA, proteins and metabolites) and the environment that constitute an organism (Libault *et al*., 2017; Nielsen & Jewett, 2007). 'Omic' technologies (transcriptomics, proteomics and metabolomics) are high-throughput technologies required for the complete understanding of gene products (transcripts, proteins and metabolites) of a system. These are responsible of the breakthrough observed in systems biology (Libault *et al*., 2017). Among the 'omic' approaches, metabolomics is the

most recent to emerge, and constitute a valuable postgenomic tool for understanding plant metabolomes. Metabolomic studies provide the metabolic state of a biological system (cell, tissue or full organism) at a defined time point and under specific physiological conditions (Dunn & Ellis, 2005; Tugizimana *et al*., 2013). There is no single approach suitable for providing all metabolites of a system at a given time. A combination of methods and techniques is required (Fukushima *et al*., 2009; Tugizimana *et al*., 2014). As such, four solvents (extraction methods) were employed in the current study to illustrate the ability of green solvents to differentially extract metabolites from *M. oleifera* leaf tissue. This study also served to establish the foundation for subsequent metabolite profile investigations of *M. oleifera*. As already mentioned, the cell culture system represents a competent alternative for the biosynthesis of secondary metabolites. Therefore, in an attempt to investigate the capabilities of cultured cells for secondary metabolite production, we profiled metabolites extracted from auxin- and cytokinin-treated undifferentiated callus in comparison to the differentiated leaf tissue, using an UHPLC-MS-based analytical platform. In addition, *M. oleifera* cell suspension cultures were manipulated with the plant hormone, methyl jasmonate, to further investigate the *in vitro* production of secondary metabolites.

1.2 Hypothesis, aims and objectives of the project

Hypothesis

- *M. oleifera* callus cultures will undergo regeneration and organogenesis on artificial media subsequent to treatment with growth hormones (auxin and cytokinin), alone and/or in combination. Cellular differentiation / redifferentiation associated with organogenesis will be accompanied with differential production of secondary metabolites.
- *M. oleifera* cell suspension cultures will differentially produce secondary metabolites following treatment with the plant hormone methyl jasmonate.

Aims

The main aim of this study was to investigate the metabolomics changes subsequent the application of phytohormones on the cultured cells of *M. oleifera*. This was divided into three secondary aims:

- ✓ Compare metabolite profiles of *M. oleifera* dried leaves when using three different green extraction systems: aqueous two phase (ATP) extraction solvent, pressurized hot water extraction (PHWE) and natural deep eutectic solvent (NADES).
- ✓ Identify and characterize secondary metabolites in *M. oleifera* callus on media containing different concentrations of auxins and cytokinins.
- ✓ Investigate the ability of *M. oleifera* cell suspensions to respond to the phytohormone methyl jasmonate.

Objectives

- ✓ Evaluate the metabolite profile of *M. oleifera* differentiated leaf tissues obtained from the three different green solvents and compared to methanol.
- ✓ Evaluate the metabolite profile of undifferentiated *M. oleifera* callus cultured under 25 different conditions of varying auxin: cytokinin ratios.
- ✓ Evaluate the metabolic changes occurring in undifferentiated *M. oleifera* cell suspensions treated with the plant hormone methyl jasmonate.

1.3 Outline of the dissertation

Chapter 2: Literature overview.

Chapter 3: Differential extraction of phytochemicals from the multipurpose tree, *Moringa oleifera*, using green extraction solvents.

Chapter 4: Regenerative potential and metabolic profiles of *Moringa oleifera* calli as affected by different ratios of auxin and cytokinin.

Chapter 5: Metabolism of exogenously added methyl jasmonate by cell suspension cultures of *Moringa oleifera.*

Chapter 6: General conclusion

1.4 References

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Chapter 2: Literature overview

2.1 *Moringa oleifera* Lamarck (Lam.)

Plants are used by more than 70% of the world population for their medicinal and nutritional properties (Busani *et al.,* 2012; Furo & Ambali, 2012; Ferreira *et al*., 2014). *Moringa* is the sole genus of the flowering plant family Moringaceae containing 13 species: *M. arborea* native to Kenya; *M. rivae* native to Kenya and Ethiopia; *M. borziana*, native to Somalia and Kenya; *M. pygmaea* native to Somalia, *M. longituba* native to Kenya, Ethiopia and Somalia; *M. stenopetala* native to Kenya and Ethiopia; *M. ruspoliana* native to Ethiopia; *M. ovalifolia* native to Namibia and Angola; *M. drouhardii* and *M. hildebrandi* native to Madagascar; *M. peregrine* native to Red Sea areas and the Horn of Africa, *M. concanensis* and *M. oleifera* native to India (Mahmood *et al.,* 2010; Paliwal & Sharma, 2011; Ferreira *et al*., 2014; Leone *et al.,* 2015). *M. oleifera,* also known as the drumstick tree, horseradish tree and ben oil tree (Saini *et al.,* 2012; Tian *et al.,* 2015), is a deciduous, perennial and dicotyledonous tree not only cultivated in India but also in some parts of Africa (Paliwal & Sharma, 2011; Saini *et al.,* 2016). It is able to grow in many tropical and subtropical countries and usually grows very fast up to 12m in height and it is resistant to drought (Mbikay, 2012; Ferreira *et al*., 2014; Saini *et al.,* 2014; Leone *et al.,* 2015). *M. oleifera* is a highly valued plant in emerging countries due to its nutritional values (Anwar *et al.,* 2007; Pirrò *et al.,* 2016), water purification (Saini *et al.,* 2016), agro-forestry (Doerr *et al.,* 2009) and medicinal domains (Doerr *et al.,* 2009; Tian *et al.,* 2015). These properties are attributed to all parts of the plant: leaves, flowers, pods (fruits) and seeds as shown in **Figure 2.1** (Tian *et al.,* 2015).

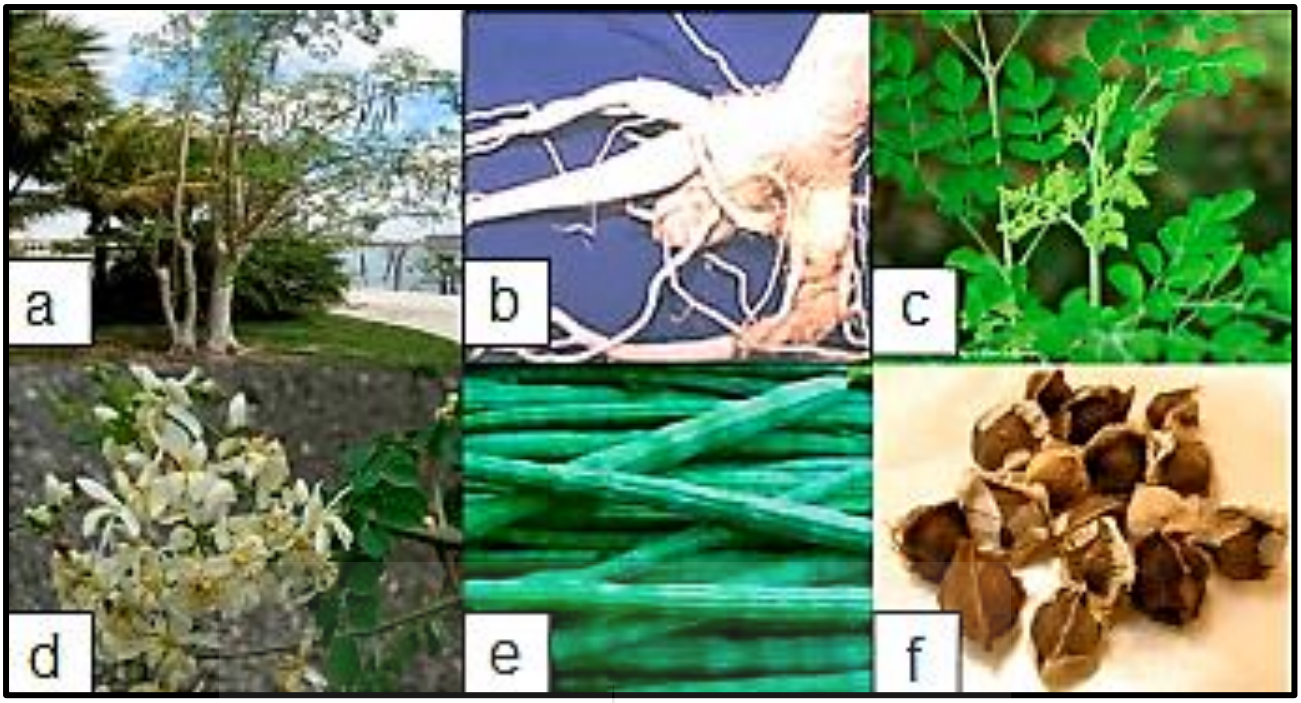

Figure 2.1: Vegetative and reproductive parts of *Moringa oleifera*. The tree (a), roots (b), leaves (c), flowers (d), pods or fruits (e) and seeds (f) (taken from Paliwal & Sharma, 2011).

2.1.1 *Moringa oleifera* as a nutritious plant

M. oleifera is an edible plant considered as an exceptionally nutritious crop that has long been used to fight against anaemia and malnutrition amongst breastfeeding women and children (Estrella *et al.,* 2000; Anwar *et al.,* 2007; Paliwal & Sharma 2011). It is a rich source of provitamin A, vitamin B, C, D and E; minerals such as iron, phosphorous and calcium; and proteins rich in essential amino acids (Mbikay, 2012). The vitamin C content in *M. oleifera* is greater than that of the orange, and the vitamin E (alpha-tocopherol) content is similar to that of nuts (Leone *et al.,* 2015). It has been reported that *M. oleifera* flowers and pods contain mono-unsaturated fatty acids; the leaves are rich in omega 3 - and omega 6 poly-unsaturated fatty acids such linolenic acid and linoleic acid, and the seeds are a rich source of palmitoleic -, arachidic - and stearic fatty acids. *Moringa* oil is similar to that of olive oil with the exception of a lower linoleic acid content (Saini *et al.,* 2016).

2.1.2 *Moringa oleifera* as a medicinal and health-beneficial (nutraceutical) plant

The name "Miracle tree" has been given to *Moringa,* not only because of its rich nutrients content but also because of its numerous biological activities. The multipurpose tree has an impressive range of medicinal and nutraceutical properties (**Figure 2.2**) (Marrufo *et al.,* 2013; Toma & Deyno, 2014). In South Africa for instance, *M. oleifera* is used by many tribes for the treatment of infectious diseases, nervous disorder, cardiovascular - and gastrointestinal illnesses and inflammations (Förster *et al*., 2013). *M. oleifera* is also involved in the treatment of diseases such as cancer, high blood pressure, asthma, hysteria, conjunctivitis, abdominal discomfort (Farooq *et al.,* 2012; Mathur *et al.,* 2014), malaria, diseases associated with HIV/AIDS (Leone *et al.,* 2015; Makita *et al.,* 2016), typhoid fever, parasitic and skin diseases, arthritis, diabetes, toothache, haemorrhoids (Farooq *et al.,* 2012; Leone *et al.,* 2015), chronic hyperglycemia and dyslipidemia (Mbikay, 2012). Moreover, *M. oleifera* has anti-tumour - (Guevara *et al.,* 1999), anti-oxidant - (Nouman *et al.,* 2016), anti-inflammatory - (Araújo *et al.,* 2013; Mathur *et al.,* 2014; Leone *et al.,* 2015), anti-hypertensive - (Saini *et al.,* 2013), anti-urolithiatic - (Mathur *et al.,* 2014), anti-spasmodic - (Debnath & Guha, 2007), anti-epileptic - (Maske *et al.,* 2012), cholesterol lowering - (Ghasi *et al.,* 2000) and diuretic activities (Sujatha & Patel, 2017). Anti-microbial - and anti-pyretic activities of *Moringa* have also been reported by Adetitun *et al.,* (2013) and Hukkeri *et al.,* (2006) respectively.

2.1.2.1 Anti-cancer properties of *Moringa oleifera*

Anti-cancer activities associated with extracts of the plant have been reported (Tiloke *et al.,* 2013; Elsayed *et al.,* 2016). For example, this was demonstrated by an increase in oxidative stress leading to apoptosis of lung cancer cells. In 2015, Jung *et al.,* (2015) reported the possible use of water extracts of *M. oleifera* as a potential natural drug against liver - and lung cancers. In the same year, Al-Asmari *et al.,* also reported the role of *M. oleifera* leaves and bark in breast - and colorectal cancers (Tiloke *et al*., 2013; Al-Asmari *et al*., 2015; Jung *et al*., 2015).

2.1.2.2 Anti-microbial properties of *Moringa oleifera*

Anti-microbial properties of *M. oleifera* have been reported (Pal *et al*., 1995; Busani *et al*., 2012). Different solvent-based extracts of *M. oleifera* were found to contain bactericidal activity against

both Gram-positive and Gram-negative bacteria (Pal *et al.,* 1995; Vieira *et al*., 2010; Busani *et al.,* 2012; Alozie *et al.,* 2015).

2.1.2.3 Anti-inflammatory properties of *Moringa oleifera*

Anti-inflammatory properties have also been reported in extracts of seeds and leaves of *M. oleifera*. This activity might be attributed to the large array of metabolites. These include: terpenoids, tannins, glucosinolates, sterols and flavonoids (discussed in detail in **section 2.2**). Over 30 anti-inflammatory compounds have been found in *M. oleifera* (Rao *et al.,* 1999; Fayazuddin *et al.,* 2013; Minaiyan *et al.,* 2014).

2.1.2.4 Anti-oxidant properties of *Moringa oleifera*

Oxidation is a chemical reaction that can occur in a living system and produces free radicals that can cause cell damage in excess. As a result, humans will suffer from degenerative diseases (such as coronary heart disease, cancer and Alzheimer's disease) and infections. These free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS include superoxide, hydroxyl radical and hydrogen peroxide whereas RNS include nitric oxide and peroxynitrite. Anti-oxidants have the ability to inhibit and neutralize these and their intake provides a protection against these oxidative-induced diseases (Sreelatha & Padma, 2009; Pakade *et al.,* 2013). Many researchers have studied the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity to determine the anti-oxidant activity of *M. oleifera* (Charoensin, 2014; Sreelatha & Padma, 2009). It was demonstrated that this plant contains significant amounts of natural anti-oxidants such as vitamin C, flavonoids and other polyphenolics (Sreelatha & Padma, 2009; Santos *et al.,* 2012; Pakade *et al.,* 2013; Charoensin, 2014). Moreover, the anti-oxidant activity of *M. oleifera* was compared with that of some vegetables (cabbage, spinach, broccoli, cauliflower and peas) and the results show that the said leaves or flower extracts have the highest radical scavenging capacity (Santos *et al.,* 2012; Pakade *et al.,* 2013).

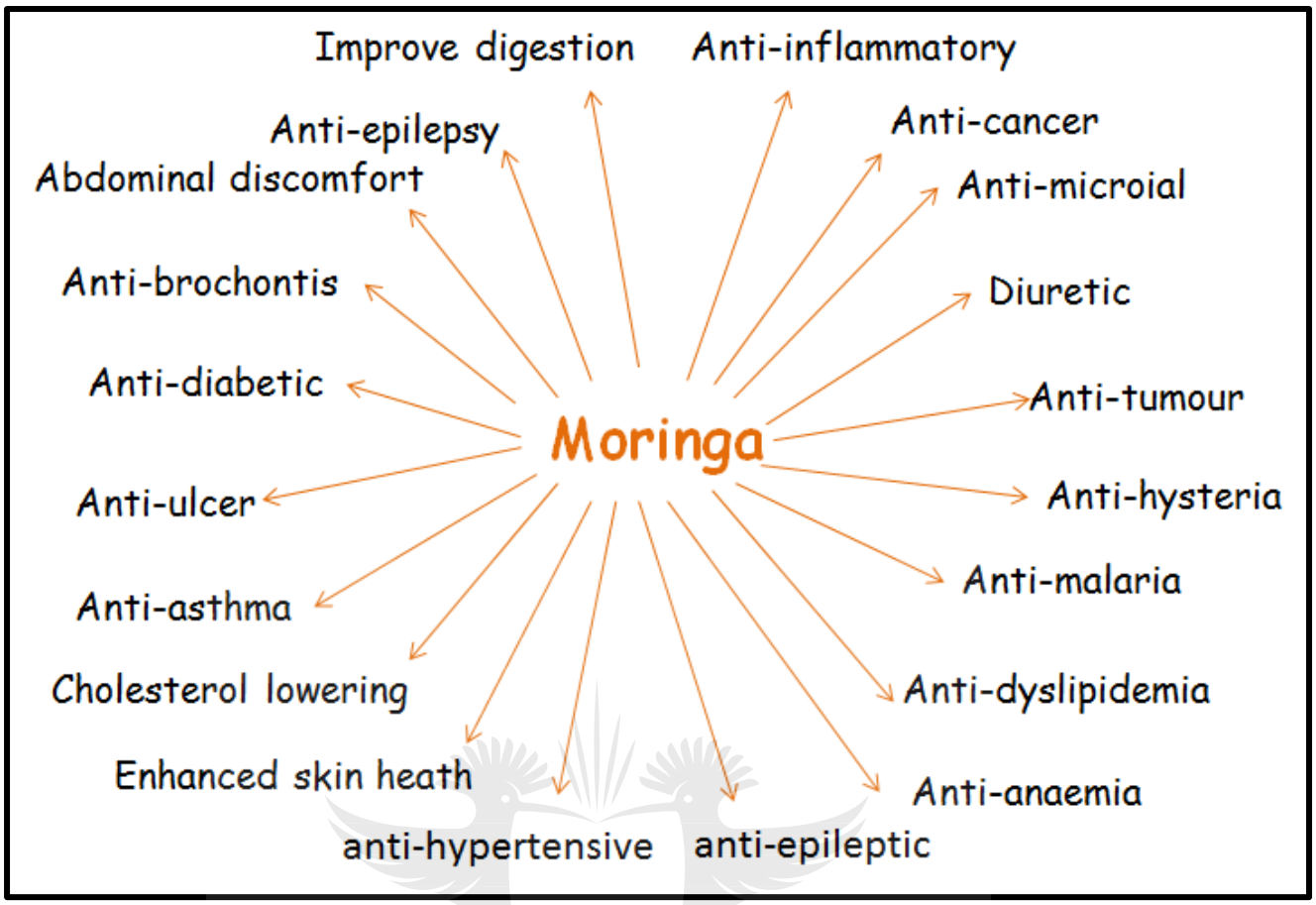

Figure 2.2: Example of health beneficial and medicinal properties of *Moringa oleifera*.

2.2 Bioactive secondary metabolites from *Moringa oleifera*

Plants produce a variety of compounds or metabolites having a wide range of properties. Some of these can be specific to a genus, species and even tissues and organs. The total number of metabolites within cell tissue or an organism is called the metabolome and constitutes the downstream event of genome transcription (Wink, 2003; du Preez & Loots, 2014). The metabolome of an organism can be divided into primary and secondary metabolites. The former represents compounds essential in basal cellular processes such as cellular division and growth, reproduction, photosynthesis, respiration, protein and nucleic acid synthesis and storage (Namdeo, 2007; Madala, 2012; Kabera *et al.*, 2014). These compounds are nucleotides, amino acids, sugars, lipids and energy sources (Aharoni & Galili, 2011). The latter, secondary or specialised metabolites (also known as Natural Products), are low molecular weight organic compounds that are not directly involved in plant basal processes, but are needed in plant interactions with the environment (Kutchan & Dixon, 2005; Namdeo, 2007; Ramawat 2013).

Natural Products can be classified based on their solubility, composition or according to the way they are synthesized. Secondary metabolites can be divided into three main groups: phenolics, terpenoids and sulphur- and nitrogen-containing compounds such as glucosinolates and alkaloids (Aharoni & Galili, 2011; Ramawat, 2013; Kabera *et al.,* 2014). Secondary metabolite precursors are derived from the primary metabolic pathways involving glycolysis and Krebs cycles, pentose phosphate pathway (PPP) and shikimate pathway **(Figure 2.3)** (Aharoni & Galili, 2011). After multiple reactions (such as hydroxylation, methylation, conjugation, prenylation and acylation), catalyzed by different enzymes belonging to different super-families (transferases, oxidoreductases, oxygenases, ligases, etc.), diverse secondary metabolite phenotypes will result (Zhang *et al.,* 2002; Vogt, 2010).

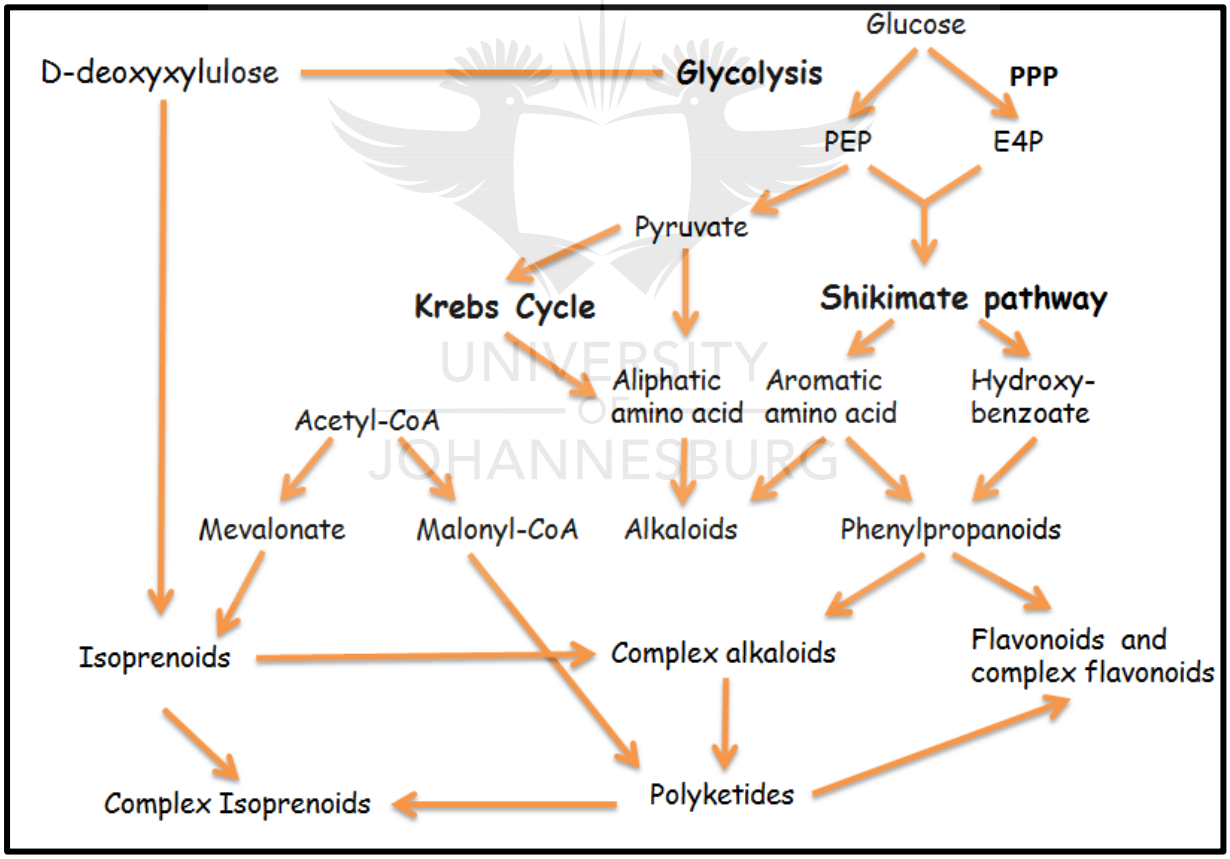

Figure 2.3: Metabolic pathways leading to the formation of some secondary metabolites. The Krebs cycle, glycoslysis, pentose phosphate - and shikimate pathways lead to precursors of phenylpropanoids, alkaloids and terpenoids. Each class of secondary metabolites can be expanded in their chemical complexity (Adapted from Aharoni & Galili, 2011).

M. oleifera is a reservoir of important secondary metabolites; the horseradish tree has been established as containing significant amounts of polyphenols and glucosinolates, and moderate or low amounts of terpenes and alkaloids **(Table 2.1)** (Anwar *et al.,* 2007; Amaglo *et al.,* 2010; Furo & Ambali, 2012; Toma & Deyno, 2014; Devisetti *et al.,* 2015; Saini *et al.,* 2016; Nouman *et al.,* 2016; Kalappurayil *et al.,* 2017; Vats & Gupta, 2017; Yadav *et al.,* 2017).

Table 2.1: *Moringa oleifera* bioactive compounds and pharmacological activities.

Group	Sub-group	Class	Pharmacological activities	References
Polyphenolics	**Phenolics**	**Hydroxycinnamic acids**	Anti-oxidative, pro-oxidant, hepatoprotective, anti-viral, anti-obese, anti-diabetic, anti-mutagenic, hypotensive.	Wood *et al.,* 1982; Yen *et al.,* 2002; Cho *et al.,* 2010; Park, 2010; *Sato et al.,* 2011; Khoza *et al.,* 2014; Devisetti *et al.,* 2015; Leone *et al.,* 2015; Nouman *et al.,* 2016; Ramabulana *et al.,* 2016
		Trans/*cis* Caffeoylquinic acids		
		Cis/*trans p*-Coumaroylquinic acids		
		Feruloylquinic acids		
		Hydroxybenzoic acids		
		Vanillic acid		
		2,3-Dihydroxybenzoic acid		
		Gallic acid		
		Ellagic acid		
	Flavonoids	Quercetin	Anti-oxidant, anti-bacterial, anti-hyperuricemia, anti-diabetic, anti-apoptotic, anti-thrombotic, anti-ischemic, anti-arrhythmic, and anti-hypertensive, vasodilator.	Hakkinen *et al.,* 1999; Amaglo *et al.,* 2010; Cushnie & Lamb, 2011; Shohaib *et al.,* 2011; Vongsak *et al.,* 2013a; Vongsak *et al.,* 2013b; Khoza *et al.,* 2014; Makita *et al.,* 2016; Nouman *et al.,* 2016; Ramabulana *et al.,* 2016; Kalappurayil *et al.,* 2017
		Quercetin glycosides		
		Kaempferol		
		Kaempferol glycosides		
		Isorhamnetin		
		Isorhamnetin glycosides		
		Apiginin glycosides		
Terpenes	**Monoterpenes**	α-terpineol	Anti-ulcer, anti-convulsant, anti-nociceptive, sedative	Kalappurayil *et al.,* 2017; Fons *et al.,* 2016

Sesquiterpenes	Nerolidol	Anti-inflammatory, analgesic, anti-schistosomal, antineoplastic, leishmanicidal	
Glucosinolates	Glucomoringin	Anti-cancer, anti-proliferative, anti-inflammatory, anti-microbial, detoxifying	Amaglo *et al.,* 2010; Toma & Deyno, 2014; Devisetti *et al.,* 2015; Leone *et al.,* 2015; Kalappurayil *et al.,* 2017
	Benzylglucosinolate (Glucotropaeolin)		
	Hydroxybenzylglucosinolate (Sinalbin)		
	Glucomoringin acetylated isomers		
Alkaloids	*N*,α-L-rhamnopyranosyl vincosamide	Analgesic, anti-pyretic, antihypo- and hypertensive central nervous stimulant and depressant, ant-iemetic, anti-cholinergic, anti-tumour, anti-malarial, oxytocic and vaso-constrictor	Toma & Deyno, 2014; Leone *et al.,* 2015; Saini *et al.,* 2016; Maqsood *et al.,* 2017; Yadav *et al.,* 2017
	Niazirin		
	Pyrrolemarumine 4''-O-α-L-rhamnopyranoside		
	Marumoside A		
	Marumoside B		
	4-(α-L-rhamnopyranosyloxy)-benzylcarbamate		
	Moringine (Benzyl amine)		
	Moringinine		

2.2.1 Polyphenolics

Polyphenolics constitute a large class of compounds abundant in the plant kingdom (Lattanzio, 2013). As the name indicates, polyphenolics are built of phenol rings and can be conjugated to one or more sugar moieties, amine, organic acids and carboxylic acids, lipids or other phenolics (Kondratyuk & Pezzuto, 2004; Pandey & Rizvi, 2009). Their biosynthesis occurs through the phenylpropanoid pathway **(Figure 2.4)** or the malonate/acetate pathway (Lattanzio, 2013).

Polyphenolics can be grouped into 4 classes according to the number of aromatic rings they possess: phenolic acids, flavonoids, stilbenes and lignans (Kondratyuk & Pezzuto, 2004; Pandey & Rizvi, 2009). Phenolic acids and flavonoids represent the main groups of polyphenols found in *M. oleifera* (Leone *et al.,* 2015) and they have been reported by many authors (Amaglo *et al.,* 2010; Vongsak *et al.,* 2013a; Vongsak *et al.,* 2013b; Leone *et al.,* 2015; Makita *et al.,* 2016; Nouman *et al.,* 2016; Kalappurayil *et al.,* 2017).

Phenolics can be further classified into benzoic acid - and cinnamic acid derivatives. Hydroxybenzoic acids have very complex structures (*e.g.* tannins) and are generally found in very low amount in plants. Hydroxycinnamic acids are more prominent in plants and comprise caffeic -, *p*-coumaric -, ferulic - and sinapic acids. These cinnamic acid derivatives can be esterified with a cyclic polyol, quinic acid, to form chlorogenic acids (Kondratyuk & Pezzuto, 2004; Manach *et al.,* 2004). Phenolic acids in *M. oleifera* include gallic acid, tannins (hydroxybenzoic acids) (Leone *et al.,* 2015) and chlorogenic acids (Amaglo *et al.,* 2010; Vongsak *et al.,* 2013a; Leone *et al.,* 2015).

Once synthesized, flavonoids are found in the plant cells vacuoles. These are water-soluble compounds that can be grouped into anthocyanins, flavones and flavonols. The total flavonoid concentration of *M. oleifera* dried leaves is higher than that of many known vegetable and fruits (Leone *et al.,* 2015). Except for the roots and seeds, all other parts of *M. oleifera* contains quercetin, kaempferol (Leone *et al.,* 2015), isorhamnetin and apiginin, all attached to a glycoside such as hexoses (sometimes benzyl and malonyl glucosides) and rutinosides (Makita *et al.,* 2016; Saini, 2016).

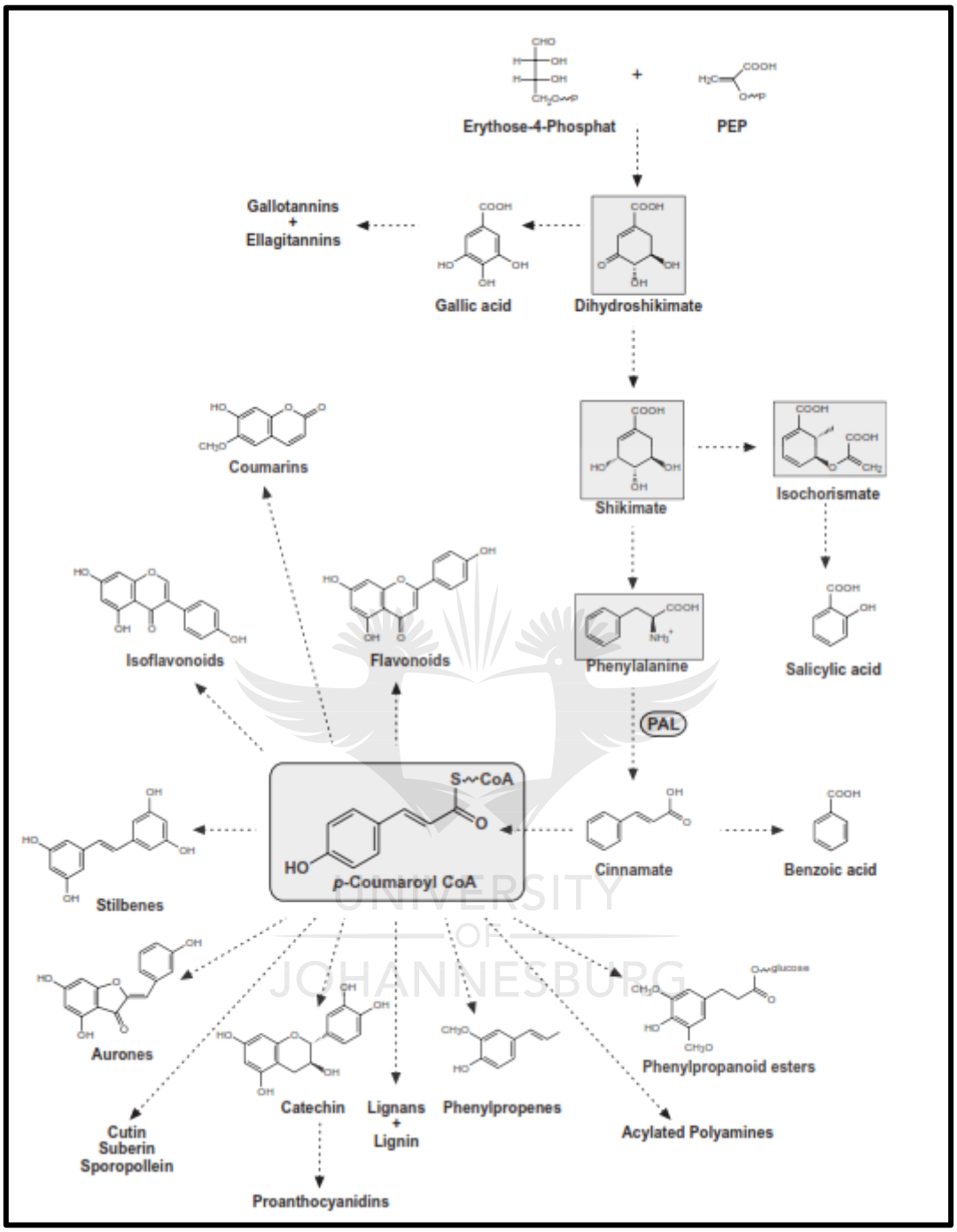

Figure 2.4: Summary of the phenylpropanoid pathway giving rise to the diversity of polyphenolics. The synthesis initiates with the shikimate pathway leading to the production of precursors such as phenylalanine. After the deamination of phenylalanine by phenylalanine ammonia lyase (PAL) to form a trans-cinnamate, reactions leading to the formation of coumaroyl-CoA (the most important intermediate in the biosynthesis of phenylpropanoids) will occur. Consequently, diverse polyphenolics will be produced (Taken from Vogt, 2010).

Polyphenolics are known to include molecules with potent anti-oxidant - (Vogt, 2010), anti-inflammatory -, anti-carcinogenic - (Kabera *et al.,* 2014), anti-diabetic -, anti-asthmatic - (Pandey & Rizvi, 2009), and anti-depressant activities (Kurkin *et al.,* 2006).

2.2.2 Glucosinolates

Glucosinolates are plant secondary metabolites commonly found in the order of Brassicales regrouping families such as the Moringaceae. These are compounds containing sulphur and nitrogen (del Carmen Martínez-Ballesta *et al.,* 2013; De Graaf *et al.,* 2015). The common core structure of glucosinolates contains a beta-D-thioglucose group and the variable side chain has amino acids as precursors **(Figure 2.5)** (Redovnikovic *et al.,* 2008; De Graaf *et al.,* 2015). Glucosinolates are biosynthesized in three steps: The first one being elongation of the amino acid chain involving the addition of alkyl groups to the side chain; the second step is the formation of the glucosinolate core structure and the last is modification of the side chain (methoxylation, sulfation, oxidation). The diversity derives from the first and third steps (Hayes *et al.,* 2008; Redovnikovic *et al.,* 2008).

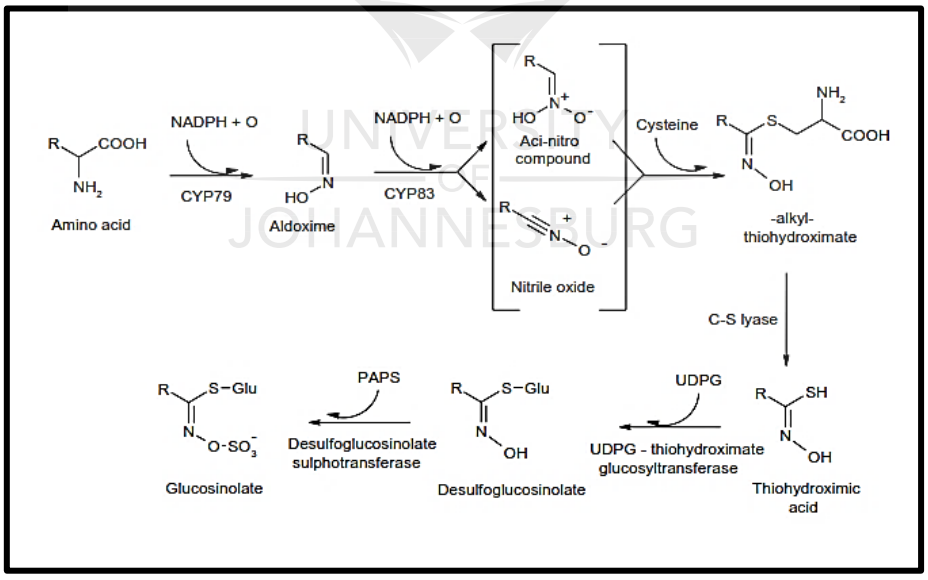

Figure 2.5: Conversion of amino acids (precursors) into glucosinolate core structure. UDPG: Uridine diphosphate glucose and PAPS: 3' phosphadenosine 5'-phosphosulphate. The R group derives from the original amino acid (Taken from Redovnikovic *et al.,* 2008).

