Use of Metabolomics to Understand Ontogenetic and Environmental Influences on the Abundance and Composition of Foliar Phenolic Compounds in Myrtaceae

Santosh Khanal

BSc, MSc

Submitted in total fulfilment of the requirements of the degree of

Doctor of Philosophy

Department of Ecology, Environment and Evolution, School of Life Sciences College of Science, Health and Engineering La Trobe University, Melbourne, Victoria, 3086 Australia

July 2020

This thesis includes work by the author that has been published or accepted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

This work was supported by a La Trobe University Postgraduate Research Scholarship.

Santosh Khanal 20th of July 2020

Table of Contents

List of figures

List of tables

List of abbreviations

ANOVA	Analysis of variance
ATSC	Australian tree seed centre
CT	Condensed tannin
ESI	Electrospray ionisation
ET	Ellagitannin
FC	Folin ciocalteu
FPCs	Formylated phloroglucinol compounds
GLM	Generalised linear model
HHDP	Hexahydroxydiphenic
HMDB	Human metabolome database
HT	hydrolysable tannin
LC-MS	Liquid chromatography-mass spectrometry
LED	Light-emitting diode
m/z	Mass by charge
MANOVA	Multivariate analysis of variance
MS/MS	Tandem mass spectrometry
PAR	Photosynthetically active radiation
PCA	Principal components analysis
PLS-DA	Partial least squares discriminant analysis
PPC	Protein precipitation capacity
ppm	Parts per million
PSII	Photosystem II
PSMs	Plant specialised metabolites
QC	Quality control
ROS	Reactive oxygen species
UPLC	Ultra-performance liquid chromatography
UV_A	Ultraviolet A
UV_B	Ultraviolet B
UV_C	Ultraviolet C
VIP	Variable importance in projection

Acknowledgements

I would first like to thank my supervisor Assoc Prof Martin Steinbauer, for your support throughout my PhD. The knowledge and experience you brought to my research were indispensable, and I feel fortunate to have had you as my supervisor during the last four years. I appreciate your prompt replies and dedication to my supervision. I would also be forever grateful to my co-supervisor, Dr Simone Rochfort (Research Leader Molecular Phenomics, Agriculture Victoria and La Trobe University). Your insight and expertise in my topic have helped me to overcome many confusions, and your constructive feedback and valuable guidance throughout these years have made my PhD a meaningful journey.

I am grateful for LTUPRS and LTUFFRS from La Trobe University that enabled me to do my PhD in Australia. I am grateful to have completed my PhD in the department where there is so much support for HDR students and research. Special thanks to my past and present Insect-Plant Interactions lab members. Looking back, I feel so lucky to have worked with such a fantastic group of people. Special mention to Thomas, Reza, Surendra, Duncan, Julia, Berenice, James, Umar, Kevin and Beryn.

Extra special thanks go to Priyanka Reddy and Elizabeth Read from AgriBio, Centre for Agri Bioscience, for always being there to help, whether for depth discussion or quick questions or just for a cup of tea. Additionally, thanks must go out to the other members of the molecular phenomics group for their support services and in troubleshooting with the equipment and software.

Lastly, I would like to thank my friends and family for all the moral support and love they gave me. I would like to express my heartfelt gratitude to my parents and my sisters Kabita and Saru for always being there to talk to me when I needed them and for supporting me on this journey from halfway around the world. A special thanks go to my wife Archana, who always supported me with a lot of love and good food in all highs and lows. I cannot

describe in words how supportive you have been during these challenging times. Thanks for being a ray of sunshine in my life. Also, special mention would go to my friends Ganesh, Pramod, Dipak, Niranjan, Vhisma and Achyut for the motivation and who have made me feel so welcome during my time here.

To those, I have named, and to those not recognised by name despite your support, I thank you all.

Abstract

Phenolic compounds are the most ubiquitous and diverse class of plant specialised metabolites and have long been suggested to have important abiotic and biotic functions. I used an unbiased metabolomics-based approach to investigate contrasting functions of phenolic compounds in *Eucalyptus camaldulensis* as well as individual species from four other genera (*Syzygium*, *Angophora*, *Callistemon*, *Corymbia*) of the family Myrtaceae. Chapter I is a review of foliar phenolic compounds as non-enzymatic adaptations to abiotic and biotic stressors. Chapter II provides a database of foliar phenolic compounds that I identified and that are referred to in my empirical or experimental chapters. In Chapter III, I consider how leaf colour and phenolic compounds in leaves of species belonging to five genera respond to seasonal changes in climatic conditions, in particular the UV-index. I found that various hydrolysable tannins and flavonoids were common to all genera and varied with leaf age and season. For Chapter IV, I studied the effect of experimentally elevated UV_A on the expression of phenolic compounds in five chemotypes of *Eucalyptus camaldulensis* subspecies *camaldulensis* spanning the lowest and highest latitudes of its range. Various hydrolysable tannins (gallotannins and ellagitannins) increased most in concentration with elevated UV_A irrespective of the latitude of the parent tree. The greatest change in the metabolites of individual leaves occurred between 3 and 6 months of age, but thereafter there was much less change. For Chapter V, canopy and understorey (sapling) leaves of *E. camaldulensis* infested with *Cardiaspina albitextura* nymphs were studied because this genus of hemipteran insect has been shown to cause photodamage-like symptoms. Concentrations of various hydrolysable tannins and galloyl glucoses were higher in younger than older leaves, especially in understorey leaves, whereas concentrations of phenylpropanoic acid derivatives were higher in canopy leaves. In Chapter VI, I group phenolic metabolites according to their putative functions and

synthesise how eucalypts respond to the abiotic and biotic stressors I considered. My findings are consistent with published literature that attributes a photoprotective function to hydrolysable tannins because they were higher in younger leaves than in older leaves and were affected by levels of UV radiation, i.e. increased hydrolysable tannins concentration in higher UV levels (index and experimental). However, my findings do not preclude protection against biotic stressors because the only insect herbivore I studied is a specialist of leaves older than six months of age.

Contributions of others to my thesis

Person	Contribution
Assoc Prof. Martin Steinbauer	Conceptualised, designed, and supervised the project. He also contributed to the interpretation of results and writing the thesis.
Dr. Simone Rochfort	Co-supervised the project. She also provided lab space and instrument access, contributed to the interpretation of results.

Chapter I

Plant phenolic compounds as non-enzymatic adaptations to abiotic and biotic stressors: a review

1.1. Introduction

Plants colonised the land during the mid-Palaeozoic era, somewhere around 400 million years ago (Cheynier *et al.*, 2013). It is hypothesised that this adaptative shift by early plants such as green algae (Charophyceae) was partly achieved by the proliferation of phenolic compounds that acted as 'phenolic light screen', that protected the plant by absorbing the excess of Ultraviolet (UV) light, (UV_A and UV_B) (Lowry *et al.*, 1980; Graham *et al.*, 2000). Excessive light stress in plants results in damage to photosystem II (PS II) by producing active oxygen species (Kasson *et al*., 2012). This occurs in two ways: (1) the donation of electrons to oxygen because of photosynthetic activity; (2) exposure of plants to UV radiation. Production of reactive oxygen species (ROS) is amplified under excess light leading to DNA damage and cell death (Hollósy, 2002; González-Pérez *et al*., 2011). Several complex mechanisms minimise this formation of active oxygen species, and they are eliminated rapidly by efficient antioxidative systems (Foyer *et al.,* 1994; Foyer & Shigeoka, 2011).

Gradually, throughout the evolution, higher plants, mainly vascular plants, have developed their ability to synthesise a complex array of polymerised phenolic compounds such as tannins and other complex phenolics ubiquitous in higher plants (Ignat *et al.,* 2011). Phenolic compounds are a broad range of metabolites with an aromatic group and one or more hydroxyl groups on their aromatic ring. They exhibit broad structural heterogeneity associated with methylation, acylation, glycosylation, and other biosynthetic processes, which alter the polarity, volatility, chemical stability of these compounds and their ability to interact with other compounds (Watson *et al.,* 2018). Some of the phenolic compounds are widely distributed throughout the plant kingdom, whilst others are limited to specific genera of the plant, making them a convenient biomarker for taxonomic studies (Cheynier *et al.,* 2013). Overall, this diversity and distribution of phenolic compounds throughout the plant kingdom suggest a broad range of physiological and ecological functions, including

protection against abiotic and biotic stress (Hammerschmidt & Schultz, 1996; Ramakrishna & Ravishankar, 2011). This greater chemical diversity may also increase the likelihood of enhanced fitness of plants by producing a rare chemical with potent and useful biological activity, and selection also favours these plant lineages (Firn & Jones, 2000). Although it has long been appreciated that plant phenolic compounds have important abiotic and biotic roles, the extent to which they act in any specific capacity depends upon the biological value to the plant of the role(s) they serve and the value of the leaf protected at a given stage of its growth, e.g., whether an expanding or fully expanded leaf (Wam *et al.,* 2017). For example, according to the optimal defence hypothesis, the limited resources of defensive compounds should be concentrated in the regions that would most increase the fitness of plants, such as younger tissues that are attractive to biotic threats (McKey, 1974).

The species-rich family Myrtaceae has vast diversity with its long-lived plants and a wide geographical distribution range (Grattapaglia *et al.,* 2012). Myrtaceae family is known to accumulate high concentration phenolic compounds and other specialised metabolites such as phloroglucinols, β-triketones, triterpenes and alkaloids. These compounds are known for their antioxidant activity due to their capacity to scavenge free radicals, quench reactive oxygen species and protect against lipid peroxidation in the plant (Reynertson *et al.,* 2008) *Eucalyptus* is the most dominant Myrtaceae genus in Australia, and together with the genera *Corymbia and Angophora*, they form a monophyletic group called 'eucalypts' (Merchant *et al.,* 2007). Various eucalypts species occupy diverse habitats of Australia, i.e., from alpine regions to dry arid regions and have experienced a range of environmental pressures such as solar radiation, drought and herbivory and thus have evolved under these environments (Bennett, 2016). These eucalypts deploy a complex array of phenolic compounds throughout their lifetime. Therefore, they are an excellent system for understanding the plant response to various abiotic and biotic factors in these long-lived

tree species (Goodger *et al*., 2013). The review chapter provides comprehensive analyses of the published literature concerning foliar phenolic compounds evoking their role against herbivores and the abiotic threat against photodamage caused by reactive oxygen species. This review focuses on the effect of UV_A radiation on plant phenolics as it forms the key focus of my research chapters, as there is a lack of research compared to UV_B.

1.2. Plant specialised metabolites: Plant phenolic compounds

Plants produce a wide array of compounds that can be subdivided into primary metabolites and plant secondary metabolites or plant specialised metabolites (PSM). Primary metabolites such as carbohydrates and proteins are vital for the plant's survival and are involved in the central process of growth and development. However, PSMs may not be directly involved in basic life functions but are involved in many essential functions for growth and development, including the interactions between the plants and their surrounding environment during both abiotic and biotic stress (Haukioja, 2005; Delgoda & Murray, 2017). PSMs are known to respond to a wide range of abiotic stress such as extremes of temperature, UV stress, moisture deficit, pH, salinity, and biotic stress such as pathogen infection and herbivory (Ramakrishna & Ravishankar, 2011; War *et al.,* 2012). The strong overlap of similar PSMs in various plant species, in addition to the diversity between plant species, raises questions about their functional importance and redundancy (Kessler & Kalske, 2018). The wide variety of the PSM is known to correlate with functional diversity. The same environment or trait may be related to different metabolites, suggesting that metabolite's functional redundancy and a metabolite can show very different or even opposite relations with the same environmental condition across the two regions, suggesting versatility of the metabolite within species (Labarrere *et al*., 2019). Over 200,000 PSMs have currently been identified, and they are mainly classified as phenolic compounds, terpenoids, polyketides, cyanogenic glycosides, alkaloids, and sulphur-containing compounds (Thirumurugan 2018; Pyne *et al.,* 2019).

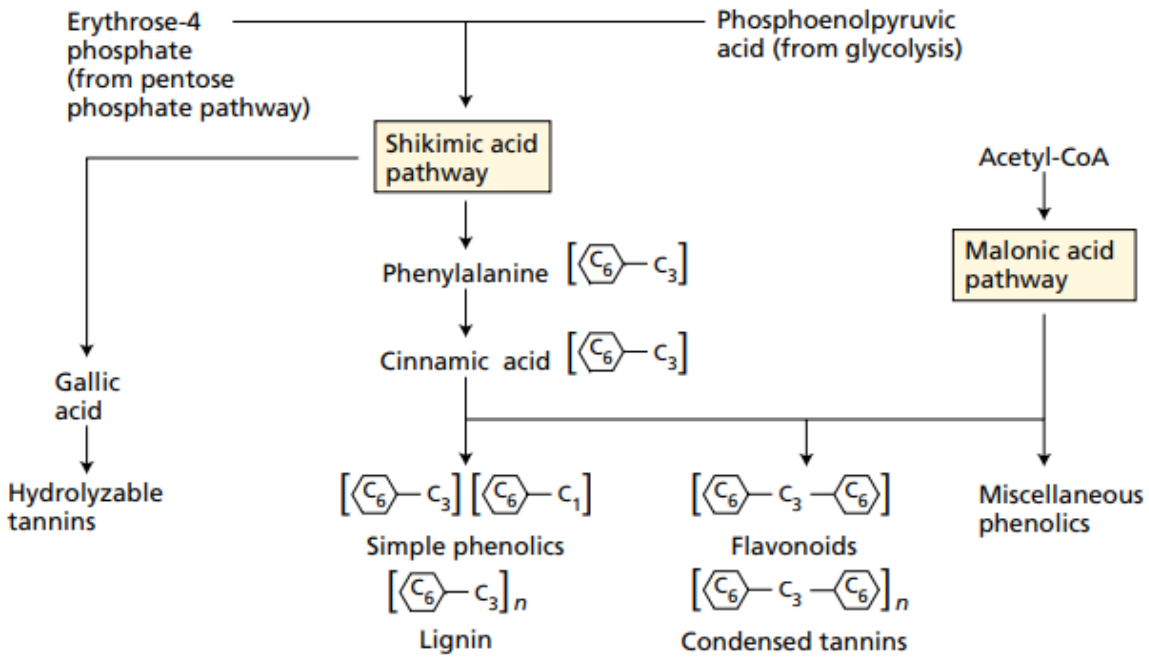


Figure 1.1. Biosynthesis of plant phenolic compounds via the (1) Shikimic acid pathway and (2) Malonic acid pathway (Taiz and Zeiger, 2002).

Phenolic compounds are among the most widely distributed groups of PSMs (Herrero *et al.*, 2013). They are organic compounds with at least one aromatic ring with one or more hydroxyl groups (Harborne, 1984). Over 8,000 phenolic compounds, collectively known as polyphenolics, are currently identified in plants (Vuolo *et al.*, 2019). These phenolic compounds are synthesised from two different routes via the (1) shikimate and (2) acetate/malonate pathways (Figure 1.1) (Strack, 1997; Robards, 2003). The majority of plant phenolic compounds are produced by the shikimate pathway (Saltveit, 2017). The shikimate pathway is localised in the chloroplast and consists of seven sequential enzymatic steps (Herrmann, 1995). This pathway serves as an entry to the biosynthesis of phenylpropanoids (C_6–C_3) and various subsequent phenolic compounds. It provides a major link between primary and secondary metabolism in higher plants (Tsopmo *et al.*, 2013). The major plant phenolic compounds include tannins, flavonoids, phenolics acids, stilbenes and lignans (Pandey & Rizvi, 2009). They are differentiated based on their

different patterns of aromatic ring hydroxylation and methoxylation and increasing complexity of the basic skeleton (Minatel *et al*., 2017; Tsimogiannis & Oreopoulou, 2019)

1.2.1. Classification of phenolic compounds

Several phenolic compounds have been categorised into groups based on the number of carbons and chemical structures of the aglycones (Table 1.1). These phenolic compounds can range from simple phenolic compounds with six carbons to highly polymerised compounds with multiple numbers of carbons. They can also have a highly diverse structure and occur as conjugates with mono and polysaccharides and associated with one or more phenolic groups (Harborne, 1984; Minatel *et al.,* 2017). Among these various classes of phenolic compounds, the leaf profile of family Myrtaceae leaves was dominated by tannins, phenolic acids and flavonoids. Also, these phenolic compounds are shown to have strong effects on protein precipitation capacity and oxidative activity and are described further below (Salvador *et al*., 2011).

Table 1.1. Classification of phenolic aglycones, including the number of carbon atoms (Harborne and Simmonds; 1964)

No. of C-atoms	Basic carbon skeleton	Major category
6	C_6	Simple phenols
7	C_6-C_1	Phenolic acids
8	C_6-C_2	Phenylacetic acids, Hydroxy-cinnamic acids
9	C_6-C_3	Coumarins, Isocoumarins, Chromones
10	C_6-C_4	Naphthoquinones
13	C_6-C_1-C_6	Xanthones, Stibenes
14	C_6-C_2-C_6	Anthraquinones
15	C_6-C_3-C_6	Flavonoids
18	$[C_6$-$C_3]_2$	Lignans, Neolignans
30	$[C_6$-C_3-$C_6]_2$	Biflavonoids
n	$[C_6]_n$, $[C_6$-$C_3]_n$	Lignins, Metanins
n	$[C_6$-C_3-$C_6]_n$	Tannins

1.2.1.1. Phenolic acids

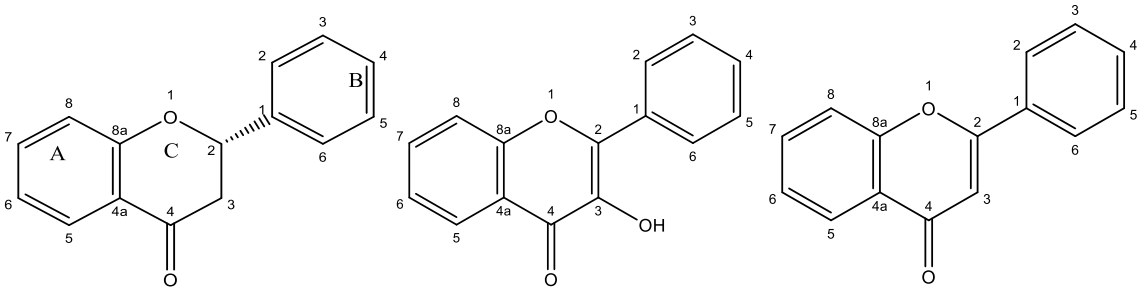
Phenolic acids are the derivatives of cinnamic and benzoic acids and have benzene rings with one or more hydroxyl substituents (Saibabu *et al.,* 2015). They are universally distributed in plants, and some of the most common phenolic acids include caffeic acid and ferulic acids (Heleno *et al.,* 2015). Phenolic acids are known for their diverse function, such as antioxidant properties (Stuper-Szablewska *et al.,* 2019), their defensive roles against herbivores (Summers & Felton, 1994), and their role in plant-microbe interactions (Mandal *et al.,* 2010). Plants are also known to accumulate various phenolic acids under abiotic stress, such as salinity stress, where an effective increase in caffeic acid and p-coumaric acid was shown at gradual salt stress (Jamalian *et al.,* 2013). They are also known to improve plant growth under drought stress and heavy metal toxicity (Rivero *et al.,* 2001). Phenolic acids also serve as precursor molecules for the various more complex

phenolic compounds such as stilbenes, chalcones, flavonoids, lignans and anthocyanins (Mandal *et al.,* 2010).

