Development of Liquid Chromatography Mass Spectrometry technique for evaluation and differentiation of plant derived isomers

By

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Declaration

I, Mbedzi Dakalo Terrence, of student number 15011401, acknowledges that I have read and understood the University's policies and rules applicable to postgraduate research, and I certify that I have, to the best of my knowledge and belief, complied with their requirements. I declare that this research thesis is my own work and it is being submitted for the degree of Master of Science in Biochemistry at The University of Venda as it has not been submitted for any degree at any other University or institution. All sources have been acknowledged and referenced accordingly.

Signature:_____ Date: 10 November 2022

Dedication

This dissertation is dedicated to my parents, Mr Thikhathali Petrus Mbedzi and Mrs Vhusiwana Salphinah Mbedzi. I will forever be grateful for having you as a pillar of my strength.

Your words “Zwivhuya zwiṅwe na zwiṅwe zwi tou konḓelelwa” will forever keep me going.

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General abstract

Cinnamic acid containing molecules have been shown to possess unprecedented pharmacological activities against various physiological disorders. Most of the plants containing these compounds have been shown to exhibit multiple bioactivities, notably against various diseases which are currently without a cure, for example human immunodeficiency virus (HIV) associated ailments. As in other plants, most of the pharmacologically relevant metabolites are produced in minute concentrations, and, as such, alternative means to produce high levels of these compounds are imperative. Herein, UV-induced photochemical conversion of cinnamic acid containing molecules is presented as a feasible means to diversify the number of bioactive metabolites in plant extracts. Naturally, UV rays from sunlight were shown to result in the formation of geometrical isomers. The presence of these isomers has resulted in analytical challenge, which has resulted in erroneous identification and isolation of an active isomer. Virtual screening and docking studies provide insights into the potential interactions between molecules and their targets, and can be useful in predicting the biological activity of compounds. This study further shown that synergistic isomerism could be beneficial during inflammation conditions, and it suggests that certain isomers of a compound may work together to enhance the desired therapeutic effect. Herein, liquid chromatography quadrupole time-of-flight mass spectrometry (LC-qTOF-MS) method capable of distinguishing between these isomers was developed.

Keyword: Cinnamic acid, Chlorogenic acids, Chicoric acids and isomers

Preface

This document has been compiled in the form of published and manuscripts under preparation.

The outline is as follows:

Chapter 1: General Introduction

Chapter 2: Literature Review

Manuscript published and under preperations

Chapter 3: Dakalo T. Mbedzi, Lutendo M. Mathomu, Msizi I. Mhlongo, Ntakadzeni E. Madala, 2022. Ultraviolet (UV) light-induced geometrical isomerisation of cinnamic acid containing molecules: A plausible, non-enzymatic approach to enhance metabolite composition of plant extracts. *Journal of Botany*, 150, 845-850.

Chapter 4: Dakalo T. Mbedzi, Ndamulelo A. Nengovhela, Lutendo M. Mathomu, Msizi I. Mhlongo, Ntakadzeni E. Madala. Synergistic isomerism of cinnamic acid containing compounds is a precursor of metabolome complexity but hinders efficient discrimination of plant metabolites: A case study of verbascoside versus isoverbascoside, *Submitted to the journal of Botany*.

Chapter 5: Dakalo T. Mbedzi, Lutendo M. Mathomu, Msizi I. Mhlongo, Ntakadzeni E. Madala: Acylation of flavonoids glycosides with cinnamic acids derivatives amplifies flavonoids chemical space in plants, *in preparation*.

Chapter 6: General conclusion

During the period of the study, the following related article was published:

Nengovhela, N., Mbedzi, D.T., Ndhlala, A.R., Mathomu, L.M., Mhlongo, M.I. and Madala, N.E., 2021. LC-MS based metabolite profiling reveals hydroxycinnamoyl conjugation as a discriminatory chemical factor between two closely related *Coccinia* species. *South African Journal of Botany*, 145, 199-206.

Executive Summary

Medicinal plants produce a wide range of natural products with significant relevance in drug discovery, human health and people's livelihood. Plant derived natural products play a significant role in providing alternative ways to treat various diseases. These natural products have been used for medicinal and pharmaceutical purposes for the past years and are deemed safer than their synthetic counterparts. *Lippia javanica* (Verbenaceae family), *Harpagophytum procumbens* (Pedaliaceae family), *Bidens pilosa* L (Asteraceae family), *Coccinia rehmannii* (Cucurbitaceae family) and *Helichrysum kraussii* (Asteraceae family) are examples of plants which have been used in traditional medicine as household remedies for various diseases. This is due to the presence of secondary metabolites that plants naturally synthesise for survival and adaptation to their environment. Examples of these metabolites include hydroxycinnamic acids (HCAs), flavonoids and chlorogenic acids (CGAs). The metabolome complexity is often amplified by isomerisation and conjugation. Highly sensitive advanced technological instrumentals that couple liquid chromatography and mass spectrometry are required to characterise and identify these metabolites, such as liquid chromatography mass spectrometry.

The study's first aim was to evaluate metabolite differences between non-UV treated and UV treated methanol extracts of *B. pilosa* using an UHPLC-qTOF-MS based platform and online tools such as principal component analysis (PCA) and cloud plot (**Chapter 3**). In addition, the study also aimed at differentiating the UV-induced geometrical isomers of selected cinnamic acid containing molecules: A case study of verbascoside and isoverbascoside using accurate mass, elution order and fragmentation pattern (**Chapter 4**). In addition, the study also aimed at deciphering different positional isomers and conjugates of hydroxycinnamic acids (HCAs) attached to different flavonoid glycosides using fragmentation pattern (**Chapter 5**).

In the current study, metabolite extraction from plants was achieved using methanol and treatment of plant leaf extracts with UV light was carried out using 254 nm. Extracted metabolites were analysed with a semi-targeted metabolomic approach using UHPLC-qTOF-MS analysis. Mass spectrometry techniques have been developed over the years as feasible to assist in characterising plant metabolites' characterisation and structural elucidation due to its high sensitivity, resolution and

detection specificity. Moreover, MS can produce accurate mass, unfragmented and fragmented data, which is important for compound characterisation and structural elucidation. The generated data was further analysed using multivariate statistical models such as principal component analysis (PCA) and XCMS (an acronym for various forms (X) of chromatography mass spectrometry).

The results revealed that plants receiving excess UV light possess a wide variety of secondary metabolites as compared to non-treated counterparts. Wide range of metabolites was noted in the treated sample (**Chapter 3**). This is due to the formation of new metabolites from existing ones. In addition, the computed PCA score plot and cloud plot provided a significant visual representation of the differences between leaf extracts of *B. pilosa* before and after UV-light exposure.

Most of the secondary metabolites containing cinnamic acid are prone to UV-induced geometrical isomerisation. One of such examples is verbascoside which exist with its structurally related isomer known as isoverbascoside due to caffeoyl migration from 4′ to 6′ positions on the glucose moiety of the phenylethanoid derivative. Herein, we show through LC-MS analysis of wild growing plant (*L. javanica*) that plants synthesise verbascoside and isoverbascoside in a *trans* conformation, but due to excessive UV-radiation, these compounds produce *cis* geometrical isomers (**Chapter 4**). The results also showed that the formation of positional and geometrical isomers in plants is a well-coordinated biochemical process with biological consequences that still need to be investigated.

Additionally, *B. pilosa, C. rehmannii and H. kraussii* were all shown to possess flavonoids with cinnamic attached to the structural backbone (**Chapter 5**). As in chlorogenic acids, the cinnamic acid moiety of these molecules were also shown to undergo positional isomerisation. Selected plant species produced flavonoids of the same chemical background (i.e quercetin or kaempferol), but the number of positional isomers differs between species owing to HCAs acylation, suggesting that this phenomenon could be used as a taxonomical marker. Most of the identified flavonoids were acylated by caffeic or coumaric acid.

In conclusion, this study further demonstrated LC-MS and multivariate statistical data analysis as effective for comparing metabolite profiles from UV-radiated and non-radiated leaf extracts. The UV-induced geometrical isomeric forms of HCA derivatives

were also efficiently discriminated based on exact mass and fragmentation differences observed in their mass spectral profiles. In addition, plants use isomerisation as a way of diversifying secondary metabolites.

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orange indicates the presence of hydroxycinnamic acid (Caffeic acid) at *m/z* 179 and 135 (Decarboxylated caffeic acid).

Figure 5.4: Representative fragmentation pattern of Kaempferol-3-*p*-coumaroylglucoside. Red cycle indicates the presence of kaempferol at *m/z* 285 and orange indicates the presence of hydroxycinnamic acid (Coumaric acid) at *m/z* 163 and 145.

Figure 5.5: Representative fragmentation pattern of quercetin-3-*p*-coumaroylglucoside. Orange colour indicate the presence of quercetin at *m/z* 300 and 301.

Figure 5.6: Representative electron spray ionisation (ESI) negative spectrum showing the fragmentation pattern of different HCAs conjugated to flavonoids glycoside. Quercetin-3-*O*-caffeoylglucoside (**A**), Kaempferol-3-*O*-caffeoylglucoside (**B**), Kaempferol-3-*p*-coumaroylglucoside (**C**) and Quircetin-3-*p*-coumaroylglucoside (**D**).

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List of Abbreviations and Acronyms

1,3-*di*CQA	1,3-*di*-caffeoylquinic acid
1,5-*di*CQA	1,5-*di*-caffeoylquinic acid
3,4-*di*CQA	3,4-*di*-caffeoylquinic acid
3,5-*di*CQA	3,5-*di*-caffeoylquinic acid
4,5-*di*CQA	4,5-*di*-caffeoylquinic acid
4CL	4-*p*-coumaric acid Coenzyme A ligase
AN1	Anthocyanin 1 transcription factor
AN2	Anthocyanin 2 transcription factor
ANOVA	Analysis of variance
ANS	Anthocyanidin synthase
API	Atmospheric pressure ionisation
BPI	Base peak intensity
C3H	*p*-coumarate 3-hydroxylase
C4H	Cinnamic acid-4-hydroxylase
CDL	Curved desolvation line
CE	Capillary electrophoresis
CE	Collision energy
CGAs	Chlorogenic acids
CHI	Chalcone isomerase
CHS	Chalcone synthase
CID	Collision-induced dissociation
CID	Collision induced dissociation
CP	Cloud plot
CRA	Chicoric acid
CTA	Caftaric acid
DFR	*di*Hydroflavonol 4-reductase
diCQA	*di*Caffeoylquinic acid
DL	Desolvation line

ESI	Electron spray ionisation
F3′H	Flavonoid 3′ hydroxylase
F3H	Flavanone 3-hydroxylase
FLS	Flavonol synthase
GC	Gas chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HCA	Hydroxycinnamic acid
HIV	Human immunodeficiency virus
HQT	Hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase
HTC	Hydroxycinnamoyl transferase
IR	Infrared
isoVB	Isoverbascoside
IT	Ion trap
LC-MS	Liquid chromatography-mass spectrometry
LC-qTOF-MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
MALDI	Matrix-assisted laser desorption ionisation
MSI	Metabolomics Standards Initiative
MVDA	Multivariate data analysis
NMR	Nuclear Magnetic Resonance
OPLS-DA	Orthogonal projection to latent structures-discriminant analysis
PAL	Phenylalanine ammonia lyase
PCA	Principal component(s) analysis
PLS	Partial least squares
PPP	Phenylpropanoid pathway
PPP	Pentose phosphate pathway
Q	Quadrupole
QA	Quinic acid

Rt	Retention time
RCF	Relative centrifugal force
TAL	Tyrosine ammonia lyase
TOF	Time of flight
UHPLC-qTOF-MS/MS	Ultra-high-performance liquid chromatography quadrupole time of flight
UV	Ultraviolet
VB	Verbascoside

List of Units

Dalton	Dalton
Da	Dalton
°C	Degree Celsius
eV	Electron volts
G	Gram
kV	Kilo volts
L/min	Litre per minute
m/v	Mass per volume
µL	Microlitre
µL/s	Microlitre per second
µm	Micrometre
mL	Millilitre
mL/min	Millilitre per minute
Mm	Millimetre
m/z	Mass to charge ratio
Ppm	Parts per million
%	Percent
Rpm	Revolution per minute
S	Seconds
v/v	Volume per volume

CHAPTER 1: GENERAL INTRODUCTION

1.1. Background

Humans have used medicinal plants for centuries for their medicinal and nutritional properties (Raskin *et al*., 2002). Many herbal drugs have been used to treat different diseases (Dhiman and Chawla, 2005). Therefore, medicinal plants can be a possible source of new alternative drugs to treat various conditions. Certain plants such as *B. pilosa, C. rehmannii* and *H. kraussii)* have been successfully used to treat and manage various diseases with minimal scientific backing. However, evidence has shown that some plants possess anti-inflammatory (Maione *et al*., 2016) and antioxidant (Fierascu *et al*., 2018) properties which can be helpful in the management of diseases such as diabetes. These are due to important biologically-active metabolites (Rubió *et al*., 2013).

Plant metabolites are small molecular weight molecules that are classified as primary and secondary metabolites based on their functions (Erb and Kliebenstein, 2020). Primary metabolites are used mainly for growth and development, while secondary metabolites are used to adapt to changing environments (Tiwari and Rana, 2015). Plant secondary metabolites are present in relatively minor concentrations under nonstressed conditions. However, these metabolites exhibit multiple pharmacological and medicinal properties against diabetes (Kawser Hossain *et al*., 2016), hypertension (Bose *et al*., 2019), obesity (Hamidpour *et al*., 2014) and syphilis (Anand *et al*., 2019). The health properties of this plant are a result of its rich phytochemical composition with examples of these secondary metabolites being phenolic compounds such as chlorogenic acids and flavonoids.

Due to excessive light from the depletion of the atmospheric ozone layer, plants are developing a chemical defensive mechanism to withstand the negative impact of extreme ultraviolet (UV) light exposure (Moyo *et al*., 2022). As such, plants are developing new metabolites and isomers from existing ones. Isomerism is the phenomenon in which more than one compound has the same chemical formula but different chemical structures whereas isobars are compounds of different elements with different atomic numbers but with the same mass number. The presence of isomers produces similar spectra and poor resolution. However, advanced analytical

approaches such as liquid chromatography-mass spectrometry (LC-MS) have been applied to distinguish between geometrical isomers of closely related molecules (Nobela *et al*., 2018).

LC-MS is one of the most widely used tools because it is highly sensitive and can cover a wide range of metabolites in a biological system (Zhou and Yin, 2016). LC-MS can also be combined with online tools such as PCA to interpret sophisticated data (Inoue *et al*., 2016). PCA allows for visualization of similarities and differences between samples by reducing the dimensionality of the dataset but preserving variance in the data. In this study, LC-MS was used to characterise closely related metabolites between various plants. Furthermore, metabolomics approaches such as multivariate data analysis statistical tools were followed to interpret the generated data.

1.2. Hypothesis

Geometrical isomers of plant metabolites are produced due to UV radiation, and UHPLC-QTOF-MS is a feasible technique to confirm their presence and identity with high confidence.

1.3. Aim

To develop a reliable LC-MS-based method for identifying and characterising positional and UV-induced geometrical isomers from *L. javanica, H. procumbens, B. pilosa*, *C. rehmannii* and *Helichrysum kraussii* plant extracts.

1.4. Objectives

1.4.1. To investigate the distribution patterns of HCA derivatives in different plants.
1.4.2. To characterise and differentiate isomeric forms of cinnamic acid containing metabolites in plants through UHPLC-QTOF-MS.
1.4.3. To investigate the effect of UV light in plant metabolites using a statistical analysis approach.
1.4.4. To evaluate the presence of UV-induced geometrical isomers in naturally growing leaves extracts.

1.5. References

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LC-MS and NMR in foodomics. *Journal of the Science of Food and Agriculture,* 96, 3876-3881.

CHAPTER 2: LITERATURE REVIEW

2.1. Medicinal plants and their chemistry

Medicinal plants are used worldwide for different therapeutic properties attributed to different chemical constituents found in various plants (Kavishankar *et al*., 2011). Depending on their functions, plants produce a broad spectrum of metabolites classified as either primary or secondary (Tiwari and Rana, 2015; Ashraf *et al*., 2018). Plant metabolites are small molecular weight molecules that are either intermediate/products of cellular metabolism (Tiwari and Rana, 2015). Primary metabolites, for example, carbohydrates, lipids, and amino acids are used mainly for growth and development (Cuadros-Inostroza *et al*., 2016), whilst secondary metabolites are used for survival in their immediate environment (Böttger *et al*., 2018; Adedeji and Babalola, 2020). Most secondary metabolites have been shown to exhibit pharmacological activities (Teo, 2015; Hussein and El-Anssary, 2018). In most cases, the composition of plant metabolome is directly correlated to its biological activities (Kumar and Pandey, 2013; Falleh *et al*., 2008). Some important biological and pharmacological activities reported from various parts of plant species include anti-cancer, anti-inflammatory, anti-bacterial and wound healing properties (Chen *et al*., 2013; de Moura Sperotto *et al*., 2018; Zhang *et al*., 2018; Atawodi and Atawodi, 2009; Nigro *et al*., 2020). Plants use metabolites to adapt to the changing environment. Mechanisms such as conjugation and isomerisation have been reported as the key players in plant adaptation to environmental stresses (Nengovhela *et al*., 2020).

2.2. Metabolite complexity

Differences in the amounts of secondary metabolites vary from one plant to the next and among different tissues of the same plant (Farah and Donangelo, 2006; Lee and Scagel, 2013). This is due to different factors that affect secondary metabolites biosynthesis, i.e., tissue age, different tissues and environmental conditions (Lee and Scagel, 2013). Environmental conditions (i.e., abiotic and biotic stresses) may increase the biosynthesis of secondary metabolites by exerting pressure in the cellular processes. Depending on the stimulus that triggers the production of different metabolites, distribution will differ among different parts of the plant (CortésRojas *et al*., 2013). Post-harvest processing such as drying, washing, and brewing plant material also influences the composition of secondary metabolites (Farah and

Donangelo, 2006; Liang and Kitts, 2016). In this study distribution of various hydroxycinnamic acid (HCA) derivatives in different plants growing in different environments was investigated.

2.3. Phenolic compounds in plants

Phenolic compounds are well-known phytochemicals found in all plants. These consist of benzoic and cinnamic acids, coumarins, tannins, lignins, lignans, and flavonoids (Khoddami *et al*., 2013). Phenolic compounds are synthesised in plants partly due to ecological and physiological pressures such as pathogen and insect attack, ultraviolet (UV) radiation, and wounding (Lattanzio *et al*., 2006). The basic structural feature of phenolic compounds is an aromatic ring bearing one or more hydroxyl groups. Plant phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule (Khoddami *et al*., 2013; Shahidi and Ambigaipalan, 2015) (Figure 2.1). Variation in phenolic acids is in the number and location of hydroxyl groups on the aromatic ring (Balasundram *et al*., 2006; Singla *et al*., 2019). Phenolic acids have two-parent structures: hydroxycinnamic and hydroxybenzoic acid.

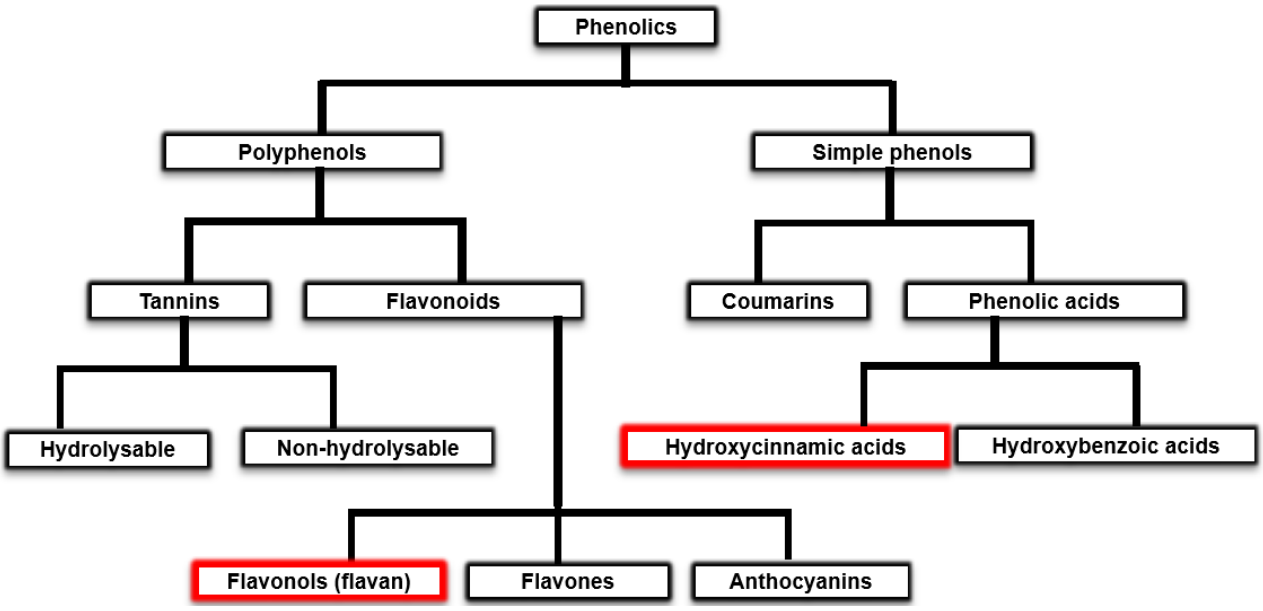

Figure 2.1: Schematic representation of different phenolic compounds arising biogenetically from either phenylpropanoid or the shikimate pathway directly providing phenylpropanoids, which can produce simple phenols and polyphenols (Adapted from Ramawat and Mérillon, (2013)). Red indicates the targeted phenolics in this study.

2.3.1. Hydroxycinnamic acids (HCAs)

HCAs are derived from cinnamic acid precursor molecule and constitute a significant subgroup of phenolic acids. HCAs consist of a chemical backbone of C_6-C_3 with C_3 side chain eighter having *cis* or *trans* configuration and hydroxyl site substituted with different functional groups (Salau *et al*., 2020). HCAs include caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid (Serafim *et al*., 2011) (Figure 2.2). They are present at high concentrations in many food products, such as in tea leaves, cocoa, wine, coffee, vegetables and fruits (El-Seedi *et al*., 2012; Bento-Silva *et al*., 2020). These molecules play a role in the biosynthesis of other molecules such as flavonoids and chalcones and can occur either as a free form or conjugated to other molecules such as quinic acid (QA), tartaric acid, amino acids, peptides and sugar derivatives (Masike *et al*., 2017; Zeiss *et al*., 2021). As a result, other secondary metabolites such as chlorogenic acids (CGAs), caftaric acids (CTAs), and chicoric acids (CRAs) are unevenly distributed across different plants and plant tissues.

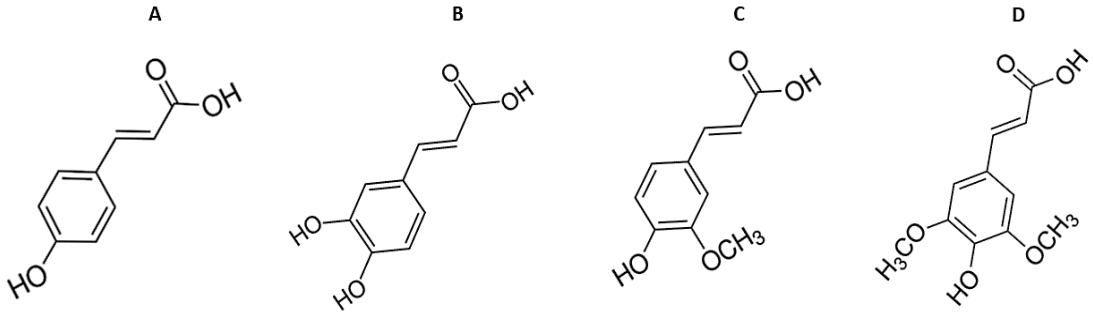

Figure 2.2: Chemical structures showing examples of HCAs derivatives. (**A**) *p*-coumaric acid, (**B**) caffeic acid, (**C**) ferulic acid, and (**D**) synapic acid.