Based on the structure of the amino acids, glucosinolates can be grouped into three classes namely, aliphatic glucosinolates (derived from methionine, isoleucine, leucine or valine),

aromatic glucosinolates (derived from phenylalanine or tyrosine) and indole glucosinolates (derived from tryptophan) (Redovnikovic *et al.,* 2008; De Graaf *et al.,* 2015). *M. oleifera* is rich in one specific aromatic glucosinolate, namely glucomoringin and acetylated derivatives (Förster *et al.,* 2015).

Plants synthesizing glucosinolates possess an enzyme called thioglucosidase or myrosinase (Redovnikovic *et al.,* 2008). During herbivore attack, the enzyme rapidly hydrolyses the glucosinolates into compounds that are more bioactive when the tissue is damaged **(Figure 2.6)**. These compounds include thiocyanate, isothiocyanate, nitriles, epithionitriles and oxazolidines (Hayes *et al.,* 2008; Dinkova-Kostova & Kostov, 2012; del Carmen Martínez-Ballesta *et al.,* 2013; De Graaf *et al.,* 2015). These compounds have anti-bacterial - and anti-cancer - or anti- proliferative properties, and are able to prevent cardiovascular diseases (Nastruzzi *et al.,* 2000; Hayes *et al.,* 2008; Jo *et al.,* 2016). They also have spasmolytic and hypotensive effects (Saini *et al.,* 2016).

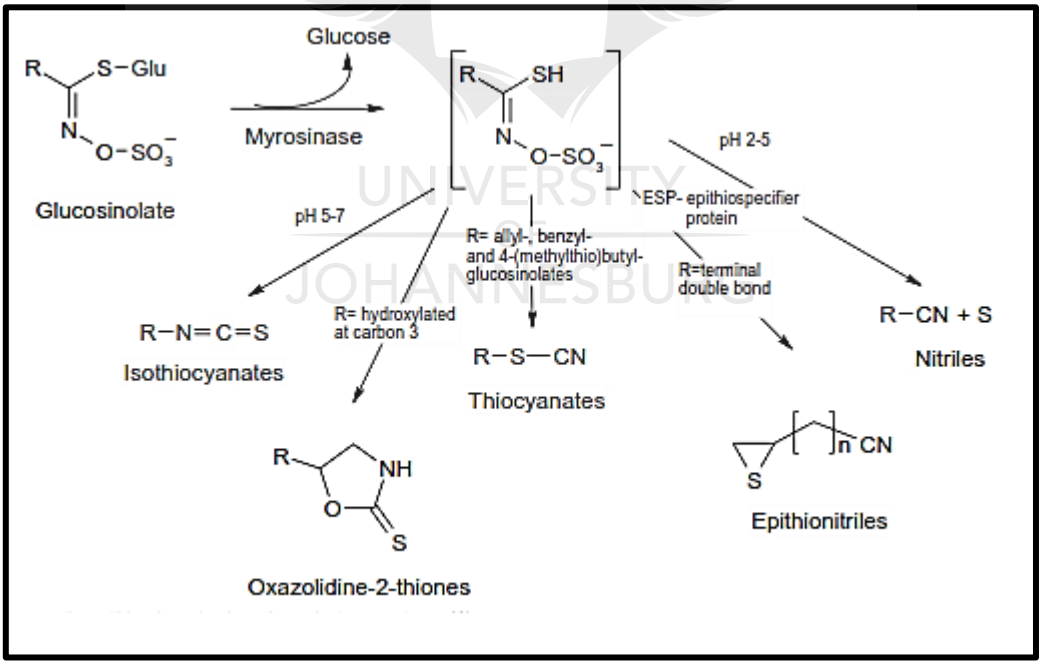

Figure 2.6: Structures of products resulting from the transformation of a glucosinolate by myrosinase (Taken from Redovnikovic *et al.,* 2008).

In *M. oleifera* leaves, the amount of glucosinolates is higher than that of many members of the Cruciferae (Brassicaceae) family (*e.g*. cabbage and broccoli). The most abundant glucosinolate in *M. oleifera* is glucomoringin (4-O-(a-L-rhamnopyranosyloxy)-

benzylglucosinolate), although glucotropaeolin is predominant in roots (Amaglo *et al.,* 2010; Saini *et al.,* 2016). Glucomoringin isomers reflecting the three position of the acetyl group at the rhamnose molecule have also been found in *M. oleifera* leaves (Leone *et al.,* 2015).

2.3 *In vitro* culture of *Moringa oleifera*

In most plant cell - and tissue cultures, the callus (originating from the Latin word *callum,* meaning hard) is a crucial starting source of cell material. Undifferentiated calli are totipotent cells, meaning they are capable to regenerate into the whole plant. These are growing masses of unorganised cells that can be manipulated in order to produce diverse compounds (Ikeuchi *et al.,* 2013). The most suitable explants for callus formation include stems, roots, leaves and cotyledons from young plants and tissue from ovary or stamens (Samuelsson & Bohlin, 2009). According to the degree of regeneration, calli can be classified into two subgroups, *i.e.* callus without organ regeneration and callus with partial organ regeneration. The former constitute of the friable or compact callus whereas the latter will constitute the shooty, embryonic or the rooty callus **(Figure 2.7)** (Ikeuchi *et al.,* 2013). The friable callus can be transferred into a liquid medium to form cell suspensions under appropriate controlled conditions (agitation, temperature, light, pH, *etc*.) (Chattopadhyay *et al.,* 2002; Hellwig *et al.,* 2004; Zhou & Wu, 2006).

In vitro growth can be carried out on artificial media containing required nutrients. The elementary medium for a simple growth includes water, a carbohydrate source such as sucrose, macro- and micro- nutrients. However, the growth can also be improved by adding very small amounts of organic compounds (vitamins, amino acids). A gelling agent such as agar can be added to solidify the culture medium. Exogenous addition of phytohormones or plant growth regulators such as auxins and cytokinins in cell culture medium can also influence the degree of growth, development or differentiation **(See section 2.3.2.1)** (Zhou & Wu, 2006; Samuelsson & Bohlin, 2009). A cell culture medium for *M. oleifera* was previously established (Shank & Lee, 2013) and the *in vitro* multiplication of the plant is being explored.

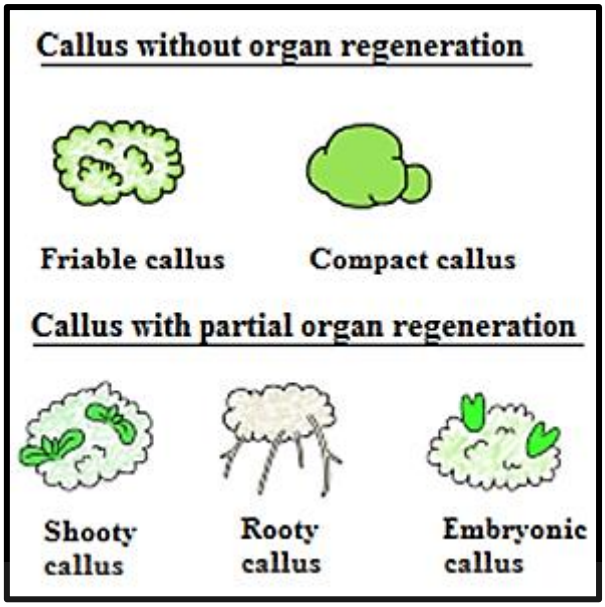

Figure 2.7: Illustration of different type of callus (Ikeuchi *et al.,* 2013).

2.3.1 *In vitro* multiplication

The increasing growth of the population as well as intense industrialization and urbanization results in an imbalance of the ecosystem. This higher demand for nutritional and medicinal plants led to the development of alternative sources thereof (Ali & Iqbal, 1999). Hence, cell - and tissue culture is a very promising technique for *in vitro* multiplication and conservation (Shahzad *et al.,* 2014; Cai *et al.,* 2015). Following one or combined external signals, a specific developmental pathway can be activated; leading to the regeneration of competent cells. However, the regenerative ability of plants differ from species to species (Sriskandarajah *et al.,* 2006). In *M. oleifera*, the regeneration and multiplication have been achieved directly by planting nodal segments of young seedlings or even mature plants onto Murashige and Skoog (MS) media enriched with auxins and cytokinins (**Figure 2.8**) (Ali & Iqbal, 1999; Islam *et al.,* 2005; Marfori, 2010; Saini *et al.,* 2012; Förster *et al.,* 2013; Shahzad *et al.,* 2014; Salem, 2016).

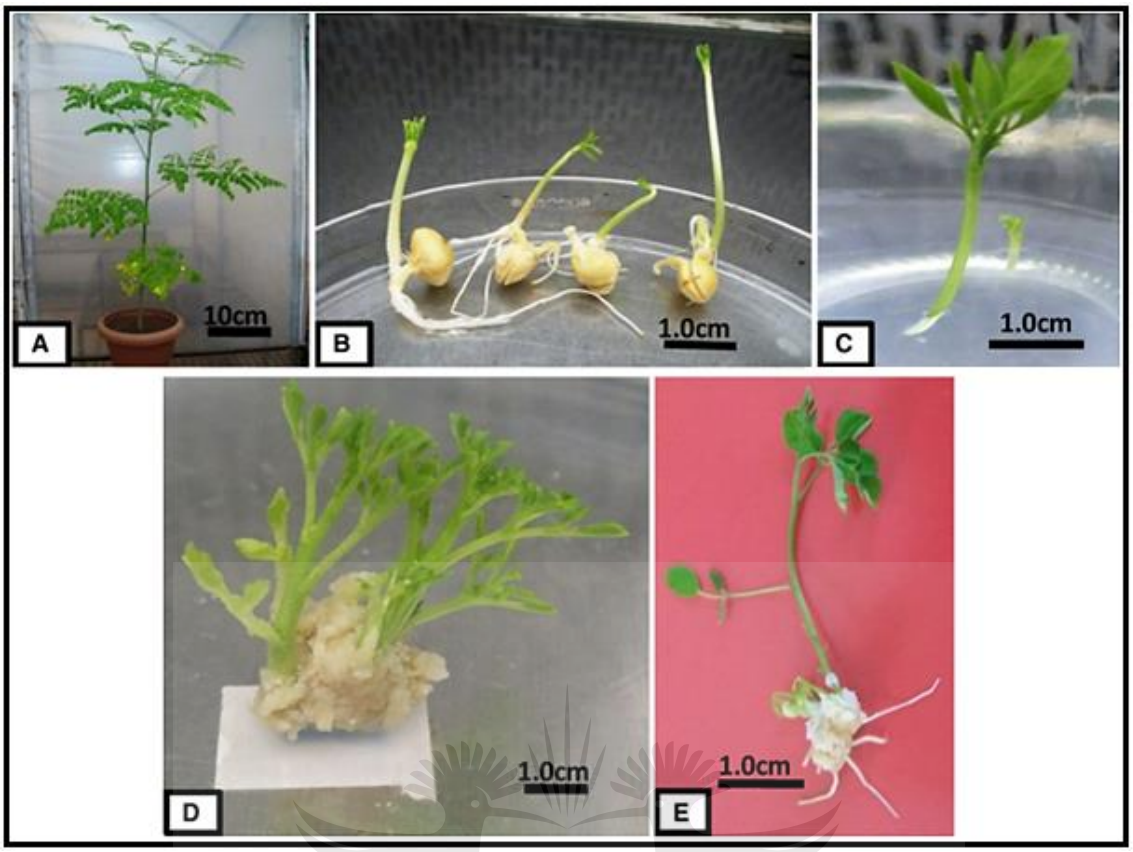

Figure 2.8: *In vitro* regeneration of *Moringa oleifera*. Nodal sections of seedlings from a *Moringa* plant grown in a greenhouse were cultured on a basal MS medium containing different hormones at different concentrations, and (a) plant grown in the green house, (b) aseptically grown seedlings, (c) shoot tips elongation, (d) multiple shoots and (e) root formation (Saini *et al.,* 2012).

2.3.2 Cultured plant cells as a source of phytochemicals

Plants synthesize a wide spectrum of natural products. Around 200,000 bioactive compounds have been identified and some have been used for many applications such as drug development in the pharmaceutical industry (DiCosmo & Misawa, 1995; Ochoa-Villarreal *et al.,* 2016). Extraction of these secondary metabolites from the source plant can be limited by its complexity and environmental instabilities. Moreover, the whole plant has a slow growth cycle and is easily affected by pest diseases (DiCosmo & Misawa, 1995; Chattopadhyay *et al.,* 2002; Mulabagal & Tsay, 2004). Essentially, the use of the whole plant for nutrition and anthropogenic activities, with the world population increasing, is leading to a speedy destruction of natural ecosystem, hence the dwindling of valuable medicinal plants such as *M. oleifera* (Shahzad *et al.,* 2014).

In order to overcome limitations of secondary metabolite production of the field-grown plant, alternative methods have been developed such as the exploitation of microbe and cultured plant cell culture. Upon comparing the microbial cell culture to the whole plant, the latter emerges as the better alternative with regard to cost, scaling and safety. Plant cell - and tissue culture is the *in vitro* growth of plant material, free of microbes in a sterile and controlled environment (Dornenburg & Knorr, 1995). Previously, plant cell - and tissue cultures were regarded as undifferentiated cells incapable of producing phytochemicals; however, Zenk and co-workers (1975) demonstrated the presence of anthraquinone in *Morinda citrifolia* cell cultures. This system has evolved as the best for the production of phytochemicals, merging advantages of both the source plant with those of the microbial cell systems (Bourgaud *et al.,* 2001; Ochoa-Villarreal *et al.,* 2016). Plant cell - and tissue culture can be developed as an abundant production platform for Natural Products, not affected by microclimate, day-to-night cycle, soil quality, or season, herbicides or pesticides, mycotoxins and fewer by-products (*e.g.* fibres, oil). Moreover, negative biological effects such as microorganisms and insects are eliminated (Rao & Ravishankar, 2002; Hellwig *et al.,* 2004; Akula & Ravishankar, 2011). Plant cell - and tissue culture is totipotent meaning a single cell in culture possess the entire genetic information of the whole plant and hence is potentially able to synthesize the same range of phytochemicals found in that plant under the appropriate conditions (Rao & Ravishankar, 2002; Pei *et al.,* 2010; Filová, 2014). The use of cell - and tissue culture can provide continuous and rapid production of secondary metabolites of constant quality and yield at low cost. It is also possible to select cultivars producing more secondary metabolites and to effectively recover intra- and extracellular products (Rao & Ravishankar, 2002; Mulabagal & Tsay, 2004). Furthermore, plant cell - and tissue culture systems are able to perform bio-transformations for the production of novel products from more abundant natural precursors, and also to perform post-translational modification comparable to those of human cells (Loyola-Vargas & Ochoa-Alejo, 2012; Rao & Ravishankar, 2002). Importantly, plant cell - and tissue culture is a powerful system for phytochemical production independent of food production, limiting the requirement of water and also considered as non-genetically modified (Ochoa-Villarreal *et al.,* 2015; Ochoa-Villarreal *et al.,* 2016).

The industrial production of plant secondary metabolites started in the 1960s. It has mostly been carried out in liquid cultures such as cell suspension cultures because of the uniformity of the culture conditions and rapid growth which is provided. For large-scale production of phytochemicals, stirred-tank and air-lift reactors are the main types of culture vessels. Although the research was very intense on this topic, only a few metabolites are commercially available. Examples of secondary metabolites and potential drugs produced by cell - and tissue culture includes shikonin produced by *Lithospermum erythrorhizon* cell culture (Yazaki *et al.,* 1999; Yazaki, 2017), ginseng saponin produced by *Panax ginseng* cell culture (Wu *et al.,* 2005; Zhou & Wu, 2006), and taxol and taxoid derivatives in cell culture of Taxus (DiCosmo & Misawa, 1995; Zhou & Wu, 2006). In addition, various flavonoids and isoflavonoids are produced by cell cultures of *Saussurea medusa* (Xu *et al.,* 2008), *Maackia amurensis*, *Glycyrrhiza uralensis* and *Panax japonicus* (Zhou & Wu, 2006). The production of metabolites by cell cultures requires a selection of a cell line that will quantitatively and qualitatively produce the desired results (Davies & Deroles, 2014). Production of secondary metabolites can be influenced by chemical - and physical factors (**Table 2.2**). Phytohormones can be used in the culture medium as growth hormones (auxins and cytokinins) or as elicitors (methyl jasmonate, salicylate, ethylene, *etc*.) and hence improve the secondary metabolite production.

Table 2.2: Factors affecting the production of secondary metabolites in plants cell cultures (Dornenburg & Knorr, 1995).

Strain improvement	• **Selection** • **Screening**
Medium variation	• Nutrients • Phytohormones (Auxins, cytokinins, *etc*.) • Precursors • Antimetabolites
Culture conditions	• Inoculum • Temperature • Light • Agitation
Specialised techniques	• Elicitors (such as Jasmonates) • Immobilization • Permeabilization • Two-phase systems • Two-stage systems

2.3.2.1 Plant growth regulators auxins and cytokinins: roles in cell cultures and effects on secondary metabolites production

Plant growth regulators (PGRs) are considered as one of the most crucial factors responsible of cell growth and differentiation (Radić *et al.,* 2016). More precisely, auxins contribute to cell growth development, cell wall acidification and stimulate the root formation. Cytokinins in the other hand promote shoots formation and expansion, and together with auxins, cell division. The regulation of cell division by both auxins and cytokinins is done at different levels. While auxins are involved in DNA replication, cytokinins are implicated in mitosis. As such, concentrations of both hormones in cell cultures should be wisely controlled (Gaspar *et al.,* 1996; George *et al.,* 2008; Manzur *et al.,* 2014).

As previously mentioned, these PGRs also play an important role in the plant secondary metabolism (Raj *et al.,* 2015). However the mechanism employed is not well understood. Auxins and cytokinins have been successfully exploited in different studies to increase the production of secondary metabolites such as phenolics (Radić *et al.,* 2016), flavonoids (Bota & Deliu, 2015) and alkaloids (Narasimhan & Nair, 2004; Raj *et al.,* 2015). This often depends on the type and concentrations of auxins/cytokinins applied (Raj *et al.,* 2015).

2.3.2.2 Jasmonates: elicitors for secondary metabolites production

Plants are constantly subjected to biotic - and abiotic stresses. In order to adapt to the changing environments, they accumulate signalling molecules and reprogram their gene expression. This involves activating defense-related genes and subsequent production of secondary metabolites. Plant hormones such as abscisic acid (ABA), salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and its derivatives, are sometimes used as elicitors in the production of secondary metabolites by cell cultures (Jalalpour *et al.,* 2014; Ncube *et al.,* 2016). Methyl jasmonate (MeJA), a derivative of JA, has shown great efficacy in increasing the production of terpenoids (James *et al.,* 2013; Tugizimana *et al.,* 2015; Filová & Krivosudská 2017), phenolics, alkaloids (Keinanen *et al.,* 2001; Sudha & Ravishankar, 2003) and glucosinolates in various medicinal plants (Yan *et al.,* 2013).

JA and its related members (*e.g*: MeJA, jasmonoyl-amino acids) are collectively referred to as jasmonates. They are fatty acid-derived compounds synthesized in plants through the octadecanoic acid (linolenic or linoleic acids) pathway or the hexadecadienoic pathway (Cheong & Choi, 2003; Schaller *et al*., 2004; Schaller & Stintzi, 2008). The two pathways result in the formation of 12-oxo-phytodienoicacid (OPDA) and dinor-oxo-phodienoic acid (dnOPDA) precursors of JA (**Figure 2.9**) (Miersch et al., 2007; Okada *et al*., 2015; Reyes-Díaz *et al*., 2016; Woldemariam *et al*., 2012)**.** JA derivatives can be formed through reactions such as *O*-glycosylation, hydroxylation and conjugation with amino acids (Schaller *et al*., 2004; Wasternack & Strnad, 2016). Furthermore, the enzyme carboxyl methyl transferase catalyzes the formation of MeJA in the cytosol (Cheong & Choi, 2003; Okada *et al*., 2015; Reyes-Díaz *et al*., 2016) (More details on JA synthesis are supplied in **Chapter 5)**.

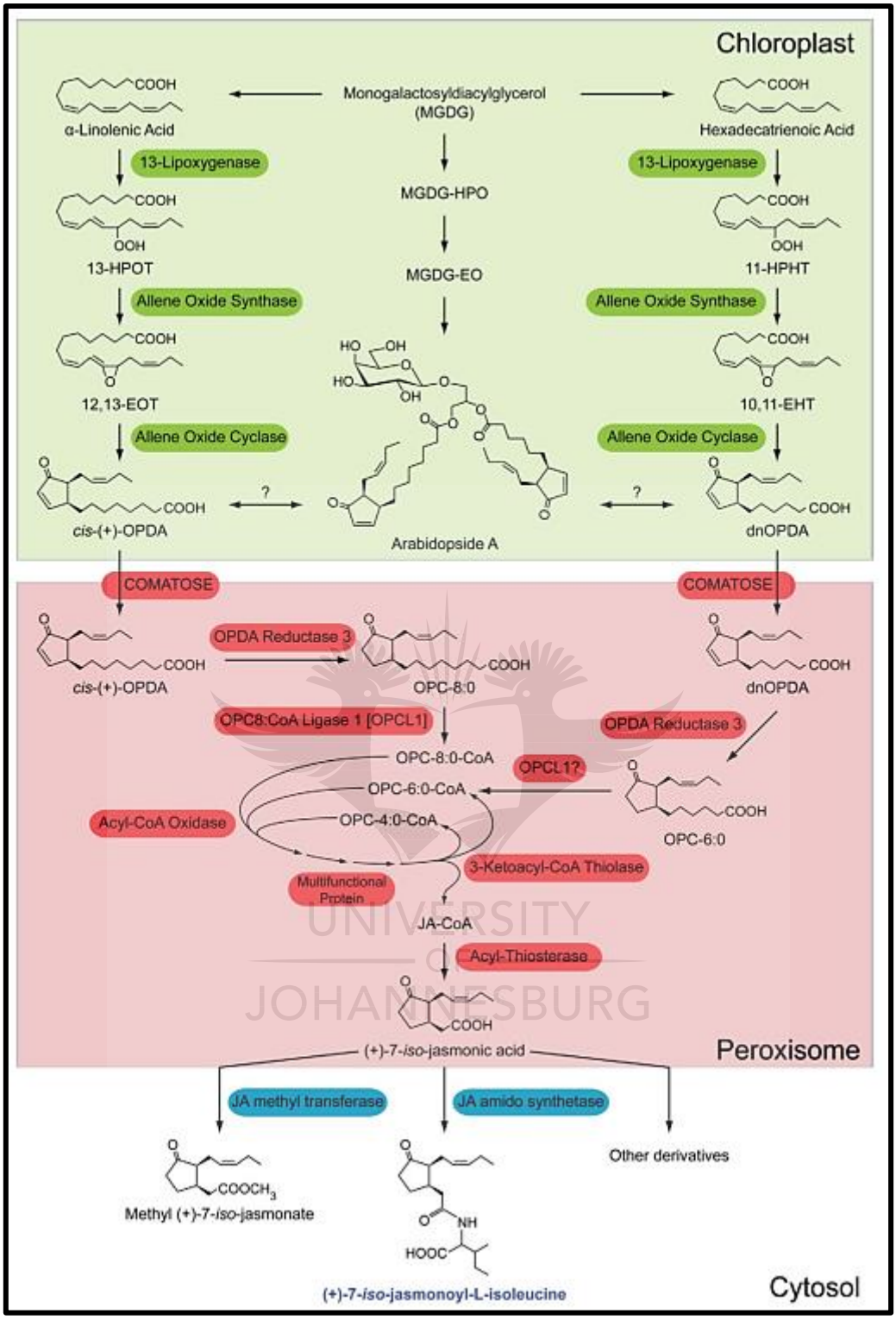

Figure 2.9: Overview of jasmonic acid biosynthesis in Arabidopsis. Linolenic acid (18:3) and the hexadecatrienoic acid (16:3) are kept in the plastid esterified to monogalactosyldiacylglycerol (MGDG). The pathway initiates in the chloroplast and the synthesis of JA takes place in the peroxisome after reduction reduction by OPDA reductase 3 (OPR3) and three cycles of β-oxidation by the enzymes acyl-CoA oxidase 1 (ACX1), multifunctional protein (MFP) and L-3-ketoacyl CoA-thiolase (KAT). JA is finally transported in the cytosol where its derivatives are produced (Taken from Acosta & Farmer, 2010).

2.4 Metabolomics in investigating secondary metabolism

The discovery of DNA structure and the definition of genetic code resulted from pronounced advances in biology pioneered mainly by molecular genetic studies (Kell *et al.,* 2016). After DNA sequencing (aiming to define the genes of a biological system (organisms, organs, tissues or cells)), the need to understand the relationship between genes and functions arose. This led to the establishment of different levels of 'omics': genomics, transcriptomics, proteomics and metabolomics (Goodacre *et al.,* 2004; Kell *et al.,* 2016) **(Figure 2.10)**. The aforementioned omics is the study of all genes of a biological system. Transcriptomics refers to all complement RNA (mRNA) molecules of an organism, organ, tissue or cell. Proteomics is the integrated study of gene products (proteins) and related biological function and processes. The last omic technology, metabolomics, is defined as a comprehensive (qualitative and quantitative) study of all small molecules present in a system in a given state and at a given time point, *i.e.* the metabolome (Goodacre *et al.,* 2004; Castillo *et al.,* 2011; Tugizimana *et al.,* 2013; Kell *et al.,* 2016). The combination of metabolomics with other 'omics' approaches provide a great contribution towards the complete biological profiling of a system (Hall, 2006; Cook & Rutan, 2014). Metabolite profiles result from anabolic and catabolic pathways are responsive to biological and environmental changes, and as such, they are correlated to the biochemical phenotype of an organism. Therefore, when compared to genomics, transcriptomics and proteomics, metabolomics provide a link between genotype and phenotype (Bhalla *et al.,* 2005; Hall *et al.,* 2006; Schrimpe-Rutledge *et al.,* 2016). This approach is a better reflection of cell functions (Goodacre *et al.,* 2004).

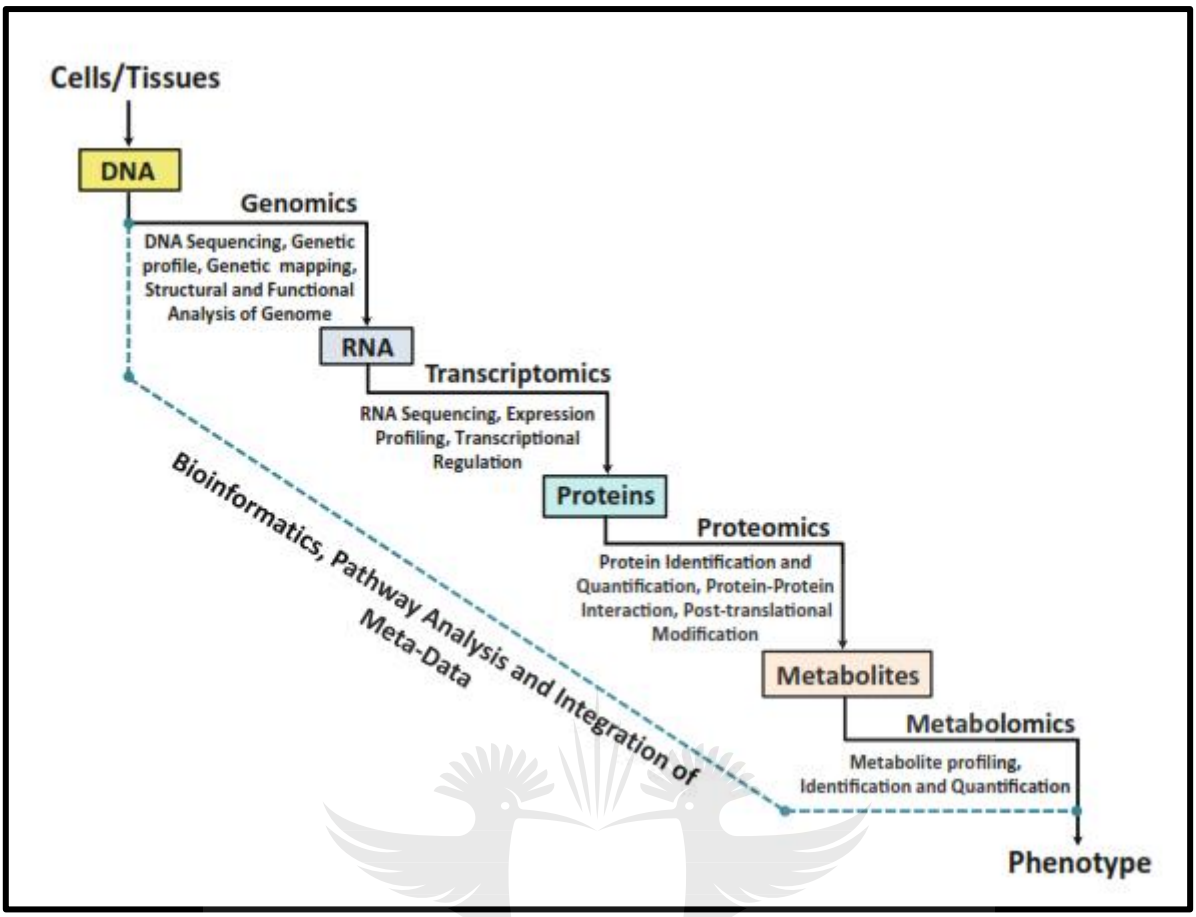

Figure 2.10: Omic approaches showing biological information flows from genomics to metabolomics, to produce a specific genotype (Ghatak *et al.*, 2017).

The plant metabolome is diverse and complex in nature. The polarity, solubility, stability and quantity vary within related organisms or even between organ types. This constitues a limitation in metabolomic studies. Using current technologies, it is not possible in a single analysis to extract and analyse the whole metabolome of a biological system. Thus, different metabolomic analyses require different strategies **(Table 2.3)** (Fiehn 2001; Sumner *et al.,* 2003; Dunn & Ellis, 2005; Hall *et al.,* 2006; Duportet *et al.,* 2012). These strategies can be used in combination to provide a comprehensive understanding of the plant metabolome (Dunn & Ellis, 2005; Tugizimana *et al.,* 2013) since all represent a point-in-time-chemistry of a biological system. In other words, such approaches give a metabolic picture of the cell at any precise time and under specidic conditions (Hall, 2006).

Table 2.3: Different metabolomic strategies and terminologies (Dunn & Ellis, 2005; Tugizimana *et al.*, 2013).

Metabolomics	Comprehensive, unbiased qualitative and quantitative study of the metabolome within a biological system, under specific conditions.
Metabonomics	Study in non-plant system, of endogenous metabolites following genetic modifications or pathophysiological stimuli.
Metabolite profiling	Qualitative and quantitative study of metabolites from a particular biochemical pathway.
Metabolite targeted analysis	Identification and quantification (using a standard protocol) of one or few pre-difined metabolites related to a specific metabolic pathway.
Metabolite fingerprinting	Classification of metabolites provide rapid analysis of sample without identification nor quantification.
Metabolite footprinting	Analysis of metabolites excreted from the intracellular compartment of a system to its extracellular matrix.

2.4.1 Workfow for plant metabolomics studies

Metabolomics is a multidisciplinary approach regrouping biochemistry, chemistry, biostatics and informatics, and aiming to characterise metabolites in a biolgical system (Fenaille *et al.,* 2017). Metabolomic strategies have been extensively employed to investigate the metabolite content of different plants (Mhlongo *et al.,* 2016; Ncube *et al.,* 2016; Gbashi *et al.,* 2017). Three main steps are involved in a metabolomic study. These are sample preparation, data acquisition and data analysis or data mining **(Figure 2.11)** (Tugizimana *et al.,* 2013; Cook & Rutan, 2014; Verpoorte *et al.,* 2014).

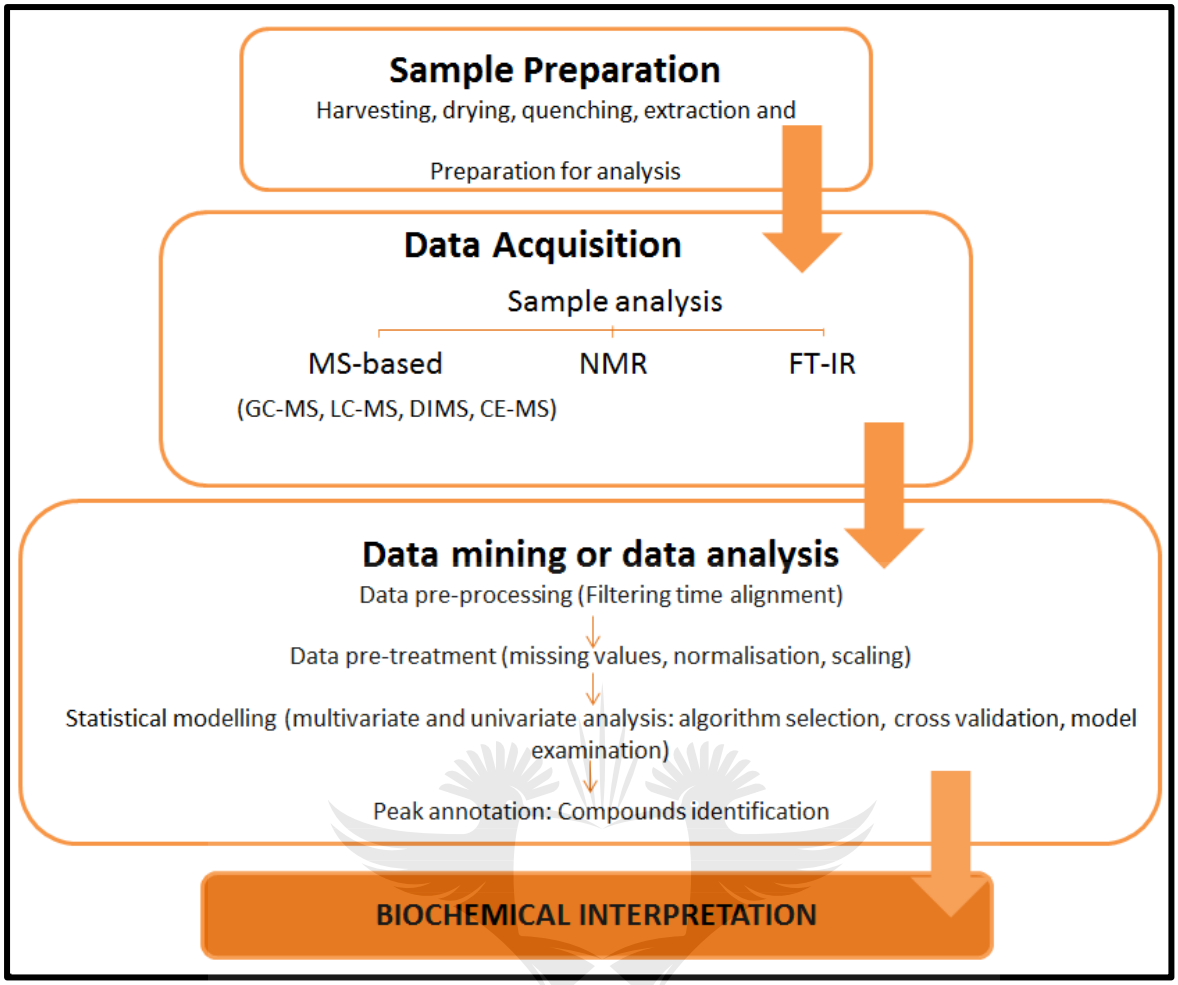

Figure 2.11: Metabolomic workflow (Tugizimana *et al.,* 2013).

2.4.1.1 Sample preparation

The integrity of a sample is a key factor determining the metabolite content and the biochemical intepretation of the data. This makes sample preparation a critical step in a metabolomic experiment. In order to obtain reproducible, robust, accurate and interpretable results, variations should be avoided as much as possible at each step of the sample preparation (Duportet *et al.,* 2012; Verpoorte *et al.,* 2014)**.** Sample preparation is divided into four steps: sample selection and harvesting, drying or quenching of enzyme activities, metabolite extraction and lastly, sample preparation for analysis (Bhalla *et al.,* 2005; Verpoorte *et al.,* 2014). Among these four steps, sample extraction and preparation for analysis are the only ones that vary according to the analytical tool chosen and the class of compounds desired (Verpoorte *et al.,* 2014). As mentioned there is no single extraction method able to cover the whole metabolome of

a system. The combination of procedures is needed to ensure a broader coverage of all metabolites, bearing in mind that different extraction methods or solvents generate different metabolite profiles and, according to the class of metabolite desired, a specific method should be used (Makkar *et al.,* 2007; Duportet *et al.,* 2012; Tugizimana *et al.,* 2013; Khoza *et al.,* 2014). In the present study, in addition to extraction with methanol as solvent, alternative 'green' extraction solvents were employed to evaluate the metabolite profile of *M. oleifera* leaves. 'Green' extraction solvents are increasingly being used in metabolomics studies as they are able to decrease the consumption of energy and ensure the purity of the product (Chemat *et al.,* 2012). The solvents/extractants investigated were aqueous two phase (ATP) extraction solvent, natural deep eutectic solvent (NADES) and pressurised hot water (PHW) **(please refer to Chapter 3)**.