1.2.1.2. Flavonoids

Flavonoids are low molecular weight specialised metabolites formed from the 15-carbon skeleton consisting of two benzene rings A and B linked via a heterocyclic pyrane ring (C) arranged in C_6-C_3-C_6 form (Table 1.1, Figure 1.2) (Panche *et al.,* 2016). They are the largest group of plant phenolic compounds with more than 6,000 naturally occurring flavonoids that have been currently identified (Panche *et al.,* 2016). Flavonoids have a common biosynthetic origin via phenylpropanoid metabolism and malonyl-coenzyme-A pathway (Kumar and Pandey, 2013). They can be divided into six subgroups: flavones, anthocyanidins, flavan-3-ols, flavonols, flavanones, and isoflavones (Ferreyra *et al*., 2012). Substitutions such as oxygenation, acylation, glycosylation, alkylation, and sulfation to the rings A and B give rise to the various compounds within each class of flavonoids (Pietta, 2000). The spectroscopic study of flavonoids exhibits two major absorption bands: Band I (320–385 nm) corresponds to B ring absorption and Band II (250–285 nm), which corresponds to A ring (Mandi, 2017). This light absorption property of the flavonoids in the visible and ultraviolet light region is responsible for the colour generation in plants and function as sunscreen, blocking the UV rays (Sisa *et al.*, 2010). Flavonoids function as antioxidants and protect the plant against high UV radiation by screening and have various roles in defence and signalling (Treutter, 2005; Panche *et al.*, 2016). During evolution, oxidative stress might have been a major factor for the distribution and/or abundance of these flavonoids, highlighting photoprotection as the most significant functional role of flavonoids (Treutter, 2005). One of the major flavonoid groups is anthocyanins, which are water-soluble pigments that give various colours to flowers, fruits, and leaves. Studies have shown flavonoids such as anthocyanins in the

mesophyll layer preventing excessive light capture by chloroplasts reducing the risk of photo-oxidative damage to leaf cells (Feild *et al*., 2001; Hoch *et al.*, 2003).



Flavanones Flavonols Flavones

.

Figure 1.2. Examples of different classes of flavonoids varying according to the degree of oxidation of the central pyran ring. They are made of 15-carbon structure connected by two benzene rings (A and B) linked via a heterocyclic pyrane ring (C). Figure adapted from Panche (2016).

1.2.1.3. Tannins

Tannins are high molecular weight phenolic compounds with a molecular weight ranging from 300 to 55,000 Da (Santos-Buelga & Scalbert, 2000; Guyot *et al.,* 2001). They are subdivided into hydrolysable (HTs), non-hydrolysable or condensed tannins (CTs), and phlorotannins. HTs have a central polyol core of polyhydric alcohol and hydroxyl group. They are esterified by gallic acid-forming gallotannins (GTs) or by hexahydroxydiphenic acid-forming ellagitannins (ETs), whilst CTs are a group of flavonoids derived from oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond (Salminen & Karonen, 2011). They are also called proanthocyanidins because, under strongly acidic conditions, they decompose to give anthocyanidins by cleavage of the C-C interflavanyl bond (Santos-Buelga & Scalbert, 2000). There is a third group of tannins known as phlorotannins which are mostly reported in algae and are oligomers and polymers of the monomeric unit phloroglucinol (1,3,5-tri-hydroxybenzene) (Shibata *et al.*, 2004).

1.2.2. Functions of plant phenolics

The primary function of plant phenolic compounds in various biotic and abiotic environments is difficult to estimate. Plant phenolic compounds have long been considered to have evolved as classic defence compounds that protect plants from herbivores (Close & McArthur, 2002). Although defence theory of plant phenolics compounds is now well established in various literature, there is also diverse literature suggesting alternative roles of these phenolics, namely protection against photodamage (Rice-Evans *et al*., 1997; Sakihama *et al*., 2002; Tattini *et al*., 2004; Agati and Tattini, 2010; Agati *et al*., 2013). While some study has shown a significant effect of herbivory on the concentration of phenolic compounds (Agrawal, 2011; Barbehenn and Constabel, 2011; Lampert *et al*., 2011), other studies have questioned their roles as defensive compounds (Jermy, 1993; Close & McArthur, 2002). Therefore, to understand the role of plant phenolic compounds, evaluation of their selective agents at an evolutionary level or ecological level is crucial.

Overall, plant phenolic compounds are known to have multiple roles in plant responses against herbivores, photodamage, and oxidative stress (Figure 1.3) (Schneider *et al.*, 2019). Also, they are known to function as signal molecules in pollination (i.e., the formation of visual cues via colour generation), seed disperser attractants, and nutrient cycling (Bhattacharya *et al.*, 2010). Some of the major functions of plant phenolics are described below:

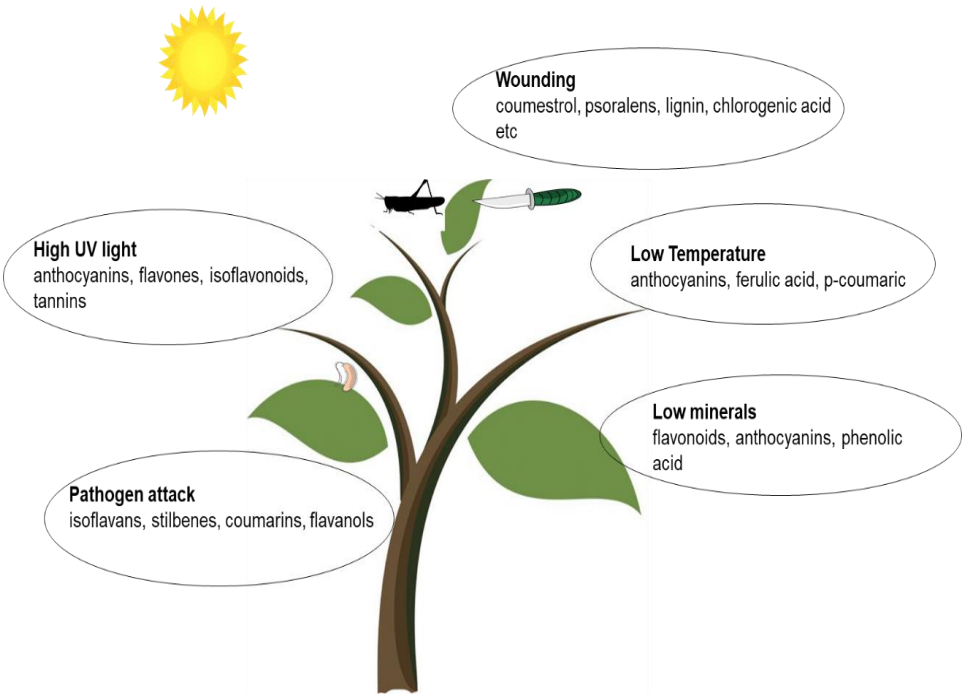


Figure 1.3. Various biotic and abiotic stress-induced phenolic compound synthesis in plants. Different phenolic compounds are induced by abiotic stress such as low temperatures, high UV, low minerals, and by biotic stress such as wounding and pathogen attack. Figure adapted from Bhattacharya (2010).

1.2.2.1. Photoprotection and oxidative response to photodamage

Solar radiation received by the Earth can be broken down into three major components of varying wavelengths, visible, UV and infrared radiation. UV radiation is characterised by the shortest wavelength ranging from 100-400 nm and is further subdivided into three regions. These regions are UV_A, UV_B and UV_C, with the longest wavelength being UV_A and the shortest being UV_C (Guan *et al.,* 2016). UV_B and UV_C radiation are very harmful to the organic tissue; however, 100% of UV_C is absorbed by the Earth's atmosphere, and the majority of UV_B radiation (approximately 90%-95%) is absorbed by the ozone layer doesn't reach the Earth's surface (Schuch *et al.,* 2017). UV_A radiation (320–400 nm) accounts for 90 to 95% of total UV radiation that reaches the Earth's surface. It comprises shortwave UV_A or UV_{A2} (320–340 nm) and long-wave UV_A or UV_{A1} (340–400 nm) (Figure

1.4) (Verdaguer *et al.*, 2017). UV radiation can vary with latitude, altitude, cloud cover, time of day, and across seasons (Ahmad, 2017.)

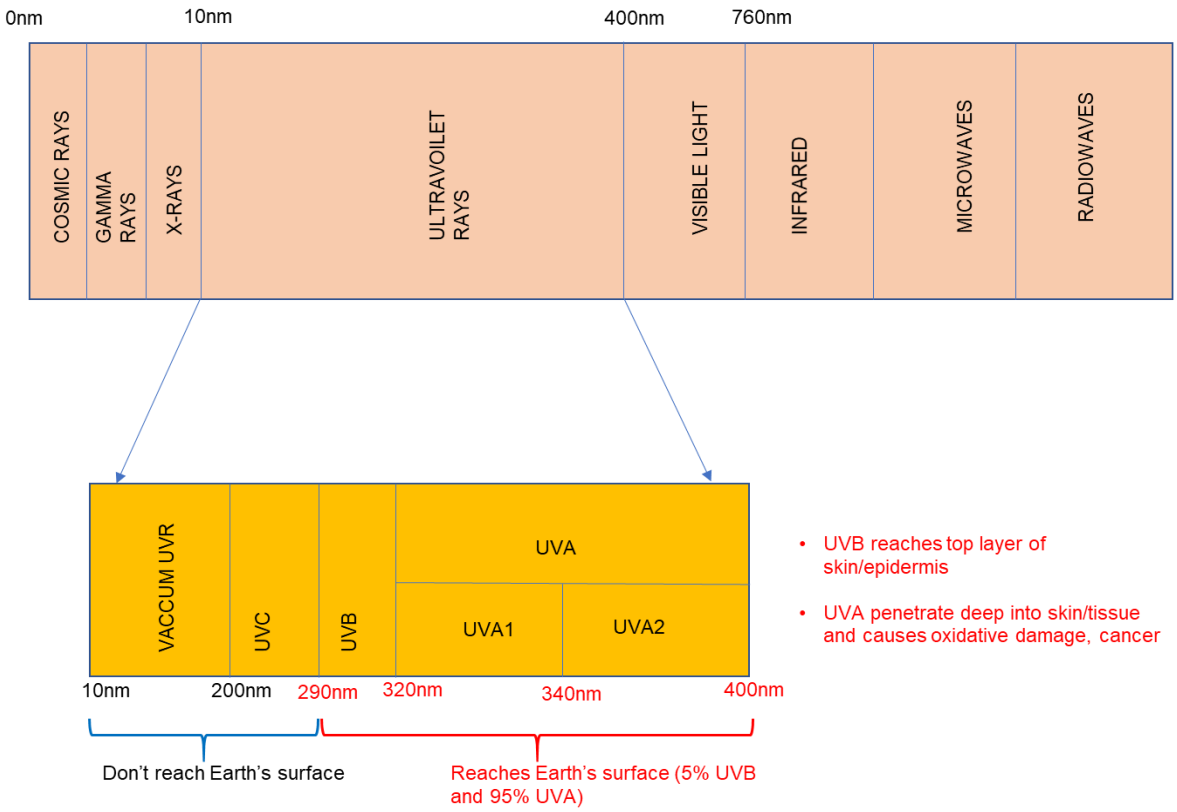


Figure 1.4. Types of UV radiation and its effects on a living organism. Figure adapted from Hollósy (2002).

The UV screening effectiveness by the leaf depends upon the pigment distribution, epidermal cell shape and cuticle thickness and leaf morphology and orientation (Vogelmann, 1989, 1993). The penetration of UV_B through the leaf is negligible, and the cuticle absorbs almost all the UVB. However, penetration of UV radiation increases in leaves with increasing wavelength, i.e., UV_A has deeper penetration (Grant *et al.*, 2003). Most of the excess UV_A radiation is screened out by phenolic compounds in the leaf epidermis, and the radicals formed by the energy of deeper penetrated UV_A can be stabilised by delocalisation by scavenging peroxide and oxygen radical (Kolb et al., 2001; Close et al., 2007; Verdaguer et al., 2017). UV_A penetrating leaf epidermis is known to initiate oxidative processes by endogenous photosensitisation through the generation of

H_2O_2, singlet oxygen and free radicals leading to cellular and structural DNA damage (Sage *et al.,* 2011). Although 95% of the UV that reaches the Earth is UV_A, compared to just 2-5% UV_B, major research has focused on the effect of UV_B because of its higher damaging potential (Frederick *et al.,* 1989; Ballaré *et al.,* 2011). Although UV_A radiation is less potent per photon than UV_B (D'Orazio *et al.,* 2013), plants are exposed 10-100 times more UV_A photons than UV_B, and it penetrates deeper into plants tissue (Hewitt, 2002). Therefore, the damaging and inhibiting effects of UV_A radiation, in addition to UV_B radiation, on the production of phenolics and the growth and photosynthesis of plants should also be considered (Nawkar *et al.,* 2013).

One of the protective mechanisms of plants against high UV radiation is the accumulation of various phenolic compounds with an appropriate UV absorptive property (Morales *et al.,* 2010; Mandi, 2017). Different phenolic compounds have their specific ranges of absorption maxima that can absorb harmful UV at various wavelengths without impacting photosynthetically active radiation. For example, hydroxycinnamic acids have an absorption maximum between 310−332 nm, flavones between 250−270 and 330−350 nm, and flavonols have between 250−270 nm and 350−390 nm (Cerovic *et al.,* 2002). Phenolic compounds such as hydroxycinnamic acids and flavonoids have been reported to occur in cell walls, absorbing as much as 60% of the incident UV radiation, allowing only 5-10% of total UV to reach mesophyll (Larcher, 1995). Further, phenolic compounds such as flavonoids and tannins are also known to act as strong antioxidant agents by quenching peroxyl radicals which may protect plants from photodamage (Figure 1.5) (Nijveldt *et al*., 2001). Various flavonoids such as anthocyanins, which are low molecular weight phenolic compounds, are known to be induced more than other phenolic compounds and are known to be active plant antioxidants *in vitro* (Pietta, 2000; Panche *et al.,* 2016). These flavonoids can quench free radicals by directly scavenging the reactive oxygen species, activating antioxidant enzymes and by increasing the antioxidant properties of other low molecular

antioxidants (Procházková *et al.*, 2011; S. Kumar & Pandey, 2013; Das & Roychoudhury, 2014). Plant experiencing stress such as low temperature, drought or salinity are known to absorb more than light than that is required for photosynthesis which is manifest of a decreased photosynthetic capacity resulting in photoinhibition (Goh *et al.*, 2012). Phenolic compounds, including phenolic acids, flavonols and anthocyanins, are known to accumulate in the epidermis of stressed and photo inhibited leaves and screen out the damaging radiation and prevent oxidative damage in cells and PSII showing their active role in photoprotection (Takahashi & Badger, 2011). Various hydrolysable tannins are known for their strong antioxidant properties and were 15-20 times more effective at quenching peroxyl radicals than simple phenolic compounds (Hagerman *et al.*; 1998). Further, studies have shown that sun adapted leaves of *Mahonia repens* (Berberidaceae) and *Eucalyptus nitens* (Myrtaceae) seedlings were found to have a higher level of hydrolysable tannins, phenolic, chlorogenic acid than shade adapted leaves suggesting that this might be direct in response to oxidative pressure (Grace *et al.* 1998; Close *et al.* 2001).

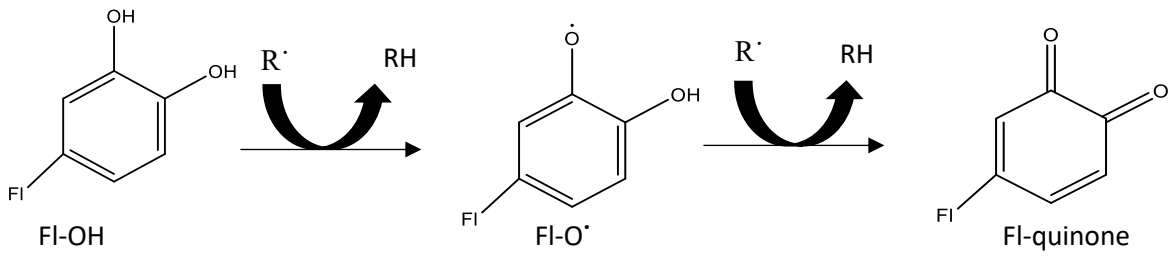


Figure 1.5. Example of the mechanism of reactive oxygen species (R˙) scavenging by flavonoid (Fl-OH) producing stable quinone structure. Figure adapted from Kumar & Pandey (2013).