2.3.2. Chlorogenic acids (CGAs)

Chlorogenic acids (CGAs) are a class of polyphenolic compounds distributed widely in plants (Upadhyay and Mohan Rao, 2013). These compounds are biosynthesised through the esterification of HCAs and QA. (Roleira *et al*., 2018). CGAs are grouped according to the number of acyl group/groups attached to QA; for instance, when one HCA is attached, they are referred to as *mono*-CGAs, if two HCAs are attached are referred to as *di*-CGAs and if three HCAs are attached are referred to as tri-CGAs. Due to the chemistry of the QA molecule (number of OH groups in particular), the formation of CGAs can result in several positional isomers (Clifford *et al*., 2003; Clifford *et al*., 2008). Apart from attaching to QA, these HCAs have been shown to also attach

to other molecules such as tartaric acid, citric acid and sugars (Masike *et al*., 2017; Nobela *et al*., 2018), thereby resulting in structurally diverse metabolomes characterised by positional isomers, geometrical isomers (*trans/cis*) and differential conjugation to various molecules. Attachment of HCA to other molecules such as flavonoids has been reported elsewhere (Nengovhela *et al*., 2020). Interestingly, the nature and chemistry of HCA derivatives have been shown to be plant-specific; as such, CGAs have been used as chemotaxonomic markers (Masike *et al*., 2017).

2.3.2.1. Biosynthesis of chlorogenic acids

There are various pathways responsible for the biosynthesis of CGAs. These include phenylpropanoid pathway (PPP) (e Silva *et al*., 2019), shikimate pathway (Kulik *et al*., 2017) and pentose phosphate pathway (PPP) (Jacobo-Velázquez and Cisneros-Zevallos, 2012). The main pathway responsible for biosynthesis of wide range of CGAs is PPP where the precursors are phenolic amino acids (phenylalanine or tyrosine and sometime tryptophan) (Figure 2.3). Generally, the first step in the PPP pathway is the breakdown of amino acids through the removal of the amino group. The carbon skeletons of the amino acids undergo further reactions to form compounds that can be used to synthesise CGAs. The enzyme tyrosine ammonia lyase (TAL) catalyses the deamination of tyrosine (Vogt, 2010) while phenylalanine ammonia lyase (PAL) catalyses the deamination of phenylalanine. As a result, *trans* cinnamic acid is formed with the C6-C3 skeleton, a basic structure of phenylpropanoids. The second step is the addition of hydroxyl group to the *trans* cinnamic acid and this reaction is catalysed by cinnamic acid-4-hydroxylase (C4H, a cytochrome P450) (Koshiro *et al*., 2007). As a results, 4-*p*-coumaric acid is formed. 4-*p*-coumaric acid is then converted to *p*-coumaroyl-CoA which is used to synthesise phenylpropanoids downstream by an enzyme 4-*p*-coumaric acid Coenzyme A ligase (4CL). The biosynthesis of chlorogenic acids downstream is still unrevealed, however, various pathways have been reported, such as shikimate and quinate pathways. The Quinate pathway involves hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) and enzymes in transesterification of HCAs and QA molecule (Moglia *et al*., 2014; Zhang *et al*., 2017). Shikimate pathway uses hydroxycinnamoyl transferase (HTC) enzyme and *p*-coumarate 3-hydroxylase (C3H) to convert *p*-coumaroyl shikimic acid to chlorogenic acid. HTC and C3H have been reported to be active on species that cannot

accumulate CGAs (André *et al*., 2009; Aseel *et al*., 2019) and overexpression of HQT in tomato was found to increase the accumulation of CGAs (Niggeweg *et al*., 2004). Elsewhere, *in vitro* studies of HCT showed that it can synthesise the 3,5-*O*-*di*-caffeoylquinic acid diester; however, the chemistry behind it is not known (Lallemand *et al*., 2012).

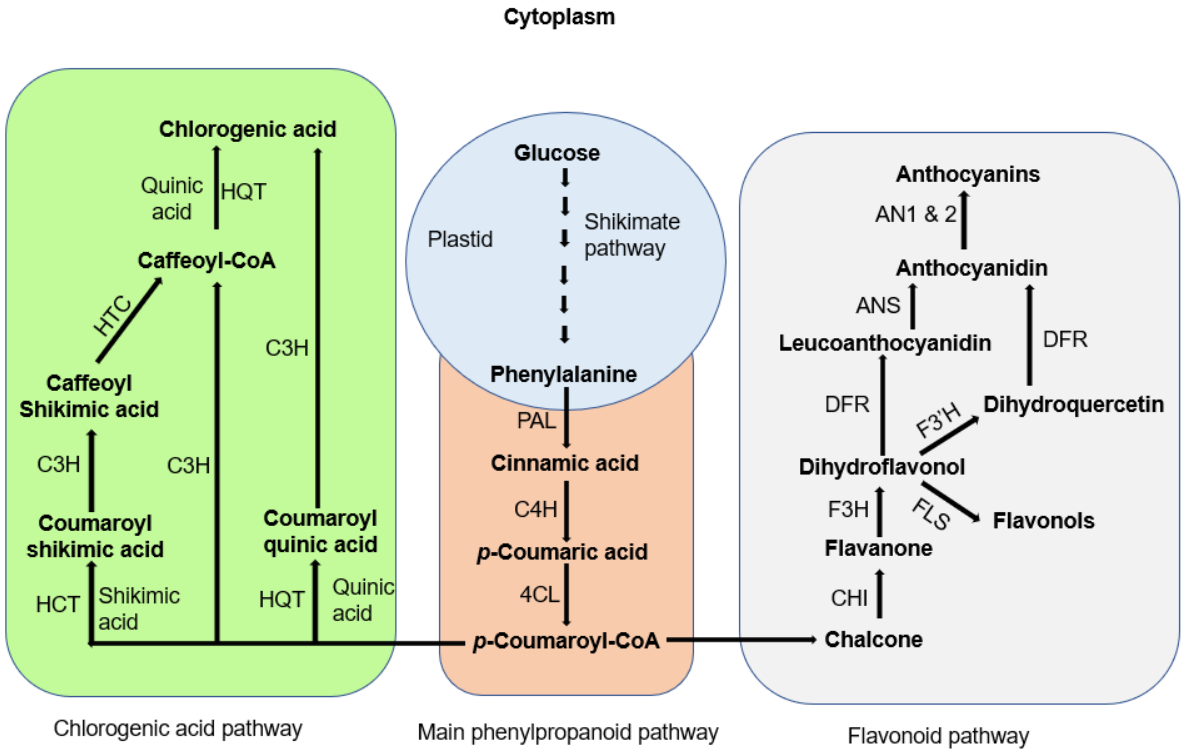

Figure 2.3: Schematic representation of proposed biosynthesis pathways of CGAs and flavonoids through phenylpropanoid pathway. Enzymes involved in biosynthesis of CGAs are: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-hydroxycinnamoyl CoA ligase (4CL), *p*-coumarate 3'-hydroxylase (C3H), hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (HQT), hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) and cinnamate 3-hydroxylase (C3H). Enzymes responsible for flavonoid pathway are; chalcone isomerase (CHI), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), flavonoid 3′ hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), anthocyanin 1 transcription factor (AN1) and anthocyanin 2 transcription factor (AN2) (adapted from Aseel *et al*., (2019)).

2.3.2.1. Medicinal and biological importance of chlorogenic acid

CGAs are demonstrated to have anti-human immunodeficiency virus (HIV) properties by binding to DNA integrase enzyme. As a result, the integration of DNA into the genome host is inhibited and HIV replication is also inhibited (Makola *et al*., 2016;

Zheng *et al*., 2017). In studies performed *in vivo* and *in vitro* CGAs, have been reported to prevent chronic and cardiovascular diseases (Meinhart *et al*., 2017). These acids have also been reported to regulate glucose and lipid metabolism (Ong *et al*., 2013). They may also confer protection of neurons as CGAs prevent non-enzymatic glycosylation (Meinhart *et al*., 2017). Other reported activities include antioxidant activity (Budryn *et al*., 2015), anti-inflammatory activity (Jaisankar and Arivarasu, 2020), cardiovascular activity (Li *et al*., 2020), hepatoprotective activity (Farah and de Paula Lima, 2019), renoprotective activity (Domitrović *et al*., 2014), neuroprotective effects (Kwon *et al*., 2010), prevention of atherosclerosis (Rouanet *et al*., 2010), scavenging free radicals (Zhang *et al*., 2013) and antiamyloidogenic effects (Porzoor *et al*., 2015). Other Medicinal and biological importance of chlorogenic acid is summarized in figure 2.4.

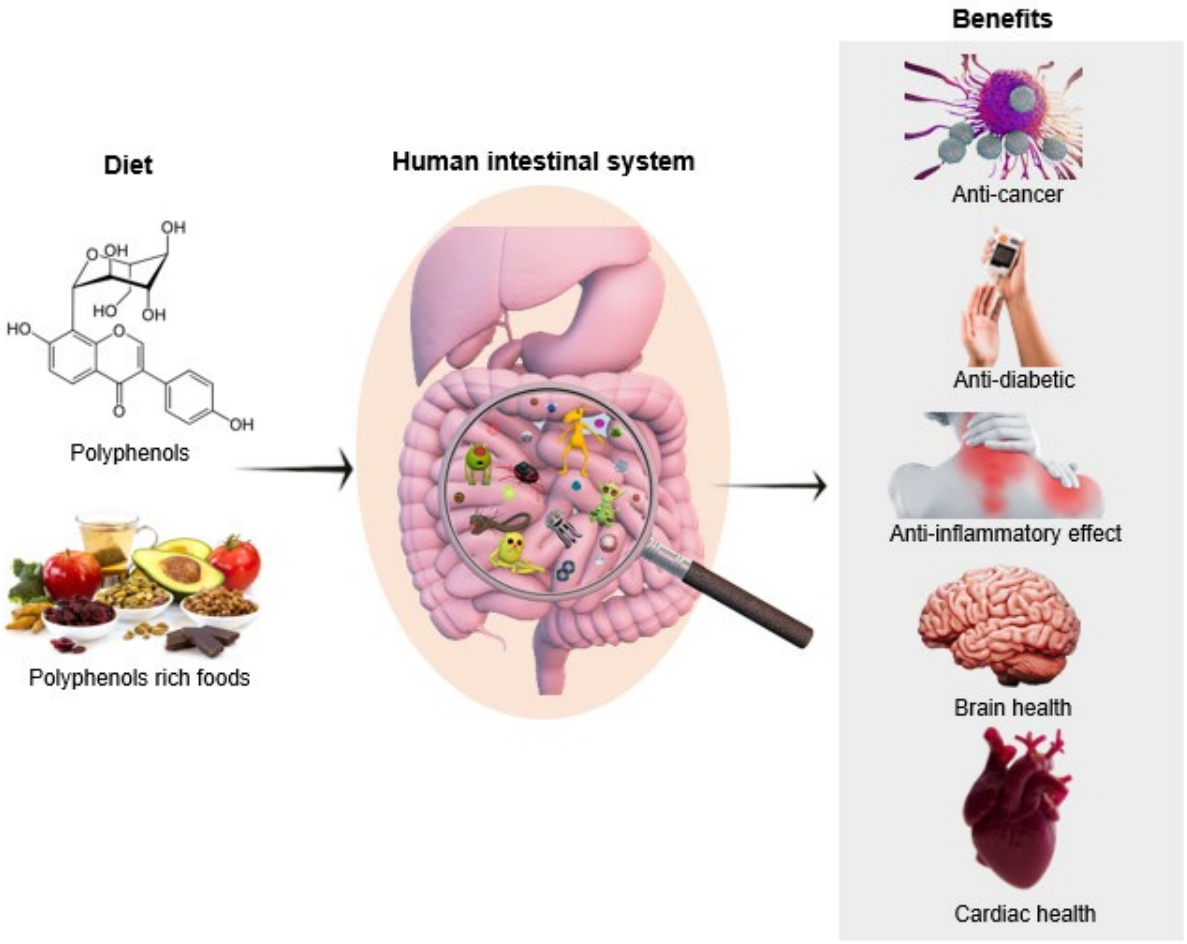

Figure 2.4: Other reported activities of CGAs (adapted from Aravind *et al*., (2021)).

2.3.3. Chicoric and caftaric acids

Similar to the esterification of HCAs to QA, HCAs can also be esterified to tartaric acid to form chicoric acid (CRA) or caftaric acid (CTA). CRA is formed by esterification of two HCA with tartaric acid (Figure 2.5a) whereas CTA formed by esterification of only one HCA with tartaric acid (Nobela *et al*., 2018) (Figure 2.5b). The abundant CRA is *di*-caffeoyltartaric acid. However, different HCAs can also esterify with tartaric acid to form vast chicoric acids i.e., caffeoyl coumaroyltartaric acid, feruloyl coumaroyltartaric acid and caffeoyl feruloylctartaric acid. CRA is reported in *Bidens pilosa* leaves and other plants *Asteraceae* family (Nüsslein *et al*., 2000). In *Echinacea purpurea*, CRA is one of the major phenolics. CTA has been reported in grapes (Khoza *et al*., 2016).

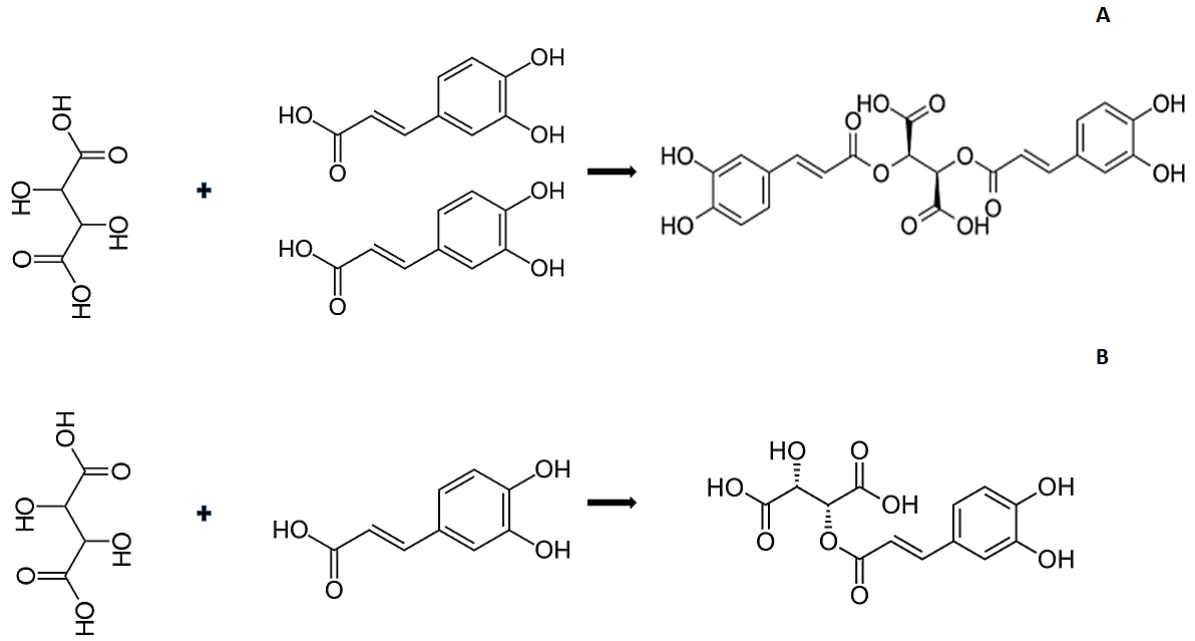

Figure 2.5: Schematic representation showing the formation of (**A**) chicoric acid from tartaric acid and two HCAs (dicaffeic acid) and formation of (**B**) caftaric acid from tartaric acid and HCA (caffeic acid).

2.3.3.1. Medicinal and biological importance of chicoric acid (CRA)

CRA is a phenolic compound with a wide range of biological activities such as antiviral, immunostimulatory, and antioxidant properties (Khoza *et al*., 2016; Kuban-Jankowska *et al*., 2016). CA is an essential hydroxycinnamoyl-tartaric acid ester that plants produce to protect themselves from biotic and abiotic stressors (Lee and Scagel, 2013). Similarly, to CGA, one of the important antiviral activities of CRA is the potential to inhibit HIV-integrase and inhibit replication of HIV (Reinke *et al*., 2004). CRA has

been shown to exhibit anti-diabetic properties as it improves glucose uptake by up-regulating the secretion of insulin and antihyperglycemic effects (Lee and Scagel, 2010). The tissue distribution of CRA in rats after gavage administration showed decreasing tendency in different tissues such as lung, kidney, heart, and brain (Wang *et al*., 2017). It also prevents methotrexate-induced kidney injury by suppressing NF-κB/NLRP3 inflammasome activation and up-regulating Nrf2 signalling (Abd El-Twab *et al*., 2019). CGAs exposure was observed to reduce neurological deficits and infarct volume in rats with cerebral ischemia-reperfusion injury (Liang *et al*., 2018). Other biological activities include anti-hepatitis B virus properties (Zhang *et al*., 2014). Elsewhere, CA has been experimentally proven to suppress the production of reactive oxygen species (Liu *et al*., 2017). CA also shows a reduction of telomerase activity and induction of apoptosis, resulting in an inhibitory effect towards colon cancer cells (Tsai *et al*., 2017). Other biological activities of CA include *in vivo* and *in vitro* stimulation of phagocytosis and the inhibition of hyaluronidase, an important enzyme in bacterial infections, thus inhibiting bacterial infections (Kuban-Jankowska *et al*., 2016).

2.3.3.2. Medicinal importance of caftaric acid

caftaric acid (CTA) is known as a phenolic derivative, and it is present in high concentrations in grape seeds and juice. It is also reported to induce coupled oxidation of other compounds (Garcia-Jares *et al*., 2015). The CTA quickly passes to the stomach and duodenum and increases the absorption of the acid in the intestinal cells. Like chicoric acid, it has an anti-inflammatory effect and antimutagenic effect in an animal model. CTA has a double effect by decreasing high blood glucose and high blood pressure so that this acid can treat diabetes and hypertension (Chukwuma *et al*., 2019).

2.3.4. Flavonoids

Flavonoids are a group of phytochemicals belonging to a class of secondary metabolites and are the most abundant and ubiquitous group of phenolic compounds identified in various flowering plants (Giada, 2013). Structurally, flavonoids are composed of two benzene rings (A and B) linked by a heterocyclic ring containing oxygen (C) (Figure 2.6) (Halbwith, 2010). These metabolites are synthesised through phenylpropanoid pathway and classified as either flavone (e.g. luteolin), isoflavones

(e.g. genistein), anthocyanidine (e.g. cyaniding), chalcones (e.g. okanin) and flavonols (e.g. kaempferol) (Figure 2.3) (Escobar-Cévoli *et al*., 2017; Kumar and Pandey 2013). Flavonoids occur widely as aglycones (flavonoids that are not conjugated). However, they usually exist as a complex mixture with different conjugates (e.g. sugars and organic acids), which dissolve in plasma or become methylated, making them lipophilic (Végh *et al*., 201).

2.3.4.1. The flavonoid biosynthetic pathway

Biosynthesis of flavonoids also starts from the phenylpropanoid pathway, where the final product is *p*-coumaroyl-CoA (Yu and Jez, 2008). This molecule enters the flavonoid pathway as chalcone through a condensation reaction with the aid of transcription factor chalcone synthase (CHS). The enzyme chalcone isomerase (CHI) then isomerises chalcone to flavone (naringenin) and then hydroxylated to produce dihydroflavonol through flavanone-3-hydroxylase (F3H). Dihydroflavonol becomes hydroxylated again to produce dihydroquircetin. These molecules, together with dihydrokaempferol are catalysed by flavonol synthase (FLS) to produce flavonols (quercetin and kaempferol). Dihydroflavonol can also undergo reduction to produce leucoanthocyanidin with the aid of enzyme dihydroflavonol-4-reductase (DFR) and becomes anthocyanidin through anthocyanidin synthase (ANS) (Figure 2.3) (Schijlen *et al*., 2004; *Bovy et al*., 2007; André *et al*., 2009; Aseel *et al*., 2019).

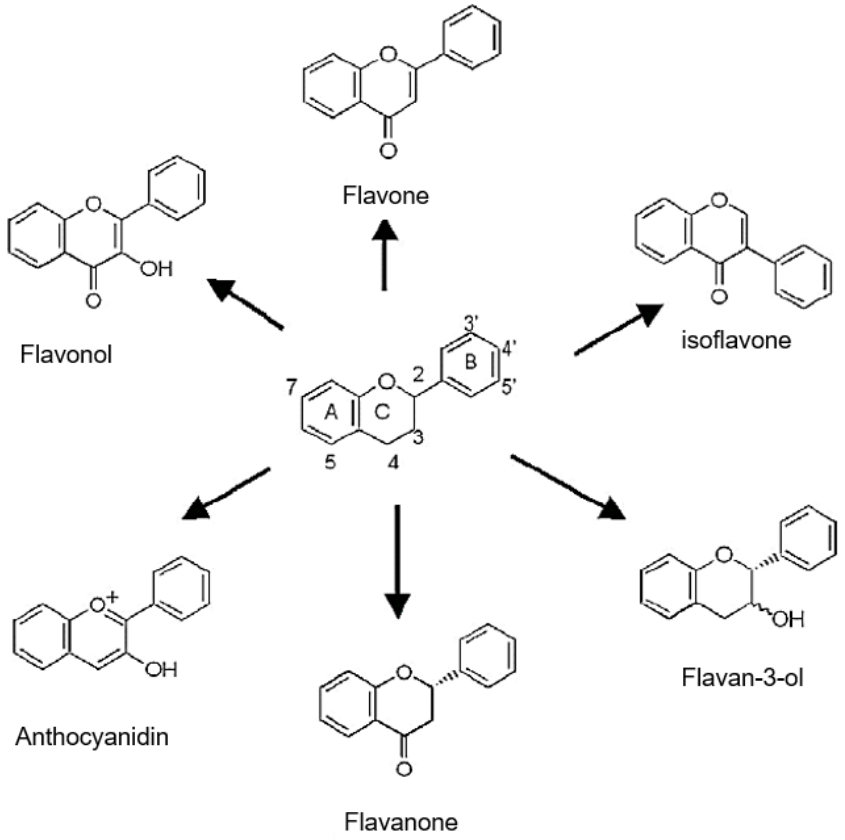

Figure 2.6: Schematic representation of chemical structures of sub-classes of flavonoids (adapted from Nishiumi *et al*., (2011)).

2.3.4.2. Pharmacological properties of flavonoids

Flavonoids have been reported to have different pharmacological activities such as anti-oxidant (Agati *et al*., 2020; Wang *et al*., 2017). The pharmacological activities of flavonoids from various sources also show that the flavonoids could provide a scaffold for developing potent anti-cancer drugs in the future (Martinez-Perez *et al*., 2014; Ekalu and Habila, 2020). Elsewhere, neohesperidin was reported potentially as an anti-aging citrus flavonoid (Guo *et al*. 2019). Consequently, numerous studies *in vitro* and in animal models have found that flavonoids have the potential to inhibit the onset and development of inflammatory diseases (Serafini *et al*., 2010; Rathee *et al*., 2009). Some dithiocarbamic esters bearing a flavanone backbone display good inhibitory properties against both Gram-positive and Gram-negative pathogens (Bahrin *et al*., 2014; Farhadi *et al*., 2019). Other properties include inhibiting cellular proliferation by modulation of multiple signaling pathways and via activation and down-regulation multiple genes (Youns and Abdel Halim Hegazy, 2017). Other studies showed that high intake of flavonoids reduces the risk of many diseases such as cardiovascular disease (David *et al*., 2016).