2.4.1.2 Data acquisition

The complexity of plant extracts was mentioned before as one of the challenges in metabolomics studies. The analytical platform used for acquisition is also considered as a limitation (Sumner *et al.,* 2003) as it is not yet possible to have a complete analysis of all metabolite with a single instrument. Depending on the biological question and the class of compounds desired, analytical platforms can be used separatly or in combination (Tugizimana *et al.,* 2013; du Preez & Loots, 2014). For instance, to analyse non-polar and volatile compounds, gas chromatography (GC) coupled with mass spectrometry (MS) can be employed. Liquid chromatography-mass spectrometry (LC-MS) can be selected for analysis of polar to mid-polar and pre-ionised compounds (du Preez & Loots, 2014). The choice of the techniques is also dependent on their selectivity and sensitivity **(Table 2.4)**. In the current study, LC-MS or more precisely ultra high performance liquid chromatography coupled with a high definition mass spectrometry (UHPLC-HDMS) was employed and will be discussed in the paragraphs below.

Table 2.4: Analytical platform for metabolomic studies (taken from Weckwerth & Morgenthal, 2005). The choice of the technique will depend on the sensitivity, the throughput, and the comprehensiveness and the biological question one would like to answer.

Analytical platform	Sensitivity	Throughput	Comprehensiveness
Liquid Chromatography-Mass Spectrometry (LC-MS)	Medium	High	High
Gas Chromatography-Mass Spectrometry (GC-MS)	High	High	High
Capillary Electrophoresis-Mass Spectrometry (CE-MS)	High	Medium	High
Liquid Chromatography-Electrochemistry-Mass Spectrometry (LC-EC-MS)	High	High	High
Nuclear Magnetic Resonance (NMR)	Low	Low–high	Low-high
Infrared Spectroscopy	Low	High	Low
Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR)	Low	Low	High
Liquid Chromatography-Ultraviolet Spectroscopy	Medium-high	High	Very low

Liquid chromatography coupled with mass spectrometry (LC-MS) is a combined system employed in plant metabolomics to separate and detect different groups of compounds such as glucosinolates, polyphenolics, terpenes, saponins, alkaloids and polyamines (Hall, 2006; Allwood *et al.,* 2011). An advantage of LC-MS is that a sample derivatisation step, even though beneficial for a better sensitivity and resolution in certain cases, is not a prerequisite. Moreover it does not require high temperature for analysis and volatility of the sample. This makes the sample preparation step simple and not time-consuming (Dunn & Ellis, 2005).

Over the years, LC has evolved to more advanced technologies such as high performance liquid chromatography and ultra high performance liquid chromatography with higher

precision. These technologies can also be coupled with high resolution, sensitivity and mass accuracy MS detectors such as time-of-flight (TOF) (Hall, 2006).

LC-MS techniques have been useful in the separation of secondary metabolites from diverse plant extracts (James *et al.,* 2013; Madala *et al.,* 2014; Mhlongo *et al.,* 2016; Masike *et al.,* 2017) including medicinal plants such as *M. oleifera* (Pachauri *et al.,* 2013; Khoza *et al.,* 2014; Förster *et al.,* 2015; Ramabulana *et al.,* 2016; Makita *et al.,* 2017).

2.4.1.3 Data mining/Data analysis

Data mining or data analysis is the last step of the metabolomic work flow. It leads to biochemical interpretation and is divided into data preprocessing, data pretreatment, statistical modelling and metabolite identification. Data preprocessing and data pretreatment processes help to eliminate all biological variations that are not predefined or induced (Tugizimana *et al.,* 2013) (*e.g*: variation that are not related to the treatment).

The data files generated during metabolomic studies are usually large in dimension and complex (Sumner *et al.,* 2003; Verpoorte *et al.,* 2010). In order to extract significant information from the data sets otained, univariate and multivariate data analysis procedures are employed with the ultimate aim to answer a biological question. Univariate data analysis is performed when only a single variable is explored at a time. The focus of univariate methods is exclusively the mean and the variance of that single variable (Saccenti *et al.,* 2014). In metabolomics, data are usually mutivariate in nature (Keun *et al.,* 2004; Saccenti *et al.,* 2014). Multivariate data analysis (MVDA) deals with all variables at once and the simultaneous relationship between them (Saccenti *et al.,* 2014). The methods employed in MVDA utilise the mean and correlations or covariances explaining the degree of relationship between variables. These MVDA methods are chemometric techniques that can provide unsupervised and supervised models, simplifying the dimension and the complexity of the data set (Verpoorte *et al.,* 2010; Tugizimana *et al.,* 2013).

Unsupervised models are non-parametric and independent of the user. For simple plant metabolomic analysis, the principal component analysis (PCA) is mostly used. PCA is based on linear clustering algorithms providing rapid, comprehensive and qualitative visualisation of similarities/dissimilarities within and between samples (Bhalla *et al.,* 2005; Trygg & Holmes, 2007; Bedair & Sumner, 2008; Tugizimana *et al.,* 2013; Cook & Rutan, 2014).

In the supervised models (sometimes called predictive models), the multivariate data obtained from the metabolite profiling are transformed into a representation of biological importance under the control of the user (supervisor). The Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) is often used to predict models in plant metabolomic studies. It is a linear regression analysis which help identify ions accountable for the variation/discrimintion among known groups. In contrast to PCA, a descriptive model, OPLS-DA is an explicative model with predictive properties (Goodacre *et al.,* 2004; Tugizimana *et al.,* 2013).

Metabolite identification is the final step in data mining. It is of great importance as biochemical interpretation depends on its output. This step is particularly challenging as metabolites do not possess a common building block or a genetic template (Schrimpe-Rutledge *et al.,* 2016). Identification can be putative or definitive/validated. In putative identification, molecular and emperical formulae are generated and compared with available compounds, spectral and metabolic pathway databases. The definitive identification, on the other hand, employs numerous molecular properties (retention index, retention time, mass spectral fragmentation fragments, *etc.*), chemical standards and *in vitro* labelling methods (Hall, 2006; Verpoorte *et al.,* 2008; Tugizimana *et al.,* 2013). **Figure 2.12** is a summary of all the steps involved in metabolite identification.

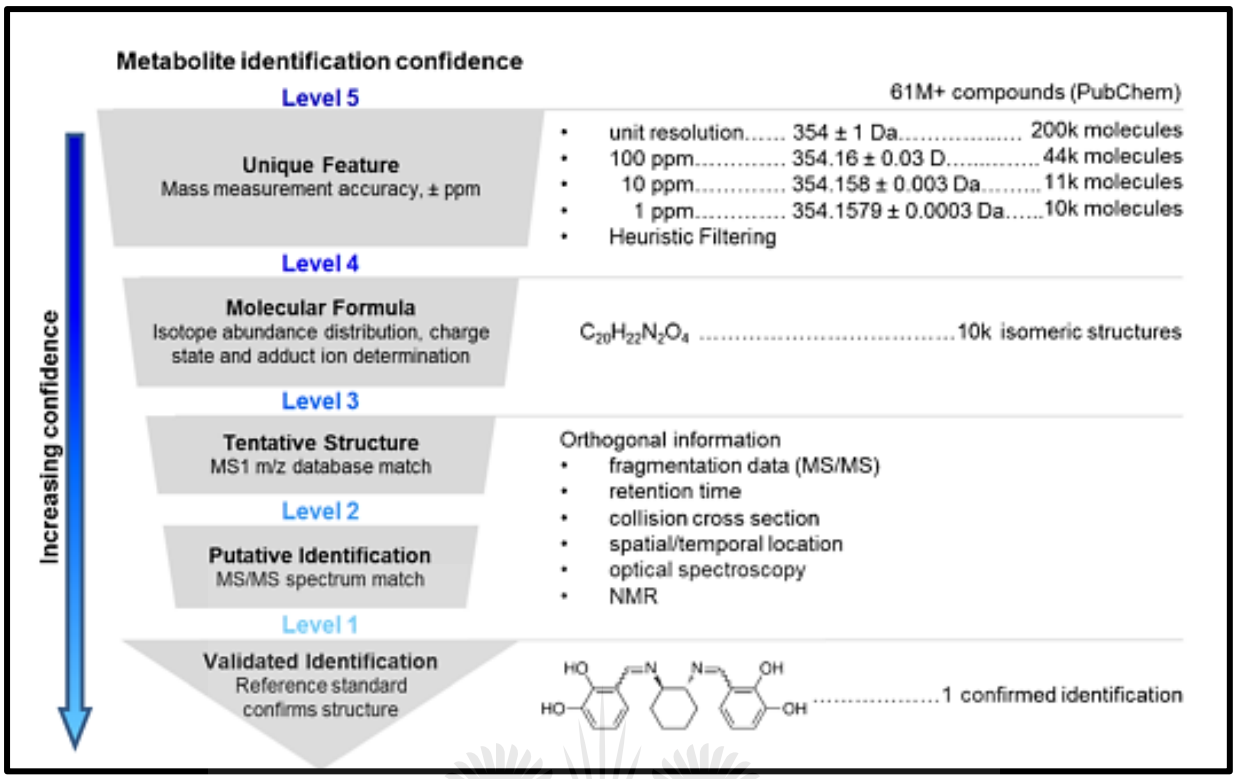

Figure 2.12: Workflow showing the level of confidence in metabolite identification using mass spectrometry. From top to bottom: the exact mass measurement for a specific feature (**Level 5**) allows a search in a database (PubChem for instance) and generation of a molecular Formula (**Level 4**), which still belongs to thousands of isomeric compounds. The tentative structures (**Level 3**) corresponds to precursor *m/z* of a metabolite in the database, and the putative identification (**Level 2**) matches to the fragmentation pattern of a metabolite in libraries. In order to obtain a validated identification (**Level 1**), additional data evidence, or results from other analytical techniques, and reference standard data under the same experimental conditions are required (taken from Schrimpe-Rutledge *et al.,* 2016).

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Chapter 3: Differential extraction of phytochemicals from the multipurpose tree, *Moringa oleifera*, using green extraction solvents

Abstract

Moringa oleifera is a tree plant with important bioactive compounds that confer its pharmacological properties. These compounds have been commonly extracted from the leaves using general methanol-based procedures. Organic solvents used for extraction purposes have negative impacts on the environment and human health. Green technologies are emerging as preferred choices for extraction as it encourages practices that are environmentally friendly and pose less or no harm to humans. In this study, the extraction-ability of pressurized hot water, aqueous two-phase (20% ammonium sulfate and ethanol) and the natural deep eutectic solvent (choline chloride and citric acid) was demonstrated on dried *M. oleifera* leaves. Each extract was analyzed on a reverse phase ultra-high performance liquid chromatography high definition quadrupole time-of-flight mass spectrometry instrument. Secondary metabolites, belonging to the class of polyphenolics (hydroxycinnamic acid and flavonoids) and glucosinolates, as well as primary metabolites were annotated in these leaf tissue extracts. In the pharmaceutical industry where extraction is one of the most crucial steps for new drug discovery, this study will provide a premise illustrating the extractability of phytochemicals with the aid of eco-friendly solvents.

Keywords: Aqueous two phase; Chlorogenic acids; Extraction; Flavonoids; Glucosinolates; *Moringa oleifera*; NADES; PHWE.

3.1 Introduction

Moringa oleifera (Lam.) is a fast-growing tree belonging to a monogeneric family called Moringaceae (Rajalakshmi *et al.,* 2017). Also known as horseradish tree or ben oil tree, *M. oleifera* is an important plant in developing countries because of its multiple applications

that include water purification (Arora *et al.,* 2013), biopesticide (Leone *et al.,* 2015), cosmetic -, pharmaceutical - (Mulugeta & Fekadu, 2014) and medicinal properties (Arora *et al.,* 2013; Toma & Deyno, 2014). All these applications are attributed to the wide range of phytochemicals produced by this plant (Mulugeta & Fekadu, 2014).

In general, phytochemicals are extracted using noxious substances (organic solvents such as methanol), with negative impacts on the environment and human health (Chemat *et al.,* 2012). As such, green technologies are progressively used in almost all scientific fields to encourage environmentally friendly practices with less or no harm on humans (Dai *et al.,* 2013; Tang *et al.,* 2016; Juneidi *et al.,* 2016). The development of green solvents such as ionic liquids, aqueous biphasic systems and pressurized hot water (Ruiz-Aceituno *et al.,* 2013), in order to substitute or reduce the use of hazardous organic solvents, has developed into an active research field (Chemat *et al.,* 2012; Heng *et al.,* 2013).

The interest in ionic liquids (ILs) is based on their low vapor pressure, high boiling point and adjustable physicochemical properties that allow extractions of compounds with varying polarity (Martinis *et al.,* 2010; Ruiz-Aceituno, 2013; Matong *et al.,* 2017). Although ILs have been used in the extraction of various micro-elements in different industries, the applications in the pharmaceutical industry has been questioned due to the toxicity observed with some of the constituents (Dai *et al.,* 2013; Matong *et al.,* 2017). For instance, pyridinium- or imidazole-based ILs are equally toxic to traditional organic solvents (Xu *et al.,* 2015; Matong *et al.,* 2017). Moreover, the irritating properties (Dai *et al.,* 2013) as well as the high production cost of synthesis also constitute drawbacks (Mulia *et al.,* 2015; Xu *et al.,* 2015; Juneidi *et al.,* 2016; Matong *et al.,* 2017). As such, the development of alternative extractants such as deep eutectic solvents (DES) has been forthcoming (Bi *et al.,* 2013; Xu *et al.,* 2015).

DES made of natural and renewable compounds are similar to ILs in term of physicochemical properties and phase behavior (Dai *et al.,* 2013; Tang *et al.,* 2016; Matong *et al.,* 2017). However, these solvents have the advantage of being easily prepared without any purification steps, inexpensive, non-inflammable, sustainable, biodegradable and recyclable to naturally derived compounds (Abbott *et al.,* 2004; Dai *et al.,* 2015; Tang *et al.,* 2016; Piemontese *et al.,* 2017). The constituents of DES include quaternary ammonium salts such

as choline chloride and hydrogen bond donors such as carboxylic acid (*e.g.* citric acid) (Matong *et al.,* 2017; Mulia *et al.,* 2015). If prepared from primary metabolites of living cells, the term NADES (natural deep eutectic solvents) is used (Wei *et al.,* 2015a; Wei *et al.,* 2015b; Piemontese *et al.,* 2017). NADES have a melting point far below those of their individual constituents (Mulia *et al.,* 2015; Juneidi *et al.,* 2016). This is mostly due to hydrogen bond formation that constitute the main driving force of NADESs (Bakirtzi *et al.,* 2016). Adding to the advantages reported on ILs and DES, NADES are best for extraction and the viscosity is adjustable (Wei *et al.,* 2015a). NADES have been reported to extract various natural products from different plants (Owczarek *et al.,* 2016). For instance, polyphenols were extracted from *Carthamus tinctorius L*. (Dai *et al.,* 2013), *Cajanus cajan* leaves (Wei *et al.,* 2015a) and different common native Greek medicinal plants (Bakirtzi *et al.,* 2016). In addition, NADES prepared from choline-chloride and lactic acid was used to extract four flavonoids (baicalin, wogonoside, baicalein and wogonin) from *Radix scutellariae* (Wei *et al.,* 2015b).

The aqueous two-phase (ATP) system is an extraction technique also considered as part of the green extraction systems (Ruiz-Aceituno *et al.,* 2013). ATP is a convenient analytical tool which can be made from short chain alcohols or hydrophilic solvents and inorganic salts (Salic *et al.,* 2011; Wu *et al.,* 2011; Guo *et al.,* 2012; D. Zhang *et al.,* 2013; Goja *et al.,* 2013). The driving forces of aqueous two-phase formation are hydrophobic and hydrogen bonds, and ionic interactions (Gu *et al.,* 2012). The partition activity of biomolecules depends on physicochemical factors such as hydrophobicity (due to the addition of salt of its ionic strength), the degree of the pH and the affinity of the biomolecule for the phases (D. Zhang *et al.,* 2013; Goja *et al.,* 2013). ATP systems are appropriate for extraction of soluble and insoluble compounds, and have the advantage of being economic in relation to conventional extraction procedures. The technique has a short processing time as well as low energy, high extraction yield, and the alcohol is easily recovered by evaporation (Wang *et al.,* 2010; Brito *et al.,* 2013; Iqbal *et al.,* 2016). ATP systems have been used to isolate secondary metabolites from different plants (Wu *et al.,* 2011; Guo *et al.,* 2012; D. Zhang *et al.,* 2013). Guo *et al.,* (2012) reported a method for rapid extraction of lithospermic acid B from *Salvia miltiorrhiza* using an ethanol/ammonium sulphate-based ATP system. Genistein and apigenin from pigeon pea roots were also efficiently extracted by an ATP system consisting of 28% ethanol and 22% dipotassium phosphate (D. Zhang *et al.,* 2013).

Pressurized hot water extraction (PHWE) is an attractive alternative to traditional extraction methods. The technique uses pure water as solvent and exploits the properties thereof. In this system the pressure is controlled and the temperature can range from 100 °C to 374 °C (Teo *et al.,* 2010; Khoza *et al.,* 2014; Plaza & Turner 2015). The PHWE procedure involves four phases: (i) increase of temperature to create desorption of compounds in the sample matrix, (ii) entry of the extraction fluid in the sample medium, (iii) partition of compounds from the sample matrix to the extraction fluid and (iv) elution of compounds into collection vials (Khoza *et al.,* 2014). In PHWE, the temperature is the key factor for extraction efficiency and selectivity. Although increasing temperature can allow extraction of several compounds, those that are thermally labile are denatured or degraded at high temperature (Teo *et al.,* 2010). One of the main advantages of PHWE is that the polarity of water at certain temperatures is comparable to that of alcohols (methanol) used in traditional extraction procedures, giving the system a broad extractability capacity (Teo *et al.,* 2010; Gbashi *et al.,* 2017). The use of water as extracting solvent represents a great advantage as it is easily available, recyclable, non-toxic and compatible with most chromatographic instruments (Teo *et al.,* 2010; Gbashi *et al.,* 2016; Gbashi *et al.,* 2017). Moreover, PHWE is less expensive and not time consuming. This technique has been used to extract secondary metabolites from different plants (Khoza *et al.,* 2014; Khoza *et al.,* 2016; Gbashi *et al.,* 2017).

In this study, different green extraction solvent (PHW, NADES and ATP) systems were evaluated in comparison to a methanol-based organic solvent extraction and utilized to demonstrate the ability to extract pharmacologically relevant secondary metabolites from *M. oleifera* dry leaf tissue.

3.2 Materials and methods

3.2.1 Sample collection

M. oleifera leaves were donated by the ARC-VOP (Institute for Vegetable and Ornamental Plants, Agricultural Research Council) at Roodeplaat, Pretoria (South Africa) and were air-dried for three days at room temperature.

3.2.2 Preparation of NADES

The eutectic solvent of choline chloride and citric acid (Sigma-Aldrich, Munich, Germany) was prepared by heating the mixture at 70 °C until a homogenous solution was obtained. In order to reduce the viscosity of the solvent, deionized water was added to the NADES mixture to give a final concentration of 40%.

3.2.3 Extraction procedures

Three extraction procedures were used: NADES -, ATP - and PHW extraction. The foliar parts of *M. oleifera* were ground in liquid nitrogen using a mortar and pestle. The fine powdered leaf material was used for each extraction procedure, which was performed in triplicate.

3.2.3.1 Methanol extraction

A mass of 2 g of *M. oleifera* powder was extracted with 20 mL (1:10 m/v ratio) of 80% aqueous methanol (MeOH, analytical grade, Romil, Cambridge, UK). The mixture was sonicated in an ultrasonic cleaning bath for 45 min at room temperature (25 °C). The samples were then centrifuged at 5100 x*g* for 20 min at 25 °C. The MeOH in the supernatant was evaporated in a 50 mL round bottom flask to approximately 1 mL at 55 °C using a rotary evaporator under reduced pressure. The 1 mL of extract was further dried to completeness in pre-weighed 2 mL Eppendorf tubes, using a speed vacuum concentrator at 45 °C. The dried extracts were weighed and reconstituted in 50% MeOH, giving a final concentration of 0.2 g/mL. Filtration was achieved through 0.22 µm nylon filters into glass chromatography vials fitted with inserts and slitted caps, and kept at -20 ˚C prior to ultra-high performance liquid chromatography - quadrupole time of flight mass spectrometry (UHPLC-qTOF-MS) analysis.

3.2.3.2 Natural deep eutectic solvent extraction

The choline chloride and citric acid-based NADES (20 mL) was added to *M. oleifera* crushed leaves at a ratio of 1:10 (m/v). The mixture was homogenised in a sonicator bath as described at 50°C for 1 h. The leaf residues were separated from the mixture by filtration using 55 mm circle filter papers (Millipore, Billerica, MA, USA) and a vacuum system. The extracts were prepared for UHPLC-qTOF-MS analysis as described above, except that the viscous nature of NADES did not allow the concentration step to be performed.

3.2.3.3 Aqueous two phase extraction

A volume of 20 mL of ammonium sulphate salt (20 %) was added to 2 g of a fine powder of *M. oleifera* leaves and the combination was homogenised in an ultrasonic bath as described at 50°C for 1 h. Following sonication, 20 mL of ultra-pure ethanol (Sigma Aldrich, Munich, Germany) was added to the mixture to create the partition and the mixture was incubated at room temperature for 4 h. The top phase of the extracts was then concentrated to a volume of approximately 1 mL using a reduced pressure rotary evaporator and dried off in a speed vacuum concentrator at 45 °C. The bottom phase was freeze dried and both phases were reconstituted with 50% ultra-pure methanol giving a final concentration of 0.2 g/mL. Each replicate was filtered and prepared for UHPLC-qTOF-MS analysis as described.

3.2.3.4 Pressurized hot water extraction

A custom made PHWE instrument was used to extract metabolites from dried, pulverized *M. oleifera* leaves. The instrument contains stainless steel tubing (with an outer diameter of 1.58 mm and an inner diameter of 0.18 mm) that connects the HPLC pump to the extraction cell (70×30 mm which is roughly 20 mL). Inside the extraction vessel, 3 g of the ground leaves and 1 g of diatomaceous earth (Sigma, Munich, Germany) was mixed. The extraction chamber was placed in a gas chromatography (GC) oven with a temperature set at 100 °C. A 6000 fluid controller HPLC pump (Waters Corporation, Manchester, UK) was used to pump pre-heated ultra-pure water through the stainless steel extraction apparatus connected inside the oven, at a constant flow rate of 3 mL/s until the collection of 25 mL of extracts. During extraction, the pressure produced by the pumps was maintained at 1000 ± 200 psi using a back-pressure regulator (Swagelok, Johannesburg, South Africa). Each replicate was freeze dried and the resulting sample was reconstituted with 50% ultra-pure methanol giving a final concentration of 0.2 g/mL. Extracts were finally filtered and prepared for UHPLC-qTOF-MS analysis.

3.2.4 Ultra-High Performance Liquid Chromatography analysis

The UHPLC-qTOF-MS system (high-definition SYNAPT G1, Waters Corporation, Manchester, UK) was used to evaluate the metabolite profile of extracts. Each extract was analyzed in triplicate to account for any analytical variability. Two microliters of each replicate was injected onto a C_8 analytical column (Acquity BEH C_8 column, 150 mm× 2.1 mm with particle

size of 1.7 µm; Waters, MA, USA) at a constant flow rate of 0.4 mL/min for a total run time of 30 min. The gradient elution was performed using a binary solvent made of deionized water (mobile phase A) and acetonitrile (Romil, Cambridge, UK) (mobile phase B). A concentration of 0.1% formic acid was added to both eluents. The elution was achieved with an initial concentration of the mobile phase B at 2% over 0.0 to 1.0 min, followed by a gradual increase of B to 3% then 8% and 50% over 1.0 to 3.0 min, 3.0 to 4.0 min and 4.0 to 25.0 min respectively. The gradient was then kept at 50% over 25.0 to 26 min followed by an increase to 95% over 26 to 27 min. Finally, re-equilibration was done over 27 to 30 min with 2% of B. The photodiode array detector (PDA, Waters Corporation, Manchester, UK) was set for a scanning range between 200-500 nm, a bandwidth resolution 1.2 nm and spectra collection rate of 20 points per second.

3.2.5 High Definition Mass Spectrometry Analysis

Metabolites were further detected using a SYNAPT G1 mass spectrometer in V-optics operating in negative electrospray ionization (ESI) mode (Waters Corporation, Manchester, UK). Leucine encephalin (50 pg/mL, 554.2615 Da) was used as reference calibrant to obtain a mass accuracy window of 0.5 mDa at a constant flow rate of 0.1 mL/min. The mode was set as centroid and the *m/z* range was 100 to 1000. The scan time was fixed to 0.2 sec and interscan delay to 0.02 sec. The sample cone voltage, capillary voltage and multichannel plate detector voltage was 30 V, 2.5 kV and 1.65 mV respectively. For the cone and desolvation gas, high purity nitrogen was used with the cone gas flow of 50 L/h and the disolvation gas flow of 550 L/h. The source temperature and the desolvation temperature were set at 120 °C and 450 °C.

3.2.6 Multivariate data analysis and compound identification

The ESI negative mode raw data were extracted and analyzed using MarkerLynx^{TM} XS software (Waters Corporation, Milford, MA, USA). Software parameters were set at a retention time (Rt) range of 0.1 to 26 min, mass tolerance 0.05 Da and intensity threshold 100. The resulting matrix was exported to SIMCA-14.0 software (Umetrics, Umea, Sweden) to perform principal component analysis (PCA). Prior the latter X-data were *Pareto*-scaled in order to reduce noise and the artefacts of the model. The evaluation of the model was done using the following diagnostic tools: the cumulative modelled variation in X matrix, R^2X

(cum) and the predictive ability parameter, Q^2 (cum). A model is robust when the values of R^2X (cum) and Q^2 (cum) are close to 1 (Tugizimana *et al.,* 2015; Mhlongo *et al.,* 2016).

The data obtained from NADES extracts were not processed for further analysis because of the viscosity of the extract that prevents its concentration.

3.3 Results and discussion

3.3.1 Chromatographic profiling

M. oleifera is known to be a "storehouse" of bioactive compounds such as glucosinolates and polyphenolics known to have very interesting pharmaceutical applications (Toma & Deyno, 2014; Saini *et al.,* 2016). In this study, different green extraction solvents were used to explore their extraction abilities for secondary metabolites from *M. oleifera*. All extracts were analyzed on an UHPLC-qTOF-MS analytical platform in ESI negative mode. Methanol extraction was used as a control. The base peak intensity (BPI) chromatograms of each extract are displayed in **Figure 3.1**. Overall, the visual inspection of each chromatogram reveals clear differences in peak intensities and presence or absence of certain peaks, suggesting that the concentration of compounds extracted from *M. oleifera* differ between extracts.

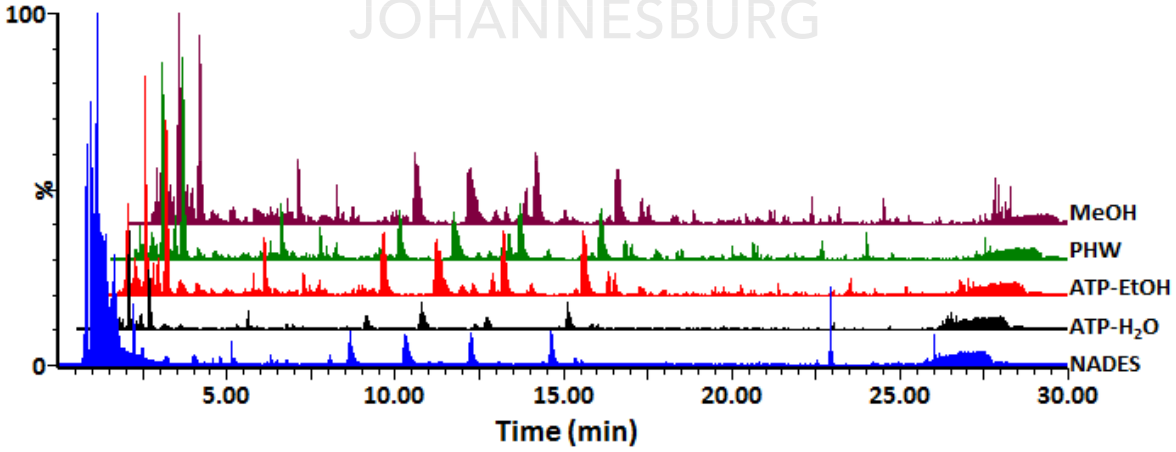

Figure 3.1: UHPLC-MS base-peak intensity (BPI) chromatograms (ESI negative mode) of extracts from dried *Moringa oleifera* leaves, extracted with different solvents. Methanol (MeOH), pressurized hot water (PHW), aqueous two phase extraction solvents (ATP-EtOH and ATP-H_2O) and natural deep eutectic solvent (NADES).

3.3.2 Multivariate analysis

In order to complement the visual inspection of chromatograms and to highlight differences and similarities between and within the samples, data obtained from UHPLC-MS analyses were further analyzed using principal component analysis (PCA) as a multivariate data analysis (MVA) tool **(Figure 3.2)**. PCA is an unsupervised chemometric model that reduces the complexity and the high-dimensionality of the data sets, providing a comprehensive and qualitative illustration of similarities or dissimilarities amongst and within the sample.

As seen on the score plot generated by the first two components (PC1 and PC2), samples are differentially clustered according to the extraction method used, revealing the differential extraction ability of each solvent. All green solvents clustered very far apart from the MeOH extract, suggesting that those solvents extract different classes of compounds or the relative concentrations of the compounds they extract are different (Tugizimana *et al.,* 2015).

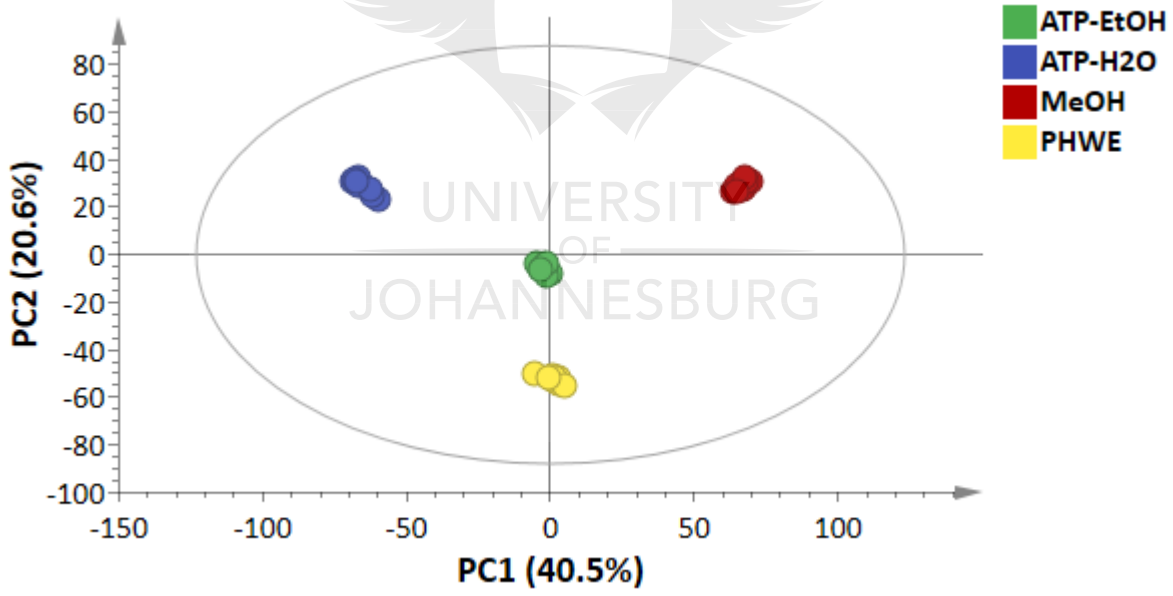

Figure 3.2: Principal component analysis (PCA) scores plot generated from the UHPLC-MS ESI negative data of *Moringa oleifera* extracted with different solvents. T1he score plot was computed from PC1 and PC2 using SIMCA-14.0 software and explains 61.1% of the total variation. Extraction method-related sample groupings can be observed in the scores space.

3.3.3 Secondary metabolite identification

The identification of all the metabolites present in **Table 3.1** was achieved as described in previous studies on *M. oleifera* (Khoza *et al.,* 2014; Rodríguez-pérez *et al.,* 2015; Makita *et*

al., 2016; Ramabulana *et al.,* 2016) and other plants (Stojakowska *et al.,* 2016; Ncube *et al.,* 2016; Khoza *et al.,* 2016; Gbashi *et al.,* 2017). The metabolite profiling of different extracts revealed 47 compounds, including two main classes of secondary metabolites namely glucosinolates and polyphenolics (*i.e.* hydroxycinnamic acid derivatives, hydroxybenzoic acid derivatives and flavonoids); and primary metabolites **(Table 3.1** and **Figure 3.4)**. Characterization of glucosinolates, chlorogenic acids and flavonoids is addressed below whereas the identification of primary metabolites as well as other secondary metabolites will be discussed in **Chapter 4 section 4.3.4** (As the focus is placed on these).

3.3.3.1 *Glucosinolates*

Glucosinolates, also referred to as β-thioglucoside N-hydroxysulphate derivatives, are polar compounds that can be divided into three groups: indolic, aliphatic and aromatic glucosinolates (Förster *et al.,* 2015; Doheny-Adams *et al.,* 2017). These are secondary metabolites linked to a wide range of biological activities (Dinkova-Kostova & Kostov 2012; Sham *et al.,* 2013), and have been reported to be a suppressing agent of tumor cell growth, indicating the ability to prevent various types of cancers (Sham *et al.,* 2013; Förster *et al.,* 2015; Ramabulana *et al.,* 2017). The comparison of all green solvents to methanol as extractant reveals that only the latter extracted L-rhamnopyranosyloxy)-benzyl glucosinolate (also known as glucomoringin, *m/z* 570 fragment shown in **Figure 3.3A**) and its three acylated isomers (*m/z* 612). By comparison, NADES was able to extract acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer II and III (*m/z* 612) **(Table 3.1).** This suggests that NADES has the ability to extract polar compounds and can therefore be employed as an extraction method of choice for glucosinolates derivatives. Surprisingly, no glucosinolates were found in the PHW extracts. Although the PHWE technique employs a polar solvent, H_2O, its inability to extract glucosinolates could be due to the destructive nature of this extraction technique. High temperature and pressure might facilitate breaking down of hydrolysis prone molecules (glucosinolates in this case), a phenomenon which has been shown elsewhere (Tyagi, 2002).

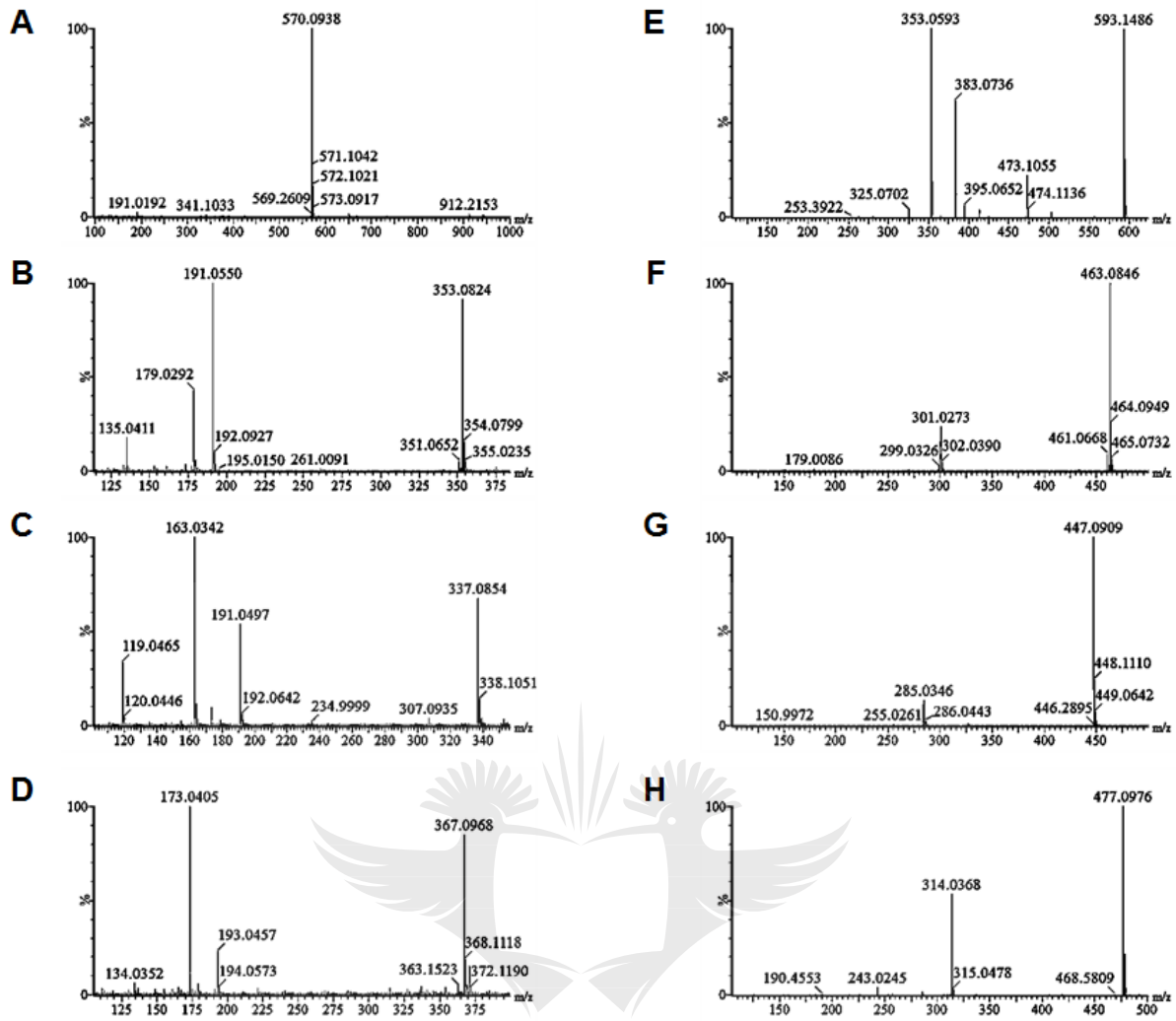

Figure 3.3: Fragmentation patterns of some secondary metabolites identified in *Moringa oleifera* leaf tissue extracted with MeOH, PHW, ATP solvents and NADES. **(A):** Glucomoringin; **(B):** 3-Caffeoyl quinic acid; **(C):** 3-*p*-Coumaroyl quinic acid; **(D):** 4-Feruloyl quinic acid; **(E):** Vicenin-2; **(F):** Quercetin hexose; **(G):** Kaempferol hexose; **(H):** Isorhamnetin hexose.