In a meta-analysis of data from 34 field studies, it was found that exposure to UV_B caused an increase up to 25% in the concentration of various UV_B absorbing compounds and increased the DNA damage up to 90% in some bryophytes and angiosperms (Newsham &

Robinson, 2009). There are comprehensive studies and reviews on the effects of ambient and supplemental UV_B radiation on plants (Conner & Neumeier, 2002; Keski-Saari *et al.,* 2005; Rozema *et al.,* 2005; Ballaré *et al.,* 2011; Xiao *et al.,* 2015) but very few studies have been conducted looking the effect of UV_A on plant phenolic compounds. A study found an increase in the concentration of anthocyanin in *Lactuca sativa* (L.) under enhanced UV_A (Li & Kubota, 2009), and another similar study found that UV_A enhanced the content of phenolics in *Perilla frutescens* (L) (Iwai *et al.,* 2010). Similarly, there was an increased concentration of phenolic compounds in two basil, *Ocimum basilicum* (L). with supplemental UV_A LED lights (Vaštakaitė *et al.,* 2015). However, another study did not find any significant effects of UV_A on the content of phenolic compounds in lettuce, *Lactuca sativa* (L.) (Tsormpatsidis *et al.,* 2008). Further, a study on *Eucalyptus nitens* (H. Deane and Maiden) seedlings showed that UV_A can alter the concentration of individual phenolic compounds such as gallotannin, stilbene, and flavonols, and their responses can be non-identical to each other (Close *et al.,* 2007). Similarly, another study showed that high irradiance (1200-1500 μmol m^{-2} s^{-1}) of UV_A (320-335 nm) promoted the accumulation of various flavonols in the epidermis of young leaves of *Mesembryanthemum crystallinum* (L.) (Ibdah *et al.,* 2002). Another study showed a decrease in total flavonoid concentration by UV_A treatment in two betulaceous species (Kotilainen *et al.,* 2008). Therefore, although UV_A is the main component of solar radiation, its effect on individual and total phenolic compounds is unclear, and attention should be given to the quantification of individual phenolic compounds rather than total phenolic content.

Overall, the literature investigating the effect of UV_A on plant physiology and biochemistry contains contradictory and/or insufficient information, such as failing to provide the UV dosage used and other factors involved such as temperature, humidity, and water (Verdaguer *et al.,* 2017). UV_A/UV_B research includes greenhouse, growth chambers and field studies, and most of the UV enhancement experiments use systems that exclude or

attenuate the UV_A component (Middleton and Teramura, 1994; Newsham and Robinson, 2009; Kataria *et al.,* 2013). Some studies themselves have questionable outcomes; for example, significant effect on treatment samples with only a fractional (2%) increase of UV_A (Tegelberg & Julkunen-Tiitto, 2001; Newsham & Robinson, 2009; Bernal *et al.,* 2015). Although there are many reported negative effects of UV_A, some qualitative datasets show the positive effect of solar UV_A radiation on higher plants, showing an increase in stomatal conductance, providing a higher yield, increased nitrogen uptake and soluble leaf proteins (Tezuka *et al.,* 1994). A UV_A exclusion study conducted on cucumber, *Cucumis sativus* (L.) (Krizek, 2004) and red-pigmented lettuce, *Lactuca sativa* (L.) (Krizek *et al.,* 1997) under UV_B, showed more significant damage and chlorosis in leaves than plants grown in combined UV_A +UV_B suggesting photo repair properties of UV_A in the presence of UV_B. Despite the varied methods and amount of UV_A radiation used, some concluding remarks can be drawn as to the effect of UV_A on plants. UV_A has been shown to affect plant biomass, the morphology of leaves, and the production of different phenolic compounds (White & Jahnke, 2002). However, further research is required to understand the complex roles of UV_A and its impact on various phenolic compounds. Moreover, UV_A levels must be measured in the experiment (i.e., photon flux, irradiance) before drawing the conclusion.

1.2.2.2. Defence against herbivores and pathogens

Plant phenolic compounds are known for their passive and active defence against herbivores by an array of responses using phenolic compounds as a constitutive and induced defence (Kant *et al.,* 2015). Constitutive defence is always present in the plant and mainly used for the prevention of diseases or infections, while the induced defence is produced at the right time, concentration, and location to be effective in herbivore resistance only after an individual has been damaged to reduce further damage (Tahvanainen *et al.,* 1985; Hammerschmidt & Schultz, 1996; Lin *et al.,* 2016).

Phenolic compounds exhibit great structural diversity, embodying a variety of functions in plant-herbivore interactions (Lin *et al.,* 2016). These defence mechanisms can be (1) induced defences or (2) feeding deterrents which make plants distasteful to insects and reduce the nutritional value of the plant, or (3) toxins that have a more negative effect on generalist herbivores and their parasitoids (Barbehenn & Constabel, 2011; Lampert *et al.,* 2011).

Tannins are complex plant phenolic compounds and are widely distributed throughout the plant kingdom (Hagerman *et al*., 1999). They are known for their roles as defensive compounds against various herbivores by deterrence and toxicity (Barbehenn *et al.,* 2006; Barbehenn & Constabel, 2011). They are considered to function as anti-herbivore agents by reducing the nutritive value of the plant by precipitating the protein in the gut of the vertebrate herbivore (Feeny, 1968; Bolwell, 1990) and by acting as prooxidants causing oxidative stress to the invertebrate herbivores and hence damaging cellular components and nutrients (Barbehenn *et al.,* 2006; Barbehenn and Constabel, 2011). The protein precipitation capacity (PPC) of tannins is known to make plant material less nutritive for vertebrate herbivores by precipitation of proteins in their digestive tract impacting their nutrient uptake (Barbehenn and Constabel, 2011). Although tannins effectively precipitate protein in the vertebrate herbivores, they may not affect the guts of the invertebrate herbivore (Salminen & Karonen, 2011). Various studies on the feeding forest tent caterpillar, *Malacosoma disstria* (Hübner), and the graminivorous grasshopper, *Aulocara ellioti* (Thomas) provided with an artificial diet containing high tannic acid, showed decreased efficiency on the conversion of digested food, reduced growth, and increased mortality in these insects, but did not reduce protein utilisation (Bernays, 1978; Karowe, 1989). The lack of protein precipitation can be explained by high gut pH (>9) in invertebrates causing ionisation of most hydroxyl groups and decreasing the hydrogen bonding, and hence hampering protein precipitation (Barbehenn and Constabel, 2011).

Therefore, the toxicity of tannins in invertebrates can be explained by the production of higher levels of reactive oxygen in the midguts of insects that leads to the occurrence of tannin oxidation, causing damage within epithelial cells (Summers & Felton, 1994; Barbehenn & Constabel, 2011). This oxidative potential of tannins is further supported by the recent *in-vitro* test of prooxidant activity of several purified tannins (Salminen and Karonen, 2011). Condensed tannins or proanthocyanidins and gallotannins are known for their higher protein precipitation activity, and ellagitannins are known for their higher prooxidant activity (Salminen, 2014). Therefore, it can be argued from various studies that the structure of tannin suggests strong specificities and mechanism for various protein, enzyme and ensure defensive roles against herbivores.

In addition to tannins, flavones, flavan 3-ols, flavonones, proanthocyanidins, flavans, and isoflavonoids have long been investigated as feeding deterrents against many insect herbivores. For example, various flavonoids from *Tephrosia villosa* (L.), *T. purpurea* (L.), and *T. vogelii* (Hook) were found to be feeding deterrents against Spodoptera *littoralis* (Bios) and *Spodoptera exempta* (Walk.) (Panche *et al.,* 2016). Similarly, isoflavonoids and flavone glycoside were also found to act as a feeding deterrent against *Helicoverpa armigera* (Hubner) and late instars of native American butterfly *Pieris napi oleracea* (L), respectively (War *et al.,* 2012). Further, various flavonols such as luteolin, quercetin 3-rhamnoside and myricetin 3-rhamnoside have been shown to deter activity in insects such as aphids (Dreyer and Jones, 1981, Sosa *et al.,* 2004). Moreover, quercetin and its glycoside rutin were found to increase the larval mortality of the tobacco armyworm *Spodoptera liture* (Mallikarjuna *et al.,* 2004). Also, flavonoids (unsubstituted B ring flavanones) in *Eucalyptus* foliage play an important role in mediating animal-plant interactions such as feeding preferences of marsupial folivores, such as koalas (Marsh *et al*., 2019). In addition to tannins and flavonoids, other phenolic compounds such as lignin which are highly branched polymers, are known to increase the leaf toughness and decrease

the nutritional quality and palatability by adding toughness to the plant cell wall (War *et al.,* 2012).

1.2.2.3. Allelopathic role and nutrient cycling

Plant phenolic compounds that leach in the soil are known to affect the nutrient cycling process of the soil by stimulating or inhibiting soil organisms such as bacteria and through various physicochemical effects on nutrients (Min *et al.,* 2015; Chomel *et al.,* 2016). Plant phenolic compounds such as tannins are known to impact nitrogen cycling in plants by forming complexes with proteins that originate from the plant or microorganisms and inhibiting or stimulating enzyme activities (Scalbert, 1991; Triebwasser *et al.,* 2012). Besides this, phenolic compounds in plants are known to inhibit the growth of other plants and hence reduce competition (Springob *et al.,* 2003). Also, various plant phenolic compounds are found to be toxic to decomposing microorganisms (Scalbert 1991; Kraus *et al.* 2003).

1.2.2.4. Role as signal molecules for pollination and seed dispersal

Certain flavonoids are known to function as attractants for pollen, seed dispersal and give colour to fruits and flowers (Woo *et al.*, 2002; Panche *et al.,* 2016). These flavonoids, such as anthocyanidins, are responsible for most of the red, blue, purple colours of flowers and fruits (Khoo *et al.,* 2017). These anthocyanidin glycosides are known to give bright colours to the flowers and accumulate at the inner epidermis of the petal before the opening of a flower bud to attract the insect for pollination and seed dispersal (Weiss, 1991). Flavonoids were also found to function as external chemical signals for symbiotic nitrogen fixation (Baker, 1992). They were also known for their various other roles as internal signal molecules and chemical messenger for the growth and development of plants (Woo *et al.,* 2002).

1.3. Family: Myrtaceae

The Myrtaceae family is a major group of angiosperms with characteristic features such as leathery leaves and oil glands. The family comprises at least 155 genera and over 3,000 species, widely distributed throughout the tropics. In Australia, 75 genera and over 1,500 species are represented predominantly in wetter regions (Chippendale GM, 1988). Myrtaceae are primarily evergreens and have a wide range of forms, from woody shrubs to tall trees (Grattapaglia *et al.,* 2012). Species of the Myrtaceae family are rich in various phenolic compounds such as tannins and phenolic glycosides (Reynertson *et al*., 2008; Mota *et al*., 2012; Tian *et al.,* 2012; Marsh *et al.,* 2017, 2020). Although the total and individual phenolics of some of the species of Myrtaceae have been quantified, their ecological significance remains an open question. Therefore, it is essential to compare variation in the composition of phenolics of various representative species of Myrtaceae against biotic and abiotic factors and identify individual phenolic compounds to weigh up the relative importance of environmental variables (such as UV) versus possible insect herbivory on the incidence of fluctuations in phenolic chemistry in these genera.

Eucalyptus is the most dominant Myrtaceae genus in Australia (McDonald *et al.,* 2009), and together with the genera *Corymbia and Angophora* form a monophyletic group called 'eucalypts' (Merchant *et al.,* 2007). Overall, eucalypts comprise over 900 species in Australia distributed in the coastal and drier parts of the country (Bennett, 2016).

1.3.1. Eucalypt ecophysiology

Eucalypts are an excellent system for comparing environmental and ontogenetic changes in phenolic compounds due to their small to large trees and production of a complex array of foliar phenolic compounds and terpenoids during their lifetime, at both the intra and interspecific level (Merchant *et al.,* 2007; McDonald *et al.,* 2009; Goodger *et al.,* 2013). Eucalypts are commonly heteroblastic, with juvenile leaves that are recognisably different from those of older trees and offering distinct variation in plant specialised metabolites,

leaf toughness and shape (Gras *et al.,* 2005). Given these differences, it is hypothesised that juvenile and adult leaves of eucalypts would present different levels of resources and different levels and combinations of putative anti-herbivore defences (Gras *et al*., 2005). Eucalypts plants are very well-defended plants because terpenoids, tannins, and associated phenolics can comprise up to 40% of the leaf dry matter (Morrow & Fox, 1989; Foley, 1992). A study has shown that jasmonic acid-mediated induced defences are largely conspicuous in their absence in eucalypts suggesting that constitutively expressed secondary metabolites interactions between insect herbivores and eucalypts may be the most important defence method in these plants (Henery *et al.,* 2008). Eucalypts are known for a rich source of biologically active terpenoids, tannins, flavonoids and phloroglucinol derivatives (Ferreira *et al.,* 2016). Further, formylated phloroglucinol compounds (FPCs) and unsubstituted B ring flavanones and tannins have been shown to play an important role in defence against herbivory and mediating animal-plant interactions such as feeding preferences of marsupial folivores, such as koalas and possums (DeGabriel *et al*., 2010; Marsh *et al.,* 2019). Attempts to relate these defence and deterrence resulting from phenolic compounds have been mostly inconclusive because most of the experiments conducted have focused on broad groups of phenolic compounds such as "total" phenolics instead of individual phenolics (Lawler *et al.,* 1998). Small variations, which are part of an evolutionary arms race in plants specialised metabolites, can cause a major effect on the palatability of plants for herbivores (Wink, 2016). Because of the wide distribution of Eucalypts with varying biotic and abiotic stress, they are a good candidate to study the production of phenolic compounds, their adaption and shifting of metabolic pathways in response to these stresses.

1.4. Metabolomics in plant research

Metabolomics is a comprehensive analysis of large numbers of metabolites present in a biological sample (Clish, 2015). Since metabolites reflect an integration of the response to

genetic, abiotic, biotic, and developmental stimuli, they can be applied to various organisms with or without modifications to understand changes in biological systems (Schwachtje *et al.,* 2019). Although the majority of metabolites are yet to be identified, metabolomics has significantly enhanced our understanding of plant behaviour under various stimuli (Hong *et al.,* 2016). Depending upon the experimental questions, quantification and identification of metabolites can be divided into two types which are targeted and untargeted metabolomics (Bingol, 2018). Targeted metabolomics is driven by a specific biochemical question or hypothesis, and untargeted metabolomics is an unbiased approach where there is a comprehensive analysis of all the measurable analytes, including unknowns. Although various novel compounds can be discovered by metabolomics, qualitative results might be limited by sample preparation and types of analytical methods used, and challenge may lie in the time required to process the extensive amounts of raw data sets, difficulty working with large molecular weight compounds and identification and characterisation of the huge number of small molecules (Vinaixa *et al*., 2012; Schrimpe-Rutledge *et al*., 2016).

The two primary analytical techniques used to facilitate the global analysis of metabolites are nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled with various separation techniques such as gas chromatography (GC) and liquid chromatography (LC) (Gowda & Djukovic, 2014). MS is the analytical technique to detect, identify, and quantitate various molecules based on their mass-to-charge ratio (m/z) (R. Kumar *et al.,* 2017). MS can also be used to investigate metabolic fluctuations of metabolites in plants and animals due to various biotic and abiotic stimuli or stressors such as disease, UV, drought and has proven to be a valuable quantitative tool in the era of modern plant metabolomics studies due to its high sensitivity and selectivity (Doerr, 2017).

Following sample processing, data generation (i.e., MS), and data cleaning, feature extraction is used to differentiate individual overlapped or closely aligned peaks.

Compounds are subsequently matched and identified by analysing spectra and chemical compound structures with metabolomics reference libraries or databases (Figure 1.6).

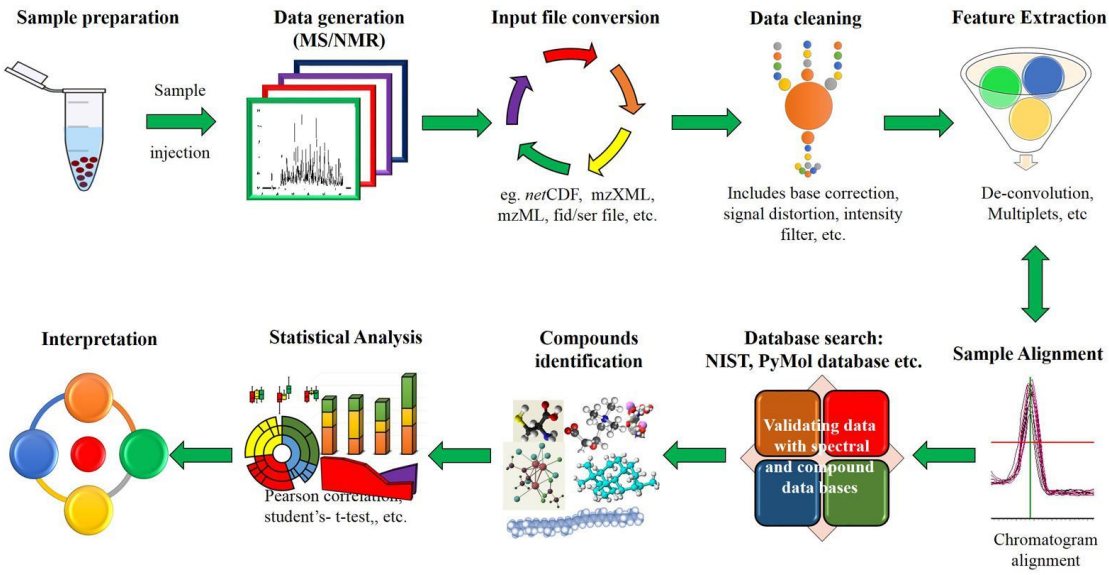


Figure 1.6. Schematic representation of the high throughput data analysis process in metabolomics. Raw data from mass spectrometer are cleaned using various filters and then aligned, and then compounds are analysed by metabolomics library search, and further statistics can be performed. Kumar (2017).