2.4. Isomerisation of phenolic compounds

2.4.1. Positional isomerisation

Positional isomerisation is a chemical modification that diversify metabolome by attaching different functional groups/molecules in different positions of a donor molecule (Xie *et al*., 2011). As a result, different molecules with the same molecular formula and atomic mass are formed with the difference in the position of acylation. Plants produce a wide range of metabolites through specialised metabolism, of which some are prone to positional isomerisation (Facchini *et al*., 2012). A common example of such metabolites is CGAs due to the presence of QA molecule that esterifies HCAs at different carbon positions (C3, C4, C5 and rarely C1). Three possible positional isomers of mono CGA can be formed (Arai *et al*., 2015; Ramabulana *et al*., 2020). Plants further amplifies their metabolome as *di*-CGAs and *tri*-CGAs can also be formed with multiple possible positional isomerism (Clifford *et al*., 2005; Ren *et al*., 2018). Elsewhere, *di*-caffeoylquinic acid has been reported form multiple positional isomers (Figure 2.7) and this structural diversity poses an analytical challenge (Zheng *et al*., 2017).

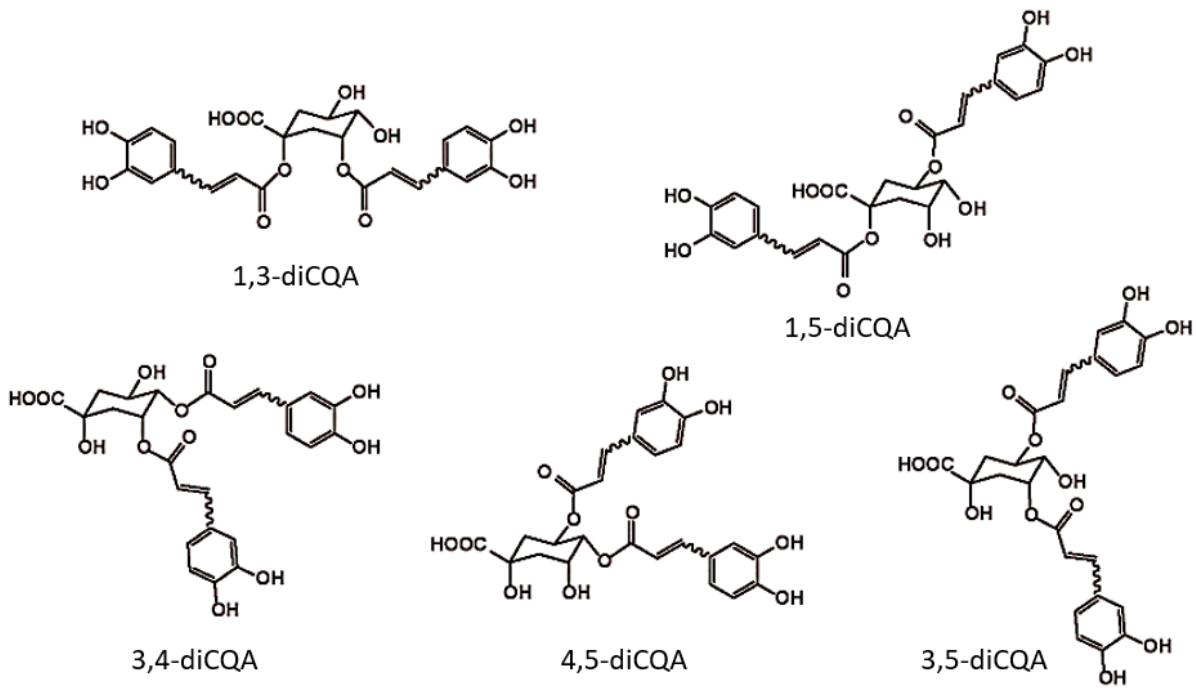

Figure 2.7: Characterisation of some chemical structures of positional isomers of the dicaffeoylquinic acid 1,3-*di*-caffeoylquinic acid (1,3-diCQA), 1,5-*di*-caffeoylquinic acid (1,5-diCQA), 3,4-*di*-caffeoylquinic

acid (3,4-diCQA), 3,5-*di*-caffeoylquinic acid (3,5-diCQA) and 4,5-*di*-caffeoylquinic acid (4,5-diCQA) (adapted from Zheng *et al*., (2017)).

2.4.2. Geometrical isomerisation and its biological significance

Geometric isomers have the same structural formulas but differ in the arrangement of groups at a single atom, at double bonds, or in rings (Trinquier, 1990). One of the optical isomers rotates the light in one direction (*cis* configuration), the other rotates the light in the opposite direction but by the same amount (*trans* configuration). The modification of chemical structures resulting from geometrical isomers poses high complexity of plant metabolome. Naturally, plant produces *trans* metabolites. However, due to UV radiation, *cis* isomers are produced (Ncube *et al*., 2014). To demonstrate an example of metabolite diversity caused by geometrical isomerisation (Figure 2.8) showing HCAs undergoing geometrical isomerisation. This increases metabolite complexity and the information about enzymes responsible for photo isomerisation is still unknown.

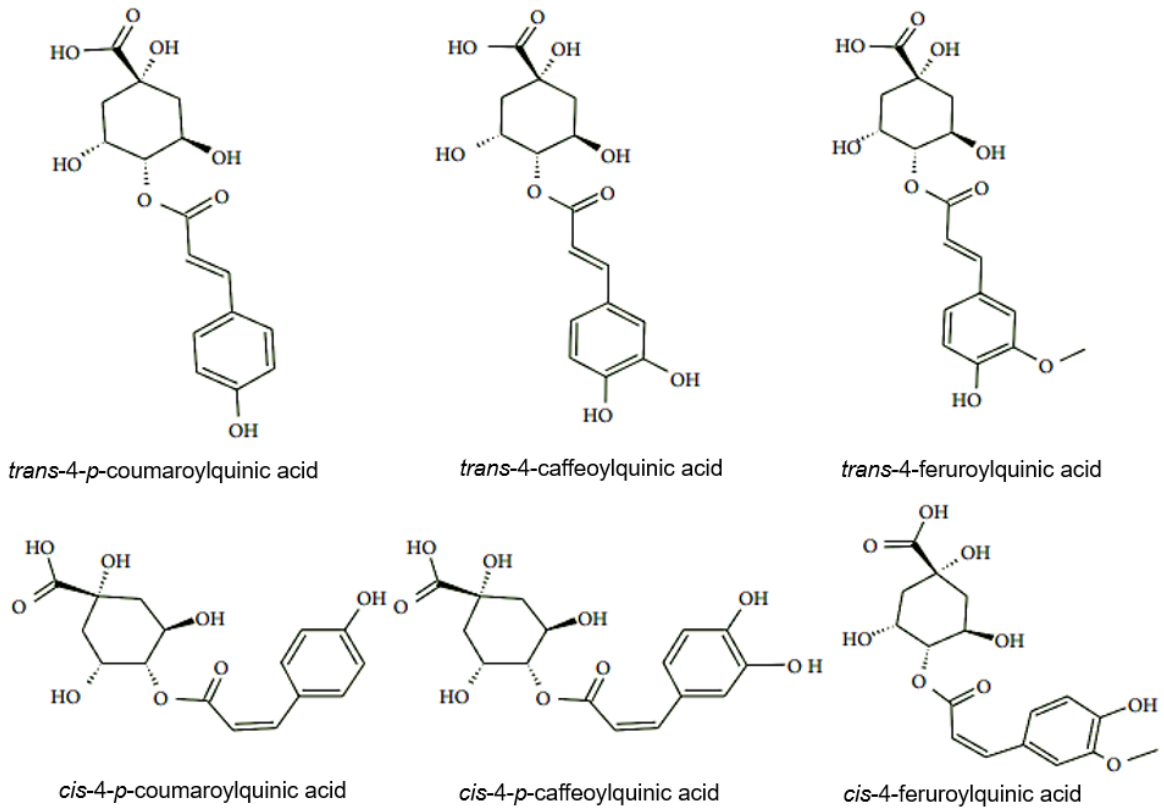

Figure 2.8: Chemical structures of geometrical isomers of chlorogenic acids (adapted from Madala *et al*., (2014)).

2.4.3. Glycoisomerisation of HCAs derivatives and different sugar moiety

A phenomenon known as glycoisomerisation is chemical modification in which molecules are acylated to different sugar moieties such as *di* and *tri*-saccharides. Previous studies reported that different sugar moieties can be conjugated to flavonoids, and multiple isomers can be formed due to a shift in position of acylation (Gonzales *et al*., 2014; Akhlaghi and Foshati, 2017) (Table 2.1). Elsewhere, glycosylation patterns of flavonoids were reported as an evolutionary strategy used by plants to diversify its metabolome and the biological effect of this isomers is not revealed (Nengovhela *et al*., 2021). Other molecules such as HCAs can further conjugate to flavonoid glycoside complex (Selma *et al*., 2009). In the presence of UV light, glycoisosemerisation can also be influenced by geometrical isomerisation. Therefore, developing an analytical approach accounting for both possible isomers is noteworthy.

Table 2.1: Glyco-isomers identified from *C. grandis* and *C. rehmannii* (adapted from Nengovhela *et al*., (2021)).

Retention time (Rt)	Molecular weight $[M-H]^-$ *(m/z)*	Molecular formula	Molecular name
6.49	739.2096	$C_{33}H_{40}O_{19}$	Kaempferol-3-rhamnoside-7-glucosyl-(1-2)-rhamnoside (Isomer 1)
6.81	739.2157	$C_{33}H_{40}O_{19}$	Kaempferol–3-rhamnoside-7-glucosyl-(1-2)-rhamnoside (Isomer 2)
6.98	609.1503	$C_{27}H_{30}O_{16}$	Quercetin-3-*O*-rutinoside (Isomer 1)
7.30	609.1423	$C_{27}H_{30}O_{16}$	Quercetin-3-*O*-rutinoside (Isomer 2)
11.66	609.1257	$C_{30}H_{26}O_{14}$	Kaempferol-3-(6-caffeoylglucoside) (Isomer 1)
12.01	609.1271	$C_{30}H_{26}O_{14}$	Kaempferol-3-(6-caffeoylglucoside) (Isomer 2)
7.79	593.1565	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*-rutinoside (Isomer 1)
8.50	593.1475	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*-rutinoside (Isomer 2)
9.21	593.1464	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*-rutinoside (Isomer 3)
12.96	593.1282	$C_{30}H_{26}O_{13}$	Kaempferol-3-*p*-coumaroylglucoside (Isomer 1)
12.98	593.1288	$C_{30}H_{26}O_{13}$	Kaempferol-3-*p*-coumaroylglucoside (Isomer 2)

2.4.4. UV-induced geometrical isomers of cinnamic acid containing molecules

The double bond of cinnamic acid allows the switching of the adjacent atoms to rearrange into the same or different side. UV-lights trigger the double bond to switch from *trans* configuration to a *cis* configuration (Figure 2.9). A potent plant-derived HIV-1 inhibitor, 3,5-*di*-caffeoylquinic acid (*di*-CQA), has been shown to undergo isomerisation upon UV exposure where the naturally occurring *trans*-*di*-CQA isomer gives rise to the *cis*-*di*-CQA isomers (Makola *et al*., 2016). The behavior of *cis* isomers of selected mono- and *di*-acyl chlorogenic acids produced by UV-irradiation has been investigated by LC–MS^n and showed the same fragmentation pattern (Clifford *et al*., 2008). Little on the effects of these geometrical isomers is known, so it is mandatory to account for different geometrical isomers. Previously, various approaches such as metal adducts have been used to differentiate geometrical isomers. However, the accurate discrimination of the geometrical isomers of these molecules has proven to be an elusive task (Makola *et al*., 2016; Zheng *et al*., 2017).

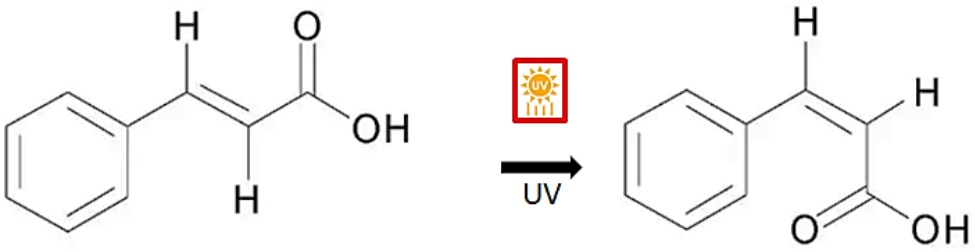

Figure 2.9: Schematic representation of geometrical isomerisation of cinnamic acid from *trans* to *cis* configuration due to UV radiation.

2.5. Metabolomics: A combination of traditional and emerging analytical tools

Metabolomics is a comprehensive approach used to describe experimental designs and strategies of global metabolite profiling of the entire metabolome. It represents the global assessment of metabolites in a biological sample and reports the closest information to the phenotype of the biological system under study (León et al., 2013; Tugizimana *et al*., 2013) (Figure 2.10). It focuses on discovering and quantifying metabolites from various organisms. Metabolomics has been used in different fields of research, such as cell biology (Chiarugi *et al*., 2012; Halama *et al*., 2013; DeBerardinis and Thompson, 2012), the discovery of new therapeutic targets (Altiné-Samey *et al*., 2021), toxicity (Booth *et al*., 2011; Ramirez *et al*., 2018), nutrition

(González-Peña and Brennan, 2019), oncology (Gao *et al*., 2021), diagnostic of different diseases (Wilkins and Trushina, 2018; Snowden *et al*., 2012) and biomarkers discovery (Lewis *et al*., 2008). It uses advanced, selective, and highly sensitive instruments to provide a global snapshot of all small molecules in cells and biological fluids (Worley and Powers, 2013). Such analytical instruments include Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography-Mass Spectrometry (GC-MS), Nuclear Magnetic Resonance (NMR) Spectrometry, and Infrared (IR) spectroscopy (Kim *et al*., 2011; Tugizimana *et al*., 2013).

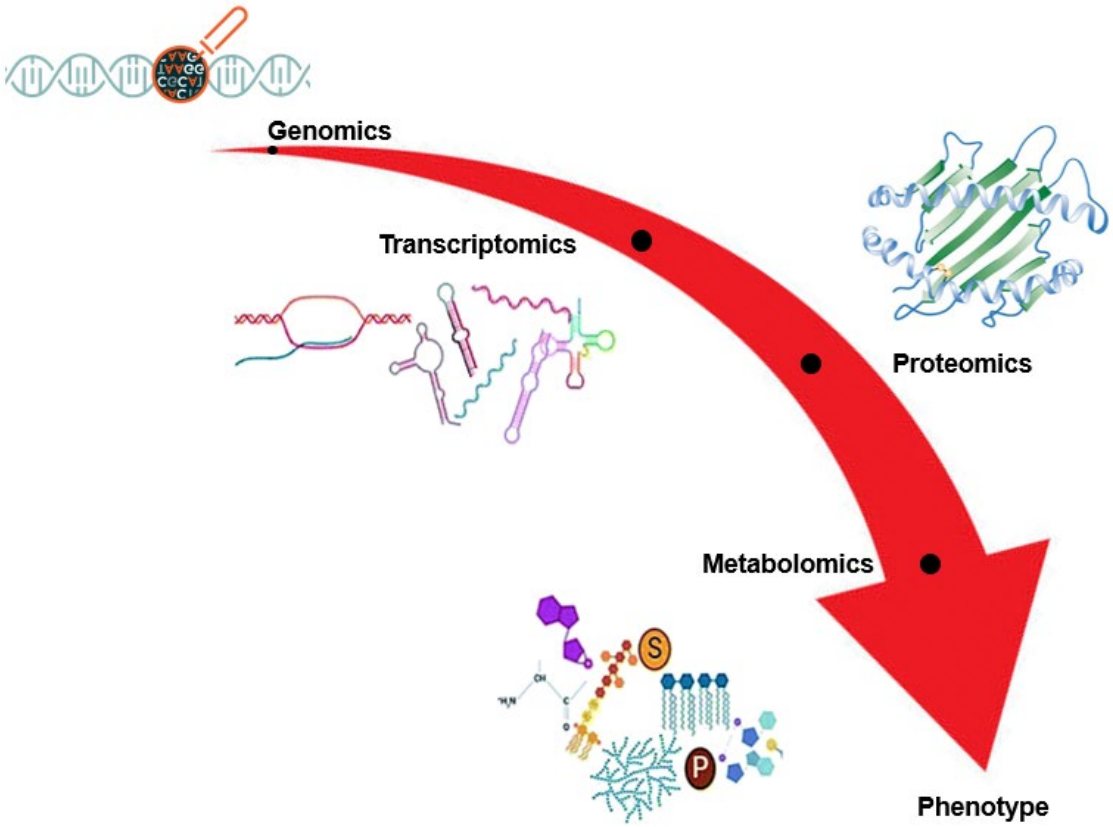

Figure 2.10: Schematic representation of the "omics" the flow of biological information from genomics to metabolomics (adapted from Muthubharathi *et al*., (2021)).

2.5.1. General metabolomics workflow

The metabolomics workflow includes various experimental steps such as sample preparation, sample processing, data acquisition, data mining and interpretation (Pezzatti *et al*., 2020; Tugizimana *et al*., 2013) (Figure 2.11). The most prerequisite for any metabolomic experiment or study is a good experimental design from sample preparation and a suitable choice of sample processing (Kim and Verpoorte, 2010;

Gong *et al*., 2017). The plant metabolome is diverse due to highly diverse chemical nature of metabolites (Tugizimana *et al*., 2013; Creydt *et al*., 2018). Therefore, no single equipment nor ptotocol can define the whole metabolome. Combining two or more metabolomic strategies helps to achieve comprehensive plant metabolome coverage. Plants produce a wide range of these compounds, posing an undisputed analytical challenge (Makita *et al*., 2017; Masike *et al*., 2017). As a result, a broad metabolic picture is achieved by combining multi parallel and complementary analytical techniques (Hall, 2006; Salem *et al*., 2016; Jorge *et al*., 2016). Different extraction methods give different metabolite profiles. Different instrument is then used to complement each other shortfalls. (Crockford *et al*., 2006; Van Der Kooy *et al*., 2009). The comparison of metabolites from different plants extracts is performed through multivariate data analysis, which quantitatively and qualitatively analyses different peaks across various plant extracts (Shulaev, 2006).

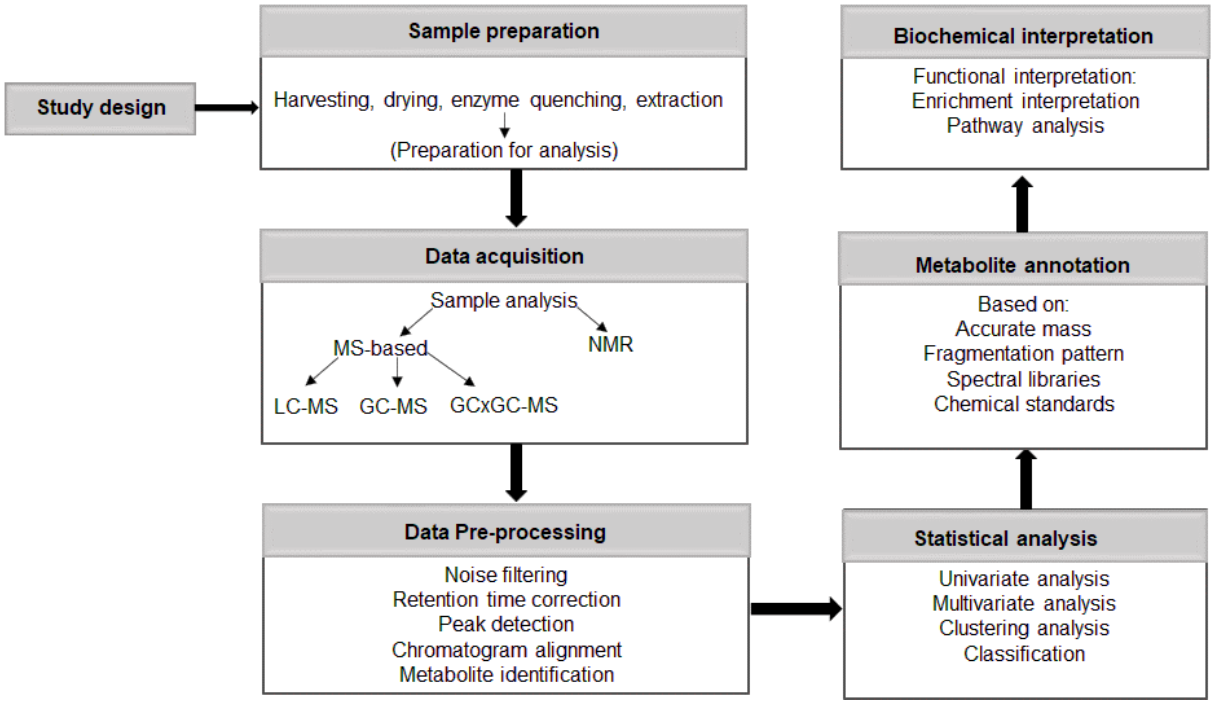

Figure 2.11: A schematic representation of a typical metabolomic study for plant metabolomics studies (adapted from Nephali *et al*., (2020)).

2.5.1.1. Sample preparation

Sample preparation is the first and crucial step in a plant metabolomic study as it entails the extraction of various metabolites (Kim and Verpoorte, 2010; Mushtaq *et al*.,

2014; Rodrigues *et al*., 2019). The steps involved in sample preparation include harvesting samples, enzyme quenching, drying, storage, and metabolites extraction (Kim *et al*., 2010; Ernst *et al*., 2014). Post harvesting, the samples are quenched by shock-freezing in liquid nitrogen. There are various types of metabolite extraction methods such as solid-phase extraction (Jakubus *et al*., 2019), micro dialysis extraction (Randewig *et al*., 2019), supercritical fluid extraction (Bader *et al*., 2020), microwave assisted extraction (Teo *et al*., 2013), liquid-liquid extraction (Liu *et al*., 2018), pressurised liquid extraction (Heavisides *et al*., 2018), ultrasound assisted extraction (Herrera-Pool *et al*., 2021) and pressurised hot water extraction (Khoza *et al*., 2016; Masike *et al*., 2017). Moreover, the choice of analytical technique for separating metabolites can influence the extraction method used (Patejko *et al*., 2017). For plant extracts, phenolic compounds are usually extracted using liquid-liquid extraction methods, using various organic solvents such as ethanol, methanol, and acetone (Garcia-Salas *et al*., 2010). It is important to note that no single approach can extract a wide range of polar and non-polar compounds (Cajka and Fiehn, 2016).

2.5.1.2. Data acquisition/sample analysis (LC-MS approach)

Plants extracts contain vast mixtures of different compounds and this poses an undisputed separation challenge for these compounds before structural elucidation. Standard techniques used for the separation of phenolic compounds are capillary electrophoresis (CE) (Priego-Capote *et al*., 2004; Lima *et al*., 2007), gas chromatography (GC) (Ma *et al*., 2016; Prazeres *et al*., 2021) and liquid chromatography (LC) (Spáčil *et al*., 2008; Setyaningsih *et al*., 2019). Unlike GC, LC has gained broad applications as it has advantages of high resolving capacity, high sensitivity, small sample volume, separation of wide range of both polar and non-polar compounds and ability to couple with other detectors such as mass spectrometer (MS) (Gika *et al*., 2014; De Vos *et al*., 2007) (Figure 2.12). The separation of compounds in LC depends on how analytes/compounds interact with two phases (stationary and mobile). However, various factors affect the separation of analytes i.e., the type of column used (Masike *et al*., 2017a), mobile phase composition (Fountain *et al*., 2010) and the column temperature (Zhou *et al*., 2016; Zhou *et al*., 2017).