3.3.3.2 *Chlorogenic acids (CGAs)*

CGAs are esters resulting from the conjugation of quinic and hydroxylated/methoxylated cinnamic acid derivatives. In this study, three CGAs were annotated from each extract of *M. oleifera* **(Table 1)**: caffeoyl quinic acid (CQA) (Mhlongo *et al.,* 2016), coumaroyl quinic acid (CoQA) (Ncube *et al.,* 2014) and feruloyl quinic acid (FQA) (Clifford *et al.,* 2003). All positional isomers and enantiomers were annotated according the MS fragmentation pattern and the order of elution as previously described (Clifford *et al.,* 2006; Madala *et al.,* 2012; J. Zhang *et al.,* 2013; Masike *et al.,* 2017). For instance, the 3CQA with a molecular mass of 354 (*m/z* 353) eluted at Rt = 1.82 min and produced fragment ions **(Figure 3.3B)** of *m/z* 191 resulting from the loss of a caffeoyl group, *m/z* 179 that represents the caffeic acid without a hydrogen and *m/z* 135, the caffeic acid that has lost a CO_2 (Clifford *et al.,* 2003;

Khoza *et al.,* 2014). In the case of 3-*p*CoQA with a precursor ion at m/z 337, the product ions were 163, corresponding to the mass of coumaric acid without a hydrogen and 119, the mass of coumaric acid without a carbon dioxide **(Figure 3.3C)**. The diagnostic ions for 4FQA were *m/z* 173 and *m/z* 193, derived from dehydrated quinic acid and ferulic acid respectively **(Figure 3.3D)**.

As seen in **Table 3.1**, the CQA and the FQA (compounds **6** and **11** respectively), eluting at the Rt 3.12 min, were co-eluting and giving the diagnostic ions of both compounds together (*m/z* 134, 135, 173, 179, 191, 193). As a result, it was difficult to conclude on the position of each cinnamic acid unit on the quinic acid, considering the fact that the ion with a mass of 173 can be diagnostic for both FQA (as for a 4FQA) and/or CQA (as for 4CQA).

Table 3.1: Pharmacologically relevant secondary metabolites of *Moringa oleifera* extracted using four different solvent systems: Methanol (MeOH), Pressurized hot water (PHW), Aqueous two-phase (ATP) and natural deep eutectic solvent (NADES).

Extracted Compounds		MeOH	PHW	ATP (EtOH)	ATP (H_2O)	NADES	Rt (min)	Precursor ion (*m/z*)	MS/MS Fragments
Glucosinolates									
1	Glucomoringin	√					1.22	570.0925	570
2	Acetyl 4-(α-L-rhamno-pyranosyloxy)-benzyl glucosinolate isomer I	√					1.62	612.1031	612
3	Acetyl 4-(α-L-rhamno-pyranosyloxy)-benzyl glucosinolate isomer II	√				√	2.18	612.103	612
4	Acetyl 4-(α-L-rhamno-pyranosyloxy)-benzyl glucosinolate isomer III	√				√	3.95	612.1051	612
Chlorogenic acids									
5	3CQA	√	√	√	√	√	1.82	353.0842	135, 179, 191
6	CQA	√	√	√	√	√	3.12	353.084	134, 135, 173, 179, 191, 193
7	*cis*-3-*p*CoQA	√	√	√	√	√	2.54	337.0895	163, 191
8	*trans*-3-*p*CoQA	√	√	√	√	√	2.7	337.0904	163, 191
9	*cis*-4-*p*CoQA	√	√	√	√	√	4.75	337.0953	163, 173
10	*trans*-4-*p*CoQA	√	√	√	√	√	5.13	337.0953	161, 173
11	FQA	√	√	√	√	√	3.08	367.0968	134, 135, 173, 179, 191, 193
12	4FQA	√	√	√	√	√	5.27	367.0968	173, 193
Flavonoids									
13	Vicenin-2 (apigenin C-diglycoside)	√	√	√	√	√	5.11	593.1443	353, 383, 473
14	Vitexin (apigenin hexose)	√	√	√	√	√	5.41	431.0925	311, 341
15	Isovitexin (apigenin C-hexose)	√	√	√	√	√	5.71	431.0508	311, 341
16	Quercetin dihexose	√	√	√	√	√	5.77	625.1436	463, 301
17	Quercetin acetyl dihexose	√	√	√	√	√	6.63	667.149	301, 463, 505
18	Quercetin rutinoside	√	√	√	√	√	8.25	609.1415	301
19	Quercetin hexose	√	√	√	√	√	8.51	463.0867	301
20	Quercetin malonyl hexose	√	√	√	√	√	10.17	549.0842	300, 505
21	Quercitin acetyl hexose isomer I	√	√	√	√	√	10.87	505.1064	300

22	Quercetin-hydroxymethylglutaroyl hexose isomer I	√	√	√	√	√	11.17	607.1278	300, 463, 505
23	Quercetin acetyl hexose isomer II	√	√	√	√	√	12.83	505.096	301
24	Quercetin-hydroxymethylglutaroyl hexose isomer II	√	√	√		√	13.22	505.0933	300, 463, 505
25	Kaempferol acetyl dihexose	√	√	√	√	√	5.19	651.1516	285, 446, 489
26	Kaempferol rutinoside	√	√	√	√		11.83	593.149	285
27	Kaempferol hexose	√	√	√	√	√	12.05	447.0916	285
28	Kaempferol malonyl hexose	√	√	√	√	√	14.44	533.0911	285, 489
29	Kaempferol-hydroxymethylglutaroyl hexose	√	√	√	√	√	15.22	591.13.96	285, 447, 489
30	Kaempferol acetyl hexose	√	√	√	√	√	16.28	489.0994	285
31	Isorhamnetin rutinoside	√	√	√			12.66	623.1624	315
32	Isorhamnetin hexose	√	√	√	√	√	12.92	477.1048	314
33	Isorhamnetin acetyl hexose	√	√	√	√	√	15.44	519.1185	315
34	Isorhamnetin-hydroxymethylglutaroyl hexose	√	√	√	√	√	16.18	621.1459	315
Derivatives of hydroxycinnamic and hydroxybenzoic acids									
35	Caffeoyl hexose		√			√	0.90	341.1357	135, 179
36	Coumaroyl hexose	√	√	√	√	√	3.53	325.0877	119, 163
37	Benzylalcohol-hexose-pentose isomer I				√		4.45	401.1419	101, 161, 269
38	Benzylalcohol-hexose-pentose isomer II	√		√	√	√	4.75	401.1431	101, 161, 269
Primary metabolites									
39	Citric acid/ Isocitric acid		√	√	√	√	0.96	191.0172	111, 133
40	Citric acid/ Isocitric acid		√	√	√	√	1.12	191.0228	111, 133
41	Phenylalanine	√	√	√	√		1.54	164.0696	147
42	Tryptophan	√	√	√	√		2.12	203.0780	116, 142, 158/159
43	Butanyol hexose pentose	√	√	√	√	√	6.43	381.1787	249, 160/161, 101/100
44	Trihydroxyoctadecadienoic acid isomer I	√	√	√	√	√	22.37	327.2164	171
45	Trihydroxyoctadecadienoic acid isomer II	√	√	√	√	√	22.49	327.2121	229, 211, 171
46	Trihydroxyoctadecadienoic acid isomer III	√	√	√	√	√	22.65	327.2172	171
47	Trihydroxyoctadecenoic acid isomer	√	√	√	√	√	23.21	329.2289	211, 171

3.3.3.3 Flavonoids

The largest number of compounds identified in *M. oleifera* belongs to the group of flavonoids. Although present at different relative concentrations, most flavonoids were extracted by all the solvents. Exceptions were observed with quercetin-hydroxymethylglutaroyl hexose absent in the water phase of the ATP system (ATP-H_2O), kaempferol rutinoside absent in NADES and isorhamnetin rutinoside not present in both NADES and ATP-H_2O. All flavonoids identified in *M. oleifera* were composed of a quercetin (*m/z* 301), kaempferol (*m/z* 285) or isorhamnetin (*m/z* 314) core attached to a sugar moiety **(Figure 3.3E-H)**. Some of these flavonoids were also identified in a study reported by Rodriguez-Perez *et al.,* (2015) and Makita *et al.,* (2016).

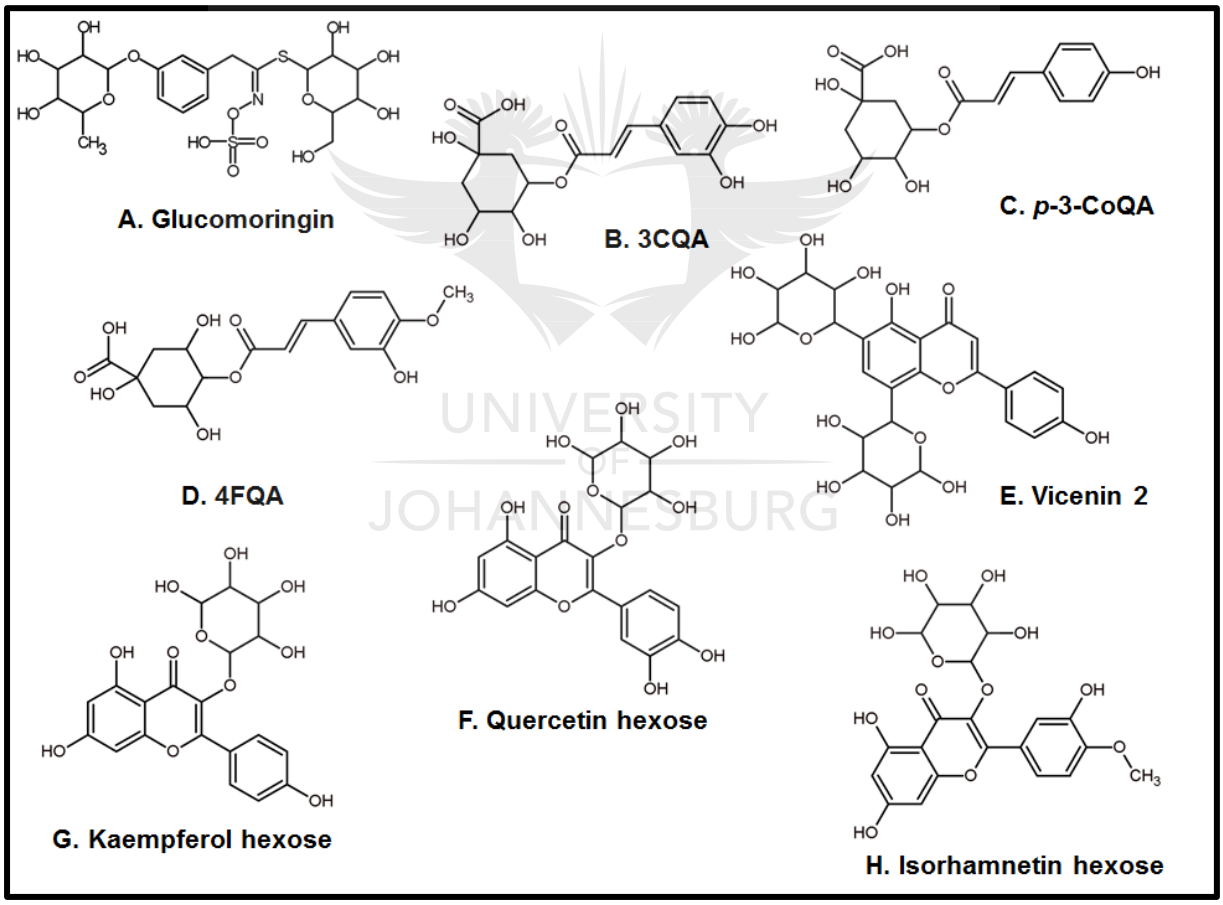

Figure 3.4: Structures of selected secondary metabolites identified in *M*oringa *oleifera* leaf tissue extracted with MeOH, PHW, ATP solvent and NADES. **(A):** Glucomoringin; **(B)**: caffeoyl quinic acid; **(C):** 3-pCoumaroyl quinic acid; **(D):** 4-Feruloyl quinic acid; **(E):** Vicenin 2; **(F):** Kaempferol hexose; **(G):** Quercetin hexose; **(H):** Isorhamnetin hexose.

3.3.4 Relative quantification and pharmacological importance of secondary metabolites

In *M. oleifera* leaves, the amount of glucosinolates is higher than that of many members of the Cruciferae (Brassicaceae) family (*e.g* cabbage, broccoli). Glucomoringin (4-O-(α-L-rhamnopyranosyloxy)-benzylglucosinolate), although not exclusive to *M. oleifera* (De Graaf *et al.,* 2015), is the most prominent glucosinolate in leaves (Amaglo *et al.,* 2010; De Graaf *et al.,* 2015; Saini *et al.,* 2016; Ramabulana *et al.,* 2017). This is also confirmed in the current study where the relative intensity of glucomoringin was found to be higher than that of the acetylated isomers of glucomoringin **(Figure 3.5A)**. These compounds, however, were suggested to be exclusively present in *M. oleifera* (Ramabulana *et al.,* 2017). Glucomoringin and its acetylated isomers were affected by the extraction technique used as it was only observed in the methanolic and NADES extracts of *M. oleifera* leaf tissue. Glucosinolates exhibit anti-microbial - (De Graaf *et al.,* 2015) and anti-inflammatory properties, and play important roles in neurological, cardiovascular (Jo *et al.,* 2016) and carcinogenic diseases (Förster *et al.,* 2015; Jo *et al.,* 2016).

The differential extraction abilities of the four solvent systems is clearly visible in the relative peak intensity graphs as shown in **Figure 3.5B** for CGAs and **3.5C** for selected flavonoids. CGAs and flavonoids are polyphenolic compounds with numerous reported pharmacological and health beneficial activities. These include anti-depressant (Kurkin *et al.,* 2006), anti-oxidant (Vongsak *et al.,* 2013), anti-bacterial, anti-diabetic, renoprotective, hepatoprotective, anti-inflammatory and anti-lipidemic activities (Quettier-Deleu *et al.,* 2000; Meng *et al.,* 2013). The relative quantification of selected polyphenolics shows that except for 3CQA, all selected compounds have higher relative concentrations in green solvents than in MeOH. Among the green solvents, the relative concentration of flavonoids seems to be higher in the H_2O phase of the ATP system. This may encourage the use of these solvents for extraction of secondary metabolites in similar research.

The extraction of compounds with known pharmaceutical importance is a crucial step in the development of new drugs. In most cases, organic solvents are employed as extractant, alone or in combination. During pharmacological studies, multiple-step procedures are

employed for the removal of such organic solvents due to their purported toxicities (Liang & Fan, 2013). However, these solvents cannot be completely eliminated and are therefore found in small amounts in the final drug product, as residual solvent (RS) or organic volatile impurities (OVI) (Grodowska & Parczewski, 2010). The use of green solvents such as NADES, PHW and ATP for extraction provides a preferred avenue to obtain pure compounds with health beneficial properties (as mentioned above). In this regard, green solvent approaches are important assets that can be used in the pharmaceutical industry. CGAs and flavonoids were previously identified in *M. oleifera* extracts achieved with PHWE (Khoza *et al.*, 2014). To the best of our knowledge, the current study is the first to present *M. oleifera* secondary metabolites extracted using NADES and ATP solvents.

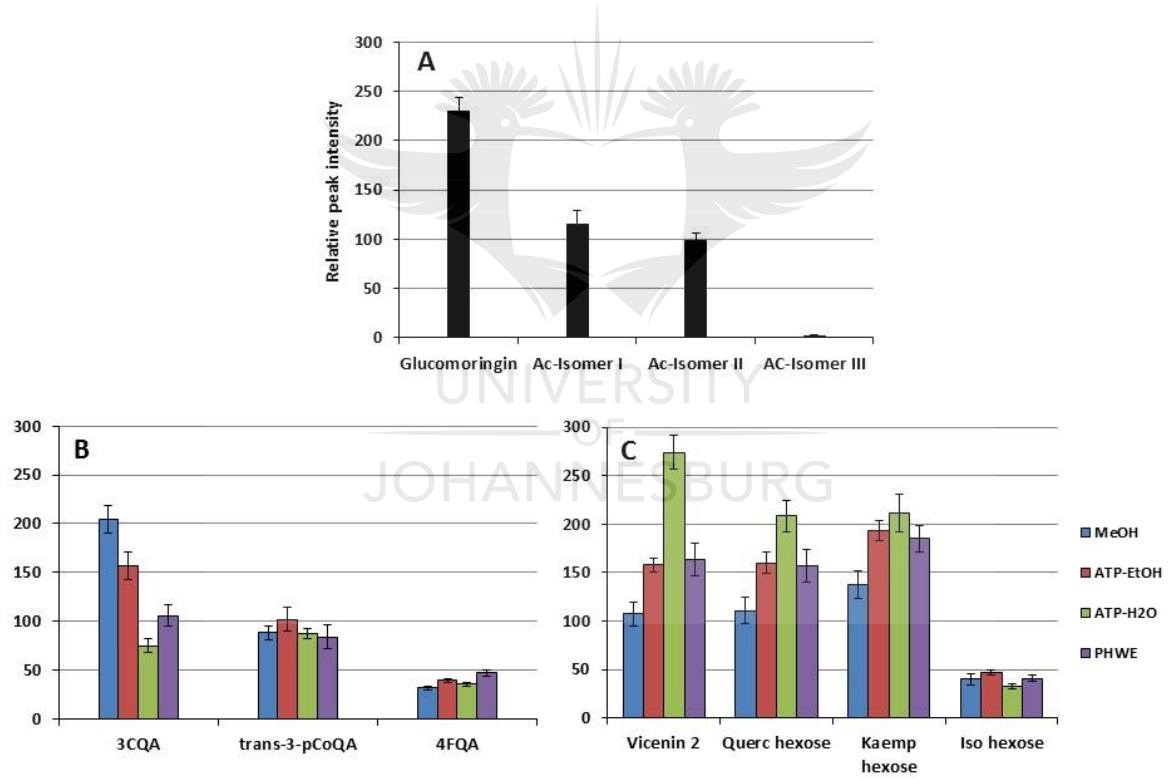

Figure 3.5: Relative peak intensities of selected secondary metabolites in *M. oleifera*, representative of the different group of compounds annotated from each extract. **(A):** Glucosinolates in methanolic extract, **(B):** Chlorogenic acids (3CQA, trans-3-pCoQA and 4FQA) and **(C):** Flavonoids (Vicenin 2, Quercetin hexose, Kaempferol hexose and Isorhamnetin hexose).

3.4 Conclusion

Although the conditions were not the same for all extractants (due to the inherent aspects of the methods), the aim of this study was to compare the type of metabolites extracted. In this study, it was demonstrated that the green solvents employed are able to extract a wide range of secondary metabolites (glucosinolates and polyphenolics such as CGAs and flavonoids) as well as primary metabolites from dried *M. oleifera* leaf tissue. This implies that the pressurized hot water, NADES (choline chloride and citric acid-based) as well as the aqueous two-phase solvent system (ammonium sulfate / ethanol) can confidently be used to extract pharmacologically relevant bioactive compounds. However, differential extraction abilities were observed. While CGAs and flavonoids were extracted with all solvent systems; glucomoringin and its acylated derivatives were only successfully extracted with methanol. The solvent systems investigated are simple, inexpensive and environmentally friendly, and can be used in diverse industries.

3.5 References

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Chapter 4: Regenerative potential and metabolic profiles of *Moringa oleifera* calli as affected by different ratios of auxin and cytokinin

Abstract

Plant cell - and tissue culture represents a renewable system essential for understanding biological processes and a valuable alternative for secondary metabolite production. The hormonal manipulation of these systems may result in redifferentiation/organogenesis and hence change in the metabolic profiles. The aim of the study was to investigate the effects of auxin (2,4-dichlorophenoxyacetic acid, 2,4-D) and cytokinin (kinetin) ratios on undifferentiated *Moringa oleifera* callus cells at a metabolomics level and to study the metabolite distribution thereof. *M. oleifera* callus, that originated from stem sections as ex-plants, was grown on media containing 25 different combinations of 2,4-D and kinetin concentrations. Methanolic extracts prepared from the calli were screened for total phenolic content (TPC, using the Folin-Ciocalteu (F-C) assay) and anti-oxidant activity (using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay). The remaining extracts were chromatographically analyzed using an UHPLC-qTOF-MS platform and multivariate data models were employed to facilitate the metabolite profiling. An unexpected result was that the callus became habituated, *i.e.* developed the ability to grow without added stimulatory plant hormones. Subsequently, no organogenesis was observed by callus grown on any of the different hormone combinations investigated. However, the TPC results exhibited different concentrations of phenolic compounds among the various treatments and was the highest (49.86 µg GAE/g wet weight) in the condition containing 1.5 mg/L of 2,4-D and 1 mg/L of kinetin. The extracts all exhibited high anti-oxidant activity with the highest (78% DPPH inhibition) in the condition containing 1 mg/L of 2,4-D and 0.5 mg/mL of kinetin. Despite the habituation phenomenon observed, the calli retained responsiveness towards external hormones and each of the 25 conditions had a unique metabolome as found by principal component analysis. This was also reflected by a number of phytochemicals (primary and secondary metabolites) that were annotated as biomarkers from

phytohormone-treated *M*. *oleifera* callus cultures. This finding demonstrates the differential influence of auxin and cytokinin on *M*. *oleifera* callus for the production of secondary metabolites.

Keywords: auxin, cytokinin, callus, *Moringa oleifera*, organogenesis, secondary metabolites.

4.1 Introduction

Totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism, and is thus a reflection of the genetic potential of a plant cell to produce the entire plant (Ezhova, 2003; Lambé *et al*., 1997; Ochoa-Villarreal *et al*., 2015). Isolated cells from differentiated tissue are generally non-dividing and quiescent; to express totipotency they have to undergo undifferentiation and then redifferentiation. Undifferentiation refers to reversion of a specialized cell or tissue to a non-specialized form. Many plants have been regenerated from single cells, but not all plant cells are totipotent; some are terminally differentiated (Lambé *et al.,* 1997).

Plant cell and tissue culture is the sterile *in vitro* growth of cells (differentiated or undifferentiated), tissues or organs under specific conditions (Thorpe, 2013). The cultivation of plant cells and tissues *in vitro* was first described by Haberlandt at the beginning of the 20th century (Haberlandt, 1902). Nowadays, plant cell - and tissue culturing is considered as routine procedures and constitutes an important asset for understanding cellular and biochemical processes (Loyola-Vargas & Ochoa-Alejo 2012; De Vijlder *et al.,* 2015). A plant tissue culture possesses the ability to regenerate organs and even the normal adult plant from a single or group of highly specialised cells, and constitute an important experimental system for studying cell function and differentiation. The fundamental starting material in most *in vitro* cultures is callus, defined as mass of undifferentiated tissue grown on solid media with the right balance of phytohormones.

Calli can be classified as compact or friable callus (without organ regeneration), shooty, embryonic or rooty callus (with partial regeneration) (Ikeuchi *et al.,* 2013). Calli can thus exhibit varying levels of differentiation following the application of plant growth regulators

(PGRs) (Radić *et al.,* 2016). The phytohormones auxins and cytokinins are the major PGRs employed for *in vitro* cultures (George *et al.,* 2008; Moubayidin *et al.,* 2009; Leljak-Levanić *et al.,* 2016). As mentioned before, auxins play an important role in cell growth development, cell wall acidification and organization of meristems resulting in either unorganized tissue or distinct organs (usually roots) (George *et al.,* 2008; Moubayidin *et al.,* 2009; Radić *et al.,* 2016). Cytokinins promote shoot formation and expansion, and together with auxins, activate cell division (Moubayidin *et al.,* 2009; Rashid & Sahebi 2015).

Auxin and cytokinin levels in tissue cultures should be carefully regulated as one can inhibit the accumulation or the action of the other (Gaspar *et al.,* 1996; George *et al.,* 2008; Manzur *et al.,* 2014), and can be used to manipulate the pattern of regeneration (Loyola-Vargas & Ochoa-Alejo, 2012). In general, a higher cytokinin to lower auxin ratio leads to shoot regeneration (caulogenesis), while a low cytokinin to higher auxin leads to root regeneration (rhizogenesis), and the same ratio of both hormones leads to growth in an unorganized manner (Moubayidin *et al.,* 2009). During long term cultivation, calli from certain plants can lose the requirement for external auxins and cytokinins, and grow in a PGR-independent manner (*i.e.* exhibit hormonal autonomy). This phenomenon is known as habituation (Lambé *et al.,* 1997; Gaspar *et al.,* 2000; Loyola-Vargas & Ochoa-Alejo 2012).

The induction of plant cell- and tissue culture with one or combined external signals may result in the activation of a specific developmental pathway and lead to the regeneration of competent cells (Sriskandarajah *et al.,* 2006). Plants have the ability to regenerate *in vitro* through direct or indirect organogenesis, in response to endogenous and exogenous stimuli. In direct organogenesis, callus is not required for regeneration while in indirect organogenesis, callus is first produced from the explant. In any case, plant *in vitro* culture allows the production of secondary metabolites through elicitation and the understanding of biochemical processes (Srivastava, 2017).

M. oleifera is a dicotyledonous tree native of India but also cultivated in some parts of Africa (Paliwal & Sharma 2011; Saini *et al.,* 2016). It grows fast in tropical and subtropical countries, at a temperature between 18 and 28 ˚C and a pH between 4.5 and 8. The edible leaves of *M. oleifera* possess large amounts of phytochemicals which confer various

nutritional, heath beneficial and medicinal properties (Vats & Gupta, 2017). The demand for nutritional and medicinal plants is increasing with the growth of the population and the intense industrialization and urbanization (Ali & Iqbal, 1999). As such, cell and tissue culture may be a very promising technique for *in vitro* multiplication and conservation of *M. oleifera* (Shahzad *et al.,* 2014) and other plants that have significance for nutrition and medicine (Cai *et al.,* 2015). The totipotent characteristic of plant cells makes plant cell and tissue culture a system of choice for secondary metabolite production as a single cell is able to produce the same range of phytochemicals found in the whole plant under appropriate regimes. Moreover, the controllable environment of *in vitro* cultures makes the synthesis of targeted compounds more effective (DiCosmo & Misawa, 1995; Rao & Ravishankar, 2002; Hellwig *et al.,* 2004; Mulabagal & Tsay, 2004; Niranjana *et al.,* 2015; Ochoa-Villarreal *et al.,* 2015; Ochoa-Villarreal *et al.,* 2016).

The focus of this specific study was to investigate the effects of auxin (2,4-dichlorophenoxyacetic acid, 2,4-D) and cytokinin (kinetin) concentrations on undifferentiated *M. oleifera* callus cells in an attempt to induce organogenesis, and to screen and study the metabolite profiles resulting from this change.

4.2 Materials and methods

4.2.1 *Moringa oleifera* callus culture

4.2.1.1 *In vitro* plantlet culture

Seeds were collected from *M. oleifera* trees cultivated in the Limpopo province of South Africa. The surface of the seeds was sterilized in a laminar airflow cabinet with 70% (v/v) ethanol for 40 sec, then 1% (v/v) sodium hypochlorite (NaOCl, ≡ 33% Jik) solution and finally rinsed three times with sterile distilled water (dH_2O). Following sterilization, seeds were germinated in Magenta jars containing 50 mL of basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Duchefa, Haarlem, Netherlands) solidified with 0.8% agar. The lids of the jars were sealed with Parafilm to avoid contamination, and placed in an incubator under a photoperiod of 12 h light and 12 h darkness, at 24.6 °C.

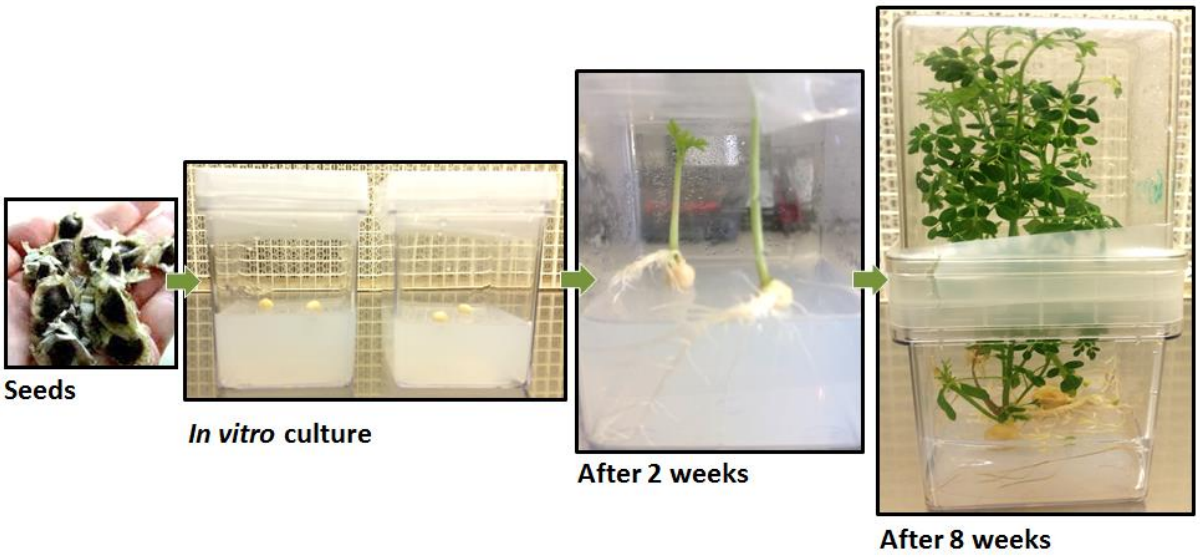

Figure 4.1: *In vitro* plantlet culture of *Moringa oleifera*. Seeds were collected from *M. oleifera* trees and surface-sterilized. After sterilization, seeds were germinated in Magenta jars and photographs of the 2 and 8 weeks old plantlets are shown above.

4.2.1.2 *Moringa oleifera* callus initiation

Using stem sections of 2 week old plantlets, callus initiation was carried out on MS medium (Murashige and Skoog, 1962) enriched with MS vitamins (riboflavin, nicotinic acid, thiamine, and glycine, Highveld Biologicals, Johannesburg, South Africa). In addition, the medium contained callus-inducing hormones (Shank & Lee, 2013); 2,4-dichlorophenoxy acetic acid (2,4-D, 2 mL of a 250 mg/L stock solution / 113.1 µM, Sigma, St Louis, MO, USA), kinetin (Kin, 2 mL of a 250 mg/L stock solution / 116.1 µM, Duchefa, Netherlands) and alpha naphthalene acetic acid (NAA, 500 µL of 2000 mg/L stock solution / 107.41 µM (NAA, Sigma, St Louis, MO, USA). The medium also contained 3% sucrose as carbon source and 0.1% hydrolysed casein (Sigma, Munich, Germany) as source of phosphate, calcium and amino acids. The pH of the media was adjusted to 5.8 and the temperature kept at ± 24.5 °C. Phytoagar (0.8%, Sigma, Munich, Germany) was added to the medium and autoclaved. The sterile medium was then left in the laminar flow cabinet to cool down and transferred into Petri dishes (85 mm) to solidify. Petri dishes were sealed with Parafilm to avoid any contamination.

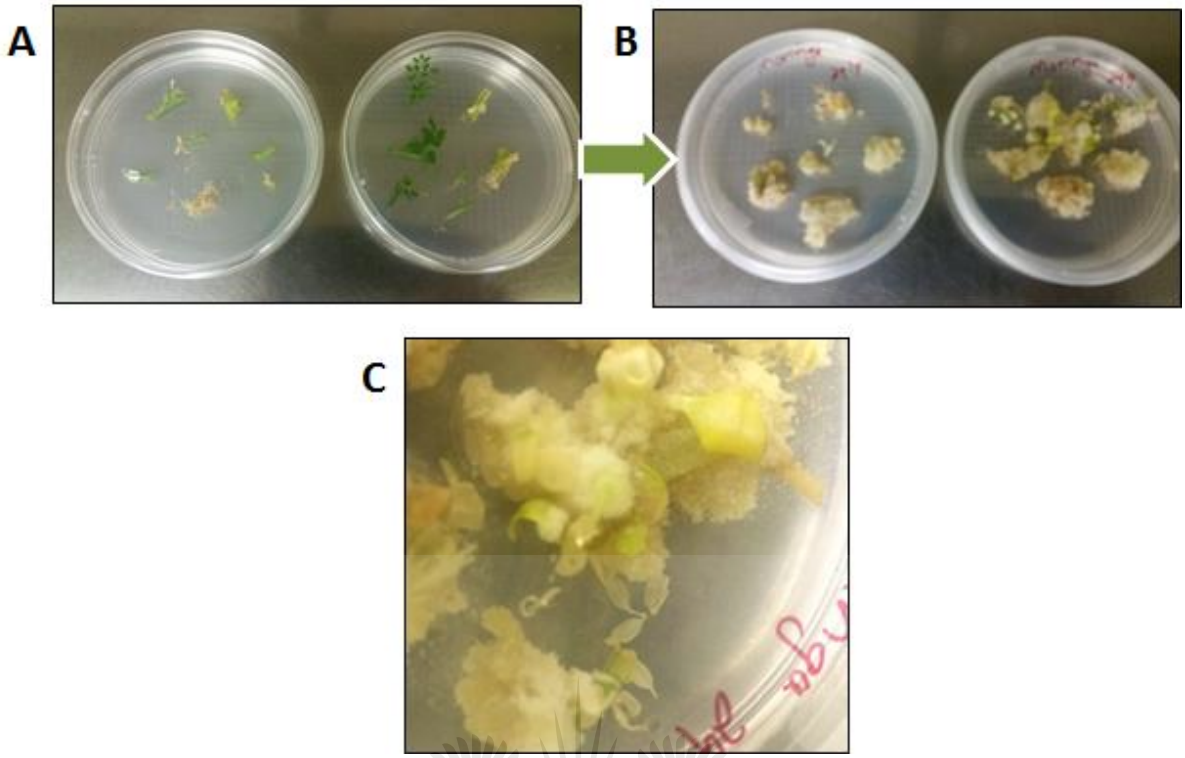

Figure 4.2: *Moringa oleifera* callus initiation. **(A)** Stem segments of *M. oleifera in vitro* plantlets, **(B)** Callus formed after three weeks of incubation, **(C)** *M. oleifera* callus.

4.2.1.3 Callus manipulation and experimental design

Calli initiated as described above were sub-cultured and multiplied before the manipulation for organogenesis took place. Concentrations (0, 0.5, 1.0, 1.5 and 2 mg/L) of plant growth hormones auxin (2,4-D) and cytokinin (kinetin) alone, and in combination (actual concentrations as represented in **Table 4.1**) were supplemented to *M. oleifera* undifferentiated cells medium (MS medium with MS vitamins, 30 g/L of sucrose and 1 g/L of casein hydrolysate). Condition **1** was regarded as the control as no hormones were added into the medium. Three replicates were assigned per treatment and the average mass of callus per plate at the start of the experiments was 2.6 g.

Table 4.1: Experimental design for the growth of *Moringa oleifera* callus under different plant growth regulator regimes.

Hormones	**Auxin (2,4-D)**					
Cytokinin (Kin)	**Conc. (mg/L)**	0.0	0.5	1.0	1.5	2.0
	0.0	Cond 1	Cond 6	Cond 11	Cond 16	Cond 21
	0.5	Cond 2	Cond 7	Cond 12	Cond 17	Cond 22
	1.0	Cond 3	Cond 8	Cond 13	Cond 18	Cond 23
	1.5	Cond 4	Cond 9	Cond 14	Cond 19	Cond 24
	2.0	Cond 5	Cond 10	Cond 15	Cond 20	Cond 25

*Cond = Condition

4.2.2 Metabolite extraction and sample preparation

Calli from each replicate were transferred into pre-weighed, sterile 50 mL Falcon tubes, working sterile in a laminar flow hood. A volume of 80% methanol (MeOH, analytical grade, Romil, Cambridge, UK) was added to *M. oleifera* calli (average 4.8 g in a 1:1 (v/m) ratio) and directly put on ice to slow down enzymatic activities. Samples were homogenized for 30 min using an ultrasonic bath then for 1 min at 25 °C using a probe sonicator (Sonopuls, Bandelin, Berlyn, Germany) set at 55% power for 20 sec. Homogenized samples were centrifuged at 5100 x*g* for 30 min at 4 °C and the supernatants carefully removed. The hydromethanolic supernatants were concentrated by vacuum evaporation using a rotary evaporator (Büchi RotavaporR-200, Flawil, Switzerland) and a centrifugal vacuum concentrator (Eppendorf/Merck, Darmstadt, Germany). Dried extracts were reconstituted with 500 µL of 50% MeOH and stored at -20 °C for future use. For ultra-high performance liquid chromatography - quadrupole time of flight mass spectrometry (UHPLC-qTOF-MS) analysis, extracts were filtered through 0.22 µm nylon filters into glass vials fitted with inserts and slitted caps.