1.5. Hypothesis and research aim

I hypothesise that long the exposure to key environmental stress factors such as light must have shaped presumed genetic differentiation to express the differential synthesis of plant phenolic compounds, and these differences in individual phenolic compounds, which could be within a species or between various species are a response to oxidative pressure and to reduce photodamage (Chapter III, IV, V). Also, I hypothesis that phenolics compounds might have negative effects on herbivores but the variation of these phenolic compounds within or between the plant species are a response to oxidative pressure, and the risk of photodamage is much significant than the risk of herbivory (Chapter V).

My research aimed to investigate the relative significance of potential contrasting roles of phenolic compounds in eucalypts specifically as well as in some other members of the family Myrtaceae. Much of the published literature concerning foliar phenolic compounds evoke a defensive role against herbivores, whereas these same PSMs have recently received attention for their possible role in protecting plants from the abiotic threat of photodamage caused by ROS. The effect of UV_A radiation on plant phenolics forms the key focus of my research chapters, as there is a lack of research compared to UV_B. While both types of UV radiation can influence the temporal and spatial expression of phenolic PSMs, their relative influence has rarely been addressed. Using a metabolomics-based empirical and experimental approach, this thesis aimed to:

1. Identify and document phenolic compounds expressed by species representative of the family Myrtaceae, namely species within the genera *Eucalyptus, Corymbia, Angophora, Syzygium and Callistemon* (Chapter II)

2. Document and quantify the influence of seasonal and ontogenetic factors on changes in leaf colour and phenolic composition for species within the genera *Eucalyptus, Corymbia, Angophora, Syzygium and Callistemon* (Chapter III)

3. Experimentally investigate the role of elevated UV_A on the expression of foliar phenolic compounds by genotypes of *Eucalyptus camaldulensis* representative of populations spanning the natural range of this tree (Chapter IV)
4. Document and quantify changes in foliar phenolics of *E. camaldulensis* affected by a senescence-inducing species of *Cardiaspina* psyllid (Chapter V)

Chapter II is intended to provide a database of foliar phenolic compounds identified during my research and that are frequently referred to in my empirical and experimental chapters. This database will also form a valuable reference for future research on the PSMs of members of the Myrtaceae.

For Chapter III, I consider how leaf colour and the phenolic compounds of young and old leaves respond to seasonal changes in solar radiation (especially ambient levels of UV_A) and temperature. This study intends to provide an overview of how the colour and phenolic composition of leaves of a range of species of Myrtaceae change naturally as they expand during different times of the year. Leaf colour is partially mediated by certain phenolic pigment compounds, which are known to reduce the quantity of photosynthetically active radiation (PAR) reaching chloroplasts. Hence, they can protect leaves against the formation of ROS.

For Chapter IV, I studied the effect of elevated UV_A on the production of foliar phenolic compounds by five genotypes of *Eucalyptus camaldulensis* subspecies *camaldulensis*. The native range of this eucalypt (commonly called River red gum) spans five degrees of latitude and, consequently, different populations could exhibit divergent responses to a range of ecological pressures. In particular, they are hypothesised to have evolved under different levels of solar radiation exposure. This study was conducted under glasshouse and ambient conditions in which potted saplings were exposed to two elevated and constant levels of UV_A under glasshouse conditions, fluctuating glasshouse UV_A or ambient

(natural) UV_A. The two latter treatments represent a procedural control and a natural reference, respectively. This study also considered the influence of leaf age on the composition of phenolic compounds expressed under the different experimental conditions.

For Chapter V, *Cardiaspina albitextura* damaged *E. camaldulensis* leaves (including canopy and understorey leaves) were studied to investigate changes in foliar phenolics in response to the photodamage-like symptoms caused by small sucking insects belonging to the order Hemiptera. It has been suggested that the senescence-inducing feeding strategy of this genus of psyllid predisposes affected eucalypt leaves to photodamage.

In my Synthesis chapter (VI), I group phenolic compounds according to their putative functional roles (based on the published literature) and attempt to synthesise how particular groups of phenolics have responded to the ecological stressors considered in each chapter.

1.6. References

Agati, G., Brunetti, C., Di Ferdinando, M., Ferrini, F., Pollastri, S., & Tattini, M. (2013). Functional roles of flavonoids in photoprotection: New evidence, lessons from the past. *Plant Physiology and Biochemistry*, *72*, 35–45.

Agrawal, A. A. (2011). Current trends in the evolutionary ecology of plant defence. *Functional Ecology*, *25*(2), 420–432.

Alonso, A., Marsal, S., & Julià, A. (2015). Analytical methods in untargeted metabolomics: state of the art in 2015. *Frontiers in Bioengineering and Biotechnology*, *3*, 23.

Baker, M. E. (1992). Evolution of regulation of steroid-mediated intercellular communication in vertebrates: Insights from flavonoids, signals that mediate plant-rhizobia symbiosis. *The Journal of Steroid Biochemistry and Molecular Biology*, *41*(3–8), 301–308.

Ballaré, C. L., Caldwell, M. M., Flint, S. D., Robinson, S. A., & Bornman, J. F. (2011). Effects of solar ultraviolet radiation on terrestrial ecosystems. Patterns, mechanisms, and interactions with climate change. *Photochemical & Photobiological Sciences*, *10*(2), 226–241.

Barbehenn, R. V., Jones, C. P., Hagerman, A. E., Karonen, M., & Salminen, J.-P. (2006). Ellagitannins have greater oxidative activities than condensed tannins and galloyl glucoses at high pH: Potential impact on caterpillars. *Journal of Chemical Ecology*, *32*(10), 2253–2267.

Barbehenn, R. V., & Peter Constabel, C. (2011). Tannins in plant-herbivore interactions. *Phytochemistry*, *72*(13), 1551–1565.

Bennett, A. F. (2016). Eucalypts, wildlife and nature conservation: From individual trees to landscape patterns. *Proceedings of the Royal Society of Victoria*, *128*(1), 71.

Bernal, M., Verdaguer, D., Badosa, J., Abadía, A., Llusià, J., Peñuelas, J., Núñez-Olivera, E., & Llorens, L. (2015). Effects of enhanced UV radiation and water availability on performance, biomass production and photoprotective mechanisms of *Laurus nobilis* seedlings. *Environmental and Experimental Botany*, *109*, 264–275.

Bernays, E. A. (1978). Tannins: An alternative viewpoint. *Entomologia Experimentalis et Applicata*, *24*(3), 244–253.

Bhattacharya, A., Sood, P., & Citovsky, V. (2010). The roles of plant phenolics in defence and communication during *Agrobacterium* and Rhizobium infection. *Molecular Plant Pathology*, *11*(5), 705–719.

Bingol, K. (2018). Recent Advances in Targeted and untargeted metabolomics by NMR and MS/NMR methods. *High-Throughput*, *7*(2).

Bolwell, G. P. (1990). Plant Polyphenols: Vegetable tannins revisited (1989). By E. Haslam. Chemistry and Pharmacology of Natural Products (J. D. Phillipson, D. C. Ayres and H. Baxter, Eds). Cambridge University Press: Cambridge, Pp. 230, *BioEssays*, *12*(9), 453–453.

Boussahel, S., Speciale, A., Dahamna, S., Amar, Y., Bonaccorsi, I., Cacciola, F., Cimino, F., Donato, P., Ferlazzo, G., Harzallah, D., & Cristani, M. (2015). Flavonoid profile, antioxidant and cytotoxic activity of different extracts from Algerian *Rhamnus alaternus* L. bark. *Pharmacognosy Magazine*, *11*(Suppl 1), S102–S109.

Cerovic, Z. G., Ounis, A., Cartelat, A., Latouche, G., Goulas, Y., Meyer, S., & Moya, I. (2002). The use of chlorophyll fluorescence excitation spectra for the non-destructive in situ assessment of UV-absorbing compounds in leaves. *Plant, Cell & Environment*, *25*(12), 1663–1676.

Cheynier, V., Comte, G., Davies, K. M., Lattanzio, V., & Martens, S. (2013). Plant phenolics: Recent advances on their biosynthesis, genetics, and ecophysiology. *Plant Physiology and Biochemistry: PPB*, *72*, 1–20.

Chippendale, G.M. (1988). '*Eucalyptus*, *Angophora* (Myrtaceae)' in A.S. George (ed.) Flora of Australia 19. Australian Government Publishing Service: Canberra.

Chomel, M., Guittonny-Larchevêque, M., Fernandez, C., Gallet, C., DesRochers, A., Paré, D., Jackson, B. G., & Baldy, V. (2016). Plant secondary metabolites: A key driver of litter decomposition and soil nutrient cycling. *Journal of Ecology*, *104*(6), 1527–1541.

Close, D. C., McArthur, C., Hagerman, A. E., Davies, N. W., & Beadle, C. L. (2007). Phenolic acclimation to ultraviolet-A irradiation in *Eucalyptus nitens* seedlings raised across a nutrient environment gradient. *Photosynthetica*, *45*(1), 36–42.

Close, Dugald C., & McArthur, C. (2002). Rethinking the role of many plant phenolics: protection from photodamage not herbivores? *Oikos*, *99*(1), 166–172. Jstor.

Conner, J. K., & Neumeier, R. (2002). The effects of UV_B radiation and intraspecific competition on growth, pollination success, and lifetime female fitness in *Phacelia campanularia* and *P. purshii* (Hydrophyllaceae). *American Journal of Botany*, *89*(1), 103–110.

Constabel, C. P., Yoshida, K., & Walker, V. (2014). Diverse ecological roles of plant tannins: Plant defence and beyond. In *recent advances in polyphenol research* (pp. 115–142). John Wiley & Sons, Ltd.

Das, K., & Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science*, *2*.

DeGabriel, J. L., Moore, B. D., Marsh, K. J., & Foley, W. J. (2010). The effect of plant secondary metabolites on the interplay between the internal and external environments of marsupial folivores. *Chemoecology*, *20*(2), 97–108.

Delgoda, R., & Murray, J. E. (2017). Chapter 7—Evolutionary perspectives on the role of plant secondary metabolites. In S. Badal & R. Delgoda (Eds.), *Pharmacognosy* (pp. 93–100). Academic Press.

Doerr, A. (2017). Global metabolomics. *Nature Methods*, *14*(1), 32–32.

D'Orazio, J., Jarrett, S., Amaro-Ortiz, A., & Scott, T. (2013). UV radiation and the skin. *International Journal of Molecular Sciences*, *14*(6), 12222–12248.

Falcone Ferreyra, M. L., Rius, S. P., & Casati, P. (2012). Flavonoids: Biosynthesis, biological functions, and biotechnological applications. *Frontiers in Plant Science*, *3*.

Feeny, P. P. (1968). Effect of oak leaf tannins on larval growth of the winter moth *Operophtera brumata*. *Journal of Insect Physiology*, *14*(6), 805–817.

Feild, T. S., Lee, D. W., & Holbrook, N. M. (2001). Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiology*, *127*(2), 566–574.

Firn, R. D., & Jones, C. G. (2000). The evolution of secondary metabolism—A unifying model. *Molecular Microbiology*, *37*(5), 989–994.

Foley, W. J. (1992). Nitrogen and energy retention and acid-base status in the common ringtail possum (*Pseudocheirus peregrinus*): Evidence of the effects of absorbed allelochemicals. *Physiological Zoology*, *65*(2), 403–421. Jstor.

Foyer, C. H., Lelandais, M., & Kunert, K. J. (1994). Photooxidative stress in plants. *Physiologia Plantarum*, *92*(4), 696–717.

Foyer, C. H., & Shigeoka, S. (2011). Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology*, *155*(1), 93–100. Frederick, J. E., Snell, H. E., & Haywood, E. K. (1989). Solar ultraviolet radiation at the Earth's surface. *Photochemistry and Photobiology*, *50*(4), 443–450.

Goodger, J. Q. D., Heskes, A. M., & Woodrow, I. E. (2013). Contrasting ontogenetic trajectories for phenolic and terpenoid defences in *Eucalyptus froggattii*. *Annals of Botany*, *112*(4), 651–659.

Gowda, G. A. N., & Djukovic, D. (2014). Overview of mass spectrometry-based metabolomics: Opportunities and challenges. *Methods in Molecular Biology (Clifton, N.J.)*, *1198*, 3–12.

Graham, L. E., Cook, M. E., & Busse, J. S. (2000). The origin of plants: Body plan changes contributing to major evolutionary radiation. *Proceedings of the National Academy of Sciences*, *97*(9), 4535–4540.

Grant, R. H., Heisler, G. M., Gao, W., & Jenks, M. (2003). Ultraviolet leaf reflectance of common urban trees and the prediction of reflectance from leaf surface characteristics. *Agricultural and Forest Meteorology*, *120*(1–4), 127–139.

Gras, E. K., Read, J., Mach, C. T., Sanson, G. D., & Clissold, F. J. (2005). Herbivore damage, resource richness and putative defences in juvenile versus adult *Eucalyptus* leaves. *Australian Journal of Botany*, *53*(1), 33–44.

Grattapaglia, D., Vaillancourt, R. E., Shepherd, M., Thumma, B. R., Foley, W., Kuelheim, C., Potts, B. M., & Myburg, A. A. (2012). Progress in Myrtaceae genetics and genomics: *Eucalyptus* as the pivotal genus. *Tree Genetics & Genomes*, *8*(3), 463–508.

Guan, W., Peng, X., & Lu, S. (2016). Effects of solar UV radiation and temperature on the morphology and photosynthetic performance of *Chaetoceros curvisetus*. *Photosynthetica*, *54*(2), 219–225.

Guyot, S., Marnet, N., & Drilleau, J. (2001). Thiolysis-HPLC characterisation of apple procyanidins covering a large range of polymerisation states. *Journal of Agricultural and Food Chemistry*, *49*(1), 14–20.

Hammerschmidt, R., & Schultz, J. C. (1996). Multiple defences and signals in plant defence against pathogens and herbivores. In J. T. Romeo, J. A. Saunders, & P. Barbosa (Eds.), *Phytochemical Diversity and Redundancy in Ecological Interactions* (pp. 121–154). Springer US.

Harborne, J. B. (1984). Phenolic compounds. In J. B. Harborne (Ed.), *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis* (pp. 37–99). Springer Netherlands.

Haukioja, E. (2005). Plant defences and population fluctuations of forest defoliators: Mechanism-based scenarios. *Annales Zoologici Fennici*, *42*(4), 313–325. Jstor.

Heleno, S. A., Martins, A., Queiroz, M. J. R. P., & Ferreira, I. C. F. R. (2015). Bioactivity of phenolic acids: Metabolites versus parent compounds: A review. *Food Chemistry*, *173*, 501–513.

Henery, M. L., Wallis, I. R., Stone, C., & Foley, W. J. (2008). Methyl jasmonate does not induce changes in Eucalyptus grandis leaves that alter the effect of constitutive defences on larvae of a specialist herbivore. *Oecologia*, *156*(4), 847–859.

Herrero, M., Castro-Puyana, M., Ibáñez, E., & Cifuentes, A. (2013). Chapter 11: Compositional analysis of foods. In S. Fanali, P. R. Haddad, C. F. Poole, P. Schoenmakers, & D. Lloyd (Eds.), *Liquid Chromatography* (pp. 295–317). Elsevier.

Herrmann, K. (1995). The shikimate pathway: Early steps in the biosynthesis of aromatic compounds. *The Plant Cell*, *7*(7), 907–919.

Hewitt, P. J. (2002). Radiation at home, outdoors and in the workplace. *The Annals of Occupational Hygiene*, *46*(2), 272–272.

Hollósy, F. (2002). Effects of ultraviolet radiation on plant cells. *Micron*, *33*(2), 179–197.

Hoch, W. A., Singsaas, E. L., & McCown, B. H. (2003). Resorption protection. Anthocyanins facilitate nutrient recovery in autumn by shielding leaves from potentially damaging light levels. *Plant Physiology*, *133*(3), 1296–1305.

Hong, J., Yang, L., Zhang, D., & Shi, J. (2016). Plant metabolomics: An indispensable system biology tool for plant science. *International Journal of Molecular Sciences*, *17*(6).

Ibdah, M., Krins, A., Seidlitz, H. K., Heller, W., Strack, D., & Vogt, T. (2002). Spectral dependence of flavonol and betacyanin accumulation in *Mesembryanthemum crystallinum* under enhanced ultraviolet radiation. *Plant, Cell & Environment*, *25*(9), 1145–1154.

Ignat, I., Volf, I., & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, *126*(4), 1821–1835.

Iwai, M., Ohta, M., Tsuchiya, H., & Suzuki, T. (2010). Enhanced accumulation of caffeic acid, rosmarinic acid and luteolin-glucoside in red perilla cultivated under red diode laser and blue LED illumination followed by UV_A irradiation. *Journal of Functional Foods*, *2*(1), 66–70.

Jamalian, S., Gholami, M., & Esna-Ashari, M. (2013). Abscisic acid-mediated leaf phenolic compounds, plant growth and yield is strawberry under different salt stress regimes. *Theoretical and Experimental Plant Physiology*, *25*(4), 291–299.

Jermy, T. (1993). Evolution of insect-plant relationships—A devil's advocate approach*. *Entomologia Experimentalis et Applicata*, *66*(1), 3–12.

Kant, M. R., Jonckheere, W., Knegt, B., Lemos, F., Liu, J., Schimmel, B. C. J., Villarroel, C. A., Ataide, L. M. S., Dermauw, W., Glas, J. J., Egas, M., Janssen, A., Van Leeuwen, T., Schuurink, R. C., Sabelis, M. W., & Alba, J. M. (2015). Mechanisms

and ecological consequences of plant defence induction and suppression in herbivore communities. *Annals of Botany*, *115*(7), 1015–1051.

Karowe, D. N. (1989). Facultative monophagy as a consequence of prior feeding experience: Behavioral and physiological specialisation in *Colias philodice* larvae. *Oecologia*, *78*(1), 106–111.