There are various ionisation techniques such as atmospheric pressure ionisation (API) (Kostiainen *et al*., 2003; Trimpin and Inutan, 2013.), Electron spray ionisation (ESI)

(Wiseman *et al*., 2008; Wang *et al*., 2017) and matrix-assisted laser desorption ionisation (MALDI) (Bizzini and Greub, 2010). ESI has been deemed the effective ionisation method preference for analysing plant extracts (Yang *et al*., 2009). Ions formed as a result can be positive or negative (indicating the gaining or losing of a hydrogen atom). Generated ions separate according to their *m/z* ratios until reaching the detector. Various mass analysers such as quadrupole (Q), time of flight (TOF), and ion trap (IT) analyses the mass to charge ratios with high accuracy.

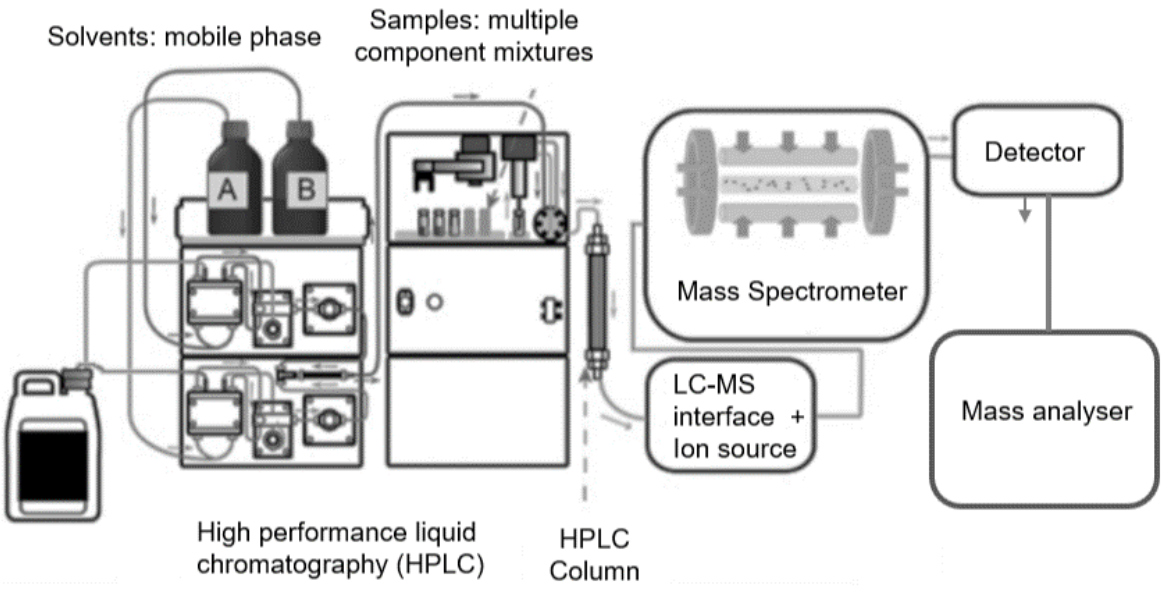

Figure 2.12. Schematic representation of HPLC-MS instrumentation (adapted from https://en.wikipedia.org/wiki/Liquid_chromatography%E2%80%93mass_spectrometry).

2.5.1.3. Data processing

Datasets generated by LC-MS-based metabolomics are very complex. As such, to clean and minimise variations in the generated data, data's pre-processing and pre-treatment steps are necessary. The pre-processing step involves noise filtering, detection of peaks and alignment, peak identification and generation of data table from the MS raw data (Katajamaa and Matej, 2005; Yu *et al*., 2013; Tugizimana *et al*., 2016). Multiple online generating tools can be applied for metabolomic data processing such as MetaboAnalyst (Xia *et al*., 2009; Liang *et al*., 2020) and XC-MS (Tang *et al*., 2021). Two statistical approaches can be applied to metabolomics data:

univariate or multivariate analysis. (Madala *et al*., 2014; Percival *et al*., 2020; Xia *et al*., 2009). The two most popular methods of Multivariate data analysis (MVDA) are principal component(s) analysis (PCA) and partial least squares (PLS) or orthogonal projection to latent structures-discriminant analysis (OPLS-DA). These statistical methods can be described as either unsupervised or supervised models (Tugizimana *et al*., 2013). Univariate statistical methods analyse one variable (a metabolite that is increased or decreased between classes) at a time, and the significance of a single peak or variable is assessed. Analysis of variance (ANOVA) and the t-test are used for univariate analysis. MVDA considers two or more variables simultaneously (Tugizimana *et al*., 2013).

2.5.1.4. Metabolite identification

Metabolite identification is the final step in metabolomic workflow analysis prior to data interpretation (Patti *et al*., 2012; Schrimpe-Rutledge *et al*., 2016). Due to metabolite complexity, MS based approach for metabolite identification encounters analytical challenges (Wu *et al*., 2012; Hu and Xu, 2013). LC coupled with other highly sensitive analytical instruments such as qTOF and the use of accurate mass and fragmentation pattern have been deemed to be sufficient for metabolite identification (Tautenhahn *et al*., 2008; Wolfender *et al*., 2015; van der Hooft *et al*., 2011; Mhlongo *et al*., 2016; Martínez-Bueno *et al*., 2019). However, different compounds may have the same fragmentation pattern (Vetere *et al*., 2018). As such, it is subjected to humans to account for multiple metabolites. Tools that minimise false positive peaks must be applied as this limits identification of different metabolites from online databases (DeFelice *et al*., 2017; Witting and Böcker, 2020). Currently, the use of generated MS based information in combination with statistical model databases such as plantMetabolomic.org, Knapsack (http://www.knapsackfamily.com/), ChemSpider (http://www.chemspider.com/) and also Pubchem (https://pubchem.ncbi.nlm.nih.gov) and molecular networking have been applied to improve the identification of multiple compounds (Tugizimana *et al*., 2013; Ramabulana *et al*., 2021).

Accurate metabolite annotation is essential for accurate downstream metabolomic interpretation. Several criteria are defined for metabolite annotations for LC-MS, such as accurate mass, Rt, and MS/MS fragmentation patterns. Accordingly, different metabolomic annotation and identification levels have been defined (Sumner *et al*.,

2007; Chaleckis *et al*., 2019). Metabolite annotation was executed based on mass spectral information from MSE and/or MS2 experiments, accurate mass information, elemental composition predictions and searches in various databases such as ChemSpider (www.chemspider.com) and Dictionary of Natural Products (www.dnp.chemnetbase.com). Mass spectral information was also compared to available literature and the metabolites were putatively identified due to the lack of available authentic standards. Metabolites were putatively identified to level 2 of the Metabolomics Standards Initiative (MSI) (Sumner *et al*., 2007).

2.6. References

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Chapter 3: Ultraviolet (UV) light-induced geometrical isomerisation of cinnamic acid containing molecules: A plausible, non-enzymatic approach to enhance metabolite composition of plant extracts

Abstract

Cinnamic acid containing molecules are widely distributed in plants, and they are known to possess several pharmacological properties. Structurally, these molecules consist of C_6-C_3 skeletal backbone, with an olefin functional group on the C_3 part. Naturally, plants produce a broad spectrum of cinnamic acid derivatives with a *trans* configuration, however, *cis* isomers are formed due to excessive UV-light exposure, thereby increasing the already complex metabolomes of plants containing these compounds. In high sunlight intensive areas, the prevalence of the *cis* isomers of cinnamic acid containing molecules is becoming more prevalent and synonymous to extracts of many plants. It is therefore imperative to develop analytical and data handling strategies to account for the presence of these molecules, which can serve as a template for rational design of pharmacologically active plant extracts or smart photo-switchable drugs. Herein, we show that a combination of ultra-high performance liquid chromatography quadrupole time of flight mass spectrometry (UHPLC-QTOF-MS) and principal component analysis (PCA) allows differentiation of plants extracts of which metabolome modification through UV-radiation has been carried out. The results of the current study show that geometrical isomerism is a feasible, non-enzymatic approach to modify metabolite composition of a plants. This simple method can be used to create “super” extracts of plants containing structurally diverse metabolites to be evaluated for various pharmacological properties in the future.

Keywords: Cinnamic acid, UHPLC-qTOF-MS, UV-light and geometrical isomerisation.

3.1. Introduction

Traditional medicine has a long history and up to 80% of the world's population has always used plants for medicinal purposes because of safe therapeutics (Jamshidi-Kia *et al*., 2018). Naturally, plants produce a wide range of secondary metabolites such as flavonoids and chlorogenic acids (CGAs) for defensive reasons against a broad spectrum of abiotic and biotic factors (Sharma *et al.,* 2019). Chlorogenic acids are a class of phytochemicals that are formed as esters between different derivatives of cinnamic acid and quinic acid molecules (Ncube *et al*., 2014; Jaiswal *et al*., 2014). these compounds have multiple biological properties such as anti-diabetes (Oboh *et al*., 2015), anti-cancer (Suberu *et al*., 2014) and controlling oxidative and inflammatory stress (Liang and Kitts, 2016).

Biosynthesis of metabolites especially cinnamic acid derivatives such as CGAs are produced through the phenylpropanoid and shikimate pathways (Kulik *et al*., 2017). However, biochemical synthesis differs from one plant to another. The most common cinnamic acid derivatives that have been reported during the biosynthesis of these molecules are caffeic acid, p-coumaric acid and ferulic acid (Heleno *et al*., 2015). Naturally, plants synthesize these molecules in the *trans* configuration; however, *cis* isomers are formed because of photoisomerisation (Clifford *et al*., 2008; Masike *et al*., 2017) due to exposure to sunlight associated UV light. As such, the structural diversity of these molecules is due to positional isomerisation and photoisomerism, which further complicates the means of analytically distinguishing between them (Ncube *et al*., 2014; Nobela *et al*., 2018).

Isomers have been shown to pose undisputed analytical challenges during LC-MS analysis because they produce the same mass to charge signals and associated fragmentation patterns (Makola *et al*., 2016). However, recent developments in analytical instruments such as the use of ion mobility, collision induced dissociation-based fragmentation and metal adduct formation and mass spectrometry allowed for efficient discrimination between trans and cis isomers of cinnamic acids (Makola *et al*., 2016; Zheng *et al*., 2017; Masike *et al*., 2017). However, these instruments are cost effective.

In the current study, LC-MS-based metabolomics approach and multivariate statistical tools such as principal component analysis (PCA) which reduce the dimensionality of

LC-MS data (Madala *et al*., 2014), were applied to differentiate between extracts treated with UV-light, thus containing additional cis isomers and non-irradiated extracts of *B. pilosa* which predominantly contains the trans isomers. The info-graphical and statistical results showed a cluster of metabolites in UV-treated sample, further highlighting geometrical isomerisation (trans/cis photo-isomerisation) as a feasible strategy deployed by plants to mitigate the effects of UV exposure.

3.2. Methods and Materials

3.2.1. Plant material

Fresh leaves of *B. pilosa* were collected from Phiphidi village, Vhembe region, Limpopo, South Africa (22°57'13.4''S 30°21'23.3''E). Plant specimen authentication was conducted with the expertise of Dr Khathutshelo Magwede and where necessary, compared to already published infographics of plant specimens (Magwede *et al*., 2019). Leaves were air dried in the shade at room temperature before being stored in dried form until metabolite extractions.

3.2.2. Methods

3.2.2.1. Metabolite extraction

Metabolite extraction from fresh leaves of *B. pilosa* was accomplished using a method reported by Ramabulana *et al*. (2020). Briefly, dried leaves were ground to fine powder with the use of a pestle and mortar. Two grams (2 g) of finely ground powder was added to a 20 mL (1:10 *m/v*) of 80% (*v/v*) aqueous methanol and mixed overnight in a rotating shaker. The homogenate was centrifuged at 5000 xg for 20 min to remove debris before the supernatant was transferred to sterile tubes. A rotary evaporator was used to concentrate the sample at 55 °C under vacuum until approximately 1 mL samples. These samples were dried overnight in a dry bath at 55 °C. The dried samples were reconstituted with 500 mL of 50% methanol and sonicated for 30 min at 30 °C with the use of a sonic bath.

3.2.2.2. Metabolites treatment with UV-light

The resulting supernatant was then diluted with methanol using a 1:1 ratio. Five tubes were used as controls and the other five tubes were subjected to UV-irradiation to induce geometrical isomerisation using an in-house UV lamp box set at 254 nm

wavelength for 24 h. After 24 h of irradiation, both the non-irradiated and the irradiated samples were filtered through a 0.22 µm nylon filter into a 2 mL vials fitted with a 0.2 mL conical bottom glass insert.

3.2.2.3. Liquid chromatography-mass spectrometry analysis

Liquid chromatography hyphenated to quadrupole-time-of-flight mass spectrometry (LC-qTOF-MS) model LC-MS 9030 instrument (Shimadzu, Kyoto, Japan), equipped with a Shim Pack Velox C_{18} column (100 mm × 2.1 mm, 2.7 µm particle size) was used. The column oven was set at 40 °C. The binary solvent mixture used consisted of 0.1% formic acid in water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B), at a constant flow rate of 0.4 mL/min. The gradient was gradually changed from 3 to 53 min to achieve the separation of intended analytes. Briefly, the gradient conditions were as follows: 0 - 3 min, 10 % B, 3 - 40 min, 60 % B, 40 - 43 min, 60 % B, 43 - 45 min, 90% B, 45 - 48 min, 90 % B, 48 - 50 min, 10 % B and back to 10 % B in 1 minute. Analyte elution was monitored using a mass spectrometer detector under the following optimized conditions: ESI negative modes; 3.5 kV interface voltage (-) and 4.5 kv (+) interface voltage; nitrogen gas was used as nebulizer at flow rate 3 L/ min, heating gas flow at 10 L/min, interface temperature at 300 °C, heat block temperature at 400 °C, DL temperature at 250 °C; 1.70 kV detector voltage.

3.2.4. Multivariate Data Analysis

UHPLC-qTOF-MS raw data was converted to centroid mzML files prior to multivariate data analysis. The data sets were then uploaded to an online tool, XCMS (https://xcmsonline.scripps.edu). Data were processed with the following parameters: minimum peak width of 5 s and maximum peak width of 20 s, cenWave of 15 ppm, retention time (Rt) range of 0.60 – 21 min, mass range of 100 Da, mass tolerance of 0.05 Da and a Rt window of 0.2 min, obiwarp settings for retention-time correction (profStep) of 1, mzwid of 0.015, minfrac of 0.5, bw of 5, statistical test of unpaired parametric t-test(welch t-test), fold change threshold of 1.5, p-value threshold of 0.01 and sample biosource of *Solanum lycopersium* (Tomato). Unsupervised statistical models computed were principal component analysis (PCA) and cloud plot (CP) which show significant differences and similarities between different samples.

3.3. Results and discussion

In this study, the distribution of metabolites from leaves extract of both treated and non-treated sample were studied using methanol extraction with the aid of highly sensitive analytical method, UHPLCqTOF-MS (Figure 3.1). Based on the base peak intensity (BPI) chromatograms of the UV-radiated sample, new metabolites were produced upon UV exposure and there is a variation in peak intensities, suggesting that plants underwent metabolome shifts thereby diversifying its metabolites as a defense mechanism against excessive UVlight exposure.

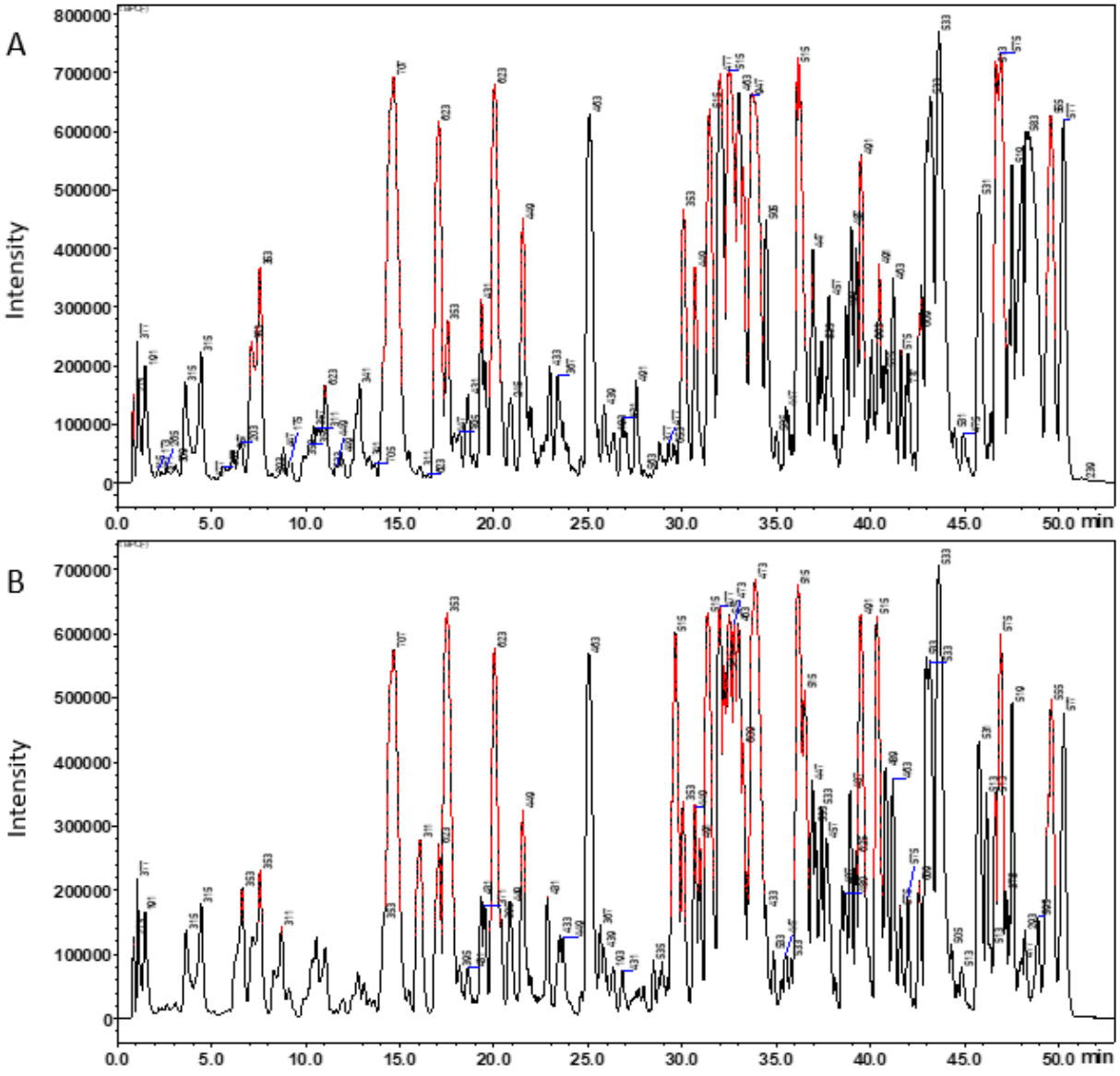

Figure 3.1: Representative UHPLC-qTOF-MS chromatograms of metabolites produced by *Bidens pilosa* before and after UV light respectively **(A)** and **(B)**.

Due to the complexity associated with plants extracts, especially after analysis with LC-MS (Ramabulana *et al*., 2021), it is essential to use statistical models to decipher any inherited patterns within the data, more especially the changes that are not so obvious with a naked eye (Madala *et al*., 2016). As such, to further study the effect of UV-radiation on the understudied plant extracts, LC-MS data obtained herein was further analyzed with multivariate data analysis method. The computed PCA score plot (Figure 3.2) provided a significant visual representation of the differences between leaf extracts of *B. pilosa* before and after UV-light exposure. PCA is an unsupervised, explorative chemometric tool for the reduction of dimensionality of complex datasets to provide insights into variations and systematic trends among sample groups (Ramabulana *et al*., 2020). The score plot showed a control sample (non-treated) with a systematic pattern where metabolites are dispersed from each other, meaning there was a slight but notable difference between samples, even though they represent the same “biological group”. However, the treated sample showed a much significant clustered pattern (Figure 3.2a). To validate the results obtained from PCA modelling, cloud plot was also computed to extract metabolite changes information of control and treated sample. The cloud plot model (Figure 3.2b) of 24 hours UV-treated and untreated sample showed a cluster pattern and a clear separation in non-treated samples, suggesting metabolomic differences in these samples. The difference is based on the metabolite intensities, where in the control samples there was a decrease in intensity (top plot in green) while in treated sample there is an increase in intensity (bottom plot in red). The size of each bubble is directly proportional to the log fold change of the feature, thereby indicating an abrupt change in metabolite composition or production due to the imposed treatment (UV light in this case).

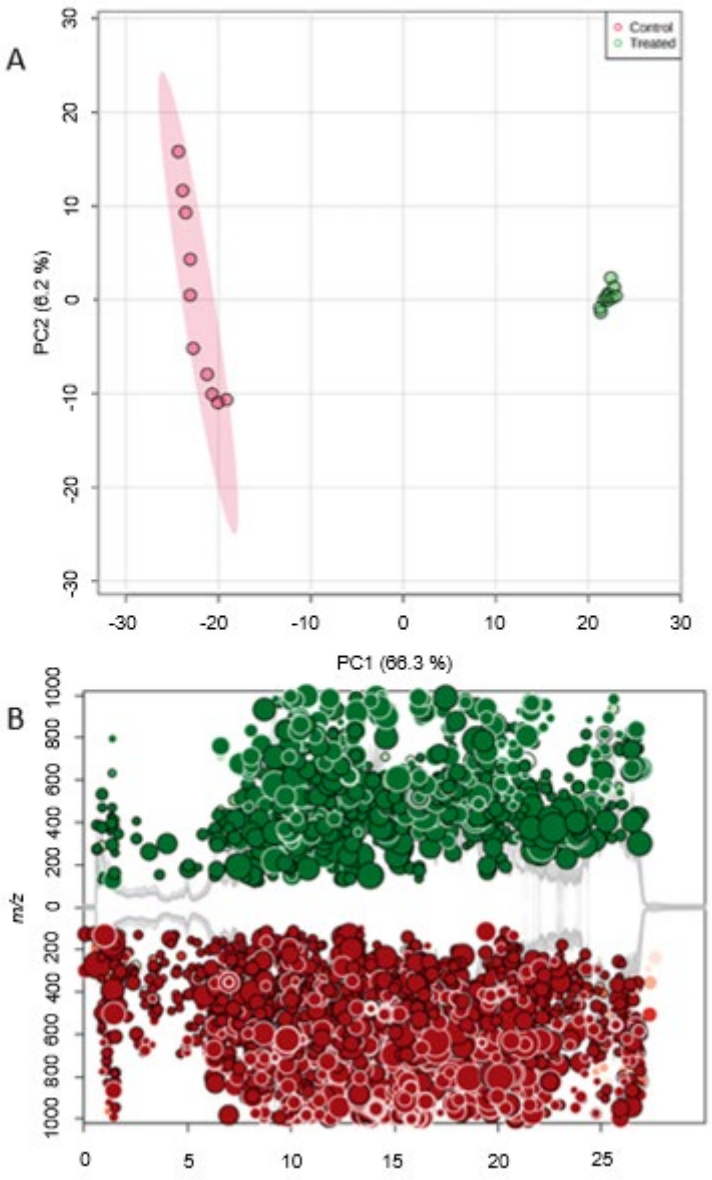

Figure 3.2: Unsupervised multivariate statistical analysis of *Bidens pilosa* L leaf extract. (**A**) A principal component analysis (PCA) scores scatterplot representing PCA 1 and PCA 2 with 72% of variation. The control sample showed scattered pattern (red) while the treated sample showed a clustered pattern (green). (**B**) indicates the cloud plot (CP) comparing the metabolite composition between non-radiated and radiated samples with p-value of ≤ 0.01 and fold change of ≥ 1.5.