4.2.3 Phytochemical screening

4.2.3.1 Total phenolic content assay

The total phenolic content was determined by the Folin-Ciocalteu (F-C) assay (Follin & Ciocalteu, 1927). The principle is based on a transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes. The reduced FC is blue in colour and detectable using a spectrophotometer at 760 nm (Ainsworth and Gillespie, 2007).

Briefly, to 100 µL of each extract, blanks (80% MeOH) and a concentration series of gallic acid/trihydroxybenzoic acid (Sigma, St Louis, Mo, USA), dissolved in 80% MeOH, in 2 mL microcentrifuge (Eppendorf) tubes, 200 µL of 10% (v/v) F-C reagent (Sigma, St Louis, Mo, USA) was added and vortexed thoroughly. A volume of 800 µL of 700 mM sodium carbonate (Na_2CO_3) was then added to the mixture and incubated at room temperature for 2 h. After incubation, 200 µL of each test sample, blank and standard (positive control) was transferred in triplicate into a 96-well microplate and the absorbance of each well was read at 765 nm using a microplate reader. Concentrations of gallic acid employed to generate the calibration curve (R^2 = 0.9954, **Figure 4.5A**) were between 42.53 µg/mL and 212.65 µg/mL (0.25 mM and 1.25 mM).

All experiments for total phenolic content were performed in triplicate. The results obtained were statistically investigated and expressed as mean ± standard deviation.

4.2.3.2 Total flavonoid content assay

The total flavonoid content was estimated by the aluminium chloride ($AlCl_3$) colorimetric method. The principle is based on the ability of $AlCl_3$ to form acid stable complexes with the C-4 keto groups and the C-3 or the C-5 hydroxyl group of flavonols and flavones. The $AlCl_3$ is also able to form labile complexes with the ortho-dihydroxyl groups of flavonoids (Kalita *et al.,* 2013).

A volume of 100 µL of 10% $AlCl_3$ solution was added to each 500 µL of sample extracts, blank and standard. After 6 min incubation, 100 µL of potassium acetate was added and

diluted with 2.8 mL of deionized water. The mixture was incubated at room temperature for 30 min. Two hundred µL of each test sample, blank and standard was transferred in triplicate into a 96-well microplate and the absorbance of each well was read at 420 nm using a microplate reader. A flavonol commonly found in plants, quercetin (concentration range of 0.5 mg/mL to 5 mg/mL = 1.47 to 14.77 µM), was used as reference standard to construct the calibration curve (R^2=0.9983, **Figure 4.7**).

All experiments for total flavonoid content were performed in triplicate. The results obtained were statistically expressed as mean ± standard deviation.

4.2.3.3 Anti-oxidant assay

The assay is based on the ability of a compound to scavenge 2,2'-diphenyl-1-picrylhydrazyl (DPPH) stable radical. The disappearance of DPPH (purple in colour) is proportional to the anti-oxidant effect of the compound and can be measured spectrophotometrically at 517 nm. Therefore, the radical scavenging activity of a compound on DPPH results in a change in colour from purple to yellow and a decrease of absorbance at 517 nm (Lee *et al*., 2007).

A volume of 150 µL of a freshly prepared solution of DPPH (Sigma, St Louis, Mo, USA) dissolved in 80% MeOH was added to a 96-well plate followed by 50 µL of each extract, blank (80% MeOH) and standard. Each sample was added to the plate in triplicate and solutions were mixed, covered and incubated in the dark for 4 h. The absorbance was then read at 517 nm. Vitamin C is a well-known anti-oxidant (Esterhuizen *et al.,* 2006) and was utilized as standard at different concentrations (varying from 8.80 µg/mL to 35.22 µg/mL = 20 µM and 200 µM) to construct the calibration curve (R^2 = 0.9913, **Figure 4.6A**). The DPPH radical inhibition activity was calculated according to the equation:

%DPPH inhibition = [(Absorbance of the blank – Absorbance of the sample) / Absorbance of the blank] x 100

All experiments for anti-oxidant assays were performed in triplicate. The results obtained were statistically investigated and expressed as mean ± standard deviation.

4.2.4 Metabolomic studies

4.2.4.1 Ultra-high performance liquid chromatography – quadrupole time-of-flight high definition mass spectrometry (UHPLC-QTOF-HDMS)

Chromatographic analyses of extracts were performed on an Acquity UHPLC system with a photodiode array (PDA) detector (Waters Corporation, Milford, MA, USA) coupled to a SYNAPT G1 high definition quadrupole time-of-flight (QTOF) mass spectrometer (Waters Corporation, Milford, MA, USA). Separation of extracts was carried out on a Waters Acquity UHPLC column (HSS T3 reverse phase column, 150 x 2.1 mm with a 1.7 µm particle size). The gradient elution was performed with eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile) at a flow rate of 0.4 mL/min. The injection volume was 3 µL. The elution was initiated with 2% of eluent B for 1 min, and was kept constant for 2 min. The gradient was introduced by increasing B to 3%, 8% then 50% for 1 min, 21 min and 1 min respectively. The eluent B concentration was finally increased to 95% for 2 min before restoration to the initial condition (2% B) for 2 min. The scanning range of the PDA detector was set between 200-500 nm, the bandwidth resolution to 1.2 nm and 20 spectra per sec for the sampling rate. Each sample was injected in triplicate and analyzed in a randomized order.

The separated compounds were further detected using a SYNAPT G1 QTOF MS system set in V-optics and operated in negative electrospray ionization (ESI) mode. The analyses were set to perform unfragmented and four fragmenting experiments (MS^E) concurrently by collision energy ramping from 10 to 40 eV. The reference calibrant was leucine encephalin (50 pg/mL, 554.2615 Da) at a flow rate of 0.1 mL/min, and a mass accuracy window of 0.5 mDa was maintained throughout the analysis. The source and dissolvation gas temperatures were set at 120 °C and 450 °C respectively, and the cone gas and dissolvation gas flows were at 50 and 550 L/h respectively. The interscan time was 0.02 sec, the scan time 0.1 sec covering a mass to charge (*m/z*) range of 100-1000. The capillary voltage was 2.5 kV, the extraction cone voltage 4 V and the sampling cone voltage 30 V.

4.2.4.2 Data processing and multivariate data analysis

The data obtained from LC-MS analyses were processed for peak finding, peak integration, alignment and Rt correction using MarkerLynx^{TM} XS software (Waters Corporation, Milford, MA, USA) with the following parameters: mass range of 100-1000 Da, mass tolerance of 0.01 Da, Rt range of 1.50- 25 min, Rt window of 0.2 min.

The datasets obtained from Markerlynx^{TM} XS software processing were analyzed by SIMCA-14 software (Soft independent modelling of class analogy - Umetrics Corporation, Umea, Sweden) for multivariate statistical analysis. In order to visualize and explain the metabolic changes between samples, principal component analysis (PCA) and hierarchical clustering analysis (HCA) were used to simplify and reduce the dimensionality of a dataset and to differentiate between treatments (Bhalla *et al.,* 2005; Moco *et al.,* 2007; Tugizimana *et al.,* 2013), and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) modelling were used to identify discriminatory biomarkers by revealing hidden biological difference from metabolite profiles under the control of an administrator (Tugizimana *et al.,* 2013; Cook & Rutan, 2014). Pre-treatment methods such as *Pareto* scaling were applied prior computing PCA and OPLS-DA models, to minimize variable redundancy and adjust for measurement errors. Diagnostic tools such as the cumulative model variation in the matrix X, R^2X (cum), which is the goodness-of-fit parameter that describes the proportion of the variance of the total variation explained by the model and the predictive ability, Q^2 were employed to evaluate generated models. Cross-validation analysis of variation (CV-ANOVA) (with a *p*-value < 0.05 indicating a good model) was utilized to further validate the generated OPLS-DA models.

4.3 Results and Discussion

4.3.1 *Moringa oleifera* callus development and growth

M. oleifera callus manipulated with plant growth hormones auxin (2,4-D) and cytokinin (kinetin) at different concentrations (**Table 4.1**) showed no organ formation in all conditions after 21 d of incubation (**Figure 4.3A**). Calli obtained after incubation were friable (or soft) and yellowish in colour (**Figures 4.3A and 4.4**). The mass of calli collected ranged from 3.086

to 6.034 g with the former associated with the smallest mass obtained in condition **21** (containing no cytokinin and the highest concentration of auxin (2 mg/L)) (**Figure 4.3B**). In contrast, the highest mass was measured in condition **8** containing 1 mg/L of cytokinin and 0.5 mg/L of auxin. The concentration and ratio of the two phytohormones thus did affect the growth rate of the callus.

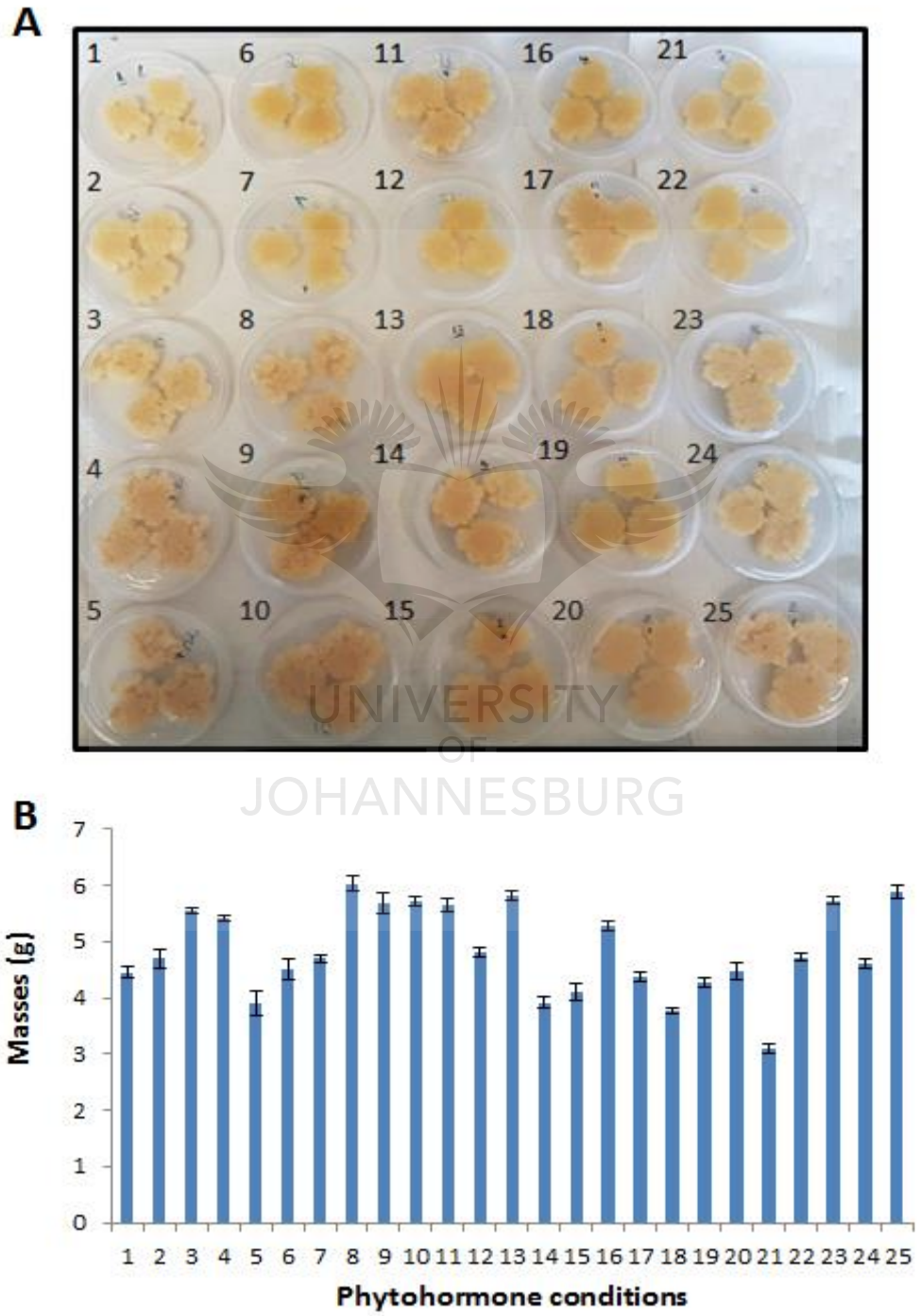

Figure 4.3: (A) *In vitro* growth of *Moringa oleifera* callus on MS media containing 2,4-D (auxin) and kinetin (cytokinin) in 25 different combinations, after 21 d of incubation. The numbers 1 to 25

correspond to the different conditions as depicted in **Table 1** above; **(B)** Bar graph representative of the masses of callus obtained in each condition.

Callus is a growing mass of unorganized cells formed over a wounded or cut plant in nature, or following induction with hormones in *in vitro* cultures (Ikeuchi *et al.,* 2013). Callus texture and appearance can vary according to the plant species and more precisely the part of the plant used as explant (Samuelsson and Bohlin, 2009; Loyola-Vargas & Ochoa-Alejo, 2012). The callus of *M. oleifera* initiated from any type of explant has been reported by Shank (2013) to be friable, soft and white to yellowish in colour. The friability of a callus presents the advantage of being appropriate for suspension cultures and favourable for secondary metabolite production (Shank & Lee, 2013).

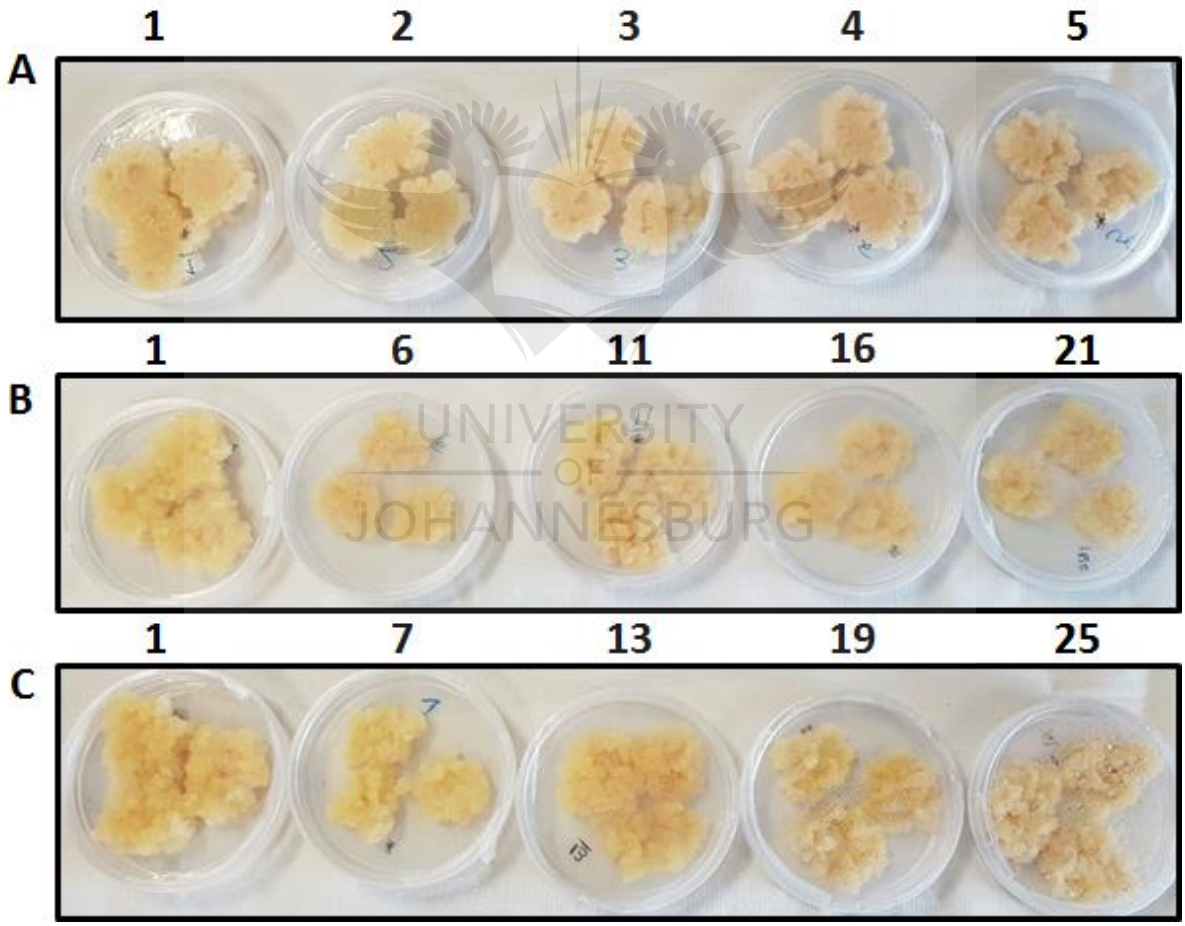

Figure 4.4: *Moringa oleifera* callus in **(A)** auxin only (0.5, 1.0, 1.5 and 2.0 mg/L), **(B)** cytokinin only (0.5, 1.0, 1.5, and 2.0 mg/L) and **(C)** combination of auxin and cytokinin at equal concentrations (0.5, 1.0, 1.5 and 2.0 mg/L). In all cases, # **1** represents the hormone-free condition (0.0 mg/L, 0 mg/L) or habituated state to which all conditions were compared to.

Surprisingly, in this study, the calli were growing well on hormone-free media (Condition **1, Figures 4.3A** and **4.4**), suggesting that *M. oleifera* cells have acquired the capability to grow in the absence of the externally added growth hormones, auxin and cytokinin. This phenomenon is known in plant tissue culture as 'habituation' (Thorpe 2013; Leljak-Levanić *et al.,* 2016). Another consideration is that the disruption of some plastid functions reduces the use of endogenous cytokinins, consequently the tissue requirements for that hormone. It was observed in a study conducted by Kaminek *et al.,* (1981) that the formation of chlorophyll was reduced in cytokinin-independent tobacco callus (Kaminek *et al.,* 1981). Although the causes of habituation are still unclear, it is speculated that it might be due to the increase in the biosynthesis of growth hormones or the decrease of the degradation rate, the altered sensitivity of cells to these hormones and the interaction of some or all of these (Kevers *et al.,* 1996; Leljak-Levanić *et al.,* 2016). Habituation is a multi-step phenomenon that occurs gradually and may lead to complete and irreversible undifferentiation of cells (Hagege, 1996; Gaspar *et al.,* 2000).

In general, plant tissue differentiation is determined by the auxin-cytokinin ratio present in the media (Förster *et al.,* 2013). The absence of organogenesis in the presence of auxin or cytokinin alone (**Figures 4.4A** and **4.4B** respectively), and in combination (**Figure 4.4C**), suggests that the *M. oleifera* callus cell line developed in this study have lost its totipotency (probably due to the habituation phenomenon). The reasons for the loss of regenerative ability of plant cells are also not well established. Nonetheless, three theories can be proposed to explain this phenomenon. The first is the genetic variation present in the original cell population (Gaspar *et al.,* 2000). This can be the result of gene mutations, recombination or migration (Griffiths *et al.,* 2000). The second theory is that during *in vitro* culture, substances promoting organogenesis may gradually diminish in the medium or a regeneration inhibitor may accumulate with time leading to the suppression of organogenesis. The last theory is the occurrence of an epigenetic variation in the cultured cells. This is manifested as changes in the manner that gene expression occurs in the cultured cells caused by the environment of culture. An example of such variation is the *in vitro* hypo- or hyper-methylation of genes involved in organogenesis, resulting in the modification of expression. Moreover, the change in metabolism, receptivity or

transduction pathways of endogenous hormones may induce loss of totipotency (Lambé *et al.,* 1997; Gaspar *et al.,* 2000).

Similarly to this study, Shahzad *et al.,* (2014) employed different hormones (Benzyl adenine: BA, Kin, 1-naphtaleneacetic acid: NAA) alone and in combination to *M. oleifera* callus to induce regeneration and did not observe organogenesis. However, in a study conducted by Marthur *et al.,* (2014) maximum plant shoot regeneration of *M. oleifera* callus was observed on media enriched with benzylaminopurine and kinetin (BAP:Kin; 2.0:2.0 mg/L). The maximum root regeneration was observed on media supplemented with indole-3-byturic acid and 1-naphtaleneacetic acid IBA:NAA (3.0:0.5 mg/L). This implies that *M. oleifera* callus regeneration depends on many factors, including the type and ratio of hormones employed and also the state of the cells (habituated/non-habituated).

4.3.2 Total phenolic – and anti-oxidant content

Each methanolic extract of *M. oleifera* callus grown under the 25 different conditions was analyzed for total phenolic content (TPC) and anti-oxidant activity. The standard curve for TPC constructed with gallic acid is represented in **Figure 4.5A** and the determination of total phenolic concentrations (in µg GAE /g wet weight) corresponding to each extract is in **Figure 4.5B**. As seen on the bar graph, there is variation in the total phenolic content following hormonal treatment of *M. oleifera* callus. This shows that the production of phenolics by *M. oleifera* calli is differentially affected by the concentration or ratio of growth hormone present in the media.

The total phenolic compound content assay reflects the reducing capacity of the extracts, and is also used as general indicator of secondary metabolite synthesis in plants (Stojakowska *et al.,* 2016). The concentration of phenolics in each sample was expressed in µg GAE / g wet weight and is presented in **Table 4.2**. The highest concentration of phenolics (49.86 µg GAE/g wet weight, highlighted in blue) was observed in condition **18** containing 1.5 mg/L of 2,4-D and 1 mg/L of kinetin. This suggests the most favourable hormone combination for phenolic compound production by *M. oleifera* callus.

An increase in phenolic compound content was observed where only 0.5 mg/mL of 2,4-D was employed (Condition **6**), followed by a decrease in condition **11**, **16** and **21** (highlighted in green). In the case of kinetin applied alone, the concentration of phenolic compounds decreased in comparison to the control. This indicates that kinetin alone does not induce the production phenolic compounds in *M. oleifera* callus.

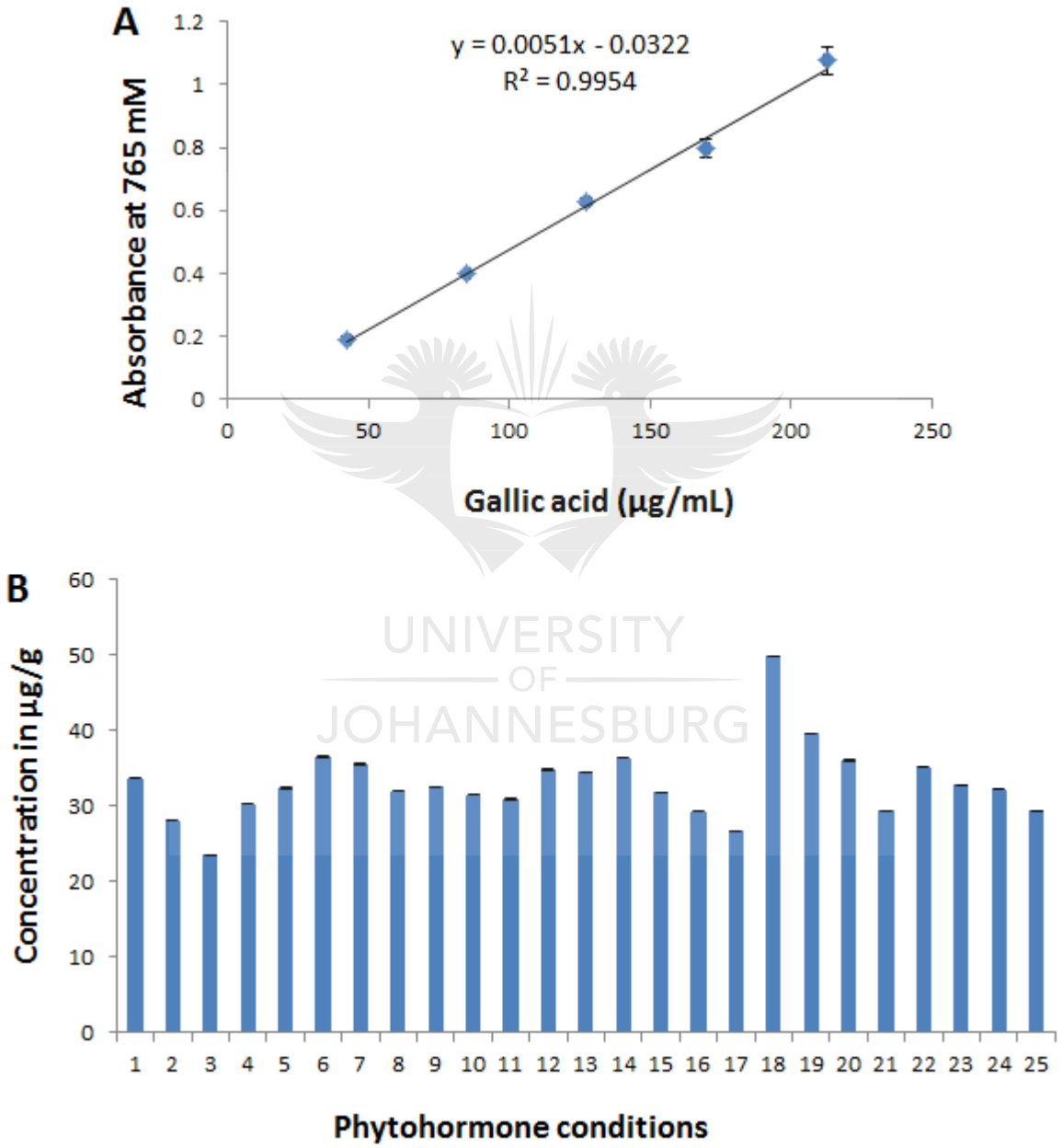

Figure 4.5: (A) Representative standard curve for 96-well assays for the total phenolic content (TPC) constructed with gallic acid at concentrations between 42.53 µg/mL and 212.65 µg/mL (0.25 mM and 1.25 mM). The standard curve equation is 0.0051x – 0.0 322 and the Pearson coefficient of determination R^2 is 0.9954. **(B)** Bar graphs for the TPC of *Moringa oleifera* callus cultured in conditions **1-25**, each with varying concentrations of 2,4-D and kinetin as indicated in **Table 4.1**.

A positive correlation is observed between the phenolic content in *M. oleifera* callus and the addition of equal concentrations of both auxin (2,4 D) and cytokinin (kinetin) (Conditions **7**, **13**, **19**) but finally decrease where concentrations of both hormones reach the highest values (condition **25**). These observations confirm the interaction existing between auxins and cytokinins (Moubayidin *et al.,* 2009). Plant growth hormones, especially auxins and/or cytokinins have been revealed to modify the phenylpropanoid/shikimate pathways responsible of the biosynthesis of the phenolic acids, possibly by regulating the enzyme activity of phenylalanine ammonia lyase and chalcone synthase (Moyo *et al.,* 2014; Thiruvengadam & Chung, 2015).

Table 4.2: Template of *Moringa oleifera* callus in 25 conditions with the corresponding total phenolic content (µg GAE/g wet weight). The highlight in yellow represents concentrations in callus grown on kinetin alone; the green highlights concentrations in callus grown on 2,4-D alone; the pink is for condition 1 (control, with no added hormones); the orange represents the increasing concentrations of kinetin and auxin in equal concentrations; the blue the highest concentration of phenolics. The arrows indicate the increase or decrease in phenolic content compared to the control.

Hormones	**Auxin (2,4-D)**					
Cytokinin (Kinetin)	**Conc. (mg/L)**	0.0	0.5	1.0	1.5	2.0
	0.0	**1** 33.66 ± 0.02	**6** ↑ 36.55 ± 0.02	**11** ↓ 30.93 ± 0.04	**16** ↓ 29.31 ± 0.03	**21** ↓ 29.39 ± 0.01
	0.5	**2** ↓ 28.05 ± 0.05	**7** ↑ 35.56 ± 0.01	**12** 34.83 ± 0.01	**17** 26.73 ± 0.01	**22** 35.18 ± 0.01
	1.0	**3** ↓ 23.56 ± 0.02	**8** 31.99 ± 0.02	**13** ↑ 34.55 ± 0.02	**18** 49.86 ± 0.03	**23** 32.78 ± 0.05
	1.5	**4** ↓ 30.30 ± 0.03	**9** 32.57 ± 0.01	**14** 36.41 ± 0.01	**19** ↑ 39.65 ± 0.02	**24** 32.25 ± 0.03
	2.0	**5** ↓ 32.37 ± 0.02	**10** 31.47 ± 0.02	**15** 31.89± 0.01	**20** 35.05 ± 0.03	**25** ↓ 31.70 ± 0.03

The standard curve for anti-oxidant activity constructed with vitamin C as standard and the quantitative determination of the anti-oxidant activities associated with the various extracts

are represented in **Figure 4.6**. In contrast to the TPC determinations, the anti-oxidant activity determination showed little variation between the 25 conditions, with the % inhibitory activity varying between 61% (condition **5** - 0 mg/L of 2,4-D and 2.0 mg/mL of kinetin) and 78% (condition **12** - 1 mg/L of 2,4-D and 0.5 mg/mL of kinetin). This indicates that the phenolic content of the cultured cells does not correlate with the determined anti-oxidant activity and suggests that the different hormone combinations and ratios have a differential effect on the secondary metabolite composition of the cultured callus. More precisely, this might imply that the anti-oxidant activity of *M. oleifera* callus is not only due to the presence and concentrations of its phenolic compounds. Anti-oxidants are systems or compounds that are able to inhibit the formation of free radicals or interrupt their propagation (by accepting or donating electrons) to prevent auto-oxidation (Lü *et al.,* 2010; Brewer, 2011; Choe & Min, 2015). They are compounds such as phenolics, ascorbic acid (vitamin C), carotenoids, protein-related compounds and sterols (Choe & Min, 2015; Nimse & Pal, 2015). These compounds are not all detected with the F-C assay and might be a reason why the variation in TPC does not always correlate to that of the anti-oxidant activity (Ghasemi *et al.,* 2009). Moreover, chemical composition and structures of phenolics present in different extract are important factors contributing to the anti-oxidant activity. This highlights the complexity of anti-oxidative properties of plant extracts which can manifest in various ways. For instance, phenolic compounds can confer anti-oxidative properties through either the hydrogen transfer (HAT) mechanism (donating a hydrogen proton) or the electron transfer (ET) mechanism (donating an electron) (Leopoldini *et al.,* 2004; Makola *et al.,* 2016). Furthermore, the effectiveness of these two mechanisms is also dependant on the chemical environment (*i.e.* polarity) (Makola *et al*., 2016). As seen in the current study, the composition of phenolic compounds in the extracts cannot easily be correlated to the anti-oxidant capacity thereof. To circumvent this, various anti-oxidant assays, varying with underlying chemical mechanism are often investigated (Sreelatha & Padma, 2009; Amzad Hossain & Shah, 2015).

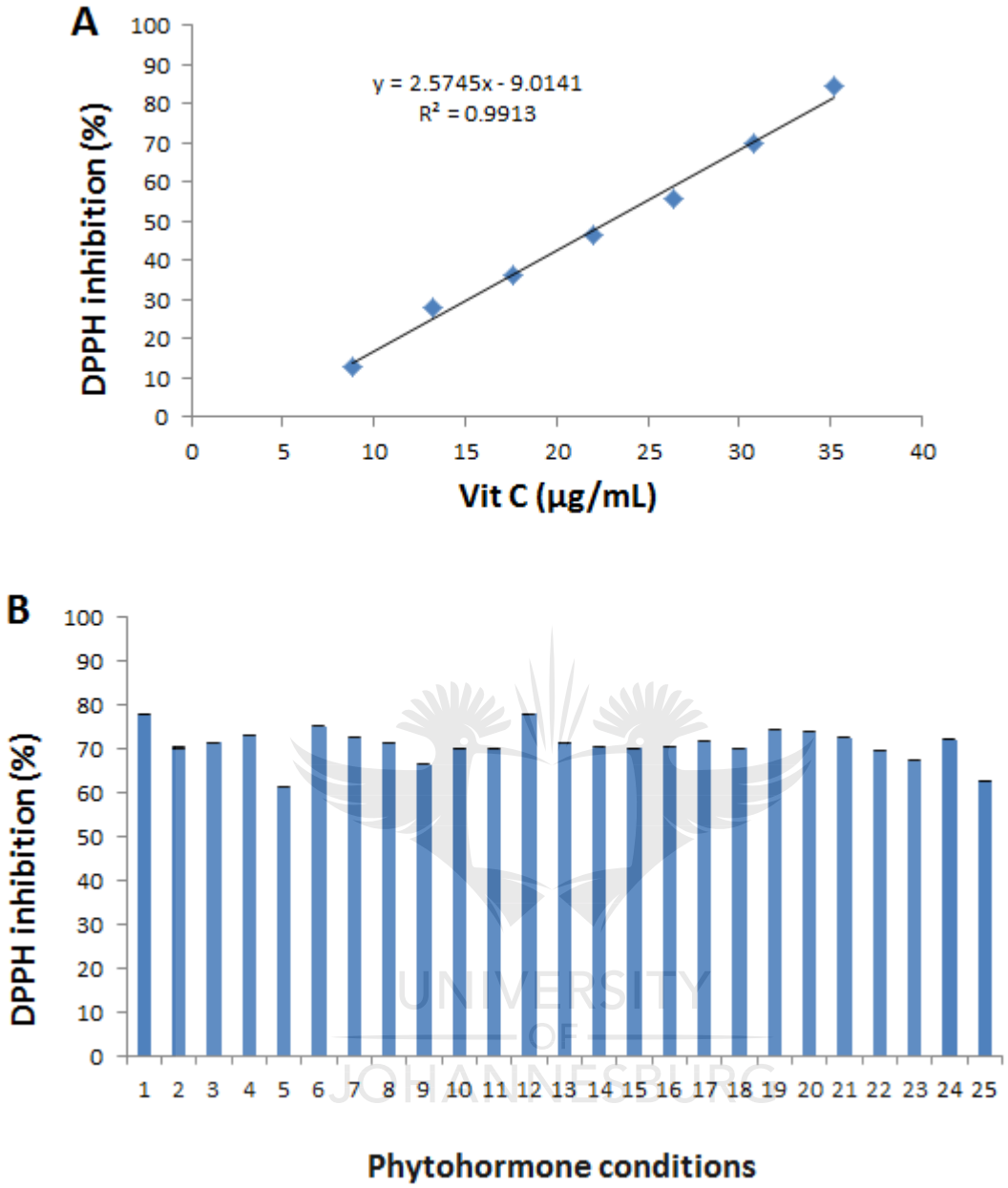

Figure 4.6: (A) Representative standard curve for 96-well assays for DPPH-scavenging activity constructed with vitamin C as standard at concentrations between 8.80 µg/mL and 35.22 µg/mL (20 µM and 200 µM). The standard curve equation is 2.5745x – 9.0141 and R^2 is 0.9913. **(B)** Bar graphs for the quantitative determination of the anti-oxidant activities associated with the various extracts of *Moringa oleifera* callus cultured in conditions **1-25**, each with varying concentrations of 2,4-D and kinetin as indicated in Table 1.

The total flavonoid compound content was also evaluated on the same extracts and the standard curve constructed with quercetin is represented below (**Figure 4.7**). The absorbance values obtained with the aluminium chloride colorimetric method were below the detection limit of the standard curve and the results regarded as non-significant (data not shown).

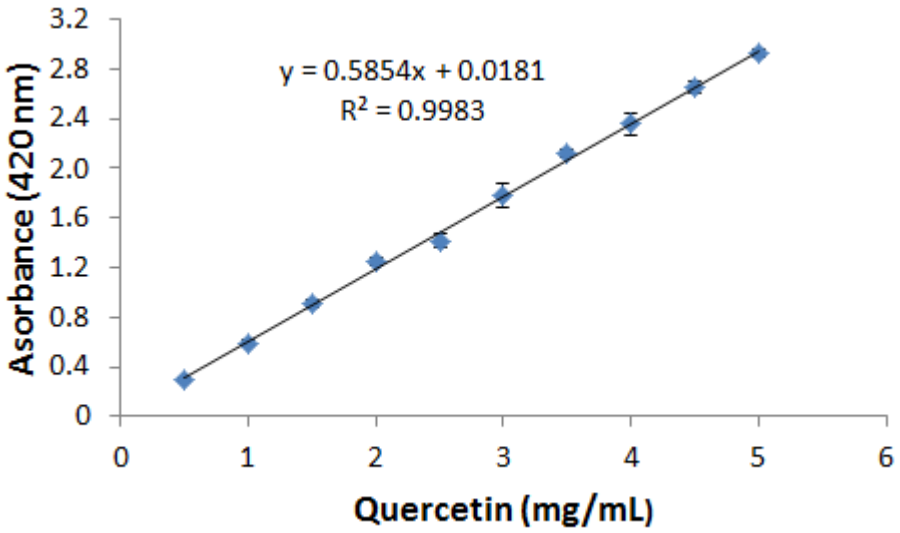

Figure 4.7: Representative standard curve for 96-well assays for total flavonoid content constructed with quercetin as standard at concentrations between 0.5 mg/mL and 5 mg/mL (1.47 mM and 14.77 mM). The standard curve equation is 0.5854x + 0.0181 and R^2 is 0.9983.