Kataria, S., Guruprasad, K. N., Ahuja, S., & Singh, B. (2013). Enhancement of growth, photosynthetic performance and yield by the exclusion of ambient UV components in C_3 and C_4 plants. *Journal of Photochemistry and Photobiology. B, Biology*, *127*, 140–152.

Keski-Saari, S., Pusenius, J., & Julkunen-Tiitto, R. (2005). Phenolic compounds in seedlings of *Betula pubescens* and *B. pendula* are affected by enhanced UV_B radiation and different nitrogen regimens during early ontogeny. *Global Change Biology*, *11*(7), 1180–1194.

Kessler, A., & Kalske, A. (2018). Plant secondary metabolite diversity and species interactions. *Annual Review of Ecology, Evolution, and Systematics*, *49*(1), 115–138.

Khoo, H. E., Azlan, A., Tang, S. T., & Lim, S. M. (2017). Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and potential health benefits. *Food & Nutrition Research*, *61*(1).

Kotilainen, T., Tegelberg, R., Julkunen-Tiitto, R., Lindfors, A., & Aphalo, P. J. (2008). Metabolite specific effects of solar UV_A and UV_B on alder and birch leaf phenolics. *Global Change Biology*, *14*(6), 1294–1304.

Krizek, D. T., Mirecki, R. M., & Britz, S. J. (1997). Inhibitory effects of ambient levels of solar UV_A and UV_B radiation on growth of cucumber. *Physiologia Plantarum*, *100*(4), 886–893.

Krumsiek, J., Bartel, J., & Theis, F. J. (2016). Computational approaches for systems metabolomics. *Current Opinion in Biotechnology*, *39*, 198–206.

Kumar, R., Bohra, A., Pandey, A. K., Pandey, M. K., & Kumar, A. (2017). Metabolomics for plant improvement: Status and prospects. *Frontiers in Plant Science*, *8*.

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An overview. *The Scientific World Journal*, *2013*.

Labarrere, B., Prinzing, A., Dorey, T., Chesneau, E., & Hennion, F. (2019). Variations of secondary metabolites among natural populations of sub-antarctic *Ranunculus* species suggest functional redundancy and versatility. *Plants (Basel, Switzerland)*, *8*(7).

Lampert, E. C., Zangerl, A. R., Berenbaum, M. R., & Ode, P. J. (2011). Generalist and specialist host-parasitoid associations respond differently to wild parsnip (*Pastinaca sativa*) defensive chemistry. *Ecological Entomology*, *36*(1), 52–61.

Lawler, I. R., Foley, W. J., Eschler, B. M., Pass, D. M., & Handasyde, K. (1998). Intraspecific variation in *Eucalyptus* secondary metabolites determines food intake by folivorous marsupials. *Oecologia*, *116*(1–2), 160–169.

Lee, N. Y., & Hyun, J.-N. (2011). LED effect on the free radical-scavenging activity of barley leaf. *Journal of Crop Science and Biotechnology*, *14*(4), 317–320.

Levin, B. R. (1971). The operation of selection in situations of interspecific competition. *Evolution*, *25*(2), 249–264.

Li, Q., & Kubota, C. (2009). Effects of supplemental light quality on growth and phytochemicals of baby leaf lettuce. *Environmental and Experimental Botany*, *67*(1), 59–64.

Lin, D., Xiao, M., Zhao, J., Li, Z., Xing, B., Li, X., Kong, M., Li, L., Zhang, Q., Liu, Y., Chen, H., Qin, W., Wu, H., & Chen, S. (2016). An overview of plant phenolic

compounds and Their importance in human nutrition and management of type 2 diabetes. *Molecules*, *21*(10).

Lowry, B., Lee, D., & Hébant, C. (1980). The origin of land plants: A new look at an old problem. *Taxon*, *29*(2–3), 183–197.

Mallikarjuna, N., Kranthi, K. R., Jadhav, D. R., Kranthi, S., & Chandra, S. (2004). Influence of foliar chemical compounds on the development of *Spodoptera litura* (Fab.) in interspecific derivatives of groundnut. *Journal of Applied Entomology*, *128*(5), 321–328.

Mandal, S. M., Chakraborty, D., & Dey, S. (2010). Phenolic acids act as signalling molecules in plant-microbe symbioses. *Plant Signaling & Behavior*, *5*(4), 359–368.

Mandi, S. S. (2017). Natural UV radiation in enhancing survival value and quality of plants. Springer.

Marsh, K. J., Kulheim, C., Blomberg, S. P., Thornhill, A. H., Miller, J. T., Wallis, I. R., Nicolle, D., Salminen, J.-P., & Foley, W. J. (2017). Genus-wide variation in foliar polyphenolics in eucalypts. *Phytochemistry*, *144*, 197–207.

Marsh, K. J., Wallis, I. R., Kulheim, C., Clark, R., Nicolle, D., Foley, W. J., & Salminen, J. (2020). New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. *The New Phytologist*, *225*(1), 488–498.

Marsh, K. J., Wallis, I. R., Kulheim, C., Clark, R., Nicolle, D., Foley, W. J., & Salminen, J.-P. (2019). New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. *The New Phytologist*.

McDonald, M. W., Brooker, M. I. H., & Butcher, P. A. (2009). A taxonomic revision of *Eucalyptus camaldulensis* (Myrtaceae). *Australian Systematic Botany*, *22*(4), 257–285.

McKey, D. (1974). Adaptive Patterns in Alkaloid Physiology. *The American Naturalist*, *108*(961), 305–320.

Merchant, A., Callister, A., Arndt, S., Tausz, M., & Adams, M. (2007). Contrasting physiological responses of six *Eucalyptus* species to water deficit. *Annals of Botany*, *100*(7), 1507–1515.

Middleton, E. M., & Teramura, A. H. (1994). Understanding photosynthesis, pigment and growth responses induced by UV_B and UV_A irradiances. *Photochemistry and Photobiology*, *60*(1), 38–45.

Min, K., Freeman, C., Kang, H., & Choi, S.-U. (2015). The regulation by phenolic compounds of soil organic matter dynamics under a changing environment. *BioMed Research International*, *2015*.

Minatel, I. O., Borges, C. V., Ferreira, M. I., Gomez, H. A. G., & Lima, C.-Y. O. C. and G. P. P. (2017). Phenolic compounds: Functional properties, impact of processing and bioavailability. *Phenolic Compounds - Biological Activity*.

Morales, L. O., Tegelberg, R., Brosché, M., Keinänen, M., Lindfors, A., & Aphalo, P. J. (2010). Effects of solar UV_A and UV_B radiation on gene expression and phenolic accumulation in *Betula pendula* leaves. *Tree Physiology*, *30*(7), 923–934.

Morrow, P. A., & Fox, L. R. (1989). Estimates of pre-settlement insect damage in Australian and North American Forests. *Ecology*, *70*(4), 1055–1060.

Mota, I., Rodrigues Pinto, P. C., Novo, C., Sousa, G., Guerreiro, O., Guerra, Â. R., Duarte, M. F., & Rodrigues, A. E. (2012). Extraction of polyphenolic compounds from *Eucalyptus globulus* Bark: Process optimisation and screening for biological activity. *Industrial & Engineering Chemistry Research*, *51*(20), 6991–7000.

Nawkar, G. M., Maibam, P., Park, J. H., Sahi, V. P., Lee, S. Y., & Kang, C. H. (2013). UV-induced cell death in plants. *International Journal of Molecular Sciences*, *14*(1), 1608–1628.

Newsham, K. K., & Robinson, S. A. (2009). Responses of plants in polar regions to UV_B exposure: A meta-analysis. *Global Change Biology*, *15*(11), 2574–2589.

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: An overview. *Journal of Nutritional Science*, *5*.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), 270–278.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products*, *63*(7), 1035–1042.

Procházková, D., Boušová, I., & Wilhelmová, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*, *82*(4), 513–523.

Pyne, M. E., Narcross, L., & Martin, V. J. J. (2019). Engineering plant secondary metabolism in microbial systems *Plant Physiology*, *179*(3), 844–861.

Ramakrishna, A., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, *6*(11), 1720–1731.

Reynertson, K. A., Yang, H., Jiang, B., Basile, M. J., & Kennelly, E. J. (2008). Quantitative analysis of antiradical phenolic constituents from fourteen edible Myrtaceae fruits. *Food Chemistry*, *109*(4), 883–890.

Rice-Evans, C., Miller, N., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, *2*(4), 152–159.

Robards, K. (2003). Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *Journal of Chromatography A*, *1000*(1), 657–691.

Roberts, L. D., Souza, A. L., Gerszten, R. E., & Clish, C. B. (2012). Targeted metabolomics. *Current Protocols in Molecular Biology*, *Chapter*, Unit 30.2.

Rozema, J., Boelen, P., & Blokker, P. (2005). Depletion of stratospheric ozone over the Antarctic and Arctic: Responses of plants of polar terrestrial ecosystems to enhanced UV_B, an overview. *Environmental Pollution (Barking, Essex: 1987)*, *137*(3), 428–442.

Sage, E., Girard, P.-M., & Francesconi, S. (2011). Unravelling UV_A-induced mutagenesis. *Photochemical & Photobiological Sciences*, *11*(1), 74–80.

Saibabu, V., Fatima, Z., Khan, L. A., & Hameed, S. (2015). Therapeutic potential of dietary phenolic acids. *Advances in Pharmacological Sciences*, *2015*.

Sakihama, Y., Cohen, M. F., Grace, S. C., & Yamasaki, H. (2002). Plant phenolic antioxidant and prooxidant activities: Phenolics-induced oxidative damage mediated by metals in plants. *Toxicology*, *177*(1), 67–80.

Salvador, M. J., de Lourenço, C. C., Andreazza, N. L., Pascoal, A. C. R. F., & Stefanello, M. É. A. (2011). Antioxidant capacity and phenolic content of four Myrtaceae plants of the south of Brazil. *Natural Product Communications*, *6*(7).

Salminen, J.-P. (2014). The chemistry and chemical ecology of ellagitannins in plant-insect interactions: From underestimated molecules to bioactive plant constituents. In *Recent Advances in Polyphenol Research* (pp. 83–113). John Wiley & Sons, Ltd.

Salminen, J.-P., & Karonen, M. (2011). Chemical ecology of tannins and other phenolics: We need a change in approach. *Functional Ecology*, *25*(2), 325–338.

Saltveit, M. E. (2017). Synthesis and metabolism of phenolic compounds in fruit and vegetable *Phytochemicals* (pp. 115–124). John Wiley & Sons, Ltd.

Santos-Buelga, C., & Scalbert, A. (2000). Proanthocyanidins and tannin-like compounds: Nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture*, *80*(7), 1094–1117.

Schneider, G. F., Coley, P. D., Younkin, G. C., Forrister, D. L., Mills, A. G., & Kursar, T. A. (2019). Phenolics lie at the centre of functional versatility in the responses of

two phytochemically diverse tropical trees to canopy thinning. *Journal of Experimental Botany*, *70*(20), 5853–5864.

Schrimpe-Rutledge, A. C., Codreanu, S. G., Sherrod, S. D., & McLean, J. A. (2016). Untargeted metabolomics strategies – Challenges and emerging directions. *Journal of the American Society for Mass Spectrometry*, *27*(12), 1897–1905.

Schuch, A. P., Moreno, N. C., Schuch, N. J., Menck, C. F. M., & Garcia, C. C. M. (2017). Sunlight damage to cellular DNA: Focus on oxidatively generated lesions. *Free Radical Biology and Medicine*, *107*, 110–124.

Schwachtje, J., Whitcomb, S. J., Firmino, A. A. P., Zuther, E., Hincha, D. K., & Kopka, J. (2019). Induced, imprinted, and primed responses to changing environments: Does metabolism store and process information? *Frontiers in Plant Science*, *10*.

Stewart, A. J., Chapman, W., Jenkins, G. I., Graham, I., Martin, T., & Crozier, A. (2001). The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. *Plant, Cell & Environment*, *24*(11), 1189–1197.

Strack, D. (1997). Phenolic metabolism. In P. M. Dey & J. B. Harborne (Eds.), *Plant Biochemistry* (pp. 387–416). Academic Press.

Stuper-Szablewska, K., Kurasiak-Popowska, D., Nawracała, J., & Perkowski, J. (2019). Quantitative profile of phenolic acids and antioxidant activity of wheat grain exposed to stress. *European Food Research and Technology*, *245*(8), 1595–1603.

Summers, C. B., & Felton, G. W. (1994). Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): Potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochemistry and Molecular Biology*, *24*(9), 943–953.

Tahvanainen, J., Helle, E., Julkunen-Tiitto, R., & Lavola, A. (1985). Phenolic compounds of willow bark as deterrents against feeding by mountain hare. *Oecologia*, *65*(3), 319–323.

Takahashi, S., & Badger, M. R. (2011). Photoprotection in plants: A new light on photosystem II damage. *Trends in Plant Science*, *16*(1), 53–60.

Tattini, M., Galardi, C., Pinelli, P., Massai, R., Remorini, D., & Agati, G. (2004). Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. *New Phytologist*, *163*(3), 547–561.

Tegelberg, R., & Julkunen-Tiitto, R. (2001). Quantitative changes in secondary metabolites of dark-leaved willow (*Salix myrsinifolia*) exposed to enhanced UV_B radiation. *Physiologia Plantarum*, *113*(4), 541–547.

Tezuka, T., Yamaguchi, F., & Ando, Y. (1994). Physiological activation in radish plants by UV_A radiation. *Journal of Photochemistry and Photobiology B: Biology*, *24*(1), 33–40.

Thirumurugan, D., Cholarajan, A., & Vijayakumar, S. S. S. R. and R. (2018). An Introductory Chapter: Secondary metabolites. *Secondary Metabolites - sources and applications*.

Tian, L.-W., Xu, M., Li, Y., Li, X.-Y., Wang, D., Zhu, H.-T., Yang, C.-R., & Zhang, Y.-J. (2012). Phenolic compounds from the branches of *Eucalyptus maideni*. *Chemistry & Biodiversity*, *9*(1), 123–130.

Treutter, D. (2005). Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant Biology*, *7*(6), 581–591.

Tsopmo, A., Awah, F. M., & Kuete, V. (2013). 12—Lignans and stilbenes from African medicinal plants. In V. Kuete (Ed.), *Medicinal Plant Research in Africa* (pp. 435–478).

Tsormpatsidis, E., Henbest, R. G. C., Davis, F. J., Battey, N. H., Hadley, P., & Wagstaffe, A. (2008). UV irradiance as a major influence on growth, development and secondary products of commercial importance in *Lollo Rosso* lettuce 'Revolution'

grown under polyethylene films. *Environmental and Experimental Botany*, *63*(1), 232–239.

Vaštakaitė, V., Viršilė, A., Brazaitytė, A., Samuolienė, G., Jankauskienė, J., Sirtautas, R., & Duchovskis, P. (2015). The effect of UV_A supplemental lighting on antioxidant properties of *Ocimum Basilicum* L. microgreens in the greenhouse *International Scientific Conference Rural Development 2017*, *0*(0), Article 0.

Verdaguer, D., Jansen, M. A. K., Llorens, L., Morales, L. O., & Neugart, S. (2017). UV_A radiation effects on higher plants: Exploring the known unknown. *Plant Science*, *255*, 72–81.

Vinaixa, M., Samino, S., Saez, I., Duran, J., Guinovart, J. J., & Yanes, O. (2012). A Guideline to univariate statistical analysis for LC/MS-Based Untargeted metabolomics-derived data. *Metabolites*, *2*(4), 775–795.

Vogelmann, T. C. (1989). Penetration of light into plants. *Photochemistry and Photobiology*, *50*(6), 895–902.

Vogelmann, T. C. (1993). Plant tissue optics. *Annual Review of Plant Physiology and Plant Molecular Biology*, *44*(1), 231–251.

Vuolo, M. M., Lima, V. S., & Maróstica Junior, M. R. (2019). Chapter 2 - Phenolic compounds: Structure, classification, and antioxidant power. In M. R. S. Campos (Ed.), *Bioactive Compounds* (pp. 33–50). Woodhead Publishing.

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., & Sharma, H. C. (2012). Mechanisms of plant defence against insect herbivores. *Plant Signaling & Behavior*, *7*(10), 1306–1320.

Watson, R. R., Preedy, V. R., & Zibadi, S. (2018). Polyphenols: prevention and treatment of human disease. Academic Press.

Weiss, M. R. (1991). Floral colour changes as cues for pollinators. *Nature*, *354*(6350), 227–229.

White, A. L., & Jahnke, L. S. (2002). Contrasting effects of UV_A and UV_B on photosynthesis and photoprotection of β-carotene in two *Dunaliella* spp. *Plant and Cell Physiology*, *43*(8), 877–884.

Wink, M. (2016). Secondary metabolites: deterring herbivores. In *ELS* (pp. 1–10). American Cancer Society.

Woo, H.-H., Kuleck, G., Hirsch, A. M., & Hawes, M. C. (2002). Flavonoids: signal molecules in plant development. In B. S. Buslig & J. A. Manthey (Eds.), *Flavonoids in Cell Function* (pp. 51–60). Springer US.

Xiao, X., Bettignies, T. de, Olsen, Y. S., Agusti, S., Duarte, C. M., & Wernberg, T. (2015). Sensitivity and acclimation of three canopy-forming seaweeds to UV_B radiation and warming. *Plos One*, *10*(12), e0143031.