Additionally, distribution of metabolites at m/z 529.135 from leaves extract of both treated and non-treated sample were studied using methanol extraction with the aid of highly sensitive analytical method, UHPLC-qTOF-MS (Figure 3.3). Based on the base peak intensity (BPI) chromatograms of the UV-radiated sample, new metabolites

were produced upon UV exposure with better resolution and there is a variation in peak intensities.

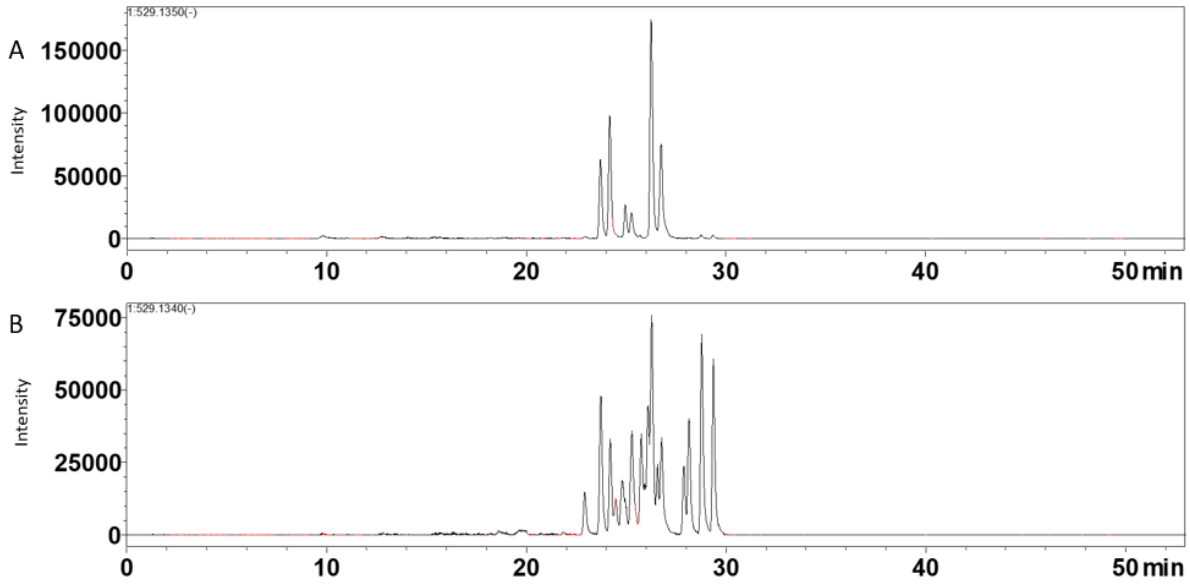

Figure 3.3: A representative UHPLC-QTOF-MS showing photo-induced geometrical isomerisation of caffeoyl-feruloyl quinic acid isolated from non-treated (**A**) and UV-treated (**B**) extracts of *B. pilosa*.

Apart from the cinnamic acid containing phytochemicals, other metabolites such as carotenes are also known to undergo photo-induced geometrical isomerisation (Sandmann, 1991). As a precursor for vitamin A synthesis, the susceptibility of these carotene molecules to light exposure can have unforeseen biological consequences. For instance, it is well known that only one geometrical isomer (11 – *cis* Retinal) is the most functional light sensing chromophore in visual pigments of a human eye (Sekharan and Morokhuma, 2011), and as such, the chemistry and biology of geometrical isomers of these molecules are expected to have underlying physiological consequences. Even though, very little is known about the geometrical isomers of cinnamic acid containing molecules, it is expected that they would also have physiological effects, especially bio-availability due to polarity switching associated with their (Clifford *et al*., 2008). It has been shown elsewhere that the *cis* isomers of acyl quinic acid bind to the sodium ions (and other alkali metals) more strongly than their *trans* counterparts (Makola *et al*., 2016a), and this could have biological consequences because another independent study had shown that acyl quinic acids interferes with the sodium ion gradient of the glucose transporter (Wolffram *et al*.,

1995), thereby further validating the potential of these compounds as drug leads towards management of diabetes (Clifford *et al*., 2020). Other research has also shown the geometrical isomers of acyl quinic acids as potential anti-HIV agents though inhibition of an integrase enzyme (Makola *et al*., 2016b). Therefore, the existence of structurally diverse isomers originating from the same molecule, could also be a premise of synergistic effects when ingested.

Surprisingly, the results of the current study also show that other molecule types (flavonoids in this case) were also affected by light exposure. Using two molecules with contrasting glycosylation degree, namely rutin and quercetin glucoside, the levels of di-glycosylated flavonoid was reduced post UV exposure whilst the levels of the mono-glycosylated was slightly increased post UV exposure (Figure 3.4). Flavonoids are known to play a significant role against excessive light exposure *in planta* (Ferreyra *et al*., 2021), through absorption of excess UV light by their electron rich phenyl rings. However, UV light is also known to cause radical induced reaction which can change the structural configuration of the entire molecule or through cleavage of certain moieties of a molecule which could explain the significant decrease in rutin concentration as observed herein (Figure 3.4a). Moreover, looking at the structures of the two flavonoids understudied herein, quercetin glucoside (Figure 3.4b) is a structural precursor of rutin and, as such, the removal of terminal rhamnose sugar moiety through an unknown UV-mediated glycosidic bond cleavage could explain the increase on the levels of quercetin glucoside, as observed herein. These preliminary results on flavonoids involvement towards mitigating UV light exposure warrants further studies in deciphering the influence of UV light exposure on other non-cinnamic acid containing molecules.

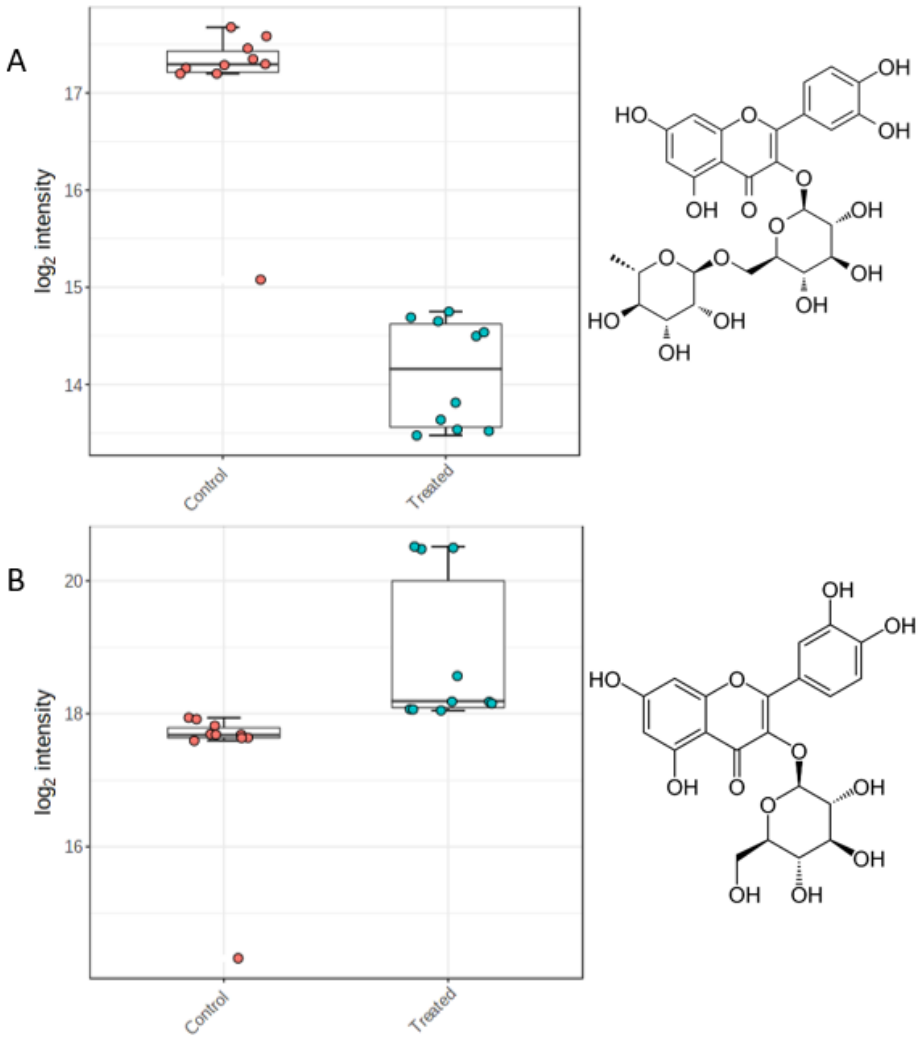

Figure 3.4: Box-whiskers plots showing the distribution of Rutin (**A**) and Quercetin-glucoside (**B**) cross the UV light-treated samples in comparison to the untreated controls. The structures of the molecules are also shown on the right-hand side of their respective graphs.

3.4. Conclusion

In the current study, it has been shown that multivariate statistics are sensitive to decipher the underlying, subtle changes in metabolite composition of plants caused by light exposure. From the results, PCA score plots were sufficient to differentiate between UV exposed extracts from untreated samples. Moreover, cloud plot was good enough to the metabolites of which the level (or geometry) has been perturbed due to UV exposure. Most cinnamic acid containing molecules were found to be the ones undergoing geometrical changes due to UV exposure, presumably due to *trans* – *cis* photoisomerisation. The use of UV-induced photoisomerisation as a plausible approach to modify the metabolome complexity of plants is presented here. An increase in metabolite composition, especially those with purported pharmacological

activities could serve as a feasible approach to enhance the biological activities thereof. Finally, a combination of UHPLC-qTOF-MS and multivariate statistics tools could suffice as a robust quality assurance method to validate the formation of the geometrical isomers. A sudden decrease in other flavonoids molecules, as seen herein, post UV-light exposure warrants further studies aimed at deciphering the chemical modifications on flavonoids chemical space due to UV light exposure.

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Chapter 4: Synergistic isomerism of cinnamic acid containing compounds is a precursor of metabolome complexity but hinders efficient discrimination of plant metabolites: A case study of verbascoside *versus* isoverbascoside

Abstract

Verbascoside (VB) is a biologically active glycoside found in selected medicinal plants. Structurally, verbascose (β-(3′,4′-dihydroxyphenyl) ethyl-*O*-α-L-rhamnopyranosyl(1→3)-β-D-(4-*O*-caffeoyl)-glucopyranoside) is formed due to acylation on the glucose moiety of the disaccharide (rutinoside) by caffeic acid and hydroxytyrosol. Isoverbascoside (isoVB) is a positional isomer of VB is formed through caffeoyl migration from 4′ to 6′ positions on the glucose moiety. Distinguishing these two compounds through typical LC-MS techniques has proven to be impossible since they have the same molecular weight and produce similar MS-fragmentation patterns. Furthermore, the caffeoyl moiety is naturally produced with the *trans* configuration; however, *cis* isomers are formed due to UV-light exposure, further complicating means of distinguishing between them. The main goal of this study was to discriminate between the geometrical isomer of VB and isoVB. Herein, UHPLC-qTOF-MS/MS was used in negative ionization mode at different collision energy. The results indicate a clear difference at a collision energy of 35 eV, where the product ion at *m/z* 161 showed a base peak in *cis* VB. This can be a diagnostic peak to differentiate the geometrical isomers of VB and isoVB. Furthermore, using methanol extracts of *Lippia javanica* plants, the results of the current study also show that VB and isoVB are prone to isomerisation which contribute to metabolome complexity. This synergistic isomerism could serve the humans positively, for instance, during inflammation conditions. However, isomerism hinders efficient discrimination and as such, other analytical techniques such as ion mobility mass spectrometry can aid in identifying VB, isoVB and their respective geometrical isomers.

Keywords: Caffeic acid, Isomerisation, *Lippia javanica,* UHPLC-QTOF-MS, Verbascoside, Isoverbascoside.

4.1. Introduction

For survival, plants produce a broad spectrum of metabolites with multiple functions. Depending on their functions, plant metabolites are classified as primary or secondary (Ashraf *et al*., 2018; Sharma *et al*., 2019). Primary metabolites are used mainly for growth and development, while secondary metabolites are used for plants' survival in their immediate environment (Ahmed *et al*., 2017; Böttger *et al*., 2020). Most secondary metabolites have been shown to exhibit pharmacological activities and as such, plants are used as a source of nutraceuticals (Leicach and Chludil, 2014; Faustino *et al*., 2019). In most cases, the composition of plant metabolome is directly correlated to its biological activities (Kumar and Pandey, 2013; Felleh *et al*., 2008; Gonulalan *et al*., 2020).

Several biochemical factors, such as environmental conditions and tissue age, have contributed to metabolome complexity (Li *et al*., 2020). Most notably, isomerisation has been shown to diversify plant metabolites (Nengovhela *et al*., 2020; Nengovhela *et al*., 2021). However, in the past, most isomers have been neglected as mere structural artefacts of one another. Analytically, isomers have also been shown to possess undisputed challenges, especially during identification and characterisation (Makola *et al*., 2016; Zheng *et al*., 2017). As such, an analytical technique such as liquid chromatography hyphenated to mass spectrometry has been used as a golden technique for isomers differentiation (Ncube *et al*., 2014; Zhou *et al*., 2017). Differentiation of positional isomers of chlorogenic acids (hydroxy-cinnamic acid derivatives) is an example where LC-MS has been used successfully to distinguish between positional and geometric isomers (Clifford *et al*., 2013; Clifford *et al*., 2008).

Cinnamic acid containing molecules, such as chlorogenic acids, is also prone to UV-induced geometrical isomerisation, resulting in *trans-cis* photoisomerisation, complicating plants' already complex metabolomes (Clifford *et al*., 2008). The formation of these geometrical isomers (*trans/cis*) has also been shown to possess analytical challenges during LC-MS analysis because they produce the same mass-to-charge signal and fragmentation patterns. However, recent developments in technology/approaches, such as metal adduct formation and ion mobility mass spectrometry, allowed for efficient discrimination between *trans* and *cis* isomers of cinnamic acids (Makola *et al*., 2016; Zheng *et al*., 2017). Other cinnamic acids

containing molecules such as chicoric acid and clovamide have been shown to undergo similar isomerisation, suggesting that cinnamic acid containing molecules are prone to this type of isomerisation (Nobela *et al*., 2018; Madala and Kabanda, 2021). More interestingly, other scientists have suggested that the formation of these geometrical isomers could be associated with climatic changes and excessive sunlight exposure (Karaköse *et al*., 2015). Taken all together, it is becoming evident that the formation of isomers in plants is a well-coordinated biochemical process with biological consequences that is yet be investigated. An example of a metabolite that is prone to multiple isomerisations is VB (Figure 4.1a) and isoVB (Figure 4.1b), which are found in various plants such *Harpagophytum procumbens* (Bae *et al*., 2014)*, Lippia javanica* (Oyourou *et al*., 2013) and *Lantana camara* (Kumar et al., 2014). VB has been associated with various pharmacological activities such as anti-cancer (Chen *et al*., 2013; Zhang *et al*., 2018), anti-inflammatory (de Moura Sperotto *et al*., 2018), anti-bacterial (Bazzaz *et al*., 2018) and wound healing properties (Nigro *et al*., 2020).

This compound has been shown to exist with its structurally related isomer known as iso-VB as shown in figure 4.1a (Li *et al*., 2005). These two compounds share similar precursor mass and fragmentation patterns during LC-MS analyses. In this study, LC-MS analyses of an environmentally growing *L. javanica* showed VB and isoVB are prone to UV-induced geometrical isomerisation, resulting in the production of their corresponding *cis* isomers. Through these findings, it is also apparent that *trans/cis* isomers of cinnamic acid containing molecules are becoming inherent parts of everyday plant extracts and their definite identification through LC-MS is mandatory.

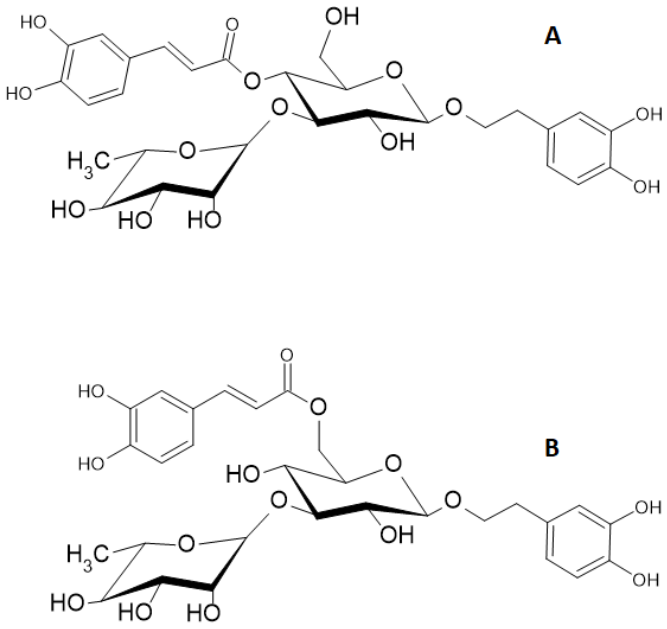


Figure 4.1: Schematic structural representation of verbascoside (**A**) and isoverbascoside (**B**) indicating positional isomerism and functional groups responsible for geometrical isomerisation. IsoVB is formed through caffeoyl migration from 4′ to 6′ positions on the glucose moiety.

4.2. Methods and Materials

4.2.1. Plant material

Fresh leaves of *L. javanica* were collected from Phiphidi village outside Thohoyandou, Venda region of Limpopo province of South Africa (22°57'13.4''S 30°21'23.3''E). Plant specimen authentication was conducted in the botany department and, where necessary, compared to already published info graphical plant specimens (Magwede *et al*., 2019). The leaves were dried at room temperature. The samples were then stored in dried form pending metabolite extractions.

4.2.2. Methods

4.2.2.1. Metabolite extraction

Authentic standards (99.6%) of VB and isoVB were purchased from Phytolab (Vestenbergsgreuth, Germany). Metabolite extraction was accomplished using the method used elsewhere (Ramabulana *et al*., 2020). Briefly, *L. javanica* plant leaves were pulverized to fine powder using pestle and mortar. Two grams (2 g) of fine ground leaves was mixed with 20 mL (1:10 *m/v*) of 80% (*v/v*) aqueous methanol and rotated overnight in an orbital shaker. The homogenate was centrifuged at 3075 RCF for 20 min to remove the debris, before the supernatant was transferred to sterile tubes. A

rotary evaporator was used to concentrate the sample at 55 °C under vacuum to approximately 1 mL of each sample. These samples were further dried overnight at 55 °C. The dried samples were reconstituted with 500 µL of 50% methanol and sonicated for 30 min at 30 °C. The samples were then filtered through a 0.22 µm nylon filter into 2 mL vials fitted with 0.2 mL conical bottom glass inserts.

4.2.2.2. Metabolites treatment with UV-light

The resulting samples were then subjected to UV-irradiation to induce geometrical isomerisation using an inhouse UV lamp box set at 254 nm wavelength for 24 hours. After 24 hours of irradiation, both the nonirradiated and the irradiated samples were filtered through a 0.22 µmm nylon filter into a 2 mL vial fitted with a 0.2 mL conical bottom glass insert.

4.2.2.3. Liquid chromatography mass spectrometry analysis

LC-QTOF-MS model LC-MS 9030 instrument with a Shim Pack Velox C_{18} column (100 mm × 2.1 mm, 2.7 µm particle size) (Shimadzu, Kyoto, Japan) and a column oven set at 40 °C, was used for sample analysis. The binary solvent mixture used consisted of 0.1 % formic acid in water (Eluent A) and 0.1 % formic acid in acetonitrile (Eluent B), at a constant flow rate of 0.3 mL/min. The gradient was gradually changed from 3 to 53 min to achieve the separation of intended analytes. Briefly, the gradient conditions were as follows: 0 - 3 min, 10 % B, 3 - 40 min, 60 % B, 40 - 43 min, 60 % B, 43 - 45 min, 90% B, 45 - 48 min, 90 % B, 48 - 50 min, 10 % B and back to 10 % B in 1 minute. Analyte elutions were monitored using a mass spectrometer detector under the following conditions: ESI negative modes; 3.5 kV interface voltage (-) and 4.5 kv (+) interface voltage; nitrogen gas was used as nebulizer at flow rate 3L/min, heating gas flow at 10 L/min; heat block temperature at 400 °C, DL temperature at 250 °C; 1.70kV detector voltage.

2.2.4. Molecular docking-based virtual screening analyses

The molecular docking virtual screening analyses were performed using PyRx software. COX-1 was converted into PDBQT file format for analysis using the PyRx software. The grid box dimensions were set to cover the surface area of COX-1 enzyme (x, y, and z axes, respectively) and the binding pocket was set to default mode. Before carrying out virtual screening with the selected ligands, all ligands were

drawn using ChemSketch and converted to PDBQT using PyMOL software. The best docking poses calculated with PyRx (with the lowest binding energy) of the co-crystallized ligand complexed with COX-1 were selected to perform the molecular docking analyses. The 2D and 3D analyses of the interaction patterns in protein-ligand complexes were performed and visualized using Discovery Studio Visualizer-2021 version. In all these analyses, the default cut-off interaction distances were used.

4.3. Results and discussion

4.3.1. LC-MS analysis

The current study used UHPLC-qTOF-MS to distinguish between the closely related cinnamic acid containing molecules using chromatographic elution order and fragmentation patterns. The fragmentation pattern of VB and isoVB were achieved using collision-induced dissociation (CID) and was sufficient to discriminate between these closely related isomers. Order of elution also aided in the identification of positional isomers thereof. As a result, VB eluted first (Figure 4.2a), whereas isoVB on the other hand, eluted last, suggesting that it is less polar compared to its positional isomer (Figure 4.2c). Upon 24 hours of UV exposure at 245 nm, additional peaks were observed, indicating geometrical isomerisation due to UV-aided photoisomerisation. Cinnamic acid containing molecules have been shown to undergo geometrical isomerisation when exposed to UV light. Interestingly, the newly formed isomers appear to have different polarity compared to their natural counterpart, suggesting a dynamic structural rearrangement that allows for possible intra-molecular forces that govern the overall dipole moments (polarity) of a molecule.

Here, geometrical isomers of VB and isoVB were examined by UHPLC-qTOF-MS (Figure 4.2b and Figure 4.2d). Without UV exposure, VB and isoVB showed a single peak each corresponding to the *trans* configuration of these compounds with a precursor mass at *m/z* 623. Herein, VB eluted at approximately 14 min whilst isoVB eluted at 16 min (Figure 4.2a and Figure 4.2c), meaning that VB is much polar than isoVB, following the principles of reverse chromatography as applied herein. However, upon UV exposure, these positional isomers formed geometric isomers, all of which eluted after their respective *trans* counterparts. Briefly, *cis* VB eluted at 17.1 min while *cis* isoVB eluted at 16.9 min (Figure 4.2b and Figure 4.2d). For both VB and isoVB, their geometrical isomers (*cis*) eluted after their *trans* counterparts, suggesting that the

cis isomers of these compounds are more non-polar than their *trans* counterparts. Therefore, for the plants that exclusively produce only VB, its *cis* VB can easily be mistaken for isoVB and *vice versa*. This is a challenge because *cis* geometrical isomers are becoming prevalent in plants growing in natural environments (Li *et al*., 2021). For example, *L. javanica* produces six metabolites with precursor ion at *m/z* 623 as shown in (Figure 4.3), even though a maximum of four isomers were expected, being two *trans* isomers of both VB and isoVB together with a pair of their *cis* isomers. It is, therefore, critical to identify which is between these positional and geometrical isomers.