4.3.3 LC-MS and multivariate data analyses (MVDA)

The methanolic extracts of *M. oleifera* callus were analyzed using UHPLC-QTOF-MS to investigate the metabolite variation following growth of callus on different concentrations and combinations of auxin (2,4-D) and cytokinin (kinetin). UHPLC-MS base peak intensity (BPI) chromatograms obtained in negative ESI mode are presented in **Figure 4.8**. The comparison of chromatograms showing samples treated with kinetin alone (**Figure 4.8A**) displays a very slight or no difference in peak intensity. In the case of 2,4-D (**Figure 4.8B**), there is difference mostly in terms of peak intensity but also with regard to the presence/absence of some peaks. **Figure 4.8C** was constructed as a summary showing overlaid chromatograms comparing the control (condition **1**), kinetin (represented by condition **3**), 2,4-D (represented by condition **11**) and a combination of both kinetin and 2,4-D (represented by condition **8**). Here, one condition was chosen per group (cytokinin, auxin and combination) to simplify observations since overlaying all 25 conditions in one figure was not very descriptive. Overall, there is a differential effect of the medium hormonal composition (especially in presence of 2,4-D alone or in combination) on the secondary metabolite production by *M. oleifera* callus. It is noteworthy to mention that the most intense peaks appear to be in the early Rt region of the chromatograms, indicating the hydrophilicity of metabolites (in that region) extracted from *M. oleifera* callus. In order to

reveal clearer differences in the metabolite profiles of *M. oleifera* callus treated with different concentration of hormones, multivariate data analyses were carried out.

Datasets resulting from metabolomic studies (more specifically LC-MS-based untargeted analysis) are high-dimensional and very complex, hence challenging to handle. In order to extract relevant information from the complexity of these datasets, modelling algorithms are generated using chemometric and bioinformatics tools and resources incorporating statistics, mathematics and science (Tugizimana *et al.,* 2016). Multivariate data analysis (MVDA) approaches or mathematical modelling methods are employed to extract significant information from the large and complex sets of data (Tugizimana *et al.,* 2013). MVDA includes two groups of analyses: unsupervised approaches such as principal component analysis (PCA) and hierarchical clustering analysis (HCA); and supervised approaches such as the orthogonal projection to latent structures-discriminant analysis (OPLS-DA) (Bhalla *et al*., 2005; Moco *et al*., 2007 and Cook & Rutan, 2014).

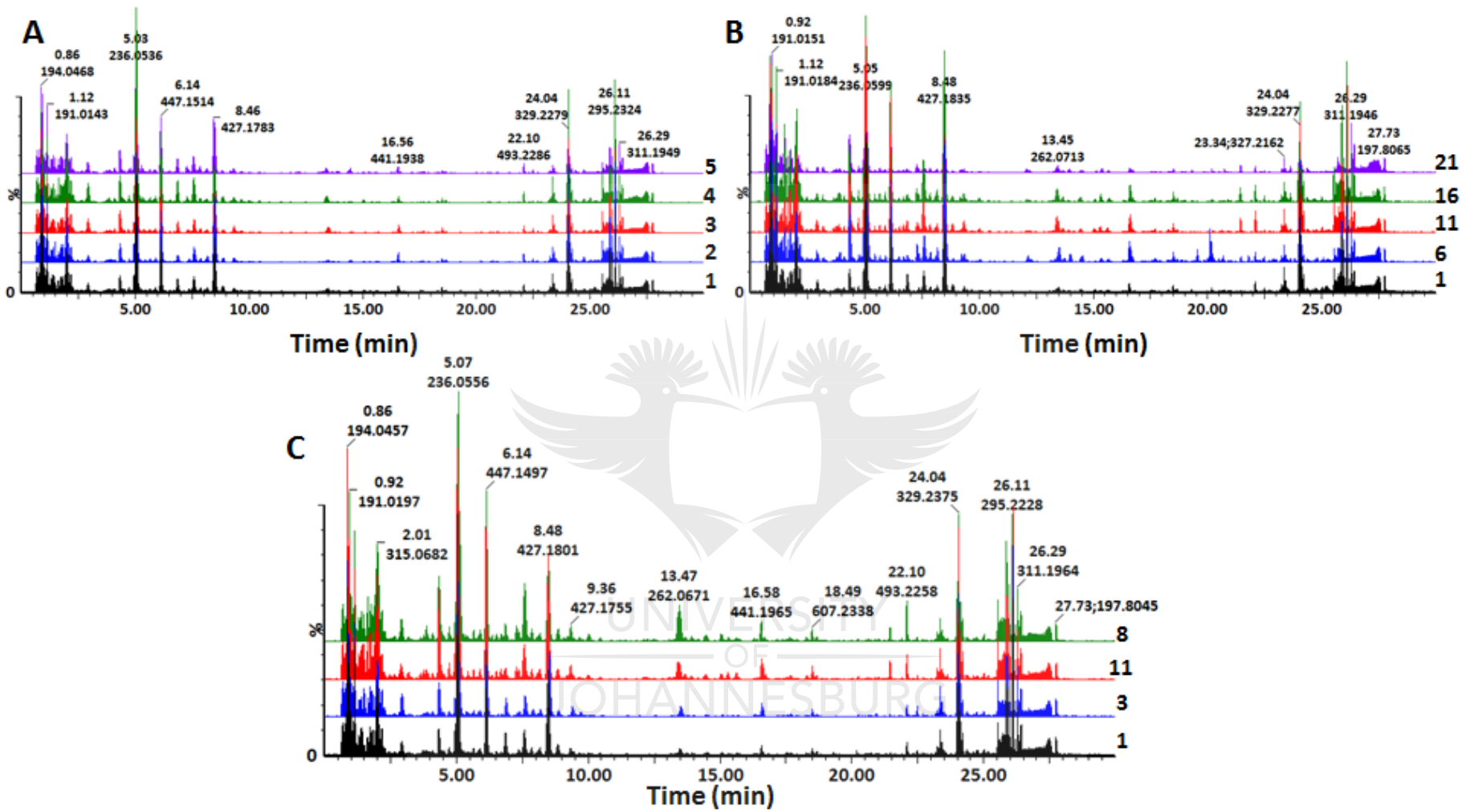

Figure 4.8: UHPLC-MS (ESI negative mode) BPI chromatograms of methanol extracts from *M. oleifera* callus. Each sample was analyzed in triplicate. **A:** Chromatograms of *M. oleifera* callus treated with cytokinin (kinetin) only (Conditions **1**. No hormone, **2**. 0.5 mg/L, **3**. 1 mg/L, **4**. 1.5 mg/L and **5**. 2 mg/L); **B:** Chromatograms of *M. oleifera* callus treated with auxin (2,4-D) only (Conditions **1**. No hormone, **6**. 0.5 mg/L, **11**. 1 mg/L, **16**. 1.5 mg/L and **21**. 2 mg/L) and **C:** Chromatograms comparing *M. oleifera* callus treated with kinetin alone (Condition **3**), 2,4-D alone (Condition **11**) and combination of both (Condition **8**) in comparison to no hormones (Condition **1**).

As seen in **Figure 4.9** representing the PCA score plots (**Figure 4.9A** and **B**) and the HCA plots (**Figure 4.9C** and **D**) of *M. oleifera* callus extracts, samples are differentially clustered according to the treatment. Kinetin-treated samples are seen to group close to the control and a clear difference is observed with 2,4-D treated samples, clustering separately from the control. This highlights the differential effect of the auxin 2,4-D in the production of secondary metabolites. Moreover, extracts containing the combination of kinetin and 2,4-D are grouped together with 2,4-D employed alone; an indication that 2,4-D is responsible for the variation between the samples. Aiming to further explore the PCA groupings at a metabolite or variable level (rather than sample level) and underline the perturbation generated by differential applications of cytokinin and auxin, HCA was performed (Tugizimana *et al.,* 2017).

The dendrograms resulting from HCA analysis emphasize on the difference between the different groups and among each sample. Two distinct main groups are observed in these data and correspond to the control and kinetin differentially separated from 2,4-D and the combination of both hormones (forming the largest cluster).

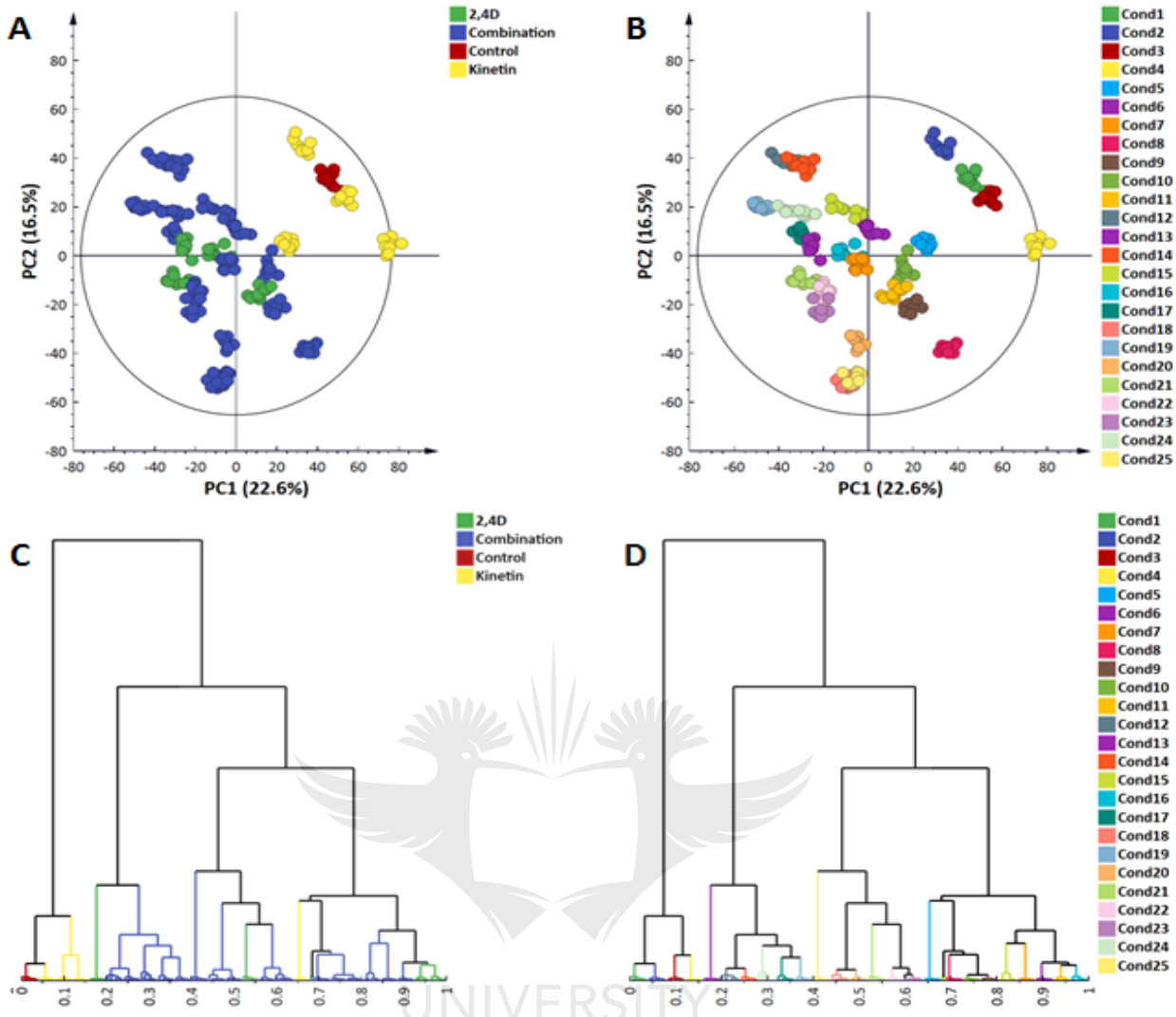

Figure 4.9: Unsupervised MVDA modelling methods. (**A**) and (**B**): Principal component analyses (PCA) showing the different grouping and separation of *M. oleifera* samples treated in triplicate with different concentration of hormones (**Table 1**) and analyzed by UHPLC-MS in triplicate in ESI neg mode. The SIMCA-14 software was used for multivariate statistical analysis. The score plot generated was coloured according to the group (**A**) and according to conditions (**B**). The model calculated 17 components and yielded $R^2X_{(cum)}$ = 73.4% and $Q_{(cum)}$ = 57.2%. (**C**) and (**D**): HCA dendograms applied on PCA (**A**) and (**B**) respectively.

The descriptive exploration of data was performed using OPLS-DA and biomarkers corresponding to each condition was extracted (**Table 4.3**). Due to the number of conditions, only examples of an OPLS-DA are shown here (**Figure 4.10A** and **C:** control *vs.* kinetin treatments, and **Figure 4.11A** and **C:** control *vs.* 2,4-D treatments). All conditions showed a similar trend whereby the control was separated from all the treatments. OPLS-DA models were validated using the receiver operator characteristic (ROC) plot (**Figure 4.10B**). The ROC plot summarizes the performance of the OPLS-DA. When the model has a

perfect discrimination, the ROC curve shows 100% specificity and sensitivity (Tugizimana *et al.,* 2017). The statistical significance and discriminability of biomarkers selected from the S-plots (features or variables) were assessed with the variable importance in projection (VIP) plot. The selected features with VIP scores > 1.0 were considered as significant in contributing to the class separation (Pavlovic *et al.,* 2011; Tugizimana *et al.,* 2016). The selected features can, therefore, be considered as important phytochemicals explaining the metabolic changes occurring in *M. oleifera* callus grown in the presence of different ratios of auxin and cytokinin.

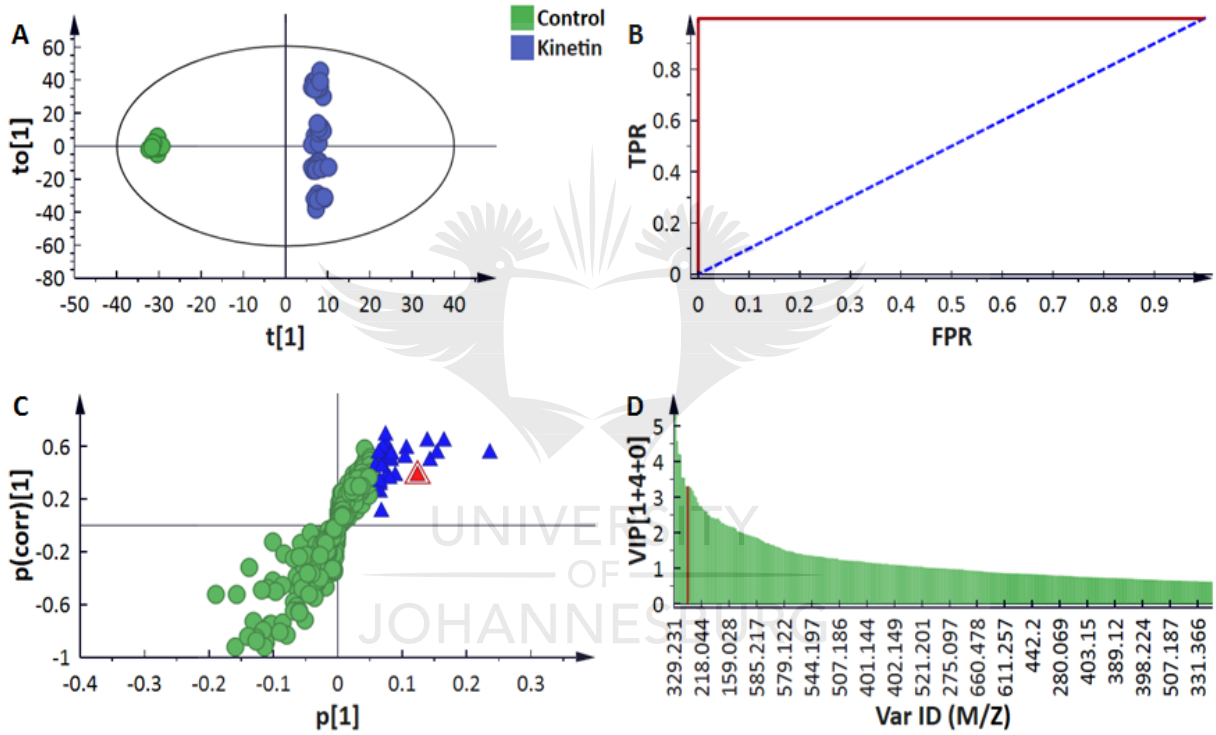

Figure 4.10: OPLS-DA modelling, variable selection and validation. The SIMCA-14 software was used for multivariate statistical analysis. (**A**): OPLS-DA score plots comparing the control against cytokinin-treated samples and showing clear separation between groups. (**B**): A typical receiver operating characteristic (ROC) curve for OPLS-DA modelling validation. (**C**): OPLS-DA loading S-plot of the same model as (**A**) showing the selected discriminant ions in blue and red, representing statistically relevant variable/features. (**D**): Variable importance in projection (VIP) plot for the same model as (**A**) and (**B**), highlighting the mathematical importance of each variable in differentiating between groups in the OPLS-DA. The variable highlighted in red (Rt = 2.91 min, *m/z* = 203.080) is also represented on the VIP plot in red and is indeed a significant feature as its score is greater than 1.

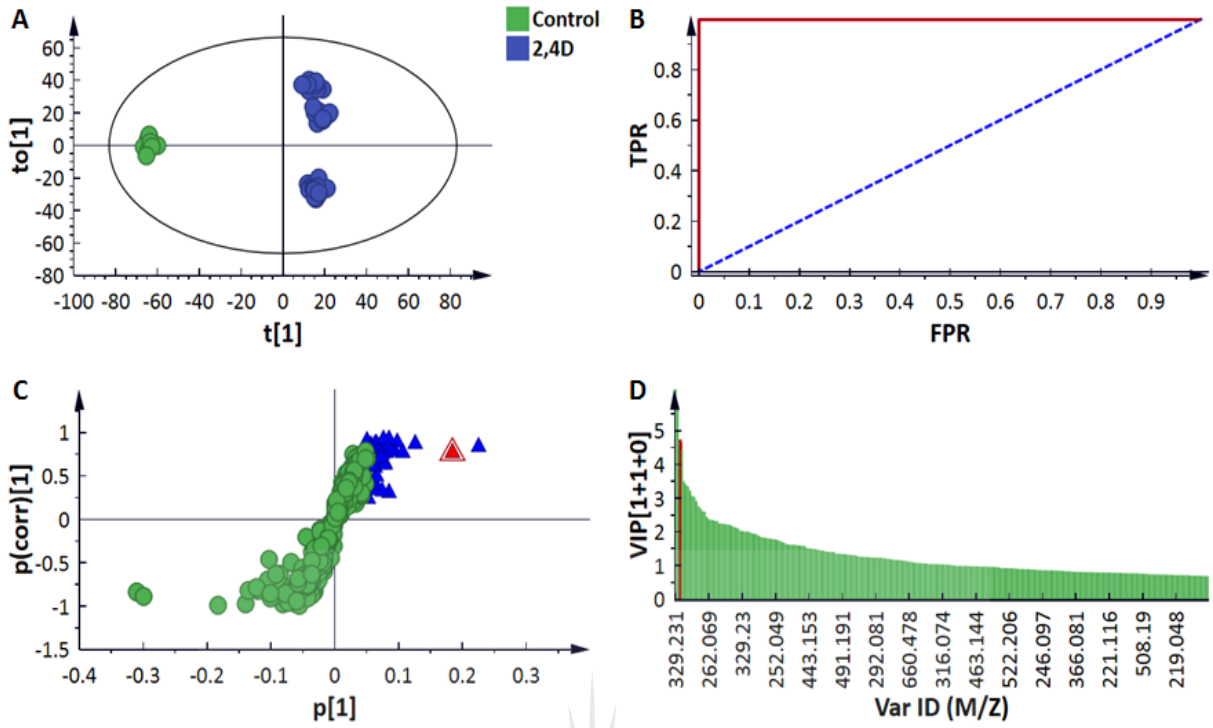

Figure 4.11: OPLS-DA modelling, variable selection and validation. The SIMCA-14 software was used for multivariate statistical analysis. (**A**): OPLS-DA score plots comparing control against auxin-treated samples and showing clear separation between groups. (**B**): A typical receiver operating characteristic (ROC) curve for OPLS-DA modelling validation. (**C**): OPLS-DA loading S-plot of the same model as (**A**) showing the selected discriminant ions in blue and red, representing statistically relevant variable/features. (**D**): Variable importance in projection (VIP) plot for the same model as (**A**) and (**B**), highlighting the mathematical importance of each variable in differentiating between groups in the OPLS-DA. The variable highlighted in red (Rt = 4.32 min, *m/z* = 278.063) is also represented on the VIP plot in red and is indeed a significant feature as its score is greater than 1.

4.3.4 Metabolite annotation

Plant metabolite identification (the last step in a metabolomic study) is a very crucial step since the biochemical interpretation of the data relies thereon. The identification of metabolites can be putative (using accurate mass and mass spectral fragmentation patterns) or definitive (using more than two molecular properties, authentic standard and *in vivo* labelling methods for confirmation) (Tugizimana *et al.,* 2013). In *M. oleifera*, a large variety of metabolites has been identified in leaves, flowers and seeds (Leone *et al.,* 2015; Rodríguez-Pérez *et al.,* 2015; Makita *et al.,* 2016; Ramabulana *et al.,* 2016; Vats & Gupta 2017; Hamany Djande *et al.,* 2018). The compounds are phenolics and flavonoids (Makita *et al.,* 2016; Nouman *et al.,* 2016; Hamany Djande *et al.,* 2018), glucosinolates (glucomoringin)

(Doerr *et al.,* 2009; Ramabulana *et al.,* 2017), terpenes and alkaloids (Furo & Ambali 2012; Saini *et al.,* 2016).

Generally, the main aim of a metabolomic study is the non-targeted identification of all metabolites produced in a specific biological system (Bhalla *et al.,* 2005). LC-MS is a technique that has widely been used in metabolomic studies for analysis of secondary metabolites. With the introduction of analytical platforms such as UHPLC-ESI-MS, this technique has become a method of choice for metabolomic approaches (Madala *et al.,* 2012). Although several studies have been reported for *M. oleifera* leaves, very little is known regarding the metabolite profiles of callus culture. In this study an UHPLC-MS platform was employed to analyze the metabolic profile(s) of *M. oleifera* cell cultures after differential treatment with the plant growth hormones, cytokinin and kinetin. The untargeted metabolite profiling of the *M. oleifera* callus revealed classes of secondary metabolites (derivatives of hydroxybenzoic acids and hydroxycinnamic acids) and primary metabolites (organic acids, amino acids, and fatty acids) (**Table 4.3**).

Table 4.3: Metabolites identified from methanolic extracts of *Moringa oleifera* callus treated with auxin (2,4-D) and cytokinin (kinetin) alone, and in combination.

Com-pound	Name	Empirical Formula	R_t	MW	Precursor ion *m/z*	MS Fragments	Class	Conditions*	Databases and references
Secondary metabolites / Phenolic compounds									
A	Caffeoyl hexose	$C_{15}H_{18}O_9$	4.79	342	341.086	179, 135	Cinnamic acid derivative	5, 6, 7, 8, 9, 10, 11, 14, 15, 16, 17, 18, 19, 20, 21, 22	DNP, Fusani *et al.*, 2016
B	Caffeoyl dihexose	$C_{21}H_{28}O_{14}$	3.76	504	503.139	341, 179, 135	Cinnamic acid derivative	9, 10, 11, 18, 19, 20, 21, 22, 23, 24, 25	DNP
C	Coumaroyl aspartate/Hydroxycinnamoyl aspartate	$C_{13}H_{13}NO_6$	4.32	279	278.063	163, 132	Cinnamic acid derivative	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25	DNP, Pereira-caro *et al.*, 2013
D	Coumaroyl hexose	$C_{15}H_{18}O_8$	4.88	326	325.091	163, 119	Cinnamic acid derivative	5, 6, 10, 12, 17, 18, 19, 20, 21, 22, 23, 24, 25	DNP
E	Protocatechuic acid hexose/ 3,4 dihydroxybenzoic acid	$C_{13}H_{16}O_9$	1.99	316	315.067	152, 153	Hydroxybenzoic acid derivative	4, 8, 9, 10, 11	Morreel *et al.*, 2014
F	Benzylalcohol-hexose-pentose isomer I	$C_{18}H_{26}O_{10}$	5.66	402	401.147	269, 161, 101	Benzoic acid intermediate	#	DNP
G	Benzylalcohol-hexose-pentose isomer II	$C_{18}H_{26}O_{10}$	5.92	402	401.140	269, 161, 101	Benzoic acid intermediate	1	DNP

H	Benzylalcohol-hexose-pentose isomer III	$C_{18}H_{26}O_{10}$	6.13	402	447.144	269, 161, 101	Benzoic acid intermediate	1	DNP
Primary metabolites									
I	Phenylalanine	$C_9H_{11}NO_2$	1.80	165	164.069	147	Amino acid	2, 3	ChemSpider, Rodriguez-Perez *et al.*, 2015
J	Tryptophan conjugate	-	2.88	275	274.091	159/158, 142, 116	Amino acid conjugate	3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 18, 20, 23, 25	In-house
K	Tryptophan	$C_{11}H_{12}N_2O_2$	2.91	204	203.080	159/158, 142, 116	Amino acid	3, 4, 5, 25	Rodriguez-Perez *et al.*, 2015
L	Citric acid/ Isocitric acid	$C_6H_8O_7$	0.92	192	191.016	173, 111	Organic acids	#	DNP, Chem-Spider Rodriguez-Perez *et al.*, 2015
M	Citric acid/ Isocitric acid	$C_6H_8O_7$	1.12	192	191.016	173, 111	Organic acids	#	DNP, Chem-Spider , Rodriguez-Perez *et al.*, 2015
N	Butanyol hexose pentose	$C_{15}H_{26}O_{11}$	8.51	382	381.171	249, 160/161, 101/100	Sugar derivative	2, 5, 6, 7, 12, 14, 15, 17, 19, 21, 22, 24	DNP
O	Trihydroxyoctadecadienoic acid isomer I	$C_{18}H_{32}O_5$	23.24	328	327.212	171	Fatty acid	#	DNP, Chem-Spider Rodriguez-Perez *et al.*, 2015

P	Trihydroxyoctadecadienoic acid isomer II	$C_{18}H_{32}O_5$	23.34	328	327.215	229, 211, 171	Fatty acid	3, 4	DNP, Chem-Spider, Rodriguez-Perez *et al.*, 2015
Q	Trihydroxyoctadecenoic acid isomer I	$C_{18}H_{34}O_5$	23.43	330	329.230	211, 171	Fatty acid	#	DNP, Chem-Spider
R	Trihydroxyoctadecadienoic acid isomer III	$C_{18}H_{32}O_5$	23.48	328	327.219	171	Fatty acid	#	DNP, Chem-Spider, Rodriguez-Perez *et al.*, 2015
S	Trihydroxyoctadecenoic acid isomer II	$C_{18}H_{34}O_5$	24.03	330	329.234	211, 171	Fatty acid	3, 4	DNP, Chem-Spider
T	Trihydroxyoctadecadienoic acid isomer IV	$C_{18}H_{32}O_5$	24.7	328	327.215	171	Fatty acid	2	DNP, Chem-Spider, Rodriguez-Perez *et al.*, 2015

*Conditions in which compounds were selected as biomarkers (numbered according to Table 4.1).
# Compounds identified during profiling (not selected as biomarkers).
DNP = Dictionary of Natural Products; web address: dnp.chemnetbase.com
Chemspider = A database of chemicals, owned by the Royal Society of Chemistry, web address: www.chemspider.com

4.3.4.1 Secondary metabolites: Phenolic compounds and derivatives

A total of six phenolic compounds (four hydroxycinnamic acid derivatives, one hydroxybenzoic acid and a benzoic acid intermediate) were annotated from *M. oleifera* callus. These were identified as biomarkers for conditions indicated in **Table 4.3**.

A precursor ion of *m/z* 341 ($[M-H]^-$, Rt = 4.79 min) exhibited MS fragment ions of *m/z* 179 corresponding to the caffeic acid resulting from the loss of a hexose moiety, and 135 corresponding to a decarboxylated caffeic acid. The compound was annotated as caffeoyl hexose based on the fragmentation pattern. The molecule was present as biomarker mostly in samples treated with 2,4-D alone (conditions **6**, **11**, **16**, **21**) and conditions containing a mixture of both 2,4-D and kinetin. A similar molecule was detected at Rt = 3.76 min with *m/z* 503 $[M-H]^-$, displaying fragments of *m/z* 341 for caffeoyl-hexose due to the loss of another molecule of hexose (162), 179 for caffeic acid and 135 for caffeoyl moiety without CO_2. The latter was characterized as caffeoyl-dihexose (Chen *et al.,* 2012; Carrillo-lopez & Yahia 2013; Ncube *et al.,* 2014; Fusani *et al.,* 2016). The up-regulation of this compound was observed in callus grown on 2,4-D alone and in combination with kinetin. A molecule with a precursor ion *m/z* 278 $[M-H]^-$ was detected at Rt 4.32 min and produced a fragment ion of *m/z* 163 corresponding to a coumaric acid residue and an intense fragment of *m/z* 132 corresponding to an aspartic acid moiety. The compound was characterized as coumaroyl aspartate (Mock *et al.,* 1993; Keller *et al.*, 1996; Pereira-Caro *et al.,* 2013) and was found to be up-regulated in all conditions in comparison to the control. The last hydroxycinnamic acid derivative with *m/z* 325 was observed at Rt 4.88 exhibiting daughter ions of *m/z* 163 (coumaric acid) due to the loss of a sugar moiety and 119 for decarboxylated coumaric acid (Bystrom *et al.,* 2011; Carrillo-Lopez & Yahia, 2013). Based on the fragmentation pattern, the compound was identified as coumaroyl hexose, and highlighted as a discriminant ion in conditions containing the highest concentration kinetin with increasing concentration of 2,4-D (conditions **5**, **10**, **20** and **25**).

Hydroxycinnamic acid derivatives are diversely distributed phytochemicals and constitute the main class of the phenylpropanoid metabolites found in nature (Macoy *et al.,* 2015). Caffeoyl-hexose has been reported in *Nicotiana tabacum* cell suspensions (Mudau, 2017)

and coumaroyl aspartate in cacao beans (Pereira-Caro *et al.,* 2013), *Arabidopsis thaliana* (Forschung *et al.,* 1993) and *Solanum tuberosum* (Keller *et al.,* 1996) cell suspension cultures. Coumaroyl hexose has been been detected in the pulp and seed extracts of *Melicoccus bijugatus* (Bystrom *et al.,* 2011). Hydroxycinnamic acids are well-known as inhibitors of lipid peroxidation and radical scavengers, and play a role in plant defense against insects and pathogens (Stojakowska *et al.,* 2016).

A molecule with a precursor ion *m/z* 315.067 $[M-H]^-$ was detected at Rt 1.99 min. The MS fragmentation pattern exhibited ions of *m/z* 153 corresponding to the protocatechuic acid ion resulting from the neutral loss of hexose (162) and *m/z* 109 for decarboxyated protocatechuic acid. The molecule was tentatively annotated as protocatechuic acid (3,4 dihydroxybenzoic acid)-hexose and identified as a significant ion in conditions **4**, **8**, **9**, **10**, **11** (Chen *et al.,* 2012; Morreel *et al.,* 2014). This hydroxybenzoic acid has been reported in many plants such as olive, onion (Semaming *et al.,* 2015), bayberries (Fang *et al.,* 2007), orange, apricot (Khoddami *et al.,* 2013) and tobacco (Mudau, 2017). Protocatechuic acid is linked to plant defenses and known to display anti-microbial (Semaming *et al.,* 2015), anti-apoptotic, anti-oxidant (Liu *et al.,* 2008) and anti-fungal (Khadem & Marles, 2010) activities.
In the same category can also be added the following compound with a precursor ion *m/z* 401 $[M-H]^-$ which eluted at the Rt = 5.66 min. The daughter ions were *m/z* 269 resulting from a neutral loss of a pentose moiety and *m/z* 161 due to the loss of both pentose and hexose moieties. The compound was annotated as benzyl alcohol hexose-pentose, and two isomers were also identified at Rt 5.92 (isomer II) and 6.13 min (isomer III) (Bystrom *et al.,* 2011; Barros *et al.,* 2012; Victoria *et al.,* 2014; Karar & Kuhnert, 2015). Benzyl alcohol hexose-pentose isomers II and III were found to be down-regulated in all conditions as compared to the control. The isomer I (Rt = 5.66 min) was not considered as a biomarker but was identified in extracts. Benzyl alcohols are benzoic acid precursors or intermediates for the synthesis of essential molecules (Widhalm & Dudareva, 2015) and have been reported in tomato (Barros *et al.,* 2012; Victoria *et al.,* 2014), Spanish lime (Bystrom *et al.,* 2011), avocado (Hurtado-Fernández *et al.,* 2011) and tobacco (Mudau, 2017) extracts. These are volatile organic compounds (VOCs) playing a role in defenses of Solanum species, *e.g.* exhibiting anti-microbial activities.

4.3.4.2 Primary metabolites

In this study, two amino acids were identified in *M. oleifera* callus. Phenylalanine (Phe) and tryptophan (Trp), with precursor ions of *m/z* 164 and 203 respectively, were characterized as previously described by Rodriguez-Perez *et al.,* (2015) in *M. oleifera* leaves. Phe and Trp were more prominent in extracts from callus grown in presence of kinetin (**2**, **3, 4**, **5**) and in highest concentrations of both kinetin and 2,4-D (**25**). Phe and Trp are aromatic amino acids, essential compounds in plants metabolism and play a role as precursors for secondary metabolite production (**Figure 4.13**). While it is possible that the aromatic amino acids (Trp) might have played a role to the determined DPPH scavenging activity, the reprogrammed cellular metabolome could have also contributed to the antioxidant properties observed.

Two compounds corresponding to the citric acid / isocitric acid isomers (*m/z* 191) eluted at Rt = 0.92 and 1.12 min, giving fragments of *m/z* 173 corresponding to the mass of a dehydrated citric/isocitric acid and *m/z* 111 resulting from the decarboxylation of dehydrated citric or isocitric acid (Rodríguez-Pérez *et al.,* 2015; Masike *et al.,* 2017). The compound was not identified as a biomarker and was detected in all conditions. Citric acid is one of the tricarboxylic acid cycle intermediates that provide cellular energy and carbon skeleton utilization by biochemical pathways (Hu *et al.,* 2016).

In the same group of organic compounds, a disaccharide was annotated with the help of the Dictionary of Natural Products and the fragmentation pattern of the molecule. The precursor ion *m/z* 381 produced fragment ions of *m/z* 249 resulting from the loss of a pentose (132), *m/z* 161 subsequent to the loss of a pentose and two carbons dioxides, *m/z* 101. The molecule was putatively annotated as butanoyl hexose pentose and extracted from OPLS-DA as a biomarker for conditions indicated in the **Table 4.3**. Lastly, four isomers of trihydroxyoctadecadienoic (*m/z* 327) were detected at Rts = 23.24, 23.34, 23.48 and 24.7 min respectively. The fragment ions *m/z* 211 and 229 resulting from the cleavage of C_{12} and C_{13} bonds and a subsequent loss of water were characteristic of the compounds (Rodríguez-Pérez *et al.,* 2015; Bao *et al.,* 2018). Similarly to this compound, two isomers of trihydroxyoctadecenoic acid (*m/z* 329) were also identified with similar fragments at Rts = 23.43 and 24.03 min.

Auxin and cytokinin treatment of *M. oleifera* callus resulted in a differential distribution of the identified compounds among samples. The occurrence of selected biomarkers between samples was highlighted using colour-coded PCAs score plots (**Figure 4.12**). As seen in the figure, coumaroyl aspartate and caffeoyl dihexose (**Figure 4.12B** and **C**) are more prominent in the extracts from callus grown on 2,4-D and combinations thereof. By comparison, protocatechuic hexose and benzyl alcohol hexose-pentose (**Figure 4.12D** and **4.12E**) were more intense in extracts from callus grown where kinetin was the dominant hormone.