Chapter II

Identification of known and unknown foliar phenolic compounds in five species of Myrtaceae (*Angophora floribunda, Callistemon salignus, Corymbia ficifolia, Eucalyptus camaldule*nsis and *Syzygium smithii*)

2.1. Introduction

The family Myrtaceae (Myrtle family) is a major group of angiosperms with at least 155 genera and over 3,000 species and is globally distributed throughout the tropics, temperate rainforest, and arid central parts of Australia (Reynertson *et al*., 2008). Various species of the Myrtaceae family are rich in various phenolic compounds such as tannins and phenolic glycosides (Reynertson *et al*., 2008; Mota *et al*., 2012; Tian *et al.,* 2012; Marsh *et al.,* 2017, 2020). *Eucalyptus* is the most dominant Myrtaceae in Australia (McDonald *et al.,* 2009), and together with the genera *Corymbia and Angophora* form a monophyletic group called 'eucalypts' (Merchant *et al.,* 2007). Eucalypts are known to have high concentrations of tannins, flavonoids, triterpenoids, saponins, alkaloids and phenolic acids (Barry *et al*., 2001; Santos *et al.,* 2011; Sobeh *et al.,* 2018). Some species of *Eucalyptus* are characterised by having up to 40% total phenolics by leaf dry weight (DW) and up to 11% of tannins DW (gallic and ellagic acid derivatives) with flavonoids as minor components (Barry *et al*., 2001). Similarly, various species of *Syzygium, Angophora, Corymbia* are known for their high concentration of hydrolysable tannins, flavonoids, anthocyanins, terpenes, and phenolic acids (Mahmoud *et al.*, 2002; Hayes *et al.,* 2014; Balyan & Sarkar, 2017; Marsh *et al.,* 2017; Chagas *et al*., 2018; Sobeh *et al*., 2018). We selected five species representing major genera representative of the Myrtaceae (namely *Angophora floribunda, Callistemon salignus, Corymbia ficifolia, Eucalyptus camaldulensis* and *Syzygium smithii*) which provides an opportunity to examine the relatedness of polyphenols and the possible influence of their common evolutionary origins.

This work represents the study that utilises the HPLC–ESI/MS/MS technique to identify the phenolic composition of five species of Myrtaceae (*Angophora floribunda, Callistemon salignus, Corymbia ficifolia, Eucalyptus camaldulen*sis and *Syzygium smithii*) to understand the phenolic profile of these plants better. Five plant species of the family Myrtaceae were selected because these species represent major genera within the family.

The phenolic compounds are identified based on their chemical structure, molecular formula, molecular mass, and various published literature, as shown in Table 2.2. These compounds were further compared with various web-based resources such as Chemspider (version 2021.0.6.0), mzCloud (https://www.mzcloud.org/), RIKEN tandem mass spectral database (ReSpect version 2013.11.11), and Human Metabolome Database (HMDB version 4.0). A customised library (Table 2.2) of compounds was created, which shows known and unique phenolic compounds and their major fragments generated through collision-induced dissociation. This chapter is descriptive and serves to set up the methodology and to define to initially define the suite of compounds that will be analysed and discussed in the rest of the thesis.

2.2. Materials and Methods

2.2.1. Study species and leaf extraction

Mature leaf samples from five fully grown plant species of the Myrtaceae family (*Angophora floribunda, Callistemon salignus, Corymbia ficifolia, Eucalyptus camaldulens*is and *Syzygium smithii*) were harvested around La Trobe University, Melbourne, Australia (37°42.6'S, 145°10.4'E) and immediately freeze-dried (Note: some sources include *Callistemon* in *Melaleuca*. Consequently, *C. salignus* is called *M. salignus* in some sources). The freeze-dried leaves were finely ground to ≤ 0.25 mm using a ball mill (Retsch MM400, Germany) at 30 Hz. 20 mg of finely ground leaf powder was weighted in duplicate into 2mL microtubes and 1mL 80:20 MeOH-H_2O (v/v) (methanol HPLC grade Sigma-Aldrich and Millipore Milli-Q water) was added to each sample, and subsequently mixed with a vortex mixer for two minutes and sonicated for 10 minutes (Unisonics, Australia). The leaf extracts were then centrifuged for 10 minutes at 15,000 rpm at room temperature (Eppendorf 5415D bench centrifuge, Hamburg, Germany). The extract was then transferred to a clean labelled microtube. The extraction procedure was

repeated a second time, and the extracts were pooled. 1mL of the extract was transferred to HPLC vials for liquid chromatography-mass spectrometry (LC-MS) analysis, and a further 5 μL of each extract were pooled to create a quality control (QC) of the sample to be analysed at the same time. The pooled QC samples were used to judge the quality and assess the analytical variance of the data.

2.2.2. Chromatographic and mass spectrometric analysis

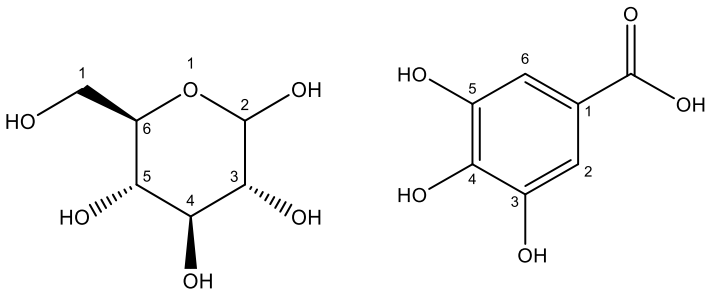
Phenolic compounds identification was performed by an ultra-high-performance liquid chromatographic system (UPLC, UHPLC+ focused, Thermo Scientific™, Waltham, MA, USA) combined with a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific™, Waltham, MA, USA) and a diode array detector. Data were collected in negative ion mode, scanning a mass range m/z of 100–1500. Negative ion mode was preferred over positive for phenolic compounds analysis for all the subgroups because of its sensitivity, clearer fragmentation patterns and less extensive fragmentation (Nováková *et al.*, 2010; Schmidt, 2016). Nitrogen was used as the sheath and auxiliary and sweep gas. The spray voltage was set at 3,600 V. The capillary temperature was set to 300°C, with the S-lens RF level set at 64 and an auxiliary gas heater temperature of 310°C. A Hypersil GOLD C_{18} column (150×2.1mm, 1.9μm, Thermo Scientific, USA) was used with the column compartment temperature set to 30°C, and a flow rate was maintained at 0.3mL/min throughout data acquisition. The mobile phase consisted of (A) 0.1% formic acid and (B) acetonitrile. A linear gradient was used beginning with 2% of B and reaching 100% of B at 15 min, then kept steady at 100% of B until 18 minutes and then returned to an initial condition where it was held for 2 min. The MS/MS analyses were carried out by automatic fragmentation, where the three most intense mass peaks were fragmented. The mass spectrometric (MS) analysis, including the prediction of chemical formula and exact mass calculation, was performed by using Thermo Xcalibur Qual Browser software version 3.0.63 (Thermo Scientific, USA).

2.3. Results

Table 2.1 summarises the various phenolic compounds characterised from each extract of *Syzygium smithii, Angophora floribunda, Callistemon salignus, Corymbia ficifolia,* and *Eucalyptus camaldule*nsis and their retention time, and deprotonated ion $[M–H]^–$, the main fragmentation ions obtained by HPLC-MS/MS. Phenolic compounds were tentatively identified based on their molecular ions and major fragments observed in MS spectra and followed by matching various literature and matching MS/MS fragment spectrum to the online library such as HMDB, mzCloud, ReSpect (Dias *et al*., 2016).

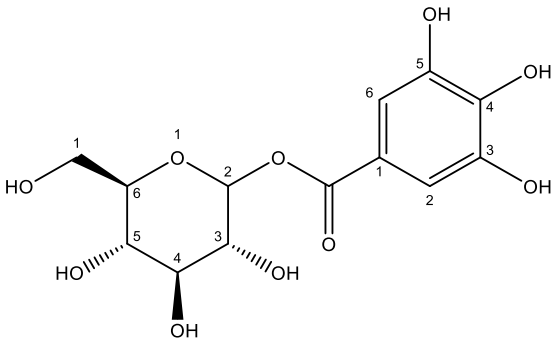
2.3.1. Neutral loss from phenolic compounds

During their MS/MS fragmentation, phenolic compounds showed common neutral losses that equate to the loss of water, hexose, gallic acid, hexahydroxydiphenoyl (HHDP), and galloyl-glucose from the precursor ion (Figure 2.1). It should be noted that glucose cannot be distinguished from any of its stereoisomers (e.g., galactose) by mass spectrometry. The same is true of other sugars, and therefore, the notation of hexose or pentose is used to describe the unassigned sugar except where the sugar identity has been well validated by published literature using NMR.

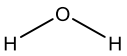


Hexose = 180 amu

Gallic acid = 170 amu



Galloyl glucose = 302 amu



Water =18 amu

Figure 2.1. Examples of typical neutral losses including water, hexose, gallic acid and galloyl glucose.

2.3.2. Identification of phenolic compounds in the negative ionisation mode

Hydrolysable tannins were previously described in the literature for various plants species (Barry *et al.*, 2001; Lee *et al.*, 2005; Salminen, 2014; R. M. Santos *et al.*, 2011; Singh *et al.*, 2016). The peak m/z 481.0617 (Comp. No. 1) was putatively identified as $[M-H]^-$ of hexahydroxydiphenoyl-glucose (HHDP-glucose) (Barry *et al.*, 2001). The fragmentation of this ion in negative ESI-MS/MS produces a typical fragment ion m/z 300.9986, which is HHDP moiety and further decarboxylation of the HHDP moiety gave a peak of 275.0194. Similarly, peak at m/z 783.0686 (Comp. No. 6) was putatively identified as di-HHDP-glucose, which gives the fragment ions at m/z 481.0619 corresponding to HHDP-glucose, fragment 300.9984 corresponding to the HHDP moiety, and 275.0194 by decarboxylation of the HHDP moiety in the MS/MS spectrum. Similarly, peak at m/z 633.0735 (Comp. No. 13), m/z 785.0843 (Comp. No. 15) and m/z 937.0947 (Comp. No.

27) were tentatively identified as HHDP-galloyl glucose, HHDP-di-galloylglucose and HHDP-tri-galloylglucose respectively (Barry *et al*., 2001). There was a neutral loss of fragment (170 amu) from m/z 937.0947 to give fragment m/z 785.0843 and further loss of galloyl (152 amu) moiety to give fragment m/z 633.0335 (Singh *et al.,* 2016). Further, loss of galloyl glucose (322 amu) gives the peak of m/z 300.9982 correspondings to $[M-H]^-$ HHDP moiety. Similarly, a peak with m/z 935.0795 (Comp. No. 32) was identified as di-HHDP-galloylglucose with neutral loss (302 amu) and further loss of galloyl glucose (322 amu) giving a fragment of m/z 300.9983 correspondings to $[M-H]^-$ HHDP moiety. The peak at m/z 1417.1458 (Comp. No. 32) was identified as deprotonated ion $[M-H]^-$ of ellagatannin dimer with characteristic fragmentation of m/z 765.0638, m/z 633.0735, m/z 300.9908 (Singab *et al.,* 2011).

Various other hydrolysable tannins such as monogalloyl-glucose, digalloyl-glucose, trigalloyl-glucose, tetragalloyl-glucose and pentagalloyl-glucose were also identified from the samples of all the plant species. These compounds showed characteristic fragment patterns at negative ionisation mode by loss of various galloyl moieties. MS/MS spectrum ions corresponding to the sequential loss of galloyl moieties (neutral loss of 152) were observed from pentagalloyl-glucose at m/z of 939.1104 (Comp. No.29), tetragalloyl-glucose at m/z 787.0984 (S.N. 24), trigalloyl-glucose at m/z 635.0891 (Comp. No. 22), digalloyl-glucose at m/z 483.0775 (Comp. No. 17), and finally from monogalloyl-glucose at m/z 331.0669 (Comp. No. 4). The peak at m/z 169.0141 (Comp. No.) and fragment m/z 125.0231 were identified as deprotonated and decarboxylated ions of the gallic acid moieties, respectively.

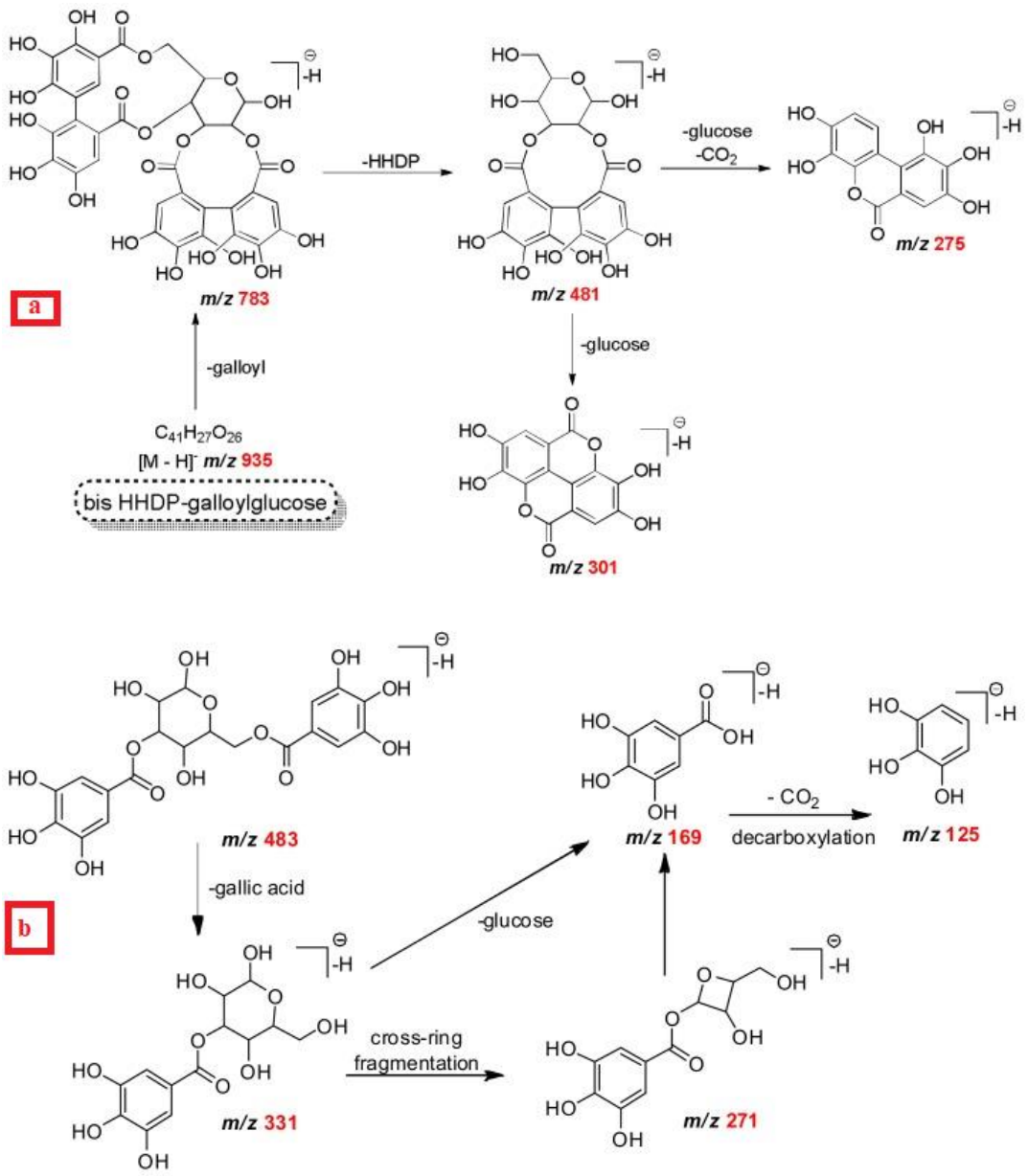


Figure 2.2. Proposed fragmentation pathways of **a**. galloyl-HHDP-glucose and **b**. digalloyl-glucose. Figure adapted from dos Santos *et al.*, (2018).

The peak at m/z 179.0344 (Comp. No. 45) was identified as a deprotonated ion $[M-H]^-$ of caffeic acid. The major fragment ion m/z 135.0346 corresponds to the loss of water and carbon dioxide from the caffeic acid. Similarly, m/z 341.0885 (Comp. No. 19) was tentatively identified as caffeic acid hexoside. Again, there is a loss of glucose (162 amu) as a neutral fragment from 341.0885, which resulted in the deprotonated ion of caffeic acid (m/z 179.0030) (Fang *et al.*, 2002). Similarly, the peak at m/z 137.0236 (Comp. No.20)

was identified as hydroxybenzoic acid with the characteristic fragment of m/z 93.0347 (Sun *et al.,* 2007). The peak at m/z 353.0874 (Comp. No. 25) was identified as caffeoylquinic acid with major fragmentation at m/z 191.0551, which is deprotonated quinic acid ion after the loss of caffeic acid moiety (Spínola *et al.,* 2015). Similarly, deprotonated ion at m/z 197.0452 (Comp. No. 35) was identified as syringic acid with the major fragment of m/z 167.0344 (Sawada *et al.,* 2012).

The peak at m/z 447.0931 (Comp. No. 18) was identified as kaempferol galactoside with the major fragment of m/z 285.0398, which corresponds to the deprotonated ion of kaempferol after the loss of neutral fragment glucose (162 amu) (Ben Said *et al.,* 2017). Similarly, peak at m/z 463.0895 (Comp. No. 42) was attributed to quercetin-glucoside having deprotonated fragment at 301.0347 (aglycone fragment) (Bentley *et al.,* 2019).

The peak at m/z 463.0518 (Comp. No. 23) was identified as ellagic acid-hexoside, which gave a characteristic fragmentation ion m/z 300.9987, which was identified as deprotonated ion $[M-H]^-$ of ellagic acid (Singh *et al.,* 2016). Similarly, peak at m/z 289.0715 (Comp. No. 26) was putatively identified as catechin, which gave characteristic fragment of m/z 245.0815 and fragment m/z 109.0282 (Sawada *et al.,* 2012). Similarly, the peak at m/z 305.0674 (Comp. No. 7) showed a fragmentation pattern like those previously described, and it was identified as gallocatechin (Justesen, 2000). Figure 2.3 shows the example of the fragmentation pattern of pentagalloyl glucose in MS. The major peaks were 769.0912 and 617.0799 and are described in Table 2.2 and Table 2.1.