Therefore, authentic standards should be used to identify these compounds with confidence. In the absence of authentic standards. VB and isoVB are predominant in plants that are used for anti-inflammation such as *H.procumbens* and can be used as a surrogate standard (McGregor *et al*., 2005; Mncwangi *et al*., 2014; Clifford *et al*., 2017). Although geometrical isomers were successfully separated, it was challenging to suggest a possible mechanism to distinguish between them through chromatographic elution order since they occupy a very narrow chromatographic space. For instance, looking at the results presented here, VB, isoVB and their respective geometical isomers can be incorectly assigned. Therefore, to assign these geometrical isomers, discriminative fragmentation patterns were archived through collision-induced dissociation (CID) approach in negative mode at different energy levels (Figure 4.4 and Figure 4.5). Elsewhere, it has been shown that chromatographic elution order of geometrical isomers is unreliable and, as such, MS-based approaches such as CID were deemed sufficient (Willems *et al*., 2016; Makita *et al*., 2017; Masike *et al*., 2017a). Following this, the CID approach was also utilized in this study. Interestingly, MS^2 analyses showed that the authentic standards of both VB and isoVB produce the same fragmentation pattern at low collision energies (15 and 25 eV). A parent ion at *m/z* 623 (Figure 4.4a and Figure 4.5a) and major daughter ions at *m/z* 461 and 161 (Figure 4.3b and Figure 4.3c) and (Figure 4.4b and Figure 4.4c) were both produced in VB and isoVB and showed consistent in mass accuracy and fragmentation pattern. The daughter ion at *m/z* 461 is due to the loss of the caffeoyl moiety (-162 Da) from the parent ion and the second daughter ion at *m/z* 161 represents dehydrated caffeoyl moiety (caffeic acid -18 Da). However, high collision energy (35 eV) showed that both VB and isoVB produced the same dehydrated

caffeoyl moiety at *m/z* 161. The peak representing this moiety had different peak intensities, which can be deemed crucial to discriminate between geometrical isomers of VB and isoVB (Figure 4.4c and Figure 4.5c). As noted elsewhere, during mass spectrometry, isomers do not produce entirely different product ions, but instead, they produce similar ions of varying intensities, and this has previously been used as a premise for distinguishing between structural isomers (Zhu *et al*., 2009; Li *et al*., 2011; Masike *et al*., 2017b).

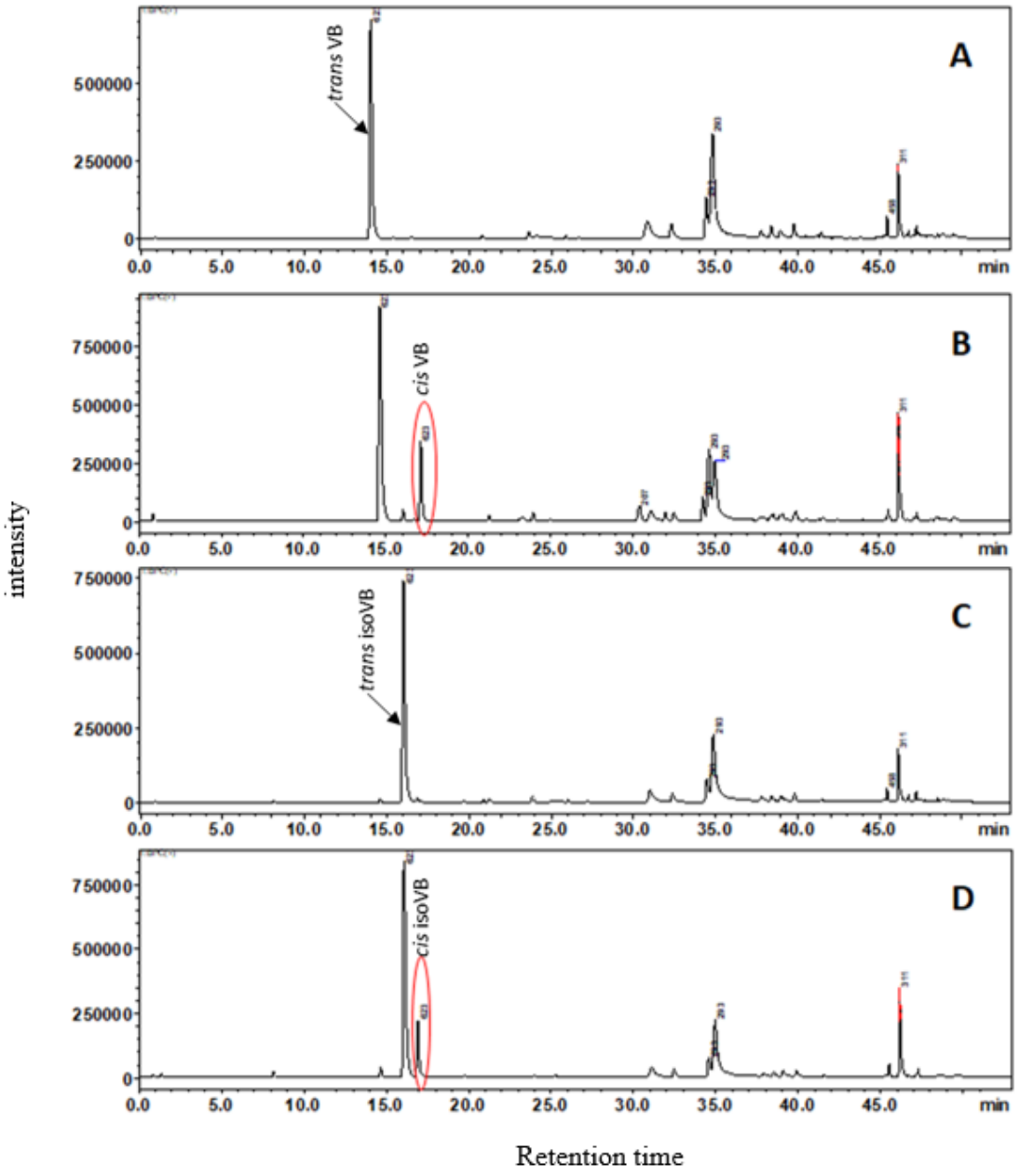


Figure 4.2: Representative UHPLC-qTOF-MS chromatogram showing distribution patterns of VB before UV radiation (**A**), After UV radiation (**B**) and isoVB before UV light exposure (**C**) and after UV light treated (**D**).

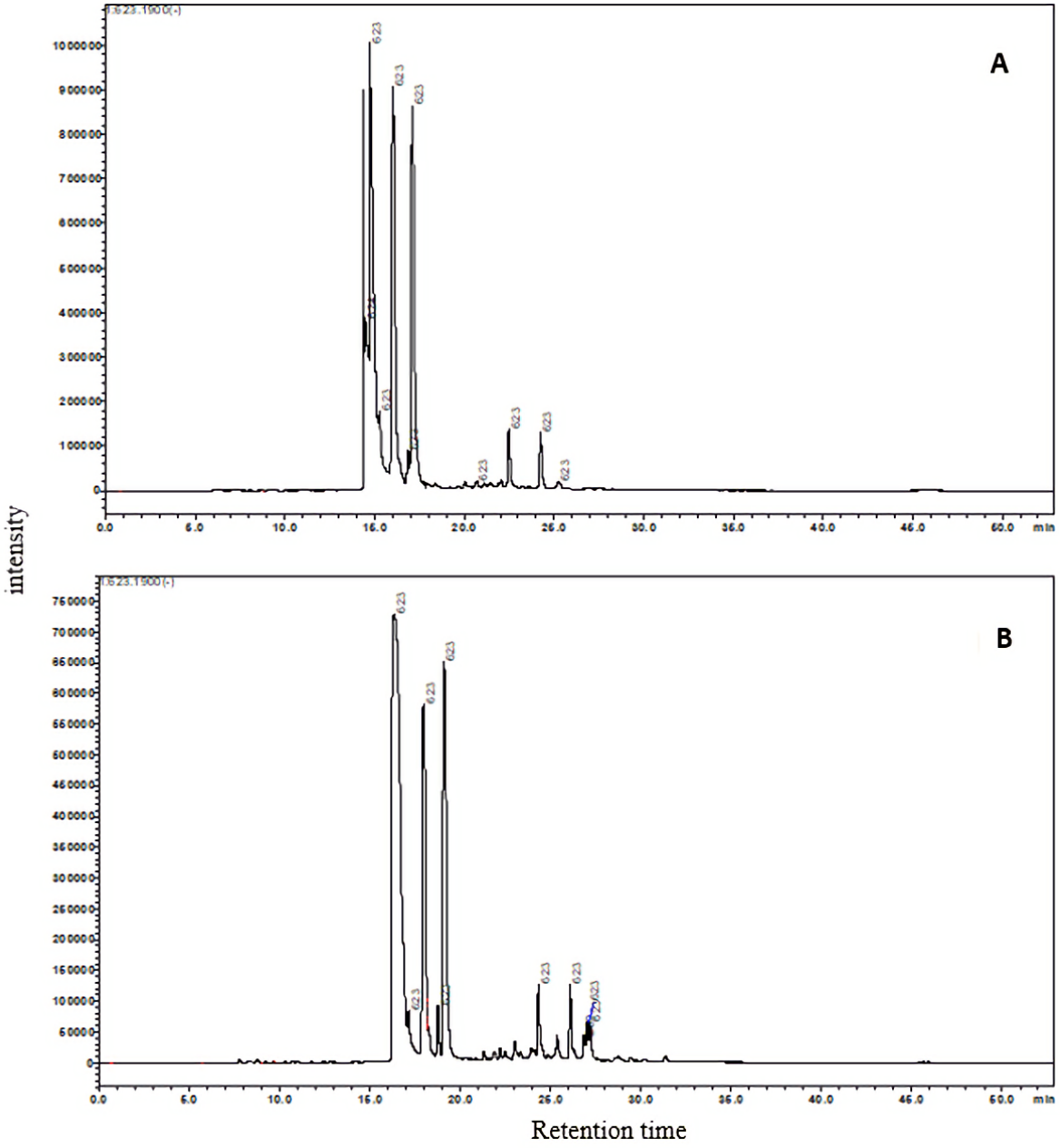


Figure 4.3: Representative UHPLC-qTOF-MS chromatograms of metabolites produced by *Lippia javanica* before and after UV light respectively (**A**) and (**B**).

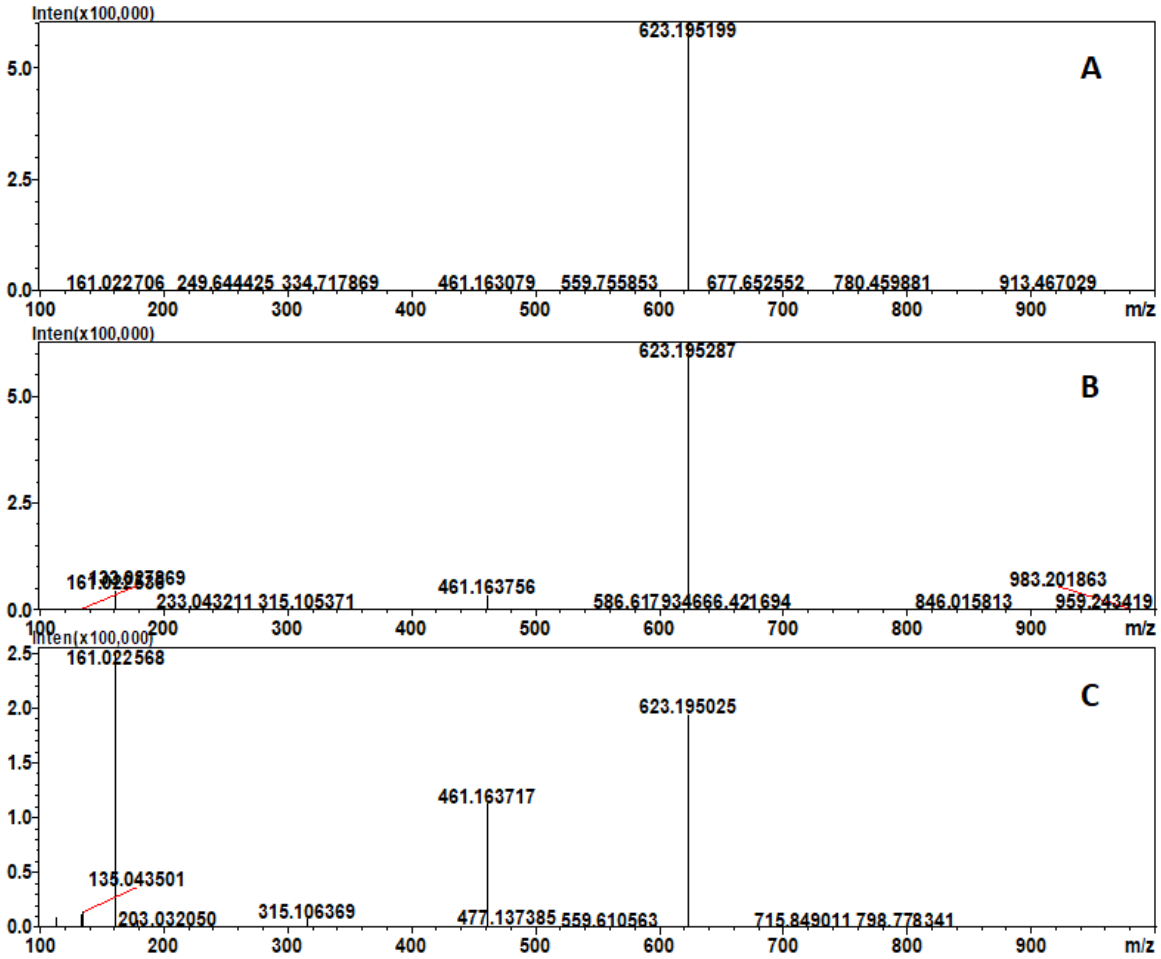


Figure 4.4: Representative UHPLC-qTOF-MS/MS spectrum showing fragmentation patterns of *cis* VB at different collision energies (CE), 15 CE (**A**), 25 CE (**B**) and 35 CE (**C**).

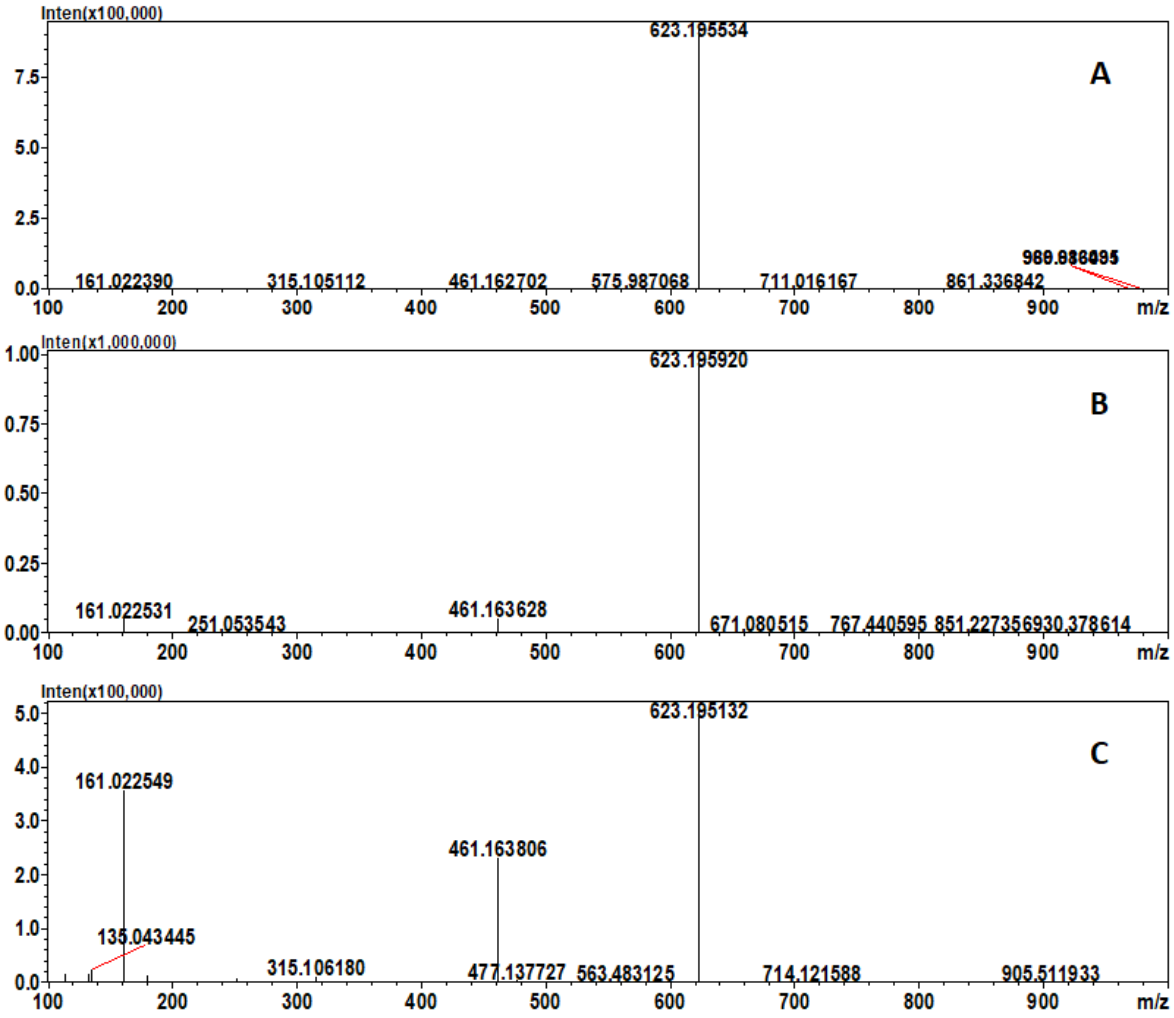


Figure 4.5: Representative UHPLC-qTOF-MS/MS spectrum showing fragmentation patterns of *cis* isoVB at different collision energies (CE), 15 CE (**A**), 25 CE (**B**) and 35 CE (**C**).

4.3.2. Molecular docking

For the present work, the enzyme constitute cyclooxygenase 1 (COX-1) enzyme was selected from the protein data bank (https;//www.rcsb.org) for molecular docking with *trans* VB and *trans* isoVB and their respective *cis* geometrical isomers. By analysing the best binding energy of the complexes, it is clear that the rhamnose part of *trans* VB is buried in the active site of COX-1 while the hydroxytyrosol and caffeic acid groups are oriented away from the catalytic site (Figure 4.6a). The active site of COX-1 is located above the Arg120/Tyr355/Gly524 constriction. Most inhibitors bind in the active site above the constriction residues; however, some have been found to interact with several residues in the lobby region that are considered to be important for inhibitory interactions (Pro86, Ile89, Leu93, and Val116) (Blobaum and Marnet, 2007). Regarding interactions, figure 4.6b shows that *trans* VB establishes three hydrogen

bonds (H-bond) where two of them are through the hydroxyl groups of rhamnose part of a disaccharide (β-ethyl-*O*-α-L-rhamnopyranosyl-β-*D*-glucopyranoside) with Asp450 and Asn382 while the other H-bond is through the hydroxyl group of aromatic ring of caffeic acid with Ser455 residue. Other two π-donor hydrogen bonds were also noted between the aromatic ring of hydroxytyrosol and His388 and between the oxygen of caffeic acid and His386. The non-polar interactions involve the aromatic ring of hydroxytyrosol with Ile444 (π-alkyl), which also form π-anion with Glu454. Notably, as shown in figure 4.6c, the orientation of *cis* VB in the catalytic site of COX-1 closely resembles that of *trans* VB, however, the hydroxytyrosol group is oriented in the opposite direction towards the outside of the catalytic site. The interaction pattern of the *cis* VB-COX-1 complex depicted in figure 4.6d reveals six hydrogen bonds interaction, of which two of them are between hydroxyl groups of aromatic ring of hydroxytyrosol with Trp387 and His388, the other three were in the two hydroxyl groups of rhamnose sugar with Asn382 and one hydroxyl group with Glu454 while the last one was in the hydroxyl group of aromatic ring of caffeic acid and Thr212. Furthermore, the non-polar interactions involve the aromatic ring with Val291 and Ala202 (π-alkyl). In addition to having a slightly different orientation in the active site of COX-1, *trans* VB and *cis* VB also establish some similar interactions. However, it is evident that a switch in a double bond of cinnamic acid (*trans* caffeic acid) structure favour the increase in the number of H-bonds between VB and COX-1. However, It is noteworthy that the presence of π-anion interaction between the aromatic ring of caffeic acid with Glu454 and π-donor hydrogen bond between the aromatic ring of hydroxytyrosol moiety and His388 in *trans* VB, showed slightly better binding energy (-9.5 kcal/mol) compared to *cis* VB (-8.6 kcal/mol) as shown in table 1. Taken together, these results show that, *cis* isoVB showed better binding compared to other isomers, emphasising the importance of geometrical isomerism in modulating inflammation. Since the energies are negatively charged, the lower the number shows better binding. Elsewhere, the presence of π-anion interaction between 6-Acetoxy-2-phenyl-5-(1,2,3-selenadiazol-4-yl) benzofuran and Asp616 residue of COX-1 enzyme has been noted (Olomola *et al*., 2020). Recently, π-anion interaction between S-ethylisothiourea and Glu377 of inducible nitric oxide synthase showed an increase in binding affinity (Phong *et al*., 2022). Other studies proved that *trans* VB reduce the expression and activity of COX and may possess significant anti-inflammatory and

analgesic effect (Mirko *et al*., 2014; Nannoni *et al*., 2020). However, geometrical isomer of *cis* isoVB showed a better anti-inflamatory activity compared to its *trans* counterpats and *trans* VB.

Conversely, *trans* isoVB showed a similar binding backbone pose compared to *cis* isoVB (Figure 4.7a and Figure 4.7c) respectively. Moreover, the 2D binding interaction of trans isoVB showed seven conventional H-bonds with Glu290, Gln289, Lys211, Thr212, Asn382, Glu454 and Val447. Additionally, an extra π-donor H-bond was also noted between the aromatic ring of caffeic acid and Trp387 residue. In this hydrogen bond type, the donor group XH is placed roughly above the center of an aromatic ring, and the X—H vector points at it (Steiner and Koellner, 2001; Stojanović *et al*., 2007). The non-polar interactions involve the aromatic ring of hydroxytyrosol with Val291(π-alkyl) and aromatic ring of caffeic acid with Ala202 and His388 (π-stacked) as shown in figure 7b, resulting in binding affinity of -9.9 kcal/mol (Table 4.1). Interestingly, *cis* isoVB result in a few H-bond interaction with Thr212, Gln289, His386 and the same non polar π-stacked interactions as *trans* isoVB (Figure 4.7d). The presence of π-anion interaction with Glu454 residue increased the binding affinity by -0.5 kcal/mol (Table 1). Suggesting that modification of chemical structures due to possible isomerisation can serve positively during the development of cyclooxygenases enzyme inhibitors (Ferreira De Freitas and Schapira, 2017).