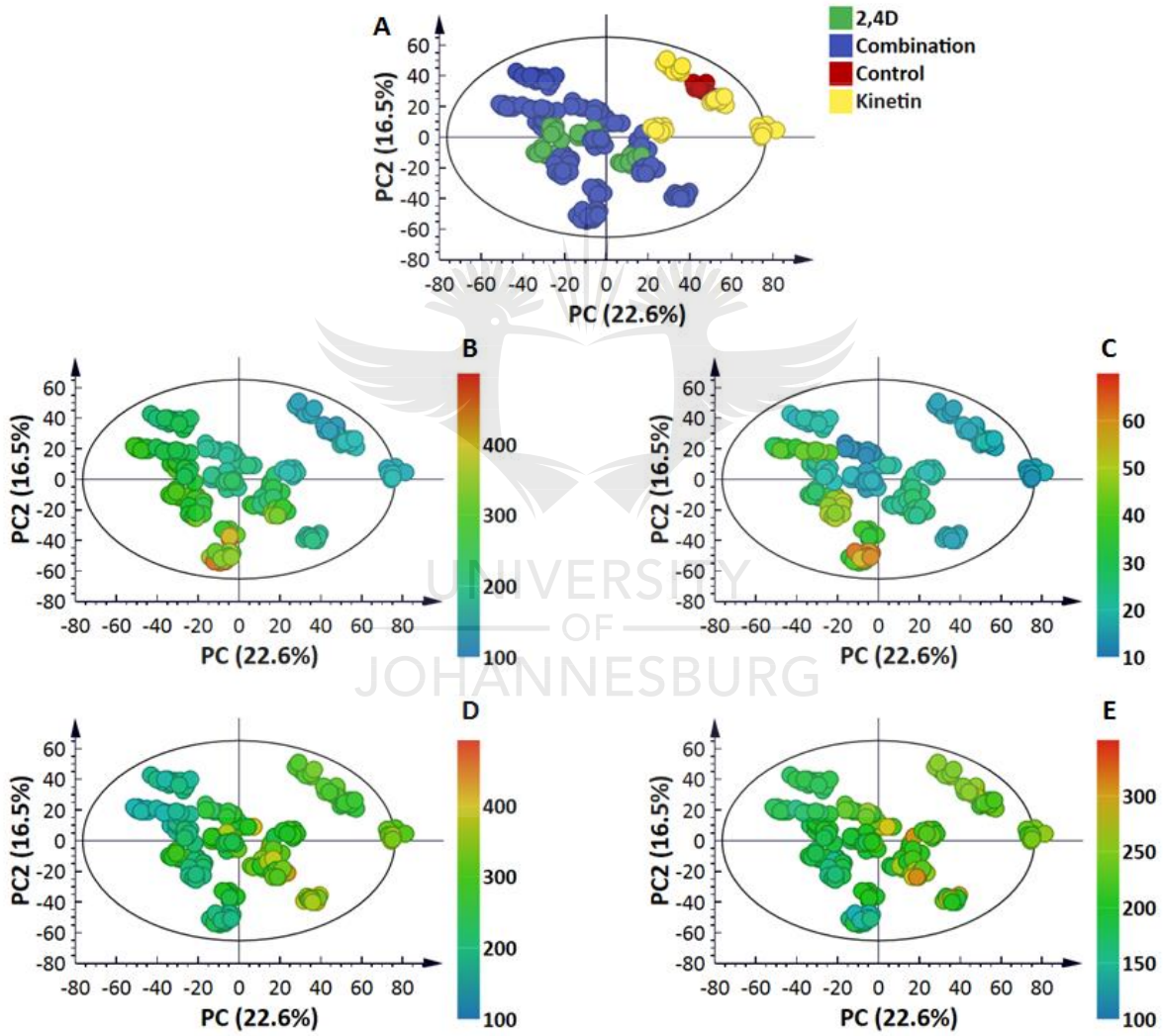

Figure 4.12: Coloured-coded principal component analysis (PCA) score plots showing the occurrence of selected compounds among *Moringa oleifera* callus treated with different concentrations of hormones **(Table 4.1)** and analyzed by UHPLC-MS in ESI neg mode. **(A):** All conditions coloured according to the group; **(B):** Coumaroyl aspartate; **(C):** Caffeoyl dihexose; **(D):** Protocatechuic acid hexose and **E:** Benzyl alcohol isomer III.

4.3.5 Comparison of metabolite profiles of differentiated and undifferentiated tissue of *Moringa oleifera*

In our recent study (Hamany Djande *et al.,* 2018), *M. oleifera* leaf tissue was extracted with different green extraction solvents in comparison to methanol that was used for the metabolite extraction of the callus grown on different concentrations of hormones (**Chapter 3**). Using an UHPLC-MS-based analytical platform, each extract revealed the presence of glucosinolates (glucomoringin), phenolics and – conjugates such as chlorogenic acid (CGA) and CGA derivatives; and flavonoids (quercetin, kaempferol, isorhamnetin and apigenin attached to one or more sugar moieties). Surprisingly, glucosinolates, CGA and flavonoids were all not detected in the callus initiated from *M. oleifera*. Metabolites associated with the early steps of the diverging phenylpropanoid pathway were however detected, *e.g.* hydroxyl benzoic acid derivatives and hydroxylated cinnamic acid derivatives conjugated to either one or two sugars moieties. Interestingly, coumaroyl aspartate and protocatechuic acid hexose were identified in callus and have not been reported in *M. oleifera* leaves thus far (Rodríguez-Pérez *et al.,* 2015). This shows that for secondary metabolite production, *M. oleifera* differentiated leaf tissue and undifferentiated callus share similar pathways, but that the sub-pathways leading to CGAs and flavonoids were repressed in callus, leading to dramatically different metabolite profiles.

The shikimate/chorismate pathway leads to the production of secondary metabolites, and it is clear that enzymes involved to the formation of the amino acids Trp and Phe (precursors of secondary metabolites) are activated in both differentiated leaf tissue and undifferentiated callus. The production of coumaroyl glycoside and caffeoyl hexose can either occur through the formation of coumaric - and caffeic acids respectively (involving enzymes such as cinnamate 4-hydroxylase (C4H)) or the conversion of *trans*-cinnamic acid to cinnamoyl glucose (involving cinnamate glucosyl tranferase). Coumaroyl-aspartate on the other hand, results from the conjugation of coumaric acid and aspartic acid. The pathway leading to the formation of CGAs and flavonoids, *via* the conversion of coumaric acid to coumaroyl-CoA with the enzyme 4-coumarate-CoA ligase (4CL), seems to be repressed in undifferentiated callus tissue as none of these metabolites was found in *M. oleifera* callus. In addition, the committing gene responsible of the biosynthesis of Phe glucosinolates (the

cytochrome P450, *CYP79A2*) in leaves, is most likely inactivated in callus cultures, resulting in the absence of glucomoringin (**Figure 4.13A** and **B**) (Tzin & Galili 2010a; Tzin & Galili 2010b; Vogt 2010; Morreel *et al.,* 2014; Widhalm & Dudareva 2015).

There are limited (or not at all) reports on metabolite profiling of callus cultures. However, similar studies on undifferentiated cell suspension cultures show similar results. For instance, non-habituated *Centella asiatica* (Ncube, 2016) and *Nicotiana tabacum* (Ncube *et al.,* 2014) cell suspensions exhibited different secondary metabolite distribution profiles compared to differentiated leaf tissues. Another example is that of intact beetroot plants and hairy root cultures showing differences in metabolite production (Georgiev *et al.,* 2010). Although the habituation phenomenon explained earlier (**Section 4.3.1**) can be responsible of the differential metabolite profiling observed in *M. oleifera* callus, the evidence behind this assumption is not established. On the contrary, it was demonstrated in *Catharanthus roseus* that habituated cells produce higher amounts of alkaloids in comparison to non-habituated cells (Merillon *et al.,* 1989).

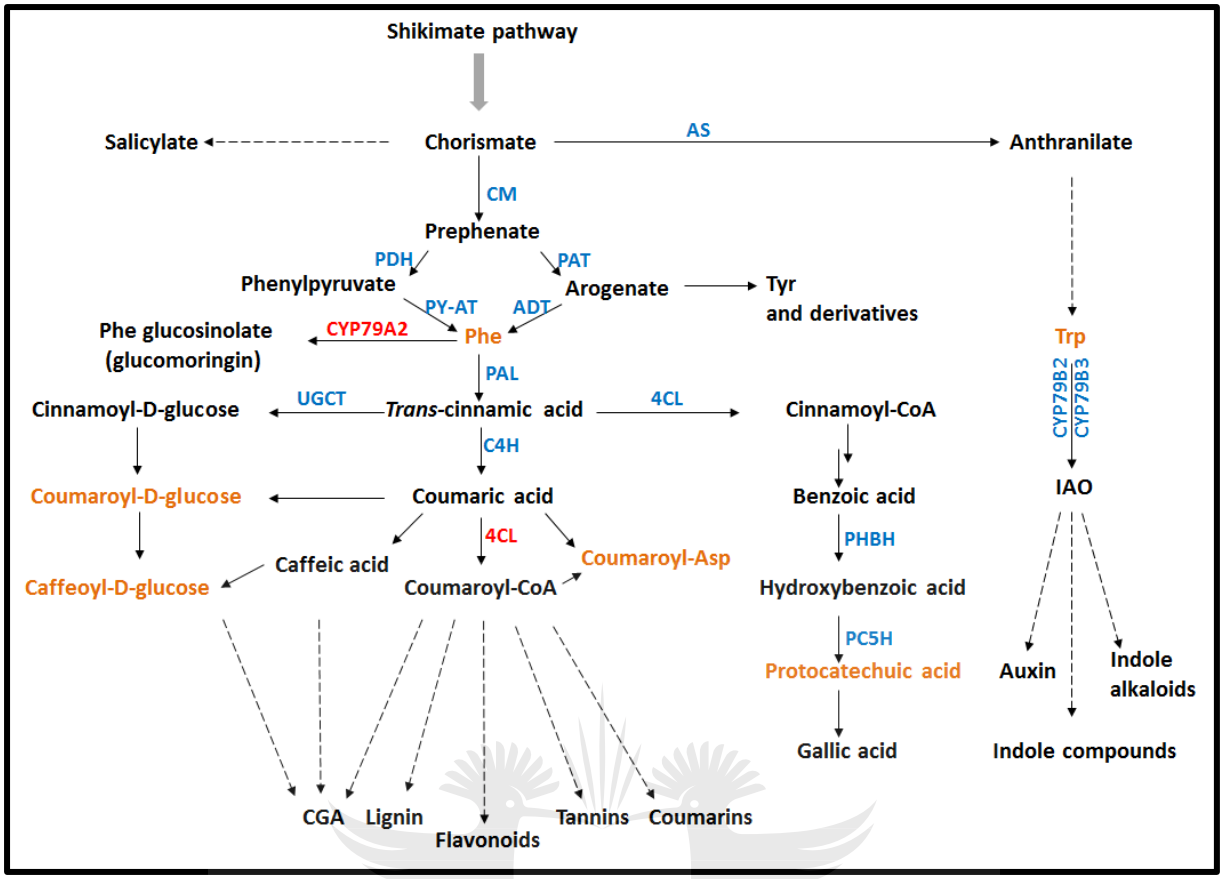

Figure 4.13A: Possible routes taken by *Moringa oleifera* leaf tissue and callus for the production of secondary metabolites. The pathway initiates with the formation of chorismate from the shikimate pathway. The arrows indicate reactions leading to the formation of metabolites. The dashed arrows designate multiple reactions before the formation of a class of compounds. Enzymes involved are: **AS**, anthranilate synthase; **CM**, chorismate mutase; **PAT**, prephenate aminotransferase; **PDH**, prephenate dehydrogenase; **ADT**, arogenate dehydratase; **PY-AT**, phenylpyruvate amino transferase; **PAL**, phenylalanine ammonia-lyase; **UGCT**, UDP glucose cinnamate glycosyl transferase; **4CL**, 4-hydroxycinnamoyl-CoA ligase; **PHBH**, p-hydroxybenzoic acid 3-hydroxylase; **PC5H**, protocatechuic acid 5-hydroxylase; **C4H**, cinnamoyl hydroxylase. Enzymes or genes in red are those that might be inactivated in callus cultures, causing the apparent lack of CGAs, flavonoids and glucosinolates.

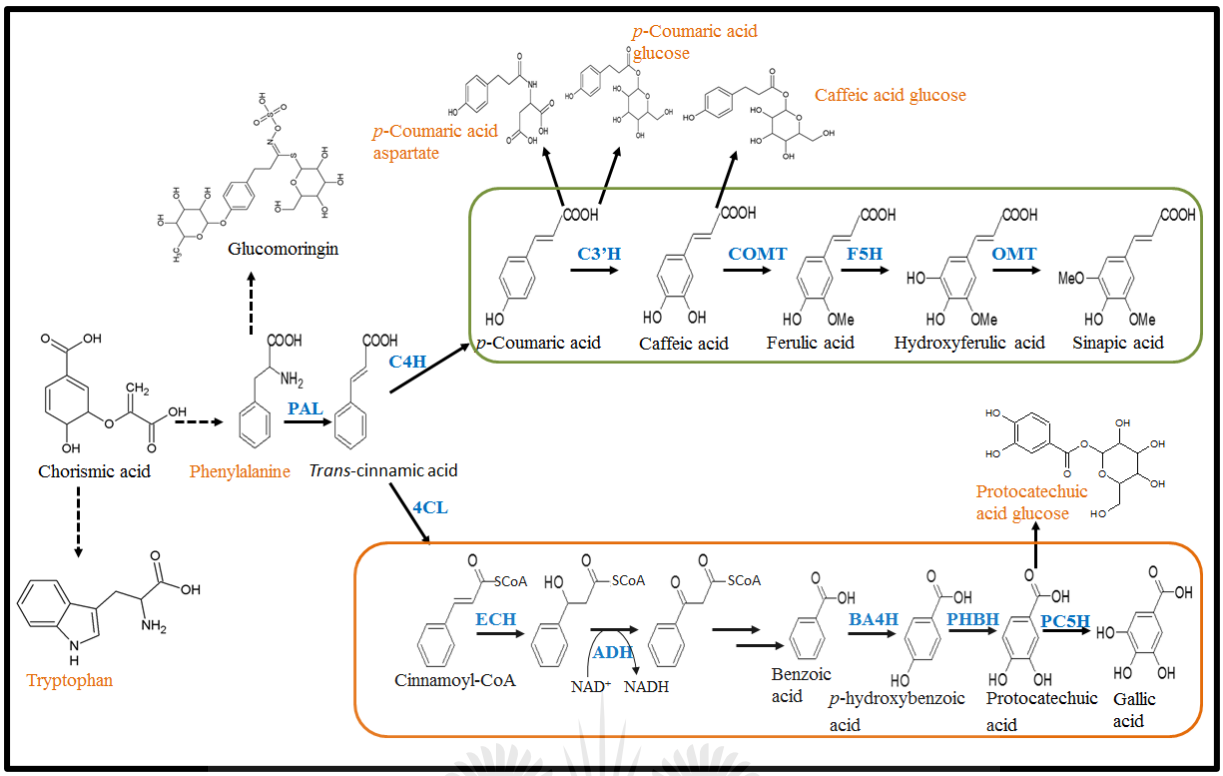

Figure 4.13B: Possible routes taken by *Moringa oleifera* for the production of secondary metabolites identified in callus tissues exposed to differing concentrations and - ratios of 2,4-D and kinetin. Names of selected biomarkers identified in the study are in brown text and abbreviated names of enzymes in blue. The pathway initiates with the formation of chorismate from the shikimate pathway. The arrows indicate reactions leading to the formation of metabolites. The dashed arrows designate multiple reactions before the formation of a class of compounds. Enzymes involved are: PAL, phenylalanine ammonia-lyase; C4H, cinnamoyl hydroxylase; C3'H, *p*-coumarate 3'-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; F5H, ferulate 5-hydroxylase; OMT, *O*-methyltransferase; 4CL, 4-hydroxycinnamoyl-CoA ligase; ECH, enoyl-CoA hydratase; ADH, cinnamoyl alcohol dehydrogenase; BA4H, benzoic acid 4-hydroxylase; PHBH, *p*-hydroxybenzoic acid 3-hydroxylase; PC5H, protocatechuic acid 5-hydroxylase.

4.4 Conclusion

This work aimed to investigate the effects of auxin (2,4-D) and cytokinin (kinetin) on the ability of undifferentiated *M. oleifera* callus cells to regenerate; and to screen and study the metabolite distribution resulting from the hormonal change in phytohormones concentrations and combination. However, following treatment with different auxin/cytokinin ratios to initiate organogenesis, no redifferentiation of roots or shoots was observed. This was probably due to the habituation phenomenon observed. *M. oleifera* calli were found to be a good source of phenolic and anti-oxidant compounds. Furthermore, metabolomic profiling of callus extracts grown on different ratios of auxin to cytokinin

revealed that the habituated cells did retain the ability to respond to external hormonal manipulation. Here, the apparent total absence of CGAs, flavonoids and glucosinolates were observed, against a background of differential accumulation of phenolic - and anti-oxidant compounds (**Figure 4.13B**). Auxins/cytokinin ratios are indeed important signals in the production of cell secondary metabolites. The phytochemical diversity and - complexity of *M. oleifera* systems could also be attributed to the genetic variation therein, and the suppression or activation of the gene expression encoding for enzymes involved in the biosynthesis of the secondary metabolites.

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Chapter 5: Metabolism of exogenously supplemented methyl jasmonate by cell suspension cultures of *Moringa oleifera*

Abstract

Jasmonic acid (JA) and its derivatives play a crucial role in plant defense signaling. It has previously been demonstrated that exogenous application of the methyl ester of JA, methyl jasmonate (MeJA), results in activation of defense-related genes and subsequent production of secondary metabolites. This implies the biotransformation of the hormone into a more active form. In the current study, *Moringa oleifera* cell suspension cultures were elicited with different concentrations of MeJA (100, 200 and 300 µM). Methanolic extracts prepared from elicited cells were analyzed with reverse phase ultra-high performance liquid chromatography (UHPLC) coupled to a quadrupole time-of-flight high definition mass spectrometer (QTOF HDMS) for jasmonate profiling. In addition to MeJA and JA, three jasmonoyl-amino acids were annotated: jasmonoylvaline (JA-Val), jasmonoylisoleucine/leucine (JA-Ile/Leu) and jasmonoylphenylalanine (JA-Phe), with characteristic fragment ions of *m/z* 116, 130, and 164 respectively. Furthermore, JA conjugated to a hexose was observed, as well as hydroxylated and carboxylated amino acid derivatives. The demethylation of MeJA and the concurrent formation of the amino acid conjugates point to active metabolism of the externally added MeJA through biotransformation and bioconversion reactions. As such, the study provides insights into the understanding of JA metabolism as a stress-related response in *M. oleifera.*

Keywords: JA, JA derivatives, MeJA, *Moringa oleifera*

5.1 Introduction

In general, the survival of a plant depends on the ability to rapidly perceive and respond to biotic and abiotic stresses by inducing defense-related molecules (subsequently secondary

metabolites) through signal transduction (Okada *et al.,* 2015; Shi *et al.,* 2016). Plant signaling molecules such as salicylic acid (SA), ethylene (ET), abscisic acid (ABA) and jasmonic acid (JA) are known to play an essential role in mediating external stresses (Ruiz-may *et al.,* 2011; Denancé *et al.,* 2013). Among the plant hormones, JA and its derivatives (*i.e.* methyl jasmonate and jasmonoyl-amino acids), collectively designated as jasmonates, are important plant regulators, and also involved in diverse processes during plant growth and development (Koornneef & Pieterse 2008; Schaller & Stintzi, 2009; Yan *et al.,* 2013). Stress stimuli such as wounding, pathogen attack, UV radiation and drought can lead to bioactive JA production in plants and hence result in the activation of processes/phenotypes modulated by JA (Goossens *et al*., 2017). The effect of JA and MeJA on gene expression (and subsequently on plant phenotypes) is indicated in different reviews (Mueller-Uri *et al*., 1988; Acosta & Farmer, 2010; James *et al.,* 2013; Tugizimana *et al.,* 2015; Mhlongo *et al*., 2016a; Huang *et al*., 2017; Howe *et al*., 2018; Wasternack & Feussner, 2018;).

Jasmonates are fatty acid-derived compounds characterized by a cyclopentanone structure (Schaller *et al.,* 2004; Wasternack, 2009; Wasternack & Song, 2017). The molecules can be synthesized through the linolenic acid (18:3) and hexadecatrienoic acid (16:3). The synthesis initiates in the chloroplast with the oxygenation of linolenic and hexadecatrienoic acids by the enzyme lipoxygenase (LOX), producing 13 (S)-hydroxyperoxy linolenic acid (13-HPOT) and 11 (S)-hydroxyperoxy linoleic acid (11-HPHT). After dehydration of 13-HPOT by a cytochrome P450 enzyme, allene oxide synthase (AOS), followed by cyclization by allene oxide cyclase (AOC), the precursor of jasmonates, 12-oxo-phytodienoicacid (OPDA, in the linolenic acid pathway) and dinor-oxo-photodienoic acid (dnOPDA, in the hexadacatrienoic acid pathway) are formed and transported to the peroxisome where JA is synthesized. The synthesis occurs after reduction of OPDA by the enzyme OPDA reductase 3 (OPR3) and three cycles of β-oxidation by the enzymes acyl-CoA oxidase 1 (ACX1), multifunctional protein (MFP) and L-3-ketoacyl CoA-thiolase (KAT) (Woldemariam *et al.,* 2012; Okada *et al.,* 2015; Wasternack and Song, 2017) **(Figure 5.1)**. JA is then transported into the cytoplasm to undergo modifications such as conjugation to amino acids (JA-Isoleucine) and esterification (MeJA) to fulfil specific biological functions (Schaller *et al.,* 2004; Acosta & Farmer, 2010; Yan *et al.,* 2013).

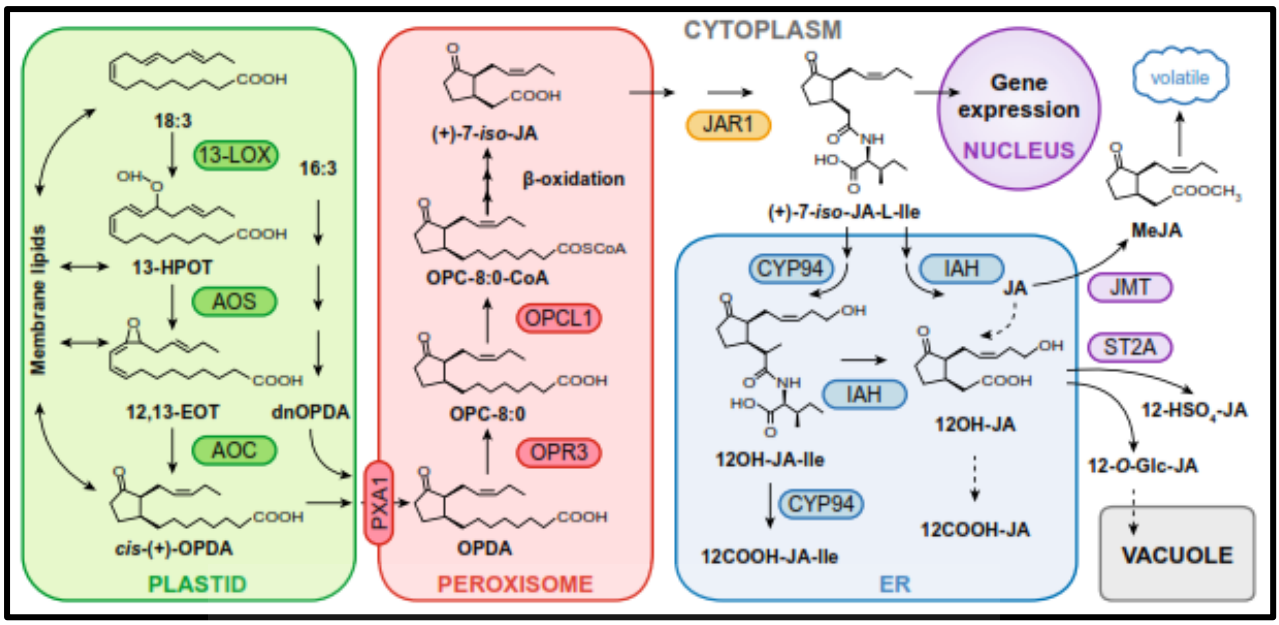

Figure 5.1: Major JA metabolic pathways. LOX: lipoxygenase; AOS: allene oxide synthase; AOC: allene oxide cyclase; PXA1: ATP-binding cassette transporter; OPR3: OPDA reductase 3; OPCL1: OPC- 8:0 Coenzyme a ligase1, JAR1: Jasmonate resistant 1; CYP94: cytochrome P450 (CYP) 94 family proteins, IAH: ILR1-like amidohydrolase; JMT: JA carboxyl methyltransferase; ST2A: hydroxyjasmonate sulfotransferase. ER: Endoplasmic reticulum. Solid arrow lines indicate the confirmed reactions and dashed arrow lines, hypothetical reactions without evidence (Koo, 2018).

The ability of the two hormones to cross biological membranes and the volatility of MeJA supports a function in long distance signal-mediating intra- and inter-plant communication, controlling aspects of plant defense responses (Koornneef & Pieterse, 2008; Tamogami *et al.*, 2012; Reyes-Díaz *et al.*, 2016). In general, JA is assumed to be biologically active. However, certain modifications of the hormone are required for optimal activities in some responses (Staswick & Tiryaki, 2004).

Plant cell- and tissue cultures are often induced with phytohormones such as MeJA to enhance the production of secondary metabolites (Namdeo, 2007; James *et al.*, 2013; Jalalpour *et al.*, 2014; Mhlongo *et al.*, 2016a). It has been demonstrated that exogenous application of MeJA to different plant cell suspension cultures results in the production of terpenes (James *et al.*, 2013; Tugizimana *et al.*, 2015; Filová & Krivosudská, 2017), phenolics and flavonoids (Tugizimana *et al.*, 2015; Mhlongo *et al.*, 2016b). Cell suspensions in particular are simple, easily controllable and fast-growing systems, and good biological materials suitable for the understanding of fundamental biosynthetic processes of

secondary metabolites (Bourgaud *et al.,* 2001; Loyola-Vargas & Ochoa-Alejo, 2012; Cimini *et al.,* 2018).

In this study, exogenous MeJA was applied to *M. oleifera* cell suspensions in order to evaluate the suitability of the system for enhanced secondary metabolite production. Using ultra-high performance liquid chromatography (UHPLC) – quadrupole time-of-flight high definition mass spectrometry (QTOF HDMS), phytochemicals resulting from the metabolism of MeJA in *M. oleifera* cell suspension cultures were detected.

5.2 Materials and methods

5.2.1 *Moringa oleifera* cell suspension cultures and elicitation

M. oleifera liquid medium consisted of the Murashige and Skoog (MS) medium, (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.1% hydrolysed casein (Sigma, Munich, Germany). The phytohormones added to the medium were: 2,4-dichlorophenoxy acetic acid (2,4-D, 2 mL of a 250 mg/L stock solution / 113.1 µM; Sigma, St Louis, MO, USA), kinetin (Kin, 2 mL of a 250 mg/L stock solution / 116.1 µM; Duchefa, Haarlem, Netherlands) and alpha naphthalene acetic acid (NAA, 500 µL of 2000 mg/L stock solution / 107.41 µM (NAA; Sigma, St Louis, MO, USA). The final pH was adjusted to 5.8.

M. oleifera cell suspensions were initiated by transferring approximately 2 g of the friable callus (initiated from stem segments as described in Chapter 4) into the Erlenmeyer flasks containing 100 mL of the sterile liquid medium. The cells in suspension were then incubated on a shaker at a speed of 130 rpm and temperature ± 25 °C, and sub-cultured into fresh medium every 7 d for a period of 3 weeks to allow the cells to adapt to growth in the liquid medium. Once successfully established the suspensions were sub-cultured every 7 d. Three days after sub-culture (corresponding to the early- to mid-log phase of the batch culture), 20 mL of the cell suspensions was transferred into pre-weighed sterile 50 mL Falcon tubes. Cells were then treated with different concentrations of MeJA (100, 200 and 300 µM; Sigma Aldrich, Munich, Germany) diluted in MS medium, and incubated for 24 h. Non-treated

samples were considered as negative controls and were harvested at 0 h and 24 h to account for any variation not related to the treatment.

5.2.2 Metabolite extraction and sample preparation

The experiment included three biological replicates. Harvested cell suspensions were centrifuged in a benchtop swinging-bucket centrifuge at 5100 x*g* for 30 min at 4 °C, to separate the media from the cells. A volume of 100% methanol (MeOH, analytical grade, Romil, Cambridge, UK) was added to the pellet (average mass 8.4 g) in a 1:1.5 (v/m) ratio and directly put on ice to slow down enzymatic activities. Samples were homogenized using an Ultra-Turrax homogenizer (CAT Scientific, Paso Robles, CA, USA) for 1 min, and centrifuged under the same conditions as above. The supernatants were carefully transferred into round bottom flasks for concentration using a rotary evaporator (Büchi RotavaporR-200, Flawil, Switzerland) set at 55 °C. The MeOH extracts were evaporated to about 1 mL and further dried to completeness in a heating block set at 55 °C under constant air flow. The dried residues were reconstituted in 50% (v/v) aqueous UHPLC-grade MeOH and filtered through 0.22 µm nylon filters into chromatographic vials fitted with inserts and slitted caps.

5.2.3 Ultra-high performance liquid chromatography (UHPLC)

Methanolic extracts prepared from cultured *M. oleifera* cells were chromatographically analyzed in triplicate and in a randomized order using an Acquity UHPLC system combined to a photodiode array (PDA) detector (Waters Corporation, Milford, MA, USA) coupled to a SYNAPT G1 high definition quadrupole time-of-flight (QTOF) mass spectrometer (Waters Corporation, Milford, MA, USA). Compounds were separated on a Waters Acquity UHPLC column (HSS T3 reverse phase - 150 x 2.1 mm with a 1.8 µm particle size). A binary solvent mixture consisting of water (eluent A) and acetonitrile (Romil, Cambridge, UK) (eluent B) containing 0.1% of formic acid (Sigma-Aldrich, Munich, Germany) was used at a flow rate of 0.4 mL/min and injection volume of 2 µL. The initial conditions were 98% A, kept constant for 3 min. A gradient was introduced to change the chromatographic conditions to 97% A for 1 min then 92% A for 21 min. The eluent A was further decreased to 50% and 5% for 1 min and 2 min respectively, then restored to the initial condition 98% A for 2 min. The PDA

detector was set to scan 200 to 500 nm (resolution 1.2 nm) and to collect 20 spectra per second.

5.2.4 Quadrupole time-of-flight high definition mass spectrometry (QTOF HDMS)

A SYNAPT G1 QTOF MS system set in V-optics and operated in negative electrospray ionisation (ESI) mode was used to further detect the LC-separated analytes. The method was set to perform unfragmented and four fragmented experiments (MSE) simultaneously by collision energy ramping from 10 to 40 eV. Leucine encephalin (50 pg/mL, 554.2615 Da) was used as calibrant with a mass accuracy window of 0.5 mDa. The mass spectrometer was operated with capillary -, sampling - and extraction cone voltages of 2.5 kV, 30 V and 4 V respectively. Nitrogen was employed as nebulization gas. The cone gas and dissolvation gas flows were at 50 and 550 L/h respectively. The dissolvation and source gas temperatures were 450 and 120 ˚C respectively. The interscan time was 0.02 sec, the scan time was set at 0.2 sec covering a mass to charge (*m/z*) range of 100-1000.

5.2.5 Data processing and multivariate data analysis

The ESI-negative raw data resulting from UHPLC-MS analyses were extracted using MarkerLynx™ XS software (Waters Corporation, Milford, MA, USA). The software parameters were set to analyze chromatograms with the Rt range of 0.70 to 26 min, Rt window of 0.20 min, mass range of 100 to 1000 Da, mass tolerance of 0.05 Da and the intensity threshold as 100. For multivariate statistical analysis, the data matrices obtained from Markerlynx™ XS software were exported to SIMCA-14 software (Soft independent modelling of class analogy - Umetrics Corporation, Umea, Sweden). The data sets were all *Pareto*-scaled to improve the models' predictive ability. Principal component analysis (PCA) was performed and validated respectively to reduce the dimensionality of the data and to visualise metabolic changes between and within samples. The corresponding loadings plot was constructed to select discriminating ions responsible of the grouping observed on the PCA score plots.

5.2.6 Annotation of biomarkers

The annotation of JA-related compounds extracted with 100% methanol after elicitation with MeJA, was performed using accurate mass data obtained from the QTOF-MS. Using a targeted approach, a putative identification was carried out as no authentic standards (except for MeJA and JA) were available (Tugizimana *et al.,* 2013). Extracted ion chromatograms (XIC) were generated for significant ions obtained from the loadings plot, and the MS fragmentation patterns were generated. From the mass spectral data of each ion, molecular formulae were calculated and submitted to online databases such as Dictionary of Natural Products (DNP, http://dnp.chemnetbase.com), Knapsack (http://kanaya.naist.jp/KNApSAcK/) and ChemSpider (http://chemspider.com). The fragment ions were also compared to previously published studies. Structures matching the molecular formulae were submitted to mass fragmentation (MassFragment) analysis using MarkerLynx™ XS software, to confirm the fragmentation patterns of annotated compounds.

As part of an orthogonal identification tool, the UHPLC triple quadrupole (QqQ) MS (Shimadzu Nexera 30; Kyoto, Japan) and operating with an optimized multiple reaction monitoring (MRM) mode, was employed to target masses of interest. A Viva C_{18} (3.0 µm, 2.1 x 100 mm; Restek, USA) analytical column was used with a binary solvent mixture consisting of eluent A (0.1% formic acid in MilliQ water) and B (0.1% formic acid in acetonitrile). The flow rate was 0.4 mL/min and the injection volume 4 µL. The gradient elution started with 5% of eluent B for 3 min. The gradient elution was introduced to change the chromatographic conditions to 6%, then 11% over 3-5 min and 5-6 min respectively. The concentration of B was further increased to 50% over 6-27 min and kept constant for 2 min. The initial conditions were restored at 29 min B and the column was allowed to re-equilibrate from 30-32 min. The targeted ions were *m/z* 209 (JA) in negative ionization mode and 225 (MeJA) in positive ionization mode.

5.3 Results and Discussion

5.3.1 Chromatographic analysis and multivariate data analysis

M. oleifera cell suspension extracts were analyzed on UHPLC-QTOF-HDMS after elicitation with MeJA. The base peak intensity (BPI) chromatograms (**Figure 5.2**) show differences in peak intensities as well as appearance and disappearance of some peaks (indicated by the orange rectangles). This clearly indicates that MeJA perturbed / altered metabolism within *M. oleifera* cells, resulting in concentration-dependent changes in the chromatographic profiles. Moreover, it is important to mention that the majority of differences is observed in the late Rt region of the chromatograms, indicating the non-polar nature of compounds resulting from the treatment.

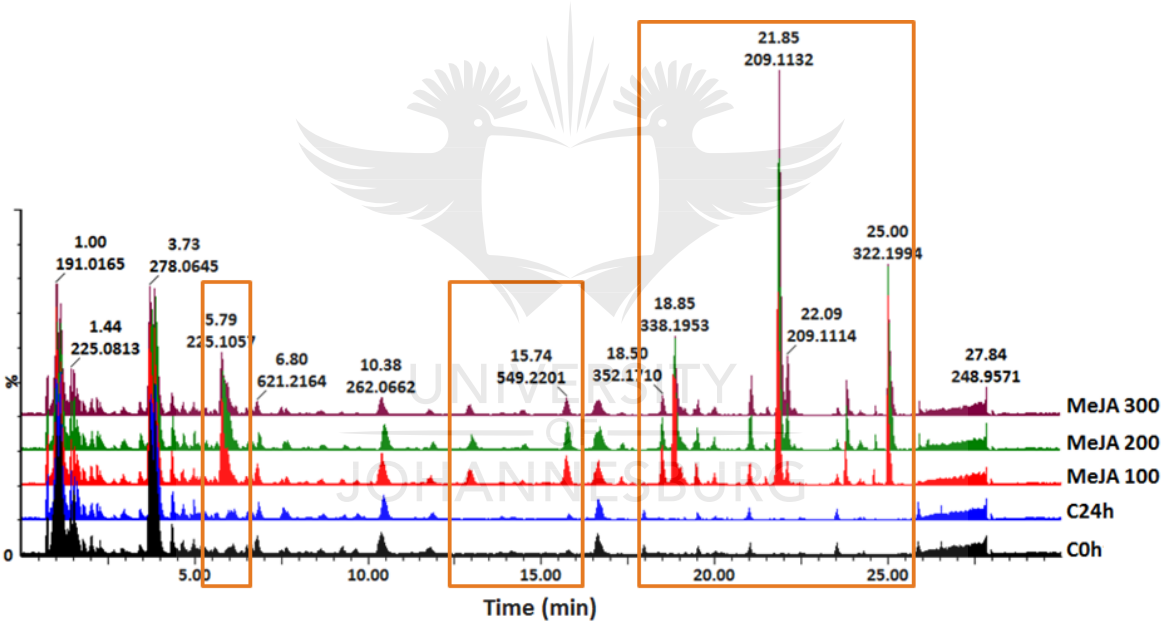

Figure 5.2: UHPLC-MS (ESI negative mode) BPI chromatograms of methanol extracts from *Moringa oleifera* cells grown in suspension. Extracts were prepared from harvested cells treated with 100, 200 and 300 µM MeJA, and incubated for 24 h. **C0h** is the non-treated control harvested before incubation and **C24h** represents the non-treated samples harvested after incubation.

In reducing the dimensionality of the data, PCA does not lose much information and presents the data in the way that underlines the similarities and the differences between and within the samples. PCA scores - and loading plots were generated to provide more interpretable observations of the BPI chromatograms (**Figure 5.2**). A four-component model explaining 80% of the total variation [R^2X(cum) = 80%] with a good predictive ability

[Q^2(cum) = 74.3%] was computed. The PCA scores plot computed with the two first components (**Figure 5.3A**) shows differential clustering between the controls and MeJA-treated samples, with little variation observed within each group and illustrating the effect of MeJA on *M. oleifera* cell suspensions. The PCA loadings plot (**Figure 5.3B**) generated helped to identify significant metabolites affecting the clustering of the samples. The features highlighted in orange correspond to some precursor ions annotated in **Section 5.3.2** (**Table 1**).