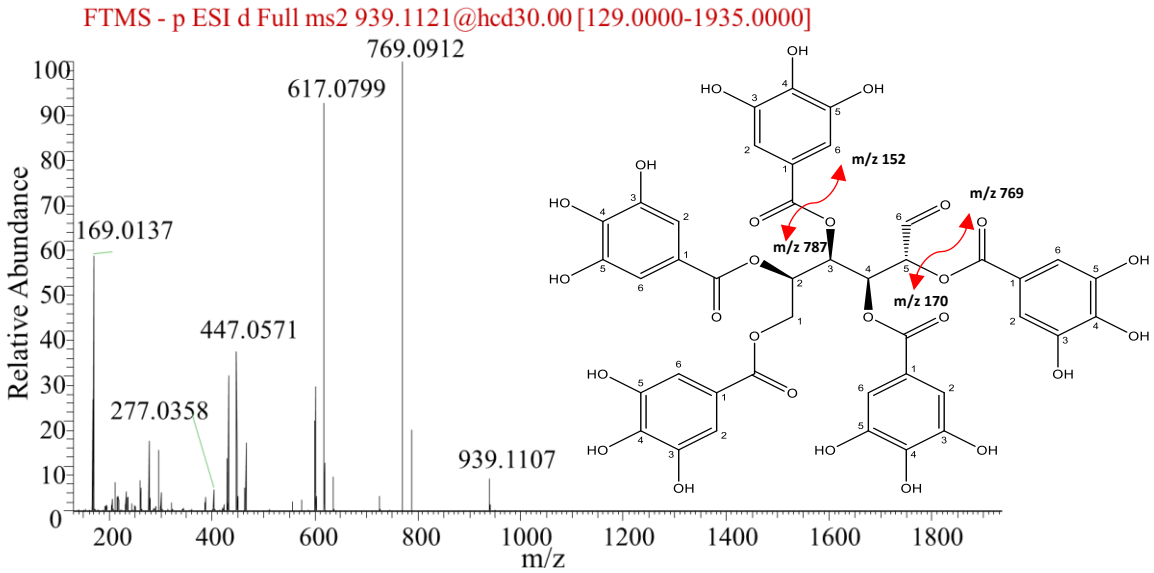


Figure 2.3. Example of MS/MS spectrum of pentagalloyl glucose showing major fragmentations.

Table 2.1. Example of molecular formula prediction and mass loss prediction based on fragmentation patterns for pentagalloyl glucose. Using Xcalibur and nitrogen rule of even electron-ion and mass tolerance of <5 ppm, m/z 939.1107 was predicted as formula $C_{41}H_{31}O_{26}$ (error <5 ppm). Similarly, fragment ion 769.0909 was predicted formed by neutral loss of 170 and index 2 was selected as a possible formula of with loss of $C_7H_6O_5$, and similarly, fragment ion 617.0799 was predicted to be formed by neutral loss of 152 from fragment ion 769.0912 and 447.05 was predicted to be formed by neutral loss of 170 from fragment 617.07. The predicted fragments are highlighted in green.

m/z	Index	Formula	Delta ppm	Neutral loss	Mass loss
939.1116	1	$C_{41}H_{31}O_{26}$	0.837		
	2	$C_{59}H_{23}O_{13}$	-2.901		
	3	$C_{66}H_{19}O_8$	3.353		
	4	$C_{23}H_{39}O_{39}$	4.575		
769.0912	1	$C_{52}H_{17}O_8$	-2.133	170	$C_7H_6O_5$
	2	$C_{34}H_{25}O_{21}$	2.431		
617.0799	1	$C_{15}H_{25}O_{24}N_2$	-0.604	152	$C_7H_4O_4$
	2	$C_{27}H_{21}O_{17}$	2.395		
	3	$C_{40}H_{13}O_6N_2$	3.226		
	4	$C_{45}H_{13}O_4$	-3.293		
447.0570	1	$C_{20}H_{15}O_{12}$	0.382	170	$C_7H_6O_5$
	2	$C_{33}H_7ON_2$	1.53		
	3	$C_8H_{19}O_{19}N_2$	-3.757		
277.0358	1	$C_{13}H_9O_7$	1.639	170	$C_7H_6O_5$
169.0136	1	$C_7H_5O_5$	-3.472	108	$C_6H_4O_2$

Table 2.2. Major phenolic compounds from leaf extract of various species of Myrtaceae inc. *Eucalyptus* (Eu), *Corymbia* (Co), *Angophora* (An), *Syzygium* (Sy) and *Callistemon* (Ca) determined by HPLC-ESI-MS/MS.

Comp. No.	$[M-H]^-$	Rt (mins)	Major fragments (m/z)	Molecular formula	Tentative identification	Eu	An	Co	Sy	Ca	Reference
1	481.06	2.06	421.0407, 300.9986(100), 275.0194	$C_{20}H_{18}O_{14}$	HHDP-glucose	+	+	+	+	+	(Santos *et al.*, 2011; Singab *et al*., 2011)
2	169.01	2.56	125.0231(100)	$C_7H_6O_5$	gallic acid	+	+	+	+	+	(Al-Sayed *et al*., 2012; Amakura *et al.*, 2009; Barry *et al*., 2001)
3	343.07	2.81	191.0551, 169.0132 (100)	$C_{14}H_{16}O_{10}$	galloyl quinic acid	+	+	+	-	+	(Singab *et al.*, 2011; Barry *et al*., 2001)
4	331.07	3.13	271.0459, 169.0132 (100), 151.0012, 125.0234	$C_{13}H_{16}O_{10}$	galloyl glucose	+	+	+	+	+	(Singab *et al.*, 2011; Barry *et al*., 2001)
5	325.06	3.19	169.0132 (100), 125.0301	$C_{14}H_{14}O_9$	galloylshikimic acid	+	+	+	+	+	(Singab *et al.*, 2011)
6	783.07	3.37	481.0619, 300.9984 (100), 275.0196	$C_{34}H_{24}O_{22}$	di-HHDP-glucose	+	+	+	+	+	(Singab *et al.*, 2011; Barry *et al.*, 2001)
7	305.07	3.38	179.0031, 125.0232 (100)	$C_{15}H_{14}O_7$	epigallocatechin	+	+	-	+	-	ReSpect, HMDB
8	466.03	3.39	450.9951, 300.9996 (100), 275.0204, 169.0138, 125.0235	$C_{41}H_{26}O_{26}$	castalagin	+	+	+	-	+	ReSpect, HMDB
9	315.07	3.43	169.0132 (100), 153.0182	$C_{13}H_{16}O_9$	phenolic glycoside	+	+	+	+	+	(Singab *et al.*, 2011)
10	739.19	3.48	289.0724, 161.0238, 125.0235, 407.0779 (100)	$C_{36}H_{36}O_{17}$	catechin derivative	-	-	+	+	-	ReSpect, HMDB

11	933.07	3.51	450.9951, 300.9996 (100), 275.0204, 169.0138, 125.0235	$C_{41}H_{26}O_{26}$	vescalagin	+	+	+	+	+	(Barry *et al.*, 2001)
12	801.08	3.54	633.07, 300.99 (100), 275.0201, 169.0129	$C_{34}H_{26}O_{23}$	unknown HHDP-glucose	-	-	-	+	+	ReSpect, HMDB
13	633.07	3.71	463.0533, 300.9985 (100), 275.0201, 169.0135	$C_{27}H_{22}O_{18}$	HHDP galloyl glucose	+	+	+	+	+	(Singab *et al.*, 2011; Barry *et al.*, 2001)
14	1417.10	3.82	765.0638, 633.0735, 300.9983 (100), 275.0201	$C_{61}H_{46}O_{40}$	ellagatannin dimer	+	-	-	-	-	(Singab *et al.*, 2011)
15	785.08	4.05	633.0736, 300.9991 (100), 275.0198, 249.0399, 169.0133	$C_{34}H_{26}O_{22}$	HHDP-di-galloylglucose	+	+	+	+	+	(Barry *et al.*, 2001)
16	183.03	4.11	168.0053 (100), 124.0153	$C_8H_8O_5$	methyl gallate	-	-	-	+	+	(Ghareeb *et al.*, 2019)
17	483.08	4.13	331.0666, 271.0457, 169.0132 (100), 125.0232	$C_{20}H_{20}O_{14}$	digalloylglucose	+	+	+	+	-	(Barry *et al.*, 2001)
18	447.09	4.16	285.0398 (100)	$C_{21}H_{20}O_{11}$	kaempferol glucoside	+	+	+	+	+	(Abd-Alla *et al.*, 1980; Puig *et al.*, 2018)
19	341.09	4.17	179.0030 (100), 161.0231	$C_{15}H_{18}O_9$	caffeic acid hexoside	+	+	+	+	+	(Santos *et al.*, 2011)
20	137.02	4.32	93.0347 (100)	$C_7H_6O_3$	hydroxybenzoic acid hexoside	-	+	+	+	+	ReSpect, HMDB
21	577.14	4.43	407.07, 289.07 (100), 125.0201	$C_{30}H_{26}O_{12}$	procyanidin B1	+	-	+	-	-	ReSpect, HMDB
22	635.09	4.44	465.0669, 313.06, 300.9984, 271.0455, 169.0129 (100)	$C_{27}H_{24}O_{18}$	trigalloyl glucose	+	+	+	+	+	(Barry *et al.*, 2001; Al-Sayed *et al.*, 2012))
23	463.05	4.58	300.9987 (100)	$C_{20}H_{16}O_{13}$	ellagic acid-hexoside	+	+	+	+	+	ReSpect, HMDB

24	787.10	4.63	635.0892, 465.0701, 300.9982, 249.0401, 169.0131 (100)	$C_{34}H_{28}O_{22}$	tetra-galloyl glucose	+	+	+	+	+	(Barry *et al*., 2001; Al-Sayed *et al*., 2012))
25	353.09	4.65	191.0551 (100), 179.0340	$C_{16}H_{18}O_9$	caffeoylquinic acid	+	+	+	+	+	ReSpect, HMDB
26	289.07	4.67	245.0815 (100), 179.0340, 109.0282	$C_{15}H_{14}O_6$	catechin	-	-	+	+	+	(Ashraf *et al*., 2015; Nasr *et al*., 2019)
27	937.09	4.95	785.0841, 465.0701, 300.9993 (100), 275.0198, 169.0136	$C_{41}H_{30}O_{26}$	HHDP-tri-galloylglucose	+	+	+	-	-	(Barry *et al.*, 2001)
28	535.07	5.02	313.0348 (100), 359.0403	$C_{23}H_{20}O_{15}$	flavonoid-3-O-glycoside	+	+	+	-	+	ReSpect, HMDB
29	939.11	5.42	769.0907, 617.0780, 465.0780, 169.0132 (100)	$C_{41}H_{32}O_{26}$	pentagalloyl glucose	+	+	+	+	+	(Barry *et al.*, 2001)
30	591.53	5.45	289.0724 (100), 301.0724	$C_{31}H_{28}O_{12}$	catechin derivative	-	+	-	+	+	ReSpect, HMDB
31	441.08	5.52	289.0715, 245.0814, 169.0132 (100), 125.0232	$C_{22}H_{18}O_{10}$	catechin gallate	+	-	+	+	+	ReSpect, HMDB
32	935.08	5.52	633.0734, 300.9983, 169.0131(100)	$C_{41}H_{28}O_{26}$	di-HHDP galloylglucose	+	+	+	+	+	(Barry *et al*., 2001)
33	163.04	5.58	119.0419 (100)	$C_9H_8O_3$	p-coumaric acid	-	-	-	+	+	
34	477.07	5.62	301.0356 (100)	$C_{21}H_{18}O_{13}$	quercetin derivative	+	+	+	-	+	(Ferreira *et al*., 2016)
35	197.05	5.79	167.0344(100)	$C_9H_{10}O_5$	syringic acid	-	-	+	+	+	ReSpect, HMDB
36	507.11	5.85	299.0566, 125.0236 (100)	$C_{23}H_{24}O_{13}$	flavonoid glycoside	+	+	+	+	+	ReSpect, HMDB
37	491.08	5.86	315.05182 (100), 299.0192, 163.03947	$C_{22}H_{20}O_{13}$	flavonoid-7-O-glucuronide	+	+	+	-	+	ReSpect, HMDB

38	461.07	5.91	285.0401 (100)	$C_{21}H_{18}O_{12}$	kaempferol glucuronide	-	-	+	+	+	ReSpect, HMDB
39	477.10	5.91	299.0192,161.0450 (100),315.0515, 313.0348	$C_{22}H_{22}O_{12}$	flavonoid glycoside	+	+	-	-	+	ReSpect, HMDB
40	1091.1	5.97	939.1103, 769.0907 (100), 617.0801, 465.0781, 169.0132	$C_{48}H_{36}O_{30}$	hexagalloyl glucose	+	+	-	-	-	(Barry *et al.*, 2001)
41	301.00	5.98	300.9988, 257.0451, 145.0312 (100)	$C_{14}H_6O_8$	ellagic acid	+	+	+	+	+	(Kim *et al.*, 2001)
42	463.09	6.01	301.0347145.03 (100), 178.9901, 151.0010	$C_{21}H_{20}O_{12}$	quercetin derivative	-	+	+	+	+	ReSpect, HMDB
43	315.01	6.41	299.9909 (100)	$C_{15}H_8O_8$	methyl ellagic acid	+	+	+	+	+	ReSpect, HMDB
44	167.03	6.57	123.0438 (100)	$C_8H_8O_4$	dihydroxybenzoic acid derivative	+	+	+	-	+	ReSpect, HMDB
45	179.03	6.65	135.0346 (100)	$C_9H_8O_4$	caffeic acid	-	+	+	+	+	(del Moral & Muller, 1970)
46	615.14	6.81	463, 300, 169.0135, 125.0234 (100)	$C_{29}H_{28}O_{15}$	quercetin galloyl hexoside	+	+	+	+	+	ReSpect, HMDB
47	301.04	7.35	178.9902, 151.0031 (100)	$C_{15}H_{10}O_7$	quercetin	+	+	+	+	-	(Martos *et al*., 2000)
48	285.04	7.37	257.0562, 151.0391 (100), 33.0280	$C_{15}H_{10}O_6$	kaempferol	+	+	+	-	+	ReSpect, HMDB

2.4. Discussion

This study shows UHPLC coupled to Q Exactive Hybrid Quadrupole-Orbitrap on the rapid detection and tentative identification of a vast range of phenolic compounds in leaf extracts of various Myrtaceae species. Forty-eight compounds were detected from five species of Myrtaceae (*Syzygium smithii, Angophora floribunda, Callistemon salignus, Corymbia ficifolia, and Eucalyptus camaldulensis*). It was found from the study that the majority of plant species in the family share similar hydrolysable tannins profiles. Various flavonoid glycosides were identified in all these species. Also, since most of the previous studies are focused on phenolic profiles of *Eucalyptus,* our study helped to identify these compounds, namely quinic acids, caffeic acids, various HHDP glucose and galloyl glucose, quercetin, catechin, ellagic acids, kaempferol glucoside in other species of Myrtaceae plants. This information may be helpful for the assessment of various Myrtaceae species according to the presence and abundance of bioactive phytochemicals.

The rapid identification and characterisation of plant phenolic compounds is difficult because of a diversity of phenolic compounds, variation in sampling method, sample preparation and instrumentation, affecting the type of phenolic compounds detected. Various previously described smaller phenolic compounds, such as resorcinol and torquatone and more lipid-soluble phenolic compounds, were not detected because our extraction and detection methods were better suited for polar compounds. Further, several non-extractable phenolic compounds are found in leaves that are usually are associated with fibre, cell wall and protein (Pérez-Jiménez and Torres, 2011). Therefore, our study only focused on extractable phenolics because the non-extractable phenolic compounds are difficult to extract through aqueous-organic solvent extraction and are not significantly released in the gut by digestive enzymes and may remain intact in the gut and hence have lesser biological activity (Pérez-Jiménez *et al.*, 2013).

2.5. References

Abd-Alla, M. F., El-Negoumy, S. I., Hosny El-Lakany, M., & Saleh, N. A. M. (1980). Flavonoid glycosides and the chemosystematics of *Eucalyptus camaldulensisis*. *Phytochemistry*, *19*(12), 2629–2632.

Albouchi, F., Avola, R., Dico, G. M. L., Calabrese, V., Graziano, A. C. E., Abderrabba, M., & Cardile, V. (2018). *Melaleuca styphelioides* Sm. polyphenols modulate interferon gamma/histamine-induced inflammation in human NCTC 2544 keratinocytes. *Molecules: A Journal of Synthetic Chemistry and Natural Product Chemistry*, *23*(10).

Al-Sayed, E., Singab, A.-N., Ayoub, N., Martiskainen, O., Sinkkonen, J., & Pihlaja, K. (2012). HPLC–PDA–ESI–MS/MS profiling and chemopreventive potential of *Eucalyptus gomphocephala* DC. *Food Chemistry*, *133*(3), 1017–1024.

Amakura, Y., Yoshimura, M., Sugimoto, N., Yamazaki, T., & Yoshida, T. (2009). Marker constituents of the natural antioxidant *Eucalyptus* leaf extract for the evaluation of food additives. *Bioscience, Biotechnology, and Biochemistry*, *73*(5), 1060–1065.

Ashraf, A., Sarfraz, R. A., Mahmood, A., & Din, M. ud. (2015). Chemical composition and in vitro antioxidant and antitumor activities of *Eucalyptus camaldulensis* Dehn. Leaves. *Industrial Crops and Products*, *74*, 241–248.

Balyan, U., & Sarkar, B. (2017). Aqueous extraction kinetics of phenolic compounds from jamun (*Syzygium cumini* L.) seeds. *International Journal of Food Properties*, *20*(2), 372–389.

Barry, K. M., Davies, N. W., & Mohammed, C. L. (2001). Identification of hydrolysable tannins in the reaction zone of *Eucalyptus nitens* wood by high performance liquid chromatography–electrospray ionisation mass spectrometry. *Phytochemical Analysis*, *12*(2), 120–127.