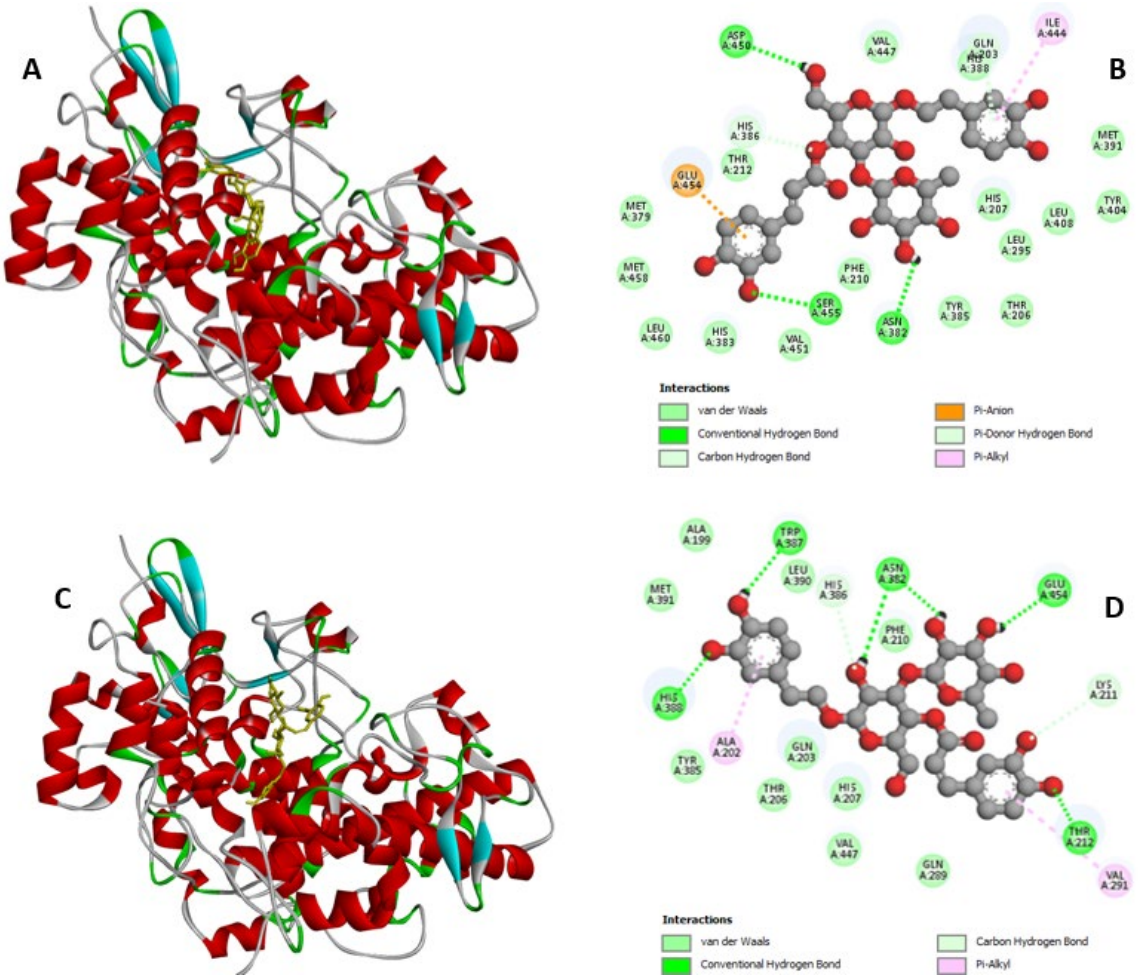


Figure 4.6: Analysis of the 3D representation of the of *trans* VB-COX-1 complex (**A**), 2D interaction diagram of the *trans* VB-COX-1 complex (**B**), 3D representation of the of the *cis* VB-COX-1 complex (**C**) and 2D interaction diagram of the *cis* VB-COX-1 complex (**D**).

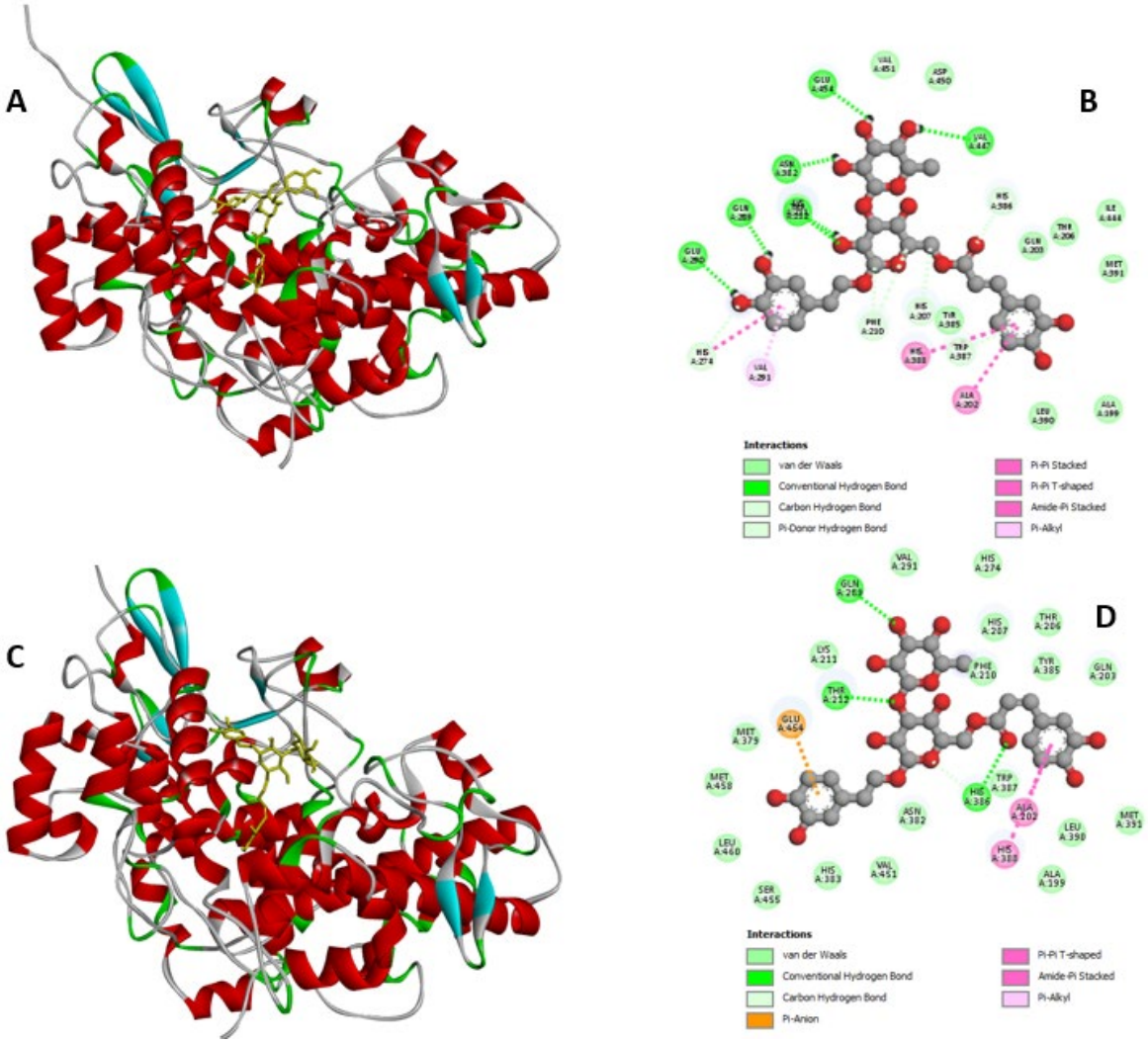
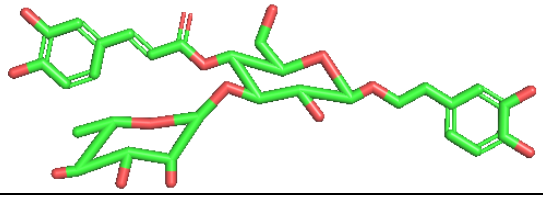
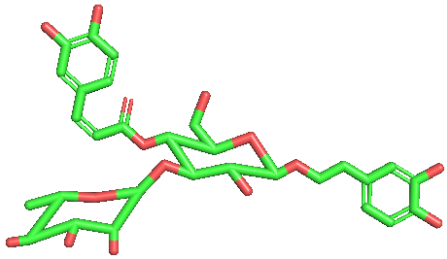
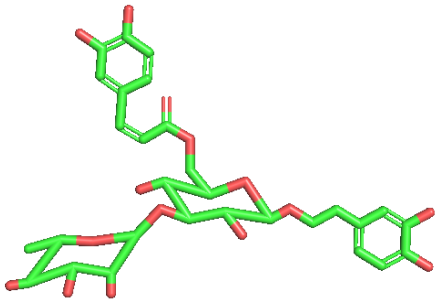


Figure 4.7: Analysis of the 3D representation of the of *trans* isoVB-COX-1 complex (**A**), 2D interaction diagram of the *trans* isoVB-COX-1 complex (**B**), 3D representation of the of the *cis* isoVB-COX-1 complex (**C**) and 2D interaction diagram of the *cis* isoVB-COX-1 complex (**D**).

Table 4.1: Chemical structures and binding energies of trans VB, trans isoVB and their respective cis geometrical isomers with COX-1 enzyme. The binding energies were obtained from calculated using PyRx and also confirmed using LeDock.

Ligand	Binding Affinity/energy kcal/mol
trans VB 	-9.5
cis VB 	-8.6
trans isoVB 	-9.9
cis isoVB 	-10.4

4.4. Conclusion

In this study, chromatographic results show that VB and isoVB form geometrical isomers upon UV radiation. However, UHPLC-MS analysis of those positional isomers shows a difference in elution order using C_{18} reverse column. VB is more polar and it elutes earlier than isoVB; and as such, chromatographic elution order was successfully used to assign these positional isomers. Moreover, to discriminate between the *cis* isomer of VB and *cis* isomer of isoVB, MS^2 analysis showed a clear difference at a collision energy of 35 eV. The ion at *m/z* 161 (dehydrated caffeic acid) showed a base peak in VB which can be a diagnostic ion peak to differentiate between *cis* VB and *cis* isoVB. The clear difference in this collision energy proved to be a premise to distinguish between these closely related isomers. To the best of our knowledge, this is the first report to differentiate geometrical isomers of VB and isoVB using MS^2 fragmentation pattern. The current study also shows synergistic isomerism of cinnamic acid containing compounds diversifying the chemical space of *L. javanica* extracts, contributing to metabolome complexity. This also poses an analytical challenge as isomers hinder the efficient discrimination of plant metabolites. In this regard, modifying chemical structures is a strategy that has allowed the development of multiple drugs, including some antibiotics. However, for the vast mixture of both positional and geometrical isomers of VB and isoVB, other analytical approaches, such as ion mobility, can aid in identifying these closely related isomers.

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Chapter 5: Acylation of flavonoids glycosides with cinnamic acids derivatives amplifies flavonoids chemical space in plants

Abstract

Flavonoids are biologically active compounds that exist as either free aglycones or glycosides. These compounds are well known for their beneficial effects on human health due to their nutraceutical properties. Analytical instruments such as liquid chromatography-mass spectrometry (LC-MS) have been deemed effective for the structural elucidation and identification of metabolites from various plants. However, different processes such as chemical modification and isomerisation have contributed to plants metabolite diversity, which poses an analytical challenge, especially during metabolite identification. The present study uses an ultra-high performance liquid chromatography coupled with quadrupole time of flight mass spectrometry (UHPLC-qTOF-MS) technique to profile flavonoid glycosides in three different plant species (*Bidens pilosa, Coccinia rehmannii* and *Helichrysum kraussii*). Herein, special emphasis was given to flavonoid glycosides acylated by hydroxycinnamic acids. The findings of the current study show that selected plant species produced flavonoids of the same chemical background, but the number of positional isomers differs between species owing to hydroxycinnamic acids acylation. Most of the identified flavonoids were acylated by either caffeic acid or coumaric acid. It can be concluded that plants use acylation of flavonoids glycosides with cinnamic acids derivatives as a biochemical strategy to amplify or diversify the flavonoid chemical space.

Keywords: Acylation, Flavonoids, Hydroxycinnamic acids and UHPLC-qTOF-MS.

5.1. Introduction

Flavonoids are natural polyphenolic compounds that are widely distributed in plants. They belong to a class of secondary metabolites and are present in fruits and vegetables. Structurally, flavonoids are composed of two benzene rings linked by a heterocyclic ring containing a pyran oxygen (Halbwith, 2010). Flavonoids are synthesised through phenylpropanoid pathway and classified into subgroups based on their chemical structures such as flavanones, flavones, flavonols, flavan-3-ols, anthocyanins and isoflavones (Kozlowska and Szostak-Wegierek, 2014). Quercetin and kaempferol are two examples of flavonoids that fall under the flavanol subclass and are reported to have different pharmacological activities, i.e. anti-inflammatory (Fraga *et al*., 2019; Maleki *et al*., 2019) and immunomodulation properties (Mendes *et al*., 2019). Flavonoids have also been shown to exhibit a pronounced effect against obesity, as indicated by their ability to lower body weight, fat mass, and plasma triglycerides/cholesterol, both *in vitro* and *in vivo* models (Rufino *et al*., 2021).

In planta, these metabolites exist as free compounds or conjugated to other molecules such as sugar and cinnamic acids derivatives (Nengovhela *et al*., 2020; Nengovhela *et al*., 2021). It can be challenging to analyse flavonoids as they usually exist as a complex mixture with different conjugates (Williamson *et al*., 2005). Additionally, the presence of multiple hydroxyl groups (-OH) in the structural skeleton of flavonoids are both important for the antioxidant capacity (Zheng *et al*., 2021) and key points for further modification resulting in *O*-methylation, glycosylation, sulfation, or acylation (Plaza *et al*., 2014), which further amplify their composition and can hinder the efficient identification thereof. Elsewhere, phenomenon referred to as glyco-isomerisation has been shown to complicate flavonoid chemical space of a plant (Nengovhela et al., 2021). Isomers have been shown to pose an analytical challenge during LC-MS analysis because they produce the same mass-to-charge signals and associated fragmentation patterns (Makola *et al*., 2016). However, elution order and subtle fragmentation differences have been used successfully to differentiate between positional isomers of other indispensable compounds from plants (Clifford *et al*., 2003). Elsewhere, mass spectrometry has been used to distinguish between positional isomers of flavonoid conjugates (Ferreres *et al*., 2010). As such, fragmentation patterns of closely related positional isomers of flavonoid glycosides can be regarded as a differential analytical tool. Moreover, conjugation of hydroxycinnamic acid with

flavonoid glycosides and other molecules has been reported elsewhere as an evolutionary strategy utilised by plants to maximise their metabolome complexity (Nengovhela *et al*., 2020). In addition, glucuronidation in flavonoid molecules generally weakened the cytoprotective capacities of flavonoids against antioxidative stress in the presence of hydrogen peroxide (H_2O_2) (Stevenson *et al*., 2008).

A recent report by Nengovhela *et al*. (2021) showed that Coccinia plants attach their hydroxycinnamic acids (HCAs) to flavonoid glycoside compounds, contributing to metabolite complexity. Herein, we showed through UHPLC-qTOF-MS analysis of methanolic extracts from plants (*B. pilosa, H. kraussii* and *C. rehmannii*) growing widely in the environment that flavonoids glycosides conjugated/acylated with HCAs are widely distributed across plants of varying background. Moreover, simple MS/MS conditions and an online informatics-based tool (SIRIUS software) are sufficient to identify these complex molecules. The results further reveal the presence of a broad spectrum of flavonoid glycosides attached to HCAs (coumaric acid and caffeic acid) on their glycosidic moieties.

5.2. Methods and Materials

5.2.1. Plant material

Fresh leaves of *B. pilosa, H. kraussii* and *C. rehmannii* were collected from Phiphidi, Vhembe region of Limpopo province in South Africa (22°57’13.4’’S 30°21’23.3’’E). Plant specimen authentication was conducted at the botany department of the university of Venda and, where necessary, compared to already published infographicals of plants specimens (Magwede *et al*., 2019). Leaves were air dried at room temperature before storage until metabolite extractions.

5.2.2. Methods

5.2.2.1. Metabolite extraction

Metabolite extraction was accomplished using a method by Ramabulana *et al*., (2020). Briefly, plant leaves of *B. pilosa, H. kraussii* and *C. rehmannii* were ground to fine powder with the use of pestle and mortar, and two grams (2 g) of fine ground leaves was added to a 20 mL (1:10 *m/v*) of 80% (*v/v*) aqueous methanol and mixed overnight in a rotating shaker. The homogenate was centrifuged at 5100 rpm for 20 min to remove debris before supernatant was transferred to sterile tubes. A rotary evaporator was used to concentrate the sample, at 55 °C under vacuum until approximately 1 mL

samples. These samples were dried overnight in a dry bath at 55 °C. The dried samples were reconstituted with 500 µL of 50 % methanol and sonicated for 30 min at 30 °C with the use of sonic bath. The samples were then filtered through a 0.22 µm nylon filter into 2 mL vials fitted with 0.2 mL conical bottom glass inserts.

5.2.2.3. Liquid chromatography mass spectrometry analysis

Liquid chromatography hyphenated to quadrupole-time-of-flight mass spectrometry (LC-qTOF-MS) model LC-MS 9030 instrument equipped with a Shim Pack Velox C_{18} column (100 mm × 2.1 mm, 2.7 µm particle size) (Shimadzu, Kyoto, Japan) and a column oven set at 40 °C, was used for sample analysis. The column oven was set at 40 °C. The binary solvent mixture used consisted of 0.1 % formic acid in water (Eluent A) and 0.1 % formic acid in acetonitrile (Eluent B), at a constant flow rate of 0.3 mL/min. The gradient method was gradually changed from 2 to 31 minto archieve the separation of intended analytes. Briefly, the gradient conditions were as follows; 0 - 2 min, 2 % B, 2 - 24 min, 60 % B, 24 - 25 min, 95 % B, 25 - 27 min, 95 % B, 27 - 28 min, 2 % B, 28 - 30 min, 2 % B and. Analyte elutions were monitored using a mass spectrometer detector under the following conditions: ESI negative modes; 3.5 kV interface voltage (-) and 4.5 kv (+) interface voltage; nitrogen gas was used as nebuliser at flow rate 3L/min, heating gas flow at 10 L/min, heat block temperature at 400 °C, CDL temperature at 250 °C; 1.70kV detector voltage.

5.3. Results and discussion

In the current study, UHPLC-qTOF-MS operated in negative ionisation mode was used to distinguish between closely related flavonoids conjugated to HCAs from three different plants leaf extracts (*B. pilosa, H. kraussii* and *C. rehmannii*) (Figure 5.1). The detailed fragmentation information (MS/MS experiment) was achieved through collision-induced dissociation (CID) approach by varying the collision energies ranging from 0 to 50 eV. Useful fragment ions and accurate mass were successfully used to discriminate between closely related isomers. The order of elution also aided in identifying positional isomers thereof. The retention time (Rt), and MS/MS data are summarised in table 5.1. Additionally, the MS/MS data was compared with results obtained through an informatics-based online software, SIRIUS which generates fragmentation trees (https://bio.informatik.uni-jena.de/sirius/). As a result, a total of 8 chromatographic peaks with typical flavonoid fragmentation patterns conjugated to

HCAs were identified with precursor ions at *m/z* 593.1389, 609.1340, and 625.1290 (Table 5.1). The results revealed the presence of flavonoid glycosides acylated by HCAs (Figure 5.1), as reported recently (Nengovhela *et al*., 2020). Interestingly, all the three plant species were described to share similar flavonoid glycoside conjugated to HCAs, suggesting that these species share a common flavonoid biosynthetic pathway.

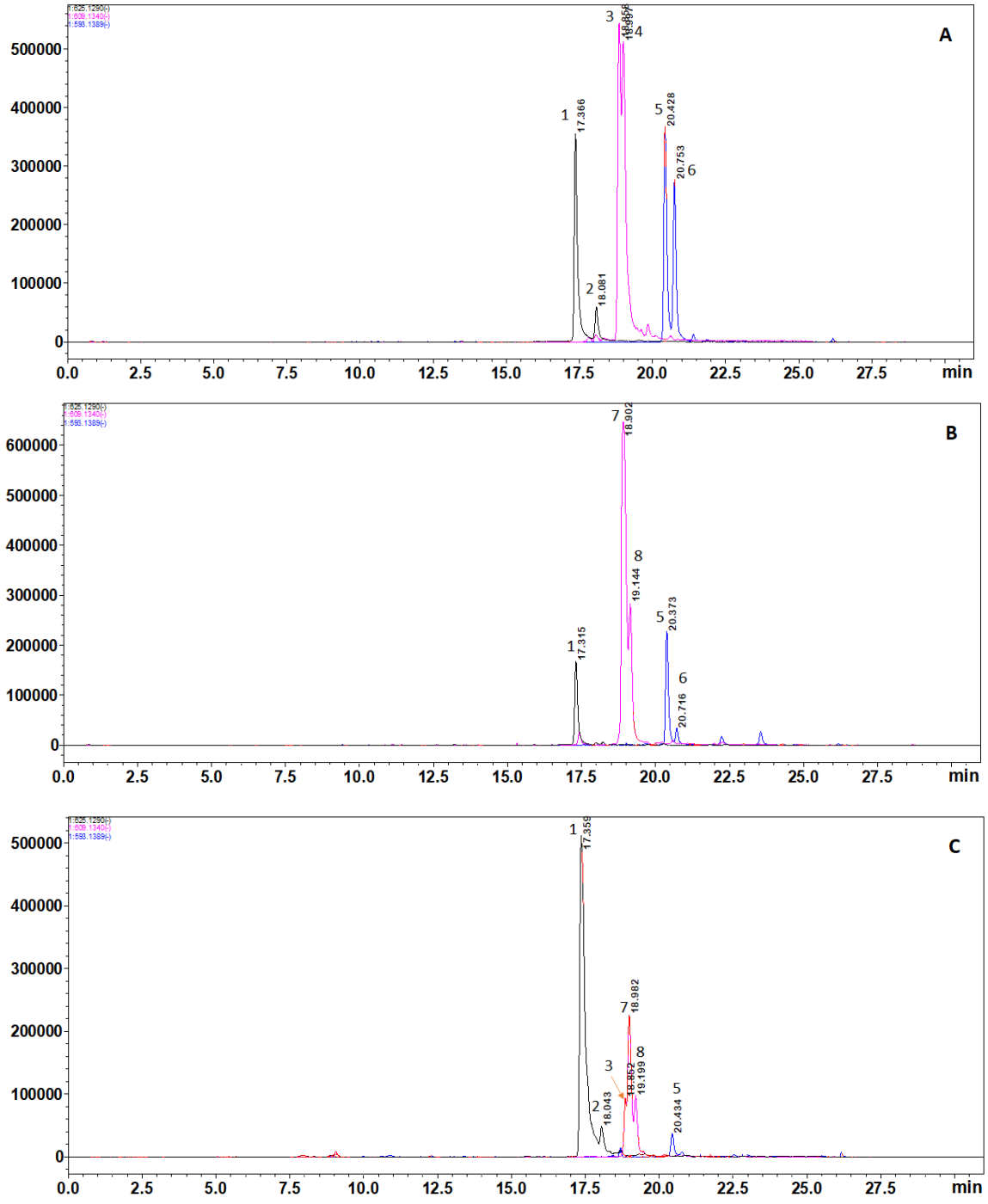

Figure 5.1: Representative ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-qTOF-MS) base peak intensity (BPI) chromatograms showing distribution pattern of flavonoids glycoside conjugated to hydroxycinnamic acids (HCAs) in methanol leaves extracts of naturally growing *C. rehmannii* (**A**), *H. kraussii* (**B**) and *B. pilosa* (**C**).