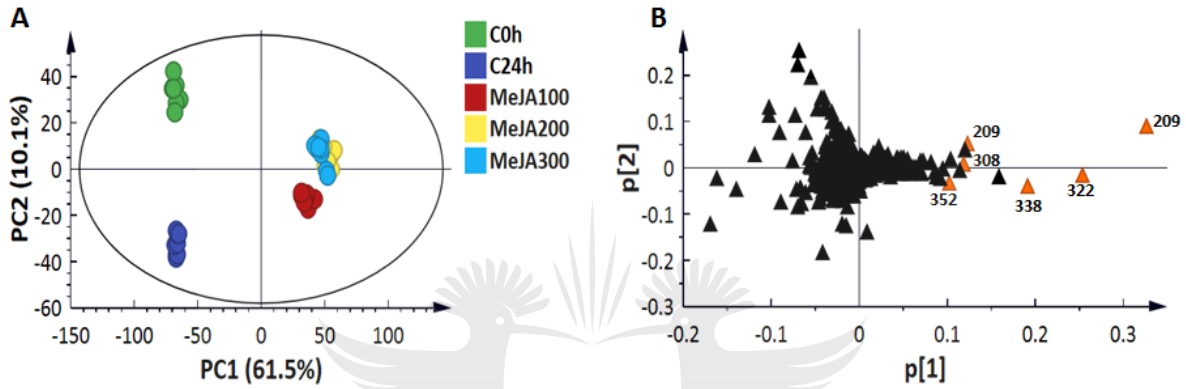

Figure 5.3: Principal component analysis (PCA) of UHPLC-MS data. **(A)**: PCA scores plot showing the grouping of samples. **(B):** The corresponding PCA loadings plot showing discriminating features/variables (*e.g*: highlighted in orange) responsible for the observed grouping. Extracts were prepared from *Moringa oleifera* cell suspensions treated with MeJA (100, 200, 300 µM) and incubated for 24 h. **C0h** represents the non-treated samples harvested before incubation and **C24h** represents the non-treated samples harvested after incubation.

5.3.2 Characterization of jasmonic acid derivatives

JA is a polyunsaturated fatty acid-derived molecule characterized by a pentacyclic ring structure (cyclopentanone) to which a carboxylic and an aliphatic side chain is attached **(Figure 5.5)** (Svoboda & Boland, 2010; Eng *et al.*, 2016). In this study, three JA-amino acids as well as JA and metabolites and derivatives thereof were annotated from *M. oleifera* cell suspensions elicited with different concentrations of MeJA (100, 200, 300 µM) (**Table 5.1**).

Table 5.1: Metabolites originating from jasmonic acid metabolism, identified from methanolic extracts of *Moringa oleifera* cell suspensions treated with 100, 200, 300 µM MeJA and incubated for 24 h.

Com-pounds	Annotation	Molecular formulae	R_t (min)	MW	*m/z*	Diagnostic fragments	Instrument and ionization mode	Peak intensity		
								100 µM	200µM	300 µM
Methyl jasmonate and Jasmonic acid										
1	Jasmonic acid (JA)	$C_{12}H_{18}O_3$	21.81	210	209.114	209	QqQ and QTOF (-)	43.6	58.4	68.3
2	Jasmonic acid (JA)	$C_{12}H_{18}O_3$	22.11	210	209.113	209	QqQ and QTOF (-)	15.1	17.2	13.6
3	Methyl jasmonate (MeJA)	$C_{13}H_{20}O_3$	-	225	224	151,147, 133	QqQ and QTOF (+)	-	-	-
Jasmonoyl-amino acid conjugates										
4	Jasmonoyl-isoleucine / leucine (JA-Ile/Leu)	$C_{18}H_{29}NO_4$	25.00	323	322.199	130	QTOF (-)	437.6	425.7	385.6
5	Dihydrojasmonoyl-isoleucine/leucine (DihydroJA-Ile)	$C_{18}H_{31}NO_4$	24.62	325	324.214	130	QTOF (-)	38.8	32.5	23.5
6	12-Carboxyjasmonoyl-isoleucine/leucine (12COOH-JA-Ile/Leu)	$C_{18}H_{27}NO_6$	18.47	353	352.172	130	QTOF (-)	105.2	76.8	52.0
7	*Trans*-12-hydroxyjasmonoyl-isoleucine/leucine (12OH-JA-Ile/Leu)	$C_{18}H_{29}NO_5$	18.83	339	338.194	130	QTOF (-)	310.4	248.8	201.8
8	*Cis*-12-hydroxyjasmonoyl-isoleucine/Leucine isomer II (12OH-JA-Ile/Leu)	$C_{18}H_{29}NO_5$	19.04	339	338.194	130	QTOF (-)	37.9	31.5	19.5
9	Jasmonoyl-valine (JA-Val)	$C_{17}H_{27}NO_4$	23.78	309	308.181	116	QTOF (-)	89.2	100.6	106.5
10	Hydroxyjasmonoyl-valine (OH-JA-Val)	$C_{17}H_{27}NO_5$	12.95	325	324.177	116	QTOF (-)	41.9	36.1	27.1

11	Jasmonoyl-phenylalanine (JA-Phe)	$C_{21}H_{27}NO_4$	25.16	357	356.183	164	QTOF (-)	6.6	13.8	19.1
12	Hydroxyjasmonoyl-phenylalanine (OH-JA-Phe)	$C_{21}H_{27}NO_5$	19.98	373	372.178	164	QTOF (-)	30.1	27.1	23.8
Jasmonic acid hexose and other Jasmonic acid-conjugates										
13	Jasmonic acid (JA) conjugate	-	15.74	540	539.216	503, 209	QTOF (-)	25.6	20.7	14.5
14	Dihydrojasmonic acid (H_2JA) conjugate 1	-	17.31	420	419.189	211	QTOF (-)	18.9	14.3	7.6
15	Dihydrojasmonic acid (H_2JA) conjugate 2	-	21.49	566	565.249	519, 355, 211	QTOF (-)	17.1	13.7	10.0
16	Jasmonic acid hexose (JA-hexose) conjugate isomer 1	-	16.55	418	417.172	407, 371, 209	QTOF (-)	26.8	39.5	29.7
17	Jasmonic acid hexose (JA-hexose) conjugate isomer 2	-	16.73	418	417.173	407, 371, 209	QTOF (-)	17.7	16.3	14.2

- Information not available using the QTOF; (-) negative ionization mode; (+) positive ionization mode

5.3.2.1 Jasmonic acid and methyl jasmonate

Two compounds were detected at Rt = 21.81 and 22.11 min with *m/z* of 209 corresponding to JA. Surprisingly, with the current MS method (QTOF HDMS), the fragmentation pattern of the extracted chromatogram revealed the presence of additional fragments of *m/z* 441 and 433 (**Figure 5.4**). The compounds were first identified as JA conjugates. However, using an authentic standard of JA with an optimized multiple reaction monitoring (MRM) method on a triple quadrupole (QqQ) instrument, it was possible to detect those peaks as JA isomers (**Figure 5.5A** and **B**). Compounds previously identified as JA conjugates were likely co-eluting with JA and hence hindering the fragmentation pattern of the latter. This shows the power of tandem MS approaches (in this case MRM) to efficiently and selectively distinguish between isomers.

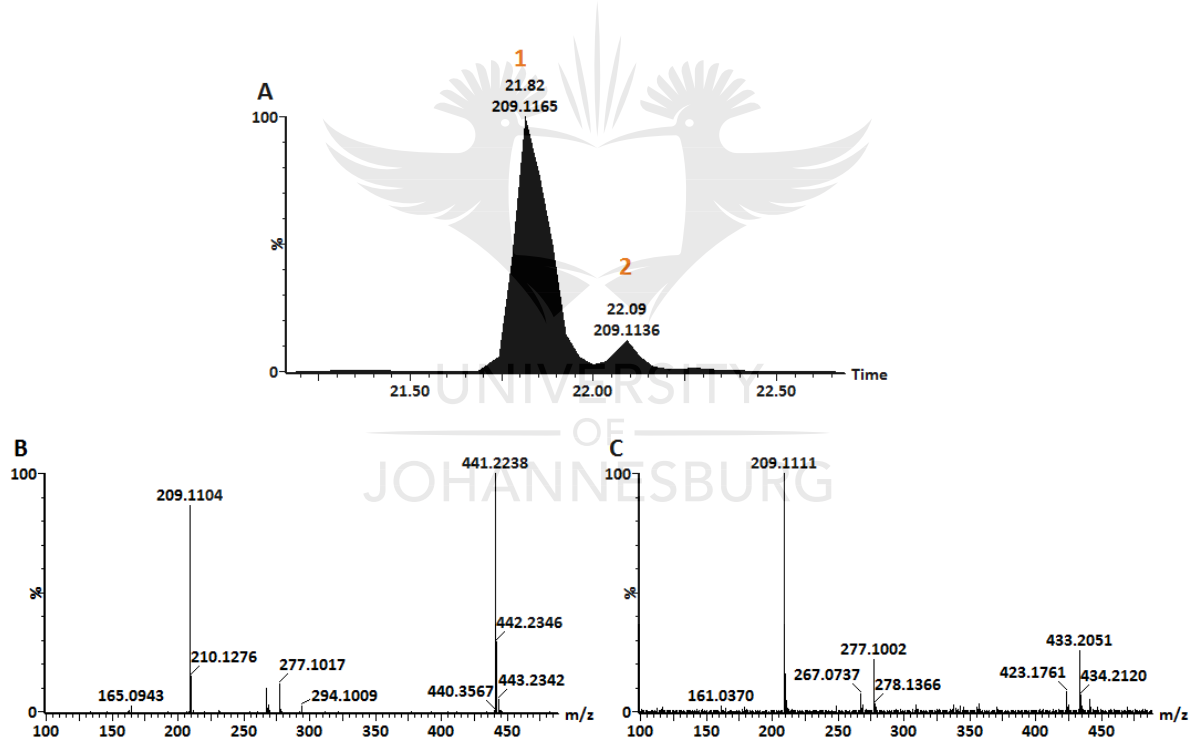

Figure 5.4: (A): UHPLC-XIC of *m/z* 209 corresponding to JA; **(B):** mass spectrum corresponding to the base peak 1; and **(C):** mass spectrum of the base peak 2.

Similarly, MeJA was also targeted and three isomers were detected in extracts of *M. oleifera* cell suspensions post-treatment (**Figure 5.5C** and **D**). MeJA and JA are molecules have been reported to exist as four stereoisomers that result from the two chiral centers (at C-3 and C-7) present in the chemical structures. These isomers are (+)-(3R,7S), (-)-(3S, 7R), (+)-(3S, 7S)

and (-)-(3R, 7R) forms (Han et al., 2001; Matencio et al., 2017). The naturally occurring plant jasmonates have been reported to be the (+)-(3R, 7R) and (+)-(3R, 7S) forms (Han et al., 2001).

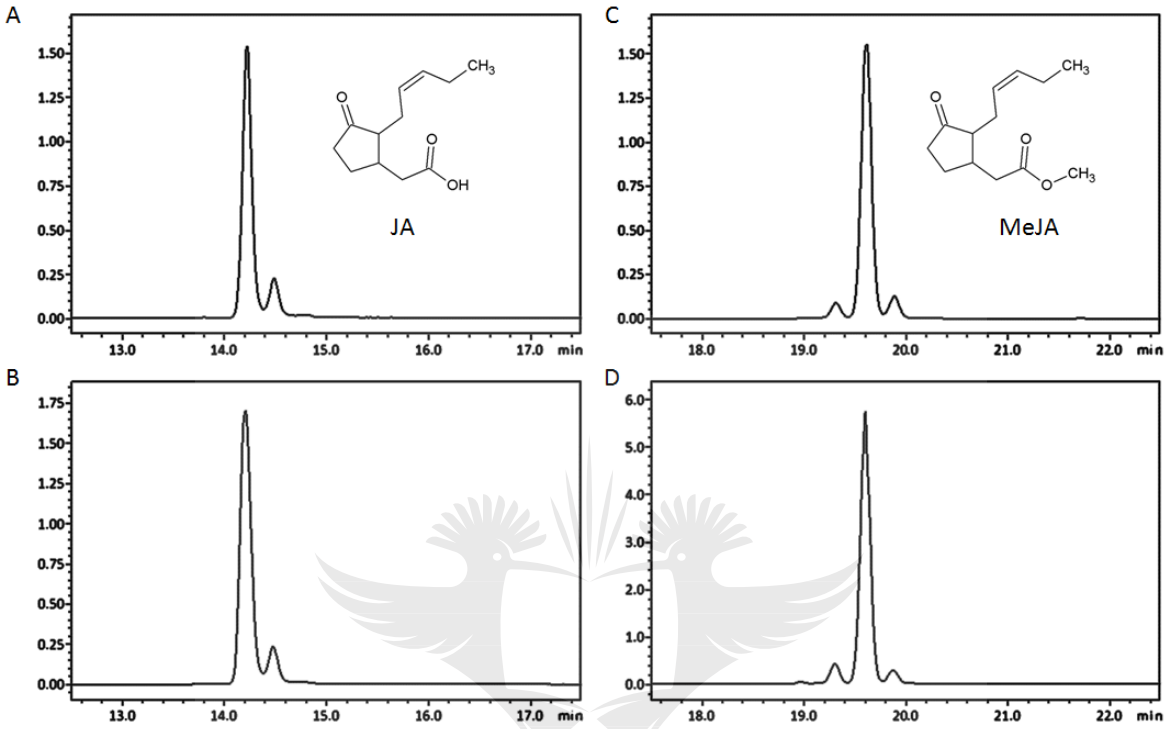

Figure 5.5: UHPLC-single ion monitoring-MS chromatograms of JA and MeJA. **(A):** JA standard; **(B):** JA in *Moringa oleifera* cell suspension sample treated with 100 µM MeJA; **(C):** MeJA standard; and **(D):** MeJA in *M. oleifera* cell suspension sample treated with 100 µM MeJA.

5.3.2.2 Jasmonoyl-isoleucine (JA-Ile) and derivatives

A precursor ion *m/z* 322 eluting at Rt = 25.00 min, exhibited a fragment ion of *m/z* 130, corresponding to isoleucine/leucine (Ile/Leu). The neutral loss of 192 corresponds to JA without a hydroxyl group ($[M\text{-}H\text{-}OH]^-$ = 210-1-17). Based on the fragmentation pattern (**Figure 5.6A**) and literature (Wang *et al.,* 2007; Göbel & Feussner 2009; Eng *et al.,* 2016), the compound was annotated as jasmonoyl-isoleucine/leucine (JA-Ile/Leu). Although JA-Ile and JA-Leu can elute at the same retention time, the compound is more likely to be JA-Ile as it is the most prominent, bioactive and studied among all the JAs (Song *et al.,* 2014). Relatedly, a compound with a precursor ion of *m/z* 324 and a fragment ion of *m/z* 130 was detected at Rt = 24.62 min and assigned as dihydrojasmonoyl-isoleucine (H_2JA-Ile). The neutral loss

between *m/z* 324 and 130 was 194 corresponding to dihydrojasmonic acid (H_2JA) without the hydroxyl group (Cross & Webster, 1970; Guranowski *et al.,* 2007).

Three compounds that shared the same fragment ion of *m/z* 130 were detected at Rts = 18.47, 18.83 and 19.04 min with *m/z* 352, 338 and 338 respectively. The last two compounds were isomers as these displayed the same mass and fragmentation patterns. The difference between *m/z* 352 and *m/z* 322 (JA-Ile) was 30, corresponding to two oxygens and two hydrogens (*m/z* 352 = *m/z* JA-Ile - 2H + 2O). The addition of oxygens can be explained by the presence of a carboxylic acid moiety on the structure of *m/z* 352 and the loss of two hydrogens is due to the formation of a double bond. In the case of *m/z* 338, the difference was one oxygen atom (*m/z* 338 = *m/z* JA-Ile + O). The compounds were thus annotated as 12-carboxyjasmonoyl-isoleucine (12COOH-JA-Ile) and 12-hydroxyljasmonoyl-isoleucine (12OH-JA-Ile) isomers I and II respectively (Glauser *et al.,* 2008; Göbel & Feussner 2009; Koo *et al.,* 2014; Widemann *et al.,* 2015). Moreover, the two isomers of 12OH-JA-Ile were annotated as *trans* and *cis* isomers respectively, as previously described by Glauser *et al.,* (2008).

5.3.2.3 Jasmonoyl-valine (JA-Val), jasmonoyl-phenylalanine (JA-Phe) and derivatives

A parent ion with *m/z* 308 was detected at Rt = 23.78. The derived fragment ion was *m/z* 116, corresponding to valine. The neutral loss of 192 was similar to the one observed with JA-Ile. The compound was characterized as jasmonoyl-valine (JA-Val) (**Figure 5.6B**). The hydroxylated derivative with *m/z* 324, giving a diagnostic product ion of 116 and the mass difference between the JA-Val and OH-JA-Val of 16 (corresponding to an oxygen atom) was annotated as hydroxyjasmonoyl-valine (OH-JA-Val) (Wang *et al.,* 2007; Göbel & Feussner 2009). Similarly, jasmonoyl-phenylalanine (JA-Phe) (**Figure 5.6C**) and hydroxyjasmonoyl-phenylalanine (OH-JA-Phe) were annotated with *m/z* 356 (Rt = 25.16 min) and 372 (Rt = 19.98 min) respectively. In both cases the fragment ion was *m/z* 164, corresponding to phenylalanine (Göbel & Feussner 2009; Widemann *et al.,* 2015).

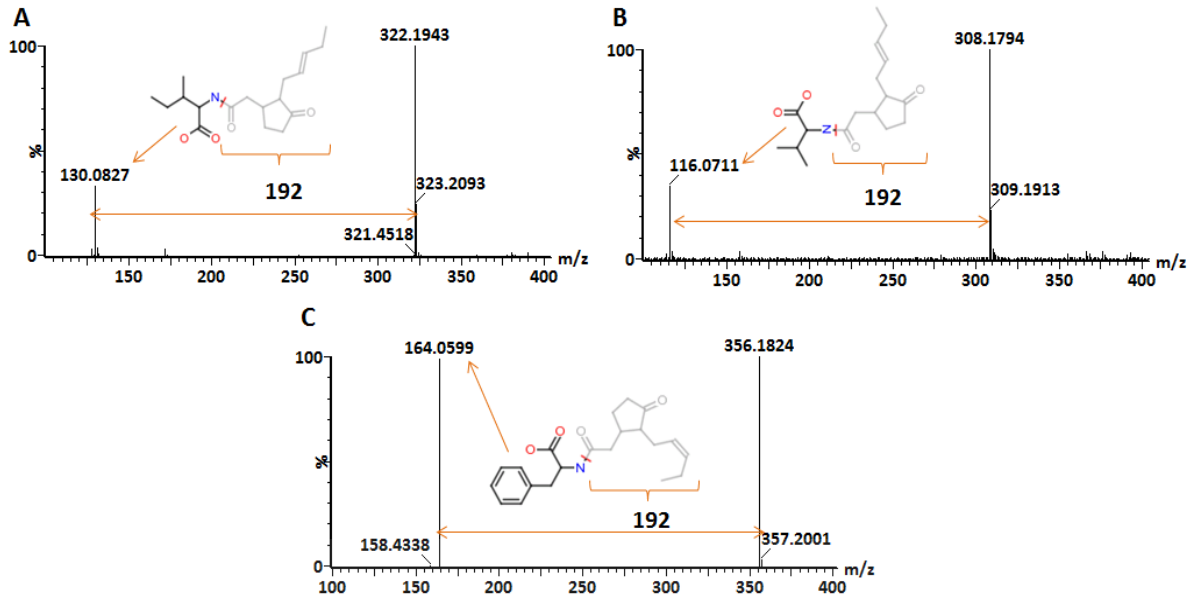

Figure 5.6: Mass fragmentation patterns of JA-conjugated amino acids characterized in methanolic extracts prepared from MeJA-elicited *Moringa oleifera* cells. **(A)**: JA-Ile; **(B)**: JA-Val; and **(C):** JA-Phe. The structures were fragmented as seen on the figure using MassFragment software. The orange arrows indicate the neutral loss of 192 corresponding to the JA without a hydroxyl group.

5.3.2.4 Jasmonic/dihydrojasmonic acid and derivatives

A compound of *m/z* 539 was detected at Rt = 15.74 min, with a daughter ion of 209 corresponding to *m/z* of JA. The compound was characterized as a JA conjugate as the *m/z* of the molecule conjugated to the JA was unknown. Two isomers with *m/z* 417 were detected at Rt = 16.55 min and 16.73 min. The diagnostic fragment ions exhibited *m/z* 209 (JA), *m/z* 371 corresponding to JA-hexose (209 + neutral loss of 162 corresponding to a hexose moiety =371) and *m/z* 407 (unknown). The compounds were annotated as JA-hexose conjugate isomer I and II. Dihydrojasmonic acid (H_2JA) is characterized by *m/z* 211, different from jasmonic acid (*m/z* 209) by two hydrogen atoms corresponding to the formation of a double bond (Göbel & Feussner, 2009; Eng *et al.*, 2016). Two H_2JA conjugates with *m/z* 419 and 565 were detected at Rt = 17.31 and 21.49 min respectively. The diagnostic ions included *m/z* 211 corresponding to H_2JA. The nature of the molecules conjugated to JA, JA-hexose and H_2JA are still to be investigated.

5.3.3 Occurrence and bioactivities of the JAs

The relative quantification of the annotated compounds shows that the concentration of 100 µM MeJA was optimal to elicit jasmonates production in *M. oleifera* cell suspensions. At

that concentration, the peak area of the majority of metabolites in **Table 5.1** is at the highest level followed by 200 µM then 300 µM. Exceptions were observed with JA-Val, JA-Phe and JA conjugate 2. The highest peak area was observed for JA-Ile/Leu (average of 437.6 at 100 µM MeJA) and the lowest relative intensity was observed for JA-Phe (average of 6.6 at 100 µM MeJA). This shows the abundance of JA-Ile/Leu in the treated cell suspensions after elicitation and suggests that this might be the active form of JA utilized by *M. oleifera* as a stress-signaling molecule. Similarly to this observation, JA-Ile was found to be the most prominent in *Arabidopsis thaliana*, with only minor amounts of JA-Val and JA-Phe present (Göbel & Feussner, 2009; Staswick & Tiryaki, 2004).

Although JA was considered as the end product and bioactive phytohormone of the octadecanoic pathway for many years, it has been proven that some JA metabolites, derivatives and biosynthetic precursors are also biologically active (Schaller & Stintzi, 2008). For instance, in Albizzia, the nyctinastic leaf movement relies on 12OH-JA-glucose (Nakamura *et al.,* 2006). JA and its *O*-glucoside were isolated from potatoes and plays a role of tuber-inducing factors (Koo, 2018). In rice the flavonoid sakuranetin is intensely elicited by JA-Phe (Tamogami *et al.,* 1997). Moreover, the latter was also found to accumulate in Arabidopsis leaves after wounding (Widemann *et al.,* 2015). As mentioned earlier, JA-Ile ((+)-7-*iso*-JA-Ile) has been reported the most bioactive compound in plant defense, even more active than the non-conjugated JA (Schaller & Stintzi, 2008; Song *et al.,* 2014). JA-Val, JA-Leu and jasmonoyl-amino cyclopropane carboxylic acid (JA-ACC), are considered to be JA storage molecules in plants (Yan *et al.,* 2013). Apart from JA-Ile and derivatives, very little is known regarding the bioactivity of other JA-amino acid conjugates in plants. However, it was suggested that they play a role in regulating the hormonal activity of JA. From the present study, the implication in plant defenses can be suggested as the presence was only observed post-elicitation with MeJA.

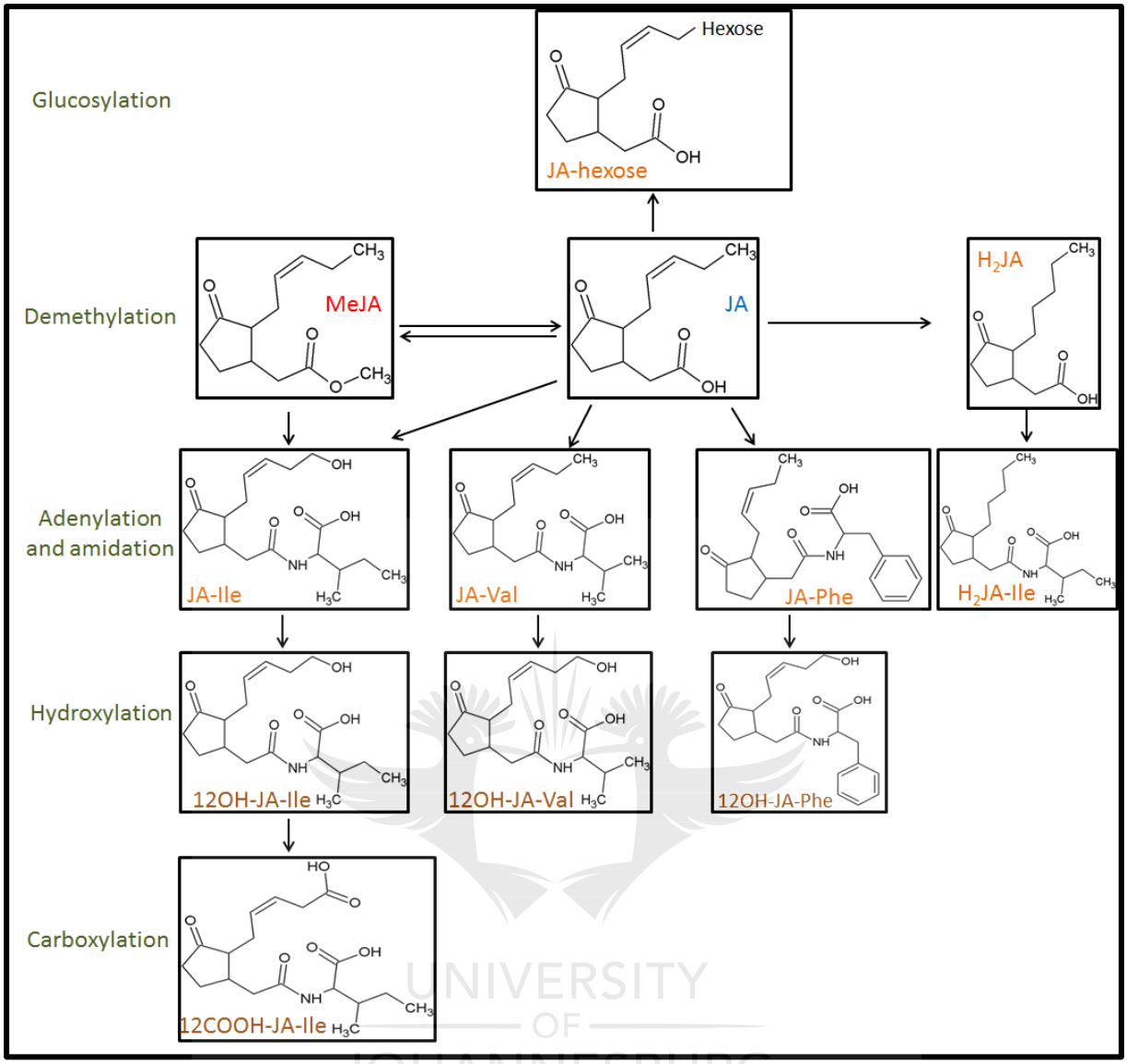

Figure 5.7: Structures of annotated metabolites in **Table 5.1** and metabolism of exogenously added MeJA (100, 200, 300 µM) to *Moringa oleifera* cell suspension cultures. Briefly, MeJA can be demethylated to form JA. Activation of the free carboxylic group of JA through adenylation allows the condensation of the JA to amino acids (Ileu, Leu, Val, Phe) through the formation of an amide bond. JA can be attached to glucose to form JA-glucosyl conjugates. H_2JA will also be conjugated to the amino acid Ileu and finally, JA-amino acids can be hydroxylated to form 12OH-JA-Ile, 12OH-JA-Val and 12OH-JA-Phe and carboxylated to 12COOH-JA-Ile.

5.3.4 Metabolism of jasmonates

The understanding of plant growth, development and response to stress requires the investigation of phytohormones such as the signaling molecule JA (Kallenbach *et al.,* 2009). Following biotic and abiotic stresses, JA rapidly accumulates in the cells. Exogenous application of MeJA can also be de-esterified to produce JA (Schaller *et al.,* 2004). In order

to prevent inhibition of plant growth and development caused by high concentrations of the hormone, the JA active forms (+)-(3R, 7R) (*cis*) can be deactivated *in vivo* by epimerization to the inactive forms (+)-(3R, 7S) (*trans*) (Glauser *et al.,* 2008; Caarls *et al.,* 2017). However, the reversibility of the epimerization results in the presence of active JA at the equilibrium.

Some modifications can occur to clear the accumulated JA in the cells, giving rise to the large group of jasmonates (Glauser *et al.,* 2008). Modifications involve the methylation of JA to form MeJA; decarboxylation to form *cis*-jasmone and *O*-glucosylation (for *O*-glucosyl-derivatives) (Wasternack & Strnad, 2016). Hydroxylation, carboxylation, sulfatation and conjugation with amino acids such as Ile, Leu, Val, Gln, Trp and Phe (Wasternack & Strnad, 2016) can also occur. Conjugation of JA to amino acids is achieved *via* adenylation of JA, followed by an energy-dependent reaction creating an amide bond between the amino acid and JA, catalyzed by the enzyme amino acid conjugate synthase, JAR1 **(Figure 5.1**) (A. Schaller & Stintzi, 2008). JA-Ile for instance can be formed directly from JA or after demethylation of MeJA for biological activity (Staswick, 2009; Hanaka *et al.,* 2015).

In general, the hormone activity is repressed by simple hydroxylation (Widemann *et al.,* 2015). In *Arabidopsis thaliana* for instance, deactivation of JA-Ile is accompanied by a shift towards oxidized derivatives, catalyzed by cytochrome P450 (CYP94) enzymes to control hormone oxidation status for signal attenuation (**Figure 5.1**) (Aubert *et al.,* 2015; Koo 2018). These authors have also suggested that JA-Ile is the metabolic hub that directs JA profile complexity and that the hormone oxidation ratio, rather than absolute changes in the levels of JA-Ile, is important in signalling. By implication, it is also possible that OH-JA-Val and OH-JA-Phe are produced to fulfil the same function of inactivating their respective precursors (JA-Val and JA-Phe).

JA-Ile can be catabolized through two pathways localized in the endoplasmic reticulum (**Figure 5.1**) (Koo *et al.,* 2014; Zhang *et al.,* 2016). The first one is the ω-oxidation pathway generating 12OH-JA-Ile and 12COOH-JA-Ile (Koo *et al.,* 2011; Heitz *et al.,* 2012; Koo, 2018), and the second one is the hydrolytic pathway resulting in JA and Ile from the hydrolysis of JA-Ile at the amide bond by the ILR1-like amidohydrolase (IAH) family (Woldemariam *et al.,* 2012; Bhosale *et al.,* 2013; Koo, 2018).

Results presented in this chapter shows that the application of MeJA to *M. oleifera* cell suspensions resulted in the conversion into JA. In order to perform specific biological functions, be cleared from the cells or stored, JA was converted into derivatives: JA-amino acids (JA-Ile, JA-Val and JA-Phe), JA-hexose and other non-identified conjugates of H_2JA and JA. Finally, JA-amino acids were then converted into the inactive forms 12OH-JA-Ile, 12OH-JA-Val, 12OH-JA-Phe 12COOH-JA-Ile (**Figure 5.7** and **5.8**). To the best of our knowledge, this study is the first investigating the metabolism of exogenously added MeJA by *M. oleifera* cell suspension cultures.

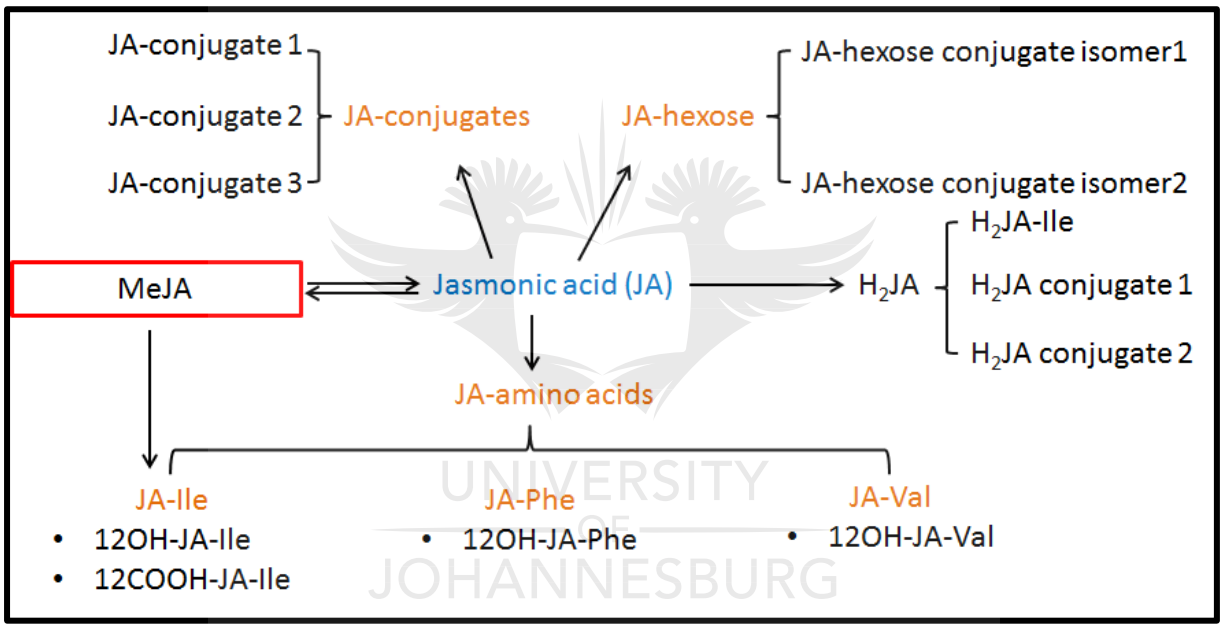

Figure 5.8: Summarizing illustration of the biotransformation and bioconversion reactions that exogenously added MeJA was subject to in *Moringa oleifera* cell suspension cultures.

5.4 Conclusion

In this study, MeJA was exogenously applied to *M. oleifera* cell suspension cultures and the resulting methanolic extracts were analyzed using UHPLC-QTOF-MS. The application of MeJA resulted in activation of biotransformation and bioconversion events, leading to the synthesis and subsequent metabolism of the JAs identified. These JA derivatives were: JA-Ile/Leu, JA-Val, JA-Phe, the hydroxylated and carboxylated forms 12OH-JA-Ile, 12OH-JA-Val, 12OH-JA-Phe, 12COOH-JA-Ile; JA and dihydroJA conjugates. The presence of JA-Ile at high

concentration re-emphasized the important role as signaling molecule in plant defense. The optimal concentration for elictation of the *M*. *oleifera* cell suspension system was 100 µM MeJA; as determined by the highest relative concentrations observed for most JAs. Jasmonates play an important role in signaling plant responsive genes after biotic or abiotic pressures and this was highlighted again in this study. The future prospects will be to study the biological role of each jasmonic acid derivative in *M*. *oleifera* by investigating the occurrence following manipulation of enzymes involved in plant defense mechanisms.

5.5 References

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Chapter 6: General conclusion

In this study, *Moringa oleifera* cell cultures were utilized to investigate the effect of different hormones on the production of secondary metabolites.

As such, different extraction methods of *M. oleifera* leaf tissue were investigated in order to establish a basis for analyzing (or metabolic profile of) metabolite production in the plant. Three different green extraction solvents (NADES, PHW and ATP) were employed and compared to the more usual methanol-extraction procedures to point out the ability of environmental friendly solvents to extract pharmacologically relevant compounds. This was successfully demonstrated as a wide range of secondary as well as primary metabolites were extracted from dried leaves of *M. oleifera*. Except for glucomoringin and its acylated derivatives, annotated only in methanolic and NADES extracts, all green solvents were able to extract CGAs and flavonoids.

The manipulation of *M. oleifera* culture medium with different ratios of the phytohormones 2,4-D and kinetin, resulted in no shoot - or root formation, perhaps because of the habituation phenomenon, implying that the cells underwent genetic – or biochemical changes and thus become non-responsive towards external stimulation. However, differential distribution of phenolic - and anti-oxidant compounds was observed, showing the ability of habituated *M. oleifera* calli, grown on different hormonal combinations, to still respond to external stimuli. Coumaroyl aspartate (more abundant where 2,4-D was employed alone or in combination with kinetin) and protocatechuic acid hexose (more abundant where kinetin was dominant) were annotated in the callus and have not yet been detected in *M. oleifera* leaf tissue. Furthermore, a difference in metabolite production was observed between undifferentiated callus and differentiated leaf tissue of the plant. The pathways leading to the formation of CGAs, flavonoids and glucosinolates were activated on leaf tissue and seemed to be inactivated / repressed in callus cultures. In an attempt to elicit the production of these metabolites in cell suspension cultures, MeJA was added to the system. Although the well-known metabolites produced by *M. oleifera* leaf tissue were not

observed, important reactions occurred, metabolizing MeJA into JA and related derivatives (JA-Val, JA-Ile/Leu, JA-Phe, JA conjugated to a hexose as well as hydroxylated and carboxylated amino acid derivatives). With the help of UHPLC-MS-based platforms for non-targeted and targeted metabolomics approaches, it was possible to explore the changes resulting from the exogenous application of phytohormones on *M*. *oleifera* cell cultures.

This study, to the best of our knowledge, is the first providing insights into the general behavior of undifferentiated *M*. *oleifera* callus and cell suspension cultures for production of phytochemicals. Although the apparent habituation of the cells in culture interfered with the aim of using the cells as bioreactors, the knowledge acquired is a step towards the understanding of metabolomic processes occurring in undifferentiated *M*. *oleifera* systems. The output of this study can be employed in many industries and opens new avenues of research.

For I know the plans I have for you, declares the LORD, plans to prosper you and not to harm you, plans to give you hope and a future.

~Jeremiah 29: 11