5.3.1. Characterisation of quercetin-3-*O*-Caffeoylglucoside

Two peaks with the precursor ion at *m/z* 625.1290 were detected in *C. rehmannii* and *B. pilosa* leaf extracts. The two peaks were termed molecule **1** and molecule **2** at Rt 17.36 and 18.08 min, respectively (Figure 5.1a and Figure 5.1c). However, only molecule **1** was detected in *H. kraussii* leaves extract (Figure 5.1b). Based on accurate mass and fragmentation pattern, **1** & **2** were identified as quercetin-3-*O*-caffeoylglucoside due to the presence of product ion at *m/z* 300, indicating the loss of hydrogen free radical from quercetin and additional peaks at *m/z* 271, 179 and 135. The peak at 179 and 135 represent deprotonated caffeic acid and decarboxylated caffeic acid moiety respectively (Table 5.1), indicating the presence of caffeic acid on this flavonoid glycoside. The MS/MS fragmentation pattern was also validated by the fragmentation tree obtained from SIRIUS (Figure 5.2 and Figure 5.6a). Since molecule **2** showed the same fragmentation pattern as molecule **1**, the slight difference in Rt suggests that these molecules are isomers of one another. Furthermore, to validate the identification of these molecules, fragmentation pattern and the predicted molecular formula were compared with already identified quercetin-3-*O*-caffeoylglucoside (Anwar *et al*., 2021; Liang and Xu, 2016). This molecule has also been reported elsewhere (Yoshida *et al*., 2008), with the same fragmentation pattern. However, to the best of our knowledge, this is the first-time positional isomers of quercetin-3-*O*-caffeoylglucoside are identified in *C. rehmannii* and *B. pilosa*.

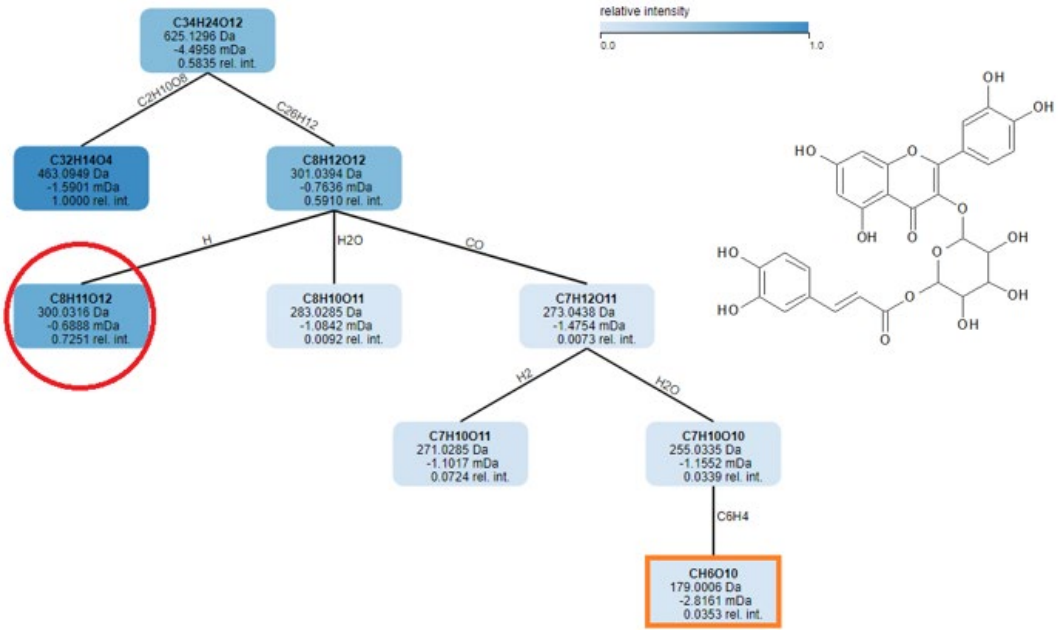

Figure 5.2: Representative fragmentation pattern of Quercetin-3-*O*-Caffeoylglucoside. Red circle indicates the presence of quercetin at *m/z* 300 and orange indicates the presence of hydroxycinnamic acid (Caffeic acid) at *m/z* 179.

5.3.2. Characterisation of Kaempferol-3-*O*-caffeoylglucoside

In *C. rehmannii* and *B. pilosa* leaf extracts, chromatographic peaks (**3** and **4**) with precursor ion $[M-H]^-$ at *m/z* 609.1340 were detected at a Rt of 18.85 and 18.99 min, respectively (Figure 5.1a), while only molecule **3** was detected in *B. pilosa* at a Rt of 18.85 min (Figure 5.1c). Furthermore, molecule **3** and **4** showed MS^2 fragment ion at *m/z* 285 representing deprotonated kaempferol and additional peaks at *m/z* 161 and 135 representing a dehydrated ($-H_2O$) and decarboxylated ($-CO_2$) caffeic acid moiety, respectively (Table 1 and Figure 5.3) and, as such, these two molecules were identified as isomers of kaempferol-3-*O*-caffeoylglucoside (Figure 5.6b).

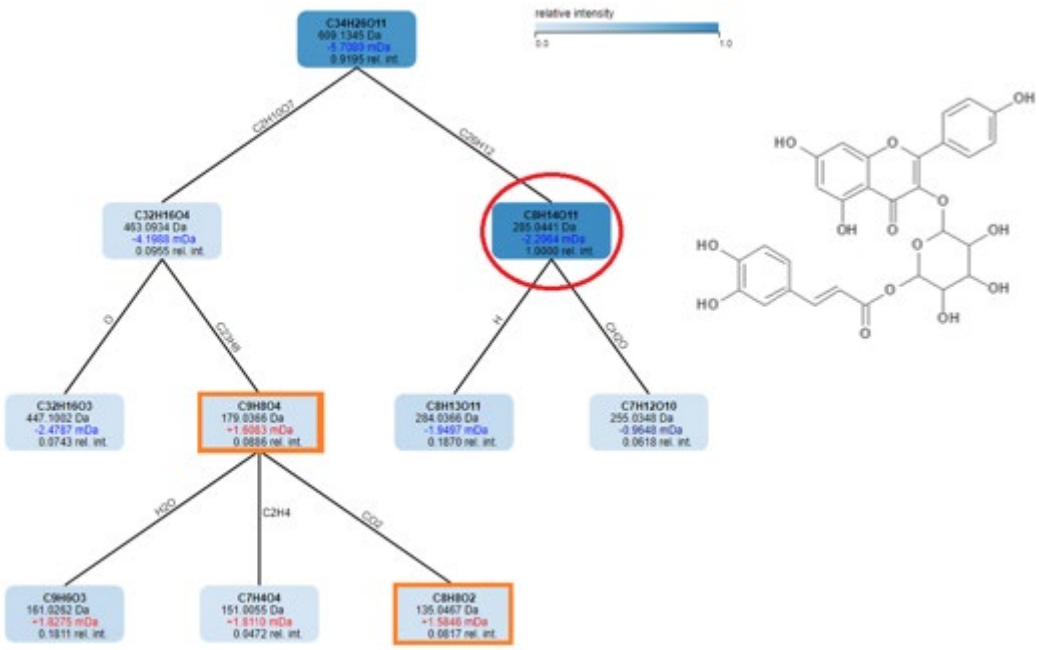

Figure 5.3: Representative fragmentation pattern of Kaempferol-3-*O*-caffeoylglucoside. Red cycle indicates the presence of kaempferol at m/z 285 and orange indicates the presence of hydroxycinnamic acid (Caffeic acid) at m/z 179 and 135 (Decarboxylated caffeic acid).

5.3.3. Characterisation of kaempferol-3-p-coumaroylglucoside

In *C. rehmannii, H. kraussii* and *B. pilosa* leaf extracts, two peaks (**5 & 6**) with precursor ion $[M-H]^-$ at *m/z* 593.1389 were detected in the chromatogram of each specie at Rt 20.4 and 20.7, 20.372 min, respectively (Figure 5.1a and Figure 5.1b), whilst in *B. pilosa* leaves extract, only one peak (**5**) was detected (Figure 5.1c). Molecules **5** and **6** showed a consistent fragmentation pattern (Table 5.1) with product ions at *m/z* 285 and additional peaks at *m/z* 255, 119 and 145 (Figure 5.4). The fragment ion at *m/z* 285 indicates the presence of kaempferol, addition peak at *m/z* 119 indicates decarboxylated coumaric acid. Based on accurate mass, chromatographic retention times (Rt) and fragmentation patterns (Figure 5.6c), these molecules were identified as isomers of kaempferol-3-*p*-coumaroylglucoside. Recently, kaempferol-3-*p*-coumaroylglucoside and its positional isomer has been reported in *C. rehmannii* (Nengovhela *et al*., 2021). To the best of our knowledge, this is the first report of kaempferol-3-*O*-coumaroylglucoside in *B. pilosa* and *H. kraussii*.

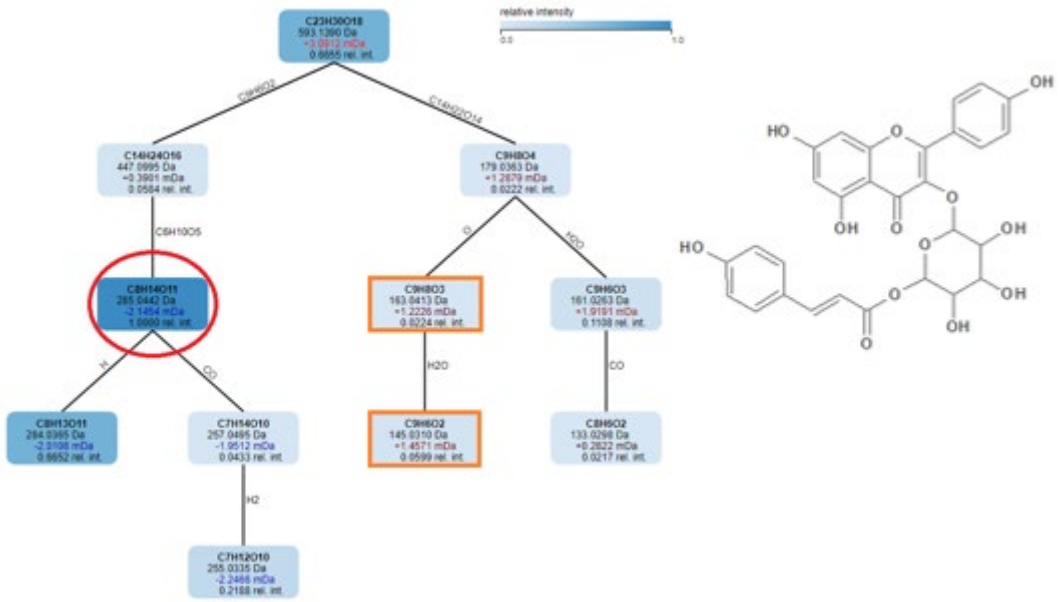

Figure 5.4: Representative fragmentation pattern of Kaempferol-3-*p*-coumaroylglucoside. Red cycle indicates the presence of kaempferol at *m/z* 285 and orange indicates the presence of hydroxycinnamic acid (Coumaric acid) at *m/z* 163 and 145.

5.3.4. Characterisation of quercetin-3-p-coumaroylglucoside

Two peaks (**7** and **8**) with precursor ion $[M-H]^-$ at *m/z* 609.1340 were detected in the chromatograms of *H. kraussii* and *B. pilosa* (Figure 5.1b) with Rt of 18.982 and 19.199 min, respectively (Figure 5.1c). Additionally, these molecules (two from *H. kraussii* and two from *B. pilosa*) also showed similar fragmentation patterns (Figure 5.5 and Table 5.1). As such, these molecules were identified as positional isomers of each other. MS^2 analysis showed a product ion at *m/z* 300 and additional peaks at *m/z* 271, 151 and 163 (Figure 5.6d), indicating the presence of coumaric acid (Davis and Brodbelt, 2008). Therefore molecule **7** and **8** were identified as isomer kaempferol-3-*p*-coumaroylglucoside (Liang and Xu, 2016).

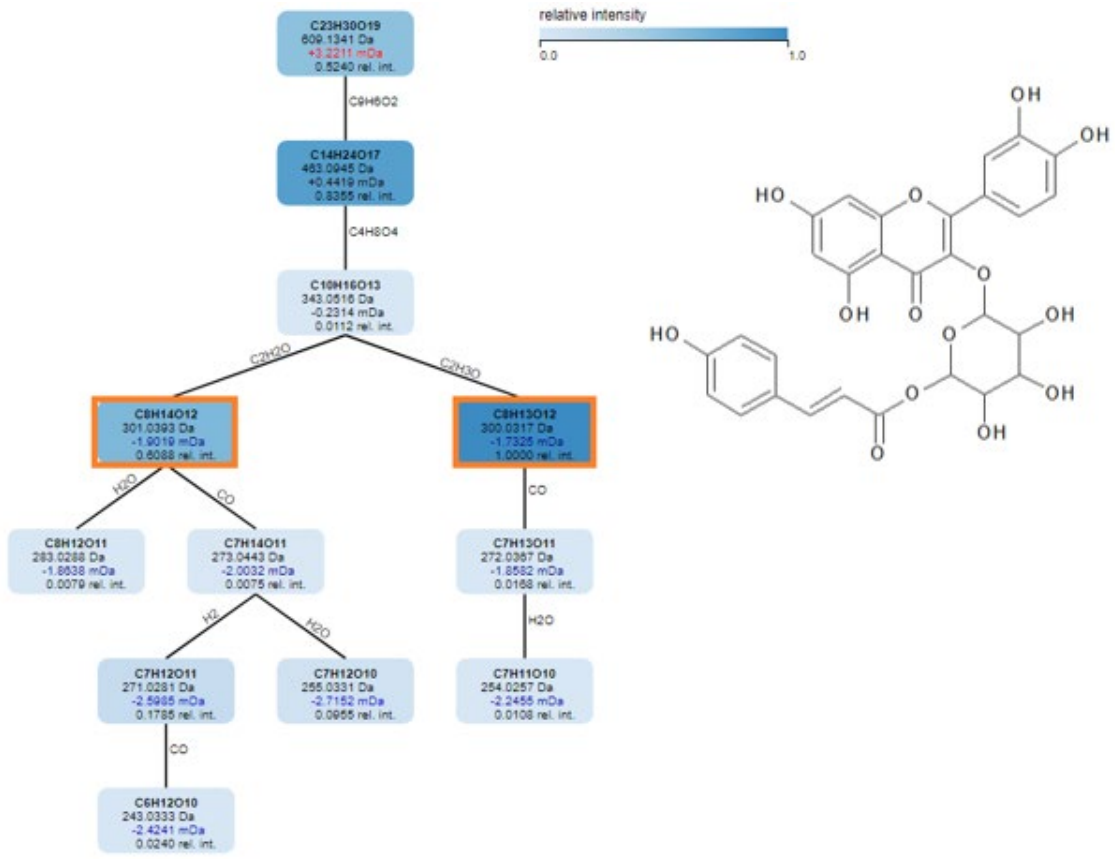

Figure 5.5: Representative fragmentation pattern of quercetin-3-*p*-coumaroylglucoside. Orange colour indicate the presence of quercetin at *m/z* 300 and 301.

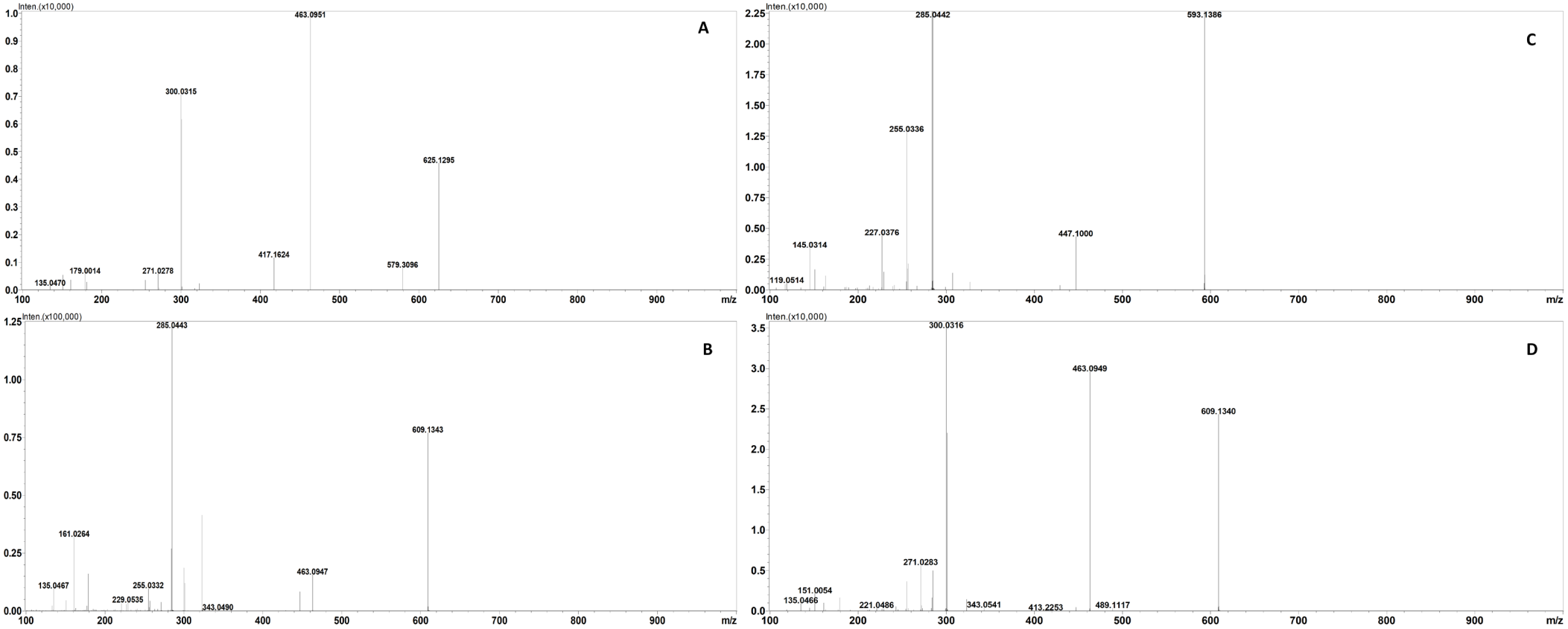

Figure 5.6. Representative electron spray ionisation (ESI) negative spectrum showing the fragmentation pattern of different HCAs conjugated to flavonoids glycoside. Quercetin-3-*O*-Caffeoylglucoside (**A**), Kaempferol-3-*O*-caffeoylglucoside (**B**), Kaempferol-3-*p*-coumaroylglucoside (**C**) and Quircetin-3-*p*-coumaroylglucoside (**D**).

Table 5.1: Characterisation of flavonoids glycoside conjugated to HCAs identified in *C. rehmannii*, *H. kraussii* and *B. pilosa.*

No	*Metabolite identity*	*Mass (m/z)*	*Molecular formula*	*Retention time*	*Fragment ions*	*C. rehmannii*	*H. kraussii*	*B. pilosa*
1	Quercetin-3-*O*-Caffeoylglucoside Isomer I	625.1290	$C_{30}H_{26}O_{15}$	17.366, 17.315, 17.359	463, 300, 271, 179, 135	✓	✓	✓
2	Quercetin-3-*O*-Caffeoylglucoside Isomer II	625.1290	$C_{30}H_{26}O_{15}$	18.081, 18.043	463, 300, 271, 179, 135	✓		✓
3	Kaempferol-3-*O*-caffeoylglucoside Isomer I	609.1340	$C_{30}H_{26}O_{14}$	18.858, 18.902, 18.852	463, 285, 255, 161, 135	✓		✓
4	Kaempferol-3-*O*-caffeoylglucoside Isomer II	609.1340	$C_{30}H_{26}O_{14}$	18.997, 19.144	463, 285, 255, 161, 135	✓		
5	Kaempferol-3-*p*-coumaroylglucoside Isomer I	593.1389	$C_{30}H_{26}O_{13}$	20.428, 20.372, 20.434	285, 250, 145, 119	✓	✓	✓
6	Kaempferol-3-*p*-coumaroylglucoside Isomer II	593.1389	$C_{30}H_{26}O_{13}$	20.753, 20.716	285, 250, 145, 119	✓	✓	
7	Quircetin-3-*p*-coumaroylglucoside isomer I	609.1340	$C_{30}H_{26}O_{14}$	18.982	300, 271, 151, 163		✓	✓
8	Quircetin-3-*p*-coumaroylglucoside isomer II	609.1340	$C_{30}H_{26}O_{14}$	19.199	300, 271, 151, 163		✓	✓

5.4. Conclusions

The UHPLC-qTOF-MS approach is a powerful strategy for separating and identifying the flavonoids glycosides conjugated to HCAs. A relatively large number of these conjugates were identified in *C. rehmannii* compared to *H. kraussii* and *B. pilosa*. A total of eight compounds were detected and tentatively identified as glycosides of quercetin or kaempferol acylated to HCAs derivatives based on fragmentation pattern. In addition, all three species produced similar flavonoids (aglycone) profiles but differed significantly in the HCAs attachment and number of positional isomers. The current results also revealed that the understudied plants use the same mechanism of diversifying flavonoids. Positional isomerisation has been deemed to be responsible for the numerous isobaric peaks of the identified molecules. The presence of these isomers could aid the plants to defend themselves against a broad spectrum of stresses encountered in the environment. The acylation and isomerisation patterns of the identified molecules might influence biological activities and bioavailability and, as such, future studies should investigate the biological/pharmacological properties of the identified compounds.

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CHAPTER 6: GENERAL CONCLUSION

This study aimed to develop a reliable LC-MS-based method for identifying and characterising positional and UV-induced geometrical isomers from various plant extracts. LC-MS-based metabolomics approaches and multivariate statistical tools were used to investigate, profile and compare closely related compounds (isomers) extracted from *Lippia javanica*. Herein, chemically diverse metabolites were differentially distributed throughout the UV-treated and non-treated samples. In this study, it was shown that multivariate statistics are sensitive to deciphering the underlying, subtle changes in the metabolite composition of plants caused by light exposure. From the results, PCA score plots were sufficient to differentiate between UV-exposed extracts from untreated samples. Moreover, cloud plot was good enough to differentiate between metabolites of which the level (or geometry) has been affected due to UV exposure. Most cinnamic acid containing molecules were found to be the ones undergoing geometrical changes due to UV exposure, presumably due to *trans-cis* photoisomerisation. The use of UV-induced photoisomerisation as a plausible approach to modify the metabolome complexity of plants is presented here.

Studied hydroxycinnamic containing molecules, including verbascoside and isoverbascoside that form geometrical isomers upon UV radiation. However, chromatographic elution order was successfully used to assign these positional isomers. To discriminate between the *cis* isomer of verbascoside and *cis* isomer of isoverbascoside, MS^2 analysis showed a clear difference at a collision energy of 35 eV. The ion at *m/z* 161 (dehydrated caffeic acid) showed a base peak which can be a diagnostic ion peak to differentiate between the two. Molecular docking also showed that these compounds including respecting geometrical isomers, binds differently to the active site of cyclooxygenase 1. However, the biological effects of these geometrical isomers are still uknown.

In addition, all the studied plants (*B. pilosa, C. rehmannii*, *H. kraussii* and *L. javanica*) were found to attach these cinnamic acids to its flavonoid glycoside molecules. A relatively large number of these conjugates were identified in *C. rehmannii* compared to *H. kraussii* and *B. pilosa.* All three species produced similar flavonoid (aglycone) profiles

but differed significantly in the HCAs attachment and number of positional isomers. The acylation and isomerisation patterns of the identified molecules might influence biological activities and bioavailability. In addition, the study highlights the importance of investigating the biological and pharmacological properties of different isomers of verbascoside and isoverbascoside. The fact that the geometrical isomers of these compounds showed differences in their ability to bind COX-1 enzyme suggests that these isomers may have different biological activities and potential therapeutic applications. Furthermore, the study suggests that UV radiation of plant extracts could be a useful tool for developing new metabolites with different pharmacological actions. UV radiation is known to induce changes in the chemical composition of plant extracts, which can lead to the formation of new compounds with potentially useful biological activities. Overall, the findings of this study may have important implications for the development of new drugs and natural products with therapeutic potential. Further research in this area could help to identify new compounds with specific pharmacological properties and may lead to the development of new treatments for a range of diseases.