Bayly, M. J., Rigault, P., Spokevicius, A., Ladiges, P. Y., Ades, P. K., Anderson, C., Bossinger, G., Merchant, A., Udovicic, F., Woodrow, I. E., & Tibbits, J. (2013). Chloroplast genome analysis of Australian eucalypts – *Eucalyptus, Corymbia, Angophora, Allosyncarpia* and *Stockwellia* (Myrtaceae). *Molecular Phylogenetics and Evolution*, *69*(3), 704–716.

Ben Said, R., Hamed, A. I., Mahalel, U. A., Al-Ayed, A. S., Kowalczyk, M., Moldoch, J., Oleszek, W., & Stochmal, A. (2017). Tentative characterisation of polyphenolic compounds in the male flowers of *Phoenix dactylifera* by liquid chromatography coupled with mass spectrometry and DFT. *International Journal of Molecular Sciences*, *18*(3).

Bentley, J., Moore, J. P., & Farrant, J. M. (2019). Metabolomic profiling of the desiccation-tolerant medicinal shrub *Myrothamnus flabellifolia* indicates phenolic variability across its natural habitat: Implications for tea and cosmetics production. *Molecules (Basel, Switzerland)*, *24*(7).

Chagas, V. T., Coelho, R. M. R. de S., Gaspar, R. S., da Silva, S. A., Mastrogiovanni, M., Mendonça, C. de J., Ribeiro, M. N. de S., Paes, A. M. de A., & Trostchansky, A. (2018). Protective effects of a polyphenol-rich extract from *Syzygium cumini* (L.) Skeels leaf on oxidative stress-induced diabetic rats. *Oxidative Medicine and Cellular Longevity*, *2018*, e5386079.

dos Santos, C., Galaverna, R. S., Angolini, C. F. F., Nunes, V. V. A., de Almeida, L. F. R., Ruiz, A. L. T. G., de Carvalho, J. E., Duarte, R. M. T., Duarte, M. C. T., & Eberlin, M. N. (2018). Antioxidative, antiproliferative and antimicrobial activities of phenolic compounds from three Myrcia Species. *Molecules: A Journal of Synthetic Chemistry and Natural Product Chemistry*, *23*(5).

Fang, N., Yu, S., & Prior, R. L. (2002). LC/MS/MS characterisation of phenolic constituents in dried plums. *Journal of Agricultural and Food Chemistry*, *50*(12), 3579–3585.

Ferreira, C. D. S., Pereyra, A., Patriarca, A., Mazzobre, M. F., Polak, T., Abram, V., Buera, M. P., & Ulrih, N. P. (2016). Phenolic compounds in extracts from *Eucalyptus globulus* leaves and *Calendula officinalis* flowers. 5.

Ghareeb, M. A., Sobeh, M., El-Maadawy, W. H., Mohammed, H. S., Khalil, H., Botros, S., & Wink, M. (2019). Chemical profiling of polyphenolics in *Eucalyptus globulus* and evaluation of its hepato-renal protective potential against cyclophosphamide induced toxicity in mice. *Antioxidants (Basel, Switzerland)*, *8*(9).

Hayes, R. A., Piggott, A. M., Smith, T. E., & Nahrung, H. F. (2014). *Corymbia* phloem phenolics, tannins and terpenoids: Interactions with a cerambycid borer. *Chemoecology*, *24*(3), 95–103.

Justesen, U. (2000). Negative atmospheric pressure chemical ionisation low-energy collision activation mass spectrometry for the characterisation of flavonoids in extracts of fresh herbs. *Journal of Chromatography. A*, *902*(2), 369–379.

Kim, J. P., Lee, I. K., Yun, B. S., Chung, S. H., Shim, G. S., Koshino, H., & Yoo, I. D. (2001). Ellagic acid rhamnosides from the stem bark of *Eucalyptus globulus.* *Phytochemistry*, *57*(4), 587–591.

Lee, J.-H., Johnson, J. V., & Talcott, S. T. (2005). Identification of ellagic acid conjugates and other polyphenolics in muscadine grapes by HPLC-ESI-MS. *Journal of Agricultural and Food Chemistry*, *53*(15), 6003–6010.

Mahmoud, I. I., Moharram, F. A., Marzouk, M. S. A., Linscheid, M. W., & Saleh, M. I. (2002). Polyphenolic constituents of *Callistemon lanceolatus* leaves. *Die Pharmazie*, *57*(7), 494–496.

Marsh, K. J., Kulheim, C., Blomberg, S. P., Thornhill, A. H., Miller, J. T., Wallis, I. R., Nicolle, D., Salminen, J.-P., & Foley, W. J. (2017). Genus-wide variation in foliar polyphenolics in eucalypts. *Phytochemistry*, *144*, 197–207.

Martos, I., Ferreres, F., Yao, L., D'Arcy, B., Caffin, N., & Tomás-Barberán, F. A. (2000). Flavonoids in monospecific *Eucalyptus* honeys from Australia. *Journal of Agricultural and Food Chemistry*, *48*(10), 4744–4748.

Mongkholrattanasit, R., Klaichoi, C., Rungruangkitkrai, N., Punrattanasin, N., Sriharuksa, K., & Nakpathom, M. (2013). Dyeing studies with *Eucalyptus*, quercetin, rutin, and tannin: A research on effect of ferrous sulfate mordant. *Journal of Textiles*, *2013*, e423842.

Nasr, A., Khan, T. S., & Zhu, G.-P. (2019). Phenolic compounds and antioxidants from *Eucalyptus camaldulensis* as affected by some extraction conditions, a preparative optimisation for GC-MS analysis. *Preparative Biochemistry & Biotechnology*, *49*(5), 464–476.

Pérez-Jiménez, J., Díaz-Rubio, M. E., & Saura-Calixto, F. (2013). Non-extractable polyphenols, a major dietary antioxidant: Occurrence, metabolic fate and health effects. *Nutrition Research Reviews*, *26*(2), 118–129.

Puig, C. G., Reigosa, M. J., Valentão, P., Andrade, P. B., & Pedrol, N. (2018). Unravelling the bioherbicide potential of *Eucalyptus globulus* Labill: Biochemistry and effects of its aqueous extract. *Plos One*, *13*(2), e0192872.

Reynertson, K. A., Yang, H., Jiang, B., Basile, M. J., & Kennelly, E. J. (2008). Quantitative analysis of antiradical phenolic constituents from fourteen edible Myrtaceae fruits. *Food Chemistry*, *109*(4), 883–890.

Salminen, J.-P. (2014). The chemistry and chemical ecology of ellagitannins in plant–insect interactions: From underestimated molecules to bioactive plant

constituents. In *Recent Advances in Polyphenol Research* (pp. 83–113). John Wiley & Sons, Ltd.

Santos, S. A. O., Freire, C. S. R., Domingues, M. R. M., Silvestre, A. J. D., & Neto, C. P. (2011). Characterisation of phenolic components in polar extracts of *Eucalyptus globulus* Labill. bark by High-Performance Liquid Chromatography–Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, *59*(17), 9386–9393.

Sawada, Y., Nakabayashi, R., Yamada, Y., Suzuki, M., Sato, M., Sakata, A., Akiyama, K., Sakurai, T., Matsuda, F., Aoki, T., Hirai, M. Y., & Saito, K. (2012). RIKEN tandem mass spectral database (ReSpect) for phytochemicals: A plant-specific MS/MS-based data resource and database. *Phytochemistry*, *82*, 38–45.

Singab, A.-N., Ayoub, N., Al-Sayed, E., Martiskainen, O., Sinkkonen, J., & Pihlaja, K. (2011). Phenolic constituents of *Eucalyptus camaldulensis Dehnh*, with potential antioxidant and cytotoxic activities. *Rec. Nat. Prod.* 5, 71-280

Singh, A., Bajpai, V., Kumar, S., Sharma, K. R., & Kumar, B. (2016). Profiling of gallic and ellagic acid derivatives in different plant parts of *Terminalia Arjuna* by HPLC-ESI-QTOF-MS/MS. *Natural Product Communications*, *11*(2).

Sobeh, M., Esmat, A., Petruk, G., Abdelfattah, M. A. O., Dmirieh, M., Monti, D. M., Abdel-Naim, A. B., & Wink, M. (2018). Phenolic compounds from *Syzygium jambos* (Myrtaceae) exhibit distinct antioxidant and hepatoprotective activities in vivo. *Journal of Functional Foods*, *41*, 223–231.

Spínola, V., Pinto, J., & Castilho, P. C. (2015). Identification and quantification of phenolic compounds of selected fruits from Madeira Island by HPLC-DAD–ESI-MS^n and screening for their antioxidant activity. *Food Chemistry*, *173*, 14–30.

Sun, J., Liang, F., Bin, Y., Li, P., & Duan, C. (2007). Screening non-coloured phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries. *Molecules*, *12*(3), 679–693.

Chapter III

Seasonal and ontogenetic changes in leaf reflectance and phenolic compounds in five species of Myrtaceae (*Angophora floribunda, Callistemon salignus, Corymbia ficifolia, Eucalyptus camaldulensis* and *Syzygium smithii*)

3.1. Introduction

Biosynthesis of plant phenolic compounds may be induced by various abiotic stresses such as extremes of temperature (Król *et al.*, 2015), solar UV radiation (Krizek *et al.,* 1997), moisture deficit (Petridis *et al.*, 2012), pH (Min *et al.*, 2015) and salinity (Valifard *et al.*, 2014) as well as by biotic stress such as pathogen infection and herbivory (Waterman, 1992; Pichersky & Lewinsohn, 2011; Ramakrishna & Ravishankar, 2011). The production of these plant phenolic compounds enhances the adaptive capacity of plants, and these changes can be qualitative and quantitative or both (Hussain *et al.,* 2008). The qualitative differences between the plant phenolic compounds and the differential responses during various growing seasons can reflect the adaptation of the plant to the various biotic and abiotic environments (Kotilainen *et al.*, 2010). Furthermore, they also provide information about plant and leaf nutritional quality, which ultimately have consequences both for the fitness of the plant and for the herbivore adaptations (Riipi *et al*., 2004).

Generally, Folin-Ciocalteu (FC) and HPLC methods are used for the estimation of total phenolic compounds (Blainski *et al*., 2013; Way *et al.*, 2020). Qualitative and quantitative determination of phenolic compounds by FC method is hampered by their structural complexity and diversity and by reaction of other reducing species in the sample matrix leading to overestimation giving a lot of false positives (Appel *et al.*, 2001; Lester *et al.*, 2012; Amorati & Valgimigli, 2015; Way *et al*., 2020). On the other hand, recent developments in high-performance liquid chromatography (HPLC) have proven to be an efficient and sensitive method for the characterisation and quantification of both individual and total phenolic compounds. Phenolic compounds can be determined more precisely using HPLC where there is the construction of enough standard curves or the use of universal detectors (Kalili & Villiers, 2011). I have only cited study using HPLC for my literature review in this chapter overcomes the shortcoming by FC reagent.

Various HPLC studies have shown that the concentration of individual foliar phenolic compounds (seasonal and ontogenetic) may fluctuate differently than total phenolic compounds, and this qualitative difference in phenolic compounds may reflect the more accurate adaptation to their environment (Salminen *et al*., 2004; Shi *et al.,* 2017; Tuominen & Salminen, 2017). For example, a study on oak leaves, *Quercus robur* L. (Fagaceae), showed that individual hydrolysable tannins and flavonoid glycosides showed a very different seasonal pattern to the total hydrolysable tannins and flavonoid glycosides (Salminen *et al*., 2004). Similarly, another study on seasonal phenolic compounds of mountain birch trees, *Betula pubescens* Ehrh. (Betulaceae) showed the content of total phenolics varied only slightly while variation in contents of individual low-molecular-weight phenolic compounds was significant (Nurmi *et al.,* 1996). Another study on walnut *Juglans sigillata* Dode (Juglandaceae) showed highly significant positive correlations between antioxidant capacity and total phenolic compounds but less significant correlations between individual phenolic compounds and antioxidant activity (Shi *et al*., 2017). Similarly, another study found higher concentrations of individual phenolic groups (hydrolysable tannins) in younger leaves of *Betula pubescens* L. (Betulaceae) (Salminen *et al.,* 2001). This variation in concentration of these foliar phenolic compounds was not only dependent upon the season (Veberic *et al.*, 2008; Aoussar *et al*., 2020; Gori *et al*., 2020), different stages of growth and development (Bhattacharya *et al.,* 2010; Sampaio *et al*., 2016) but also with environmental factors such as temperature, rainfall, and UV radiation (Kouki & Manetas, 2002; Monteiro *et al*., 2006; Kabtni *et al*., 2020).

Solar UV_B (280–315 nm) and UV_A (315–400 nm) are known to induce the synthesis of various plant phenolic compounds, but the relative effect of UV radiation can vary between different groups of phenolic compounds (Kotilainen *et al.,* 2010). Australian plants are exposed to intense UV radiation in summer because of the relatively clear skies and relative proximity to the sun, and thinning of the ozone layer, Figure 3.1 and (Roy *et al*., 1995).

Thus, these native species of well-adapted plants to extreme conditions should exhibit some sort of mechanisms to mitigate damage, such as the production of antioxidants or UV filters (Bornman *et al*., 2015). One of the protective mechanisms of plants against high ultraviolet radiation is the accumulation of various phenolic compounds such as flavonoids and hydroxycinnamic acids with appropriate UV absorptive properties (Taiz and Zeiger 1998; Morales *et al.* 2010). Further, phenolic compounds such as flavonoids and tannins act as strong antioxidant agents by quenching peroxyl radicals (Close and McArthur, 2001). Furthermore, flavonoids are also known to absorb UV radiation, thereby acting as UV filters (Stapleton & Walbot, 1994). For example, quercetin glycosides were found to be accumulated in the leaves of both *Betula pubescens* Ehrh. (Betulaceae) and *Betula pendula* Roth (Betulaceae) in response to higher UV_B (Keski-Saari *et al*., 2005). Also, concentrations of quercetin derivatives in *B. pubescens* were correlated positively with latitude and can be explained by the high antioxidant capacity of quercetin (Stark *et al*., 2008). The concentrations of chlorogenic acids, flavonoids and cinnamic acids in both tree species *Alnus incana* L. (Betulaceae) and *B. pubescens* were highest in early summer, and the concentration of various hydrolysable tannins remained similar throughout the season (Kotilainen *et al*., 2010). Similarly, there was a negative correlation between the content of hydrolysable tannins in leaves early in the season and the content of proanthocyanins late in the season; the inverse relationship between these two groups was consistent between seasons (Riipi *et al*., 2004).

A handful of earlier studies of various genera of Myrtaceae have shown the foliage of the family to be rich in tannins and phenolic glycosides (Reynertson *et al.,* 2008; Mota *et al.,* 2012; Tian *et al.,* 2012; Marsh *et al.,* 2017, 2020). A study of *Eugenia uniflora* L. (Myrtaceae) in Brazil found high levels of hydrolysable tannins during the wet season (November to March) but high flavonoids in the dry season (May to October) (Santos *et al.,* 2011). Research on seven different species of *Eucalyptus* has shown that the

concentration of total phenolic compounds and condensed tannins (CT) increased during the winter, but no other trends for other phenolics with the season or leaf age were apparent. Hence, the authors suggested that this increase in phenolics may be linked to the presence of herbivorous insects, which is difficult to accept since insect herbivores are more active in summer (Macauley & Fox, 1980). The higher phenolic compounds in winter also can be explained by an increase of phenolic compounds due to cold-induced photoinhibition, which can affect the carbon balance and is associated with loss of productivity and direct damage to photosystem II (Mattila *et al*., 2020). Studies have shown that when photosynthesis can be inhibited by cold conditions, UV light can potentially be more damaging (Close *et al.,* 2001). Phenolic compounds such as tannins can play a major role in photoprotection by quenching the reactive species and can be present in greater concentrations in winter even though the plant might be exposed to less intense solar radiation (Close *et al.*, 2001; Close & McArthur, 2002). For example, an increase in concentration concentrations of total phenolics and FPCs in *Eucalyptus microcorys* F.Muell (Myrtaceae) in colder temperature and higher elevation (Moore *et al.,* 2004) and an increase in the concentration of anthocyanins following photoinhibition (Close *et al.,* 2001; Hoch *et al.,* 2001).

Leaves of *Angophora, Corymbia* and *Eucalyptus* are heteroblastic to varying degrees, showing abrupt changes in morphology and chemistry between seedling, juvenile, intermediate and adult leaves, ultimately altering plant defence against herbivory (Loney *et al*., 2006). The leaf dimorphisms between juvenile and adult leaves may be because of environmental heterogeneity, mainly due to light heterogeneity experienced during the ontogeny of an individual (Vlasveld *et al.,* 2018). Various theories have been proposed to explain the adaptive significance of seasonal changes in leaf colour. The first is the photoprotection hypothesis which suggests that anthocyanins protect leaves from photo-inhibition and photo-oxidation (Close & Beadle, 2003) and the second is the coevolution

hypothesis which suggests that leaf redness signals high direct defences such as high PSMs and low nitrogen content to insect herbivores (Archetti, 2000; Hamilton & Brown, 2001). Herbivores are known to alter plant specialised metabolites. For example, feeding by nymphs of *Cardiaspina albitextura* (Psyllidae) can cause discolouration of *Eucalyptus camaldulensis* leaves, increasing total phenolic concentrations above the critical level, which ultimately leads to the collapse of *C. albitextura* populations (Morgan & Taylor, 1988). However, in most cases, unfavourable conditions might negatively impact the generalist herbivores while favouring the specialist herbivores due to lack of natural enemy or competitor (Thiel *et al.*, 2020).

For this chapter, I consider how leaf colour, concentration and composition of foliar phenolic compounds respond to seasonal changes in solar radiation. I hypothesise that exposure to environmental stressors, particularly UV radiation, should have shaped the genetic differentiation of species in their expression of phenolic compounds. Since physiological adaptations to potentially lethal stressors are likely to be conserved among species belonging to the same family and/or that experience similar levels of radiation, I can test the generality of these plant's chemical responses to the threat of photodamage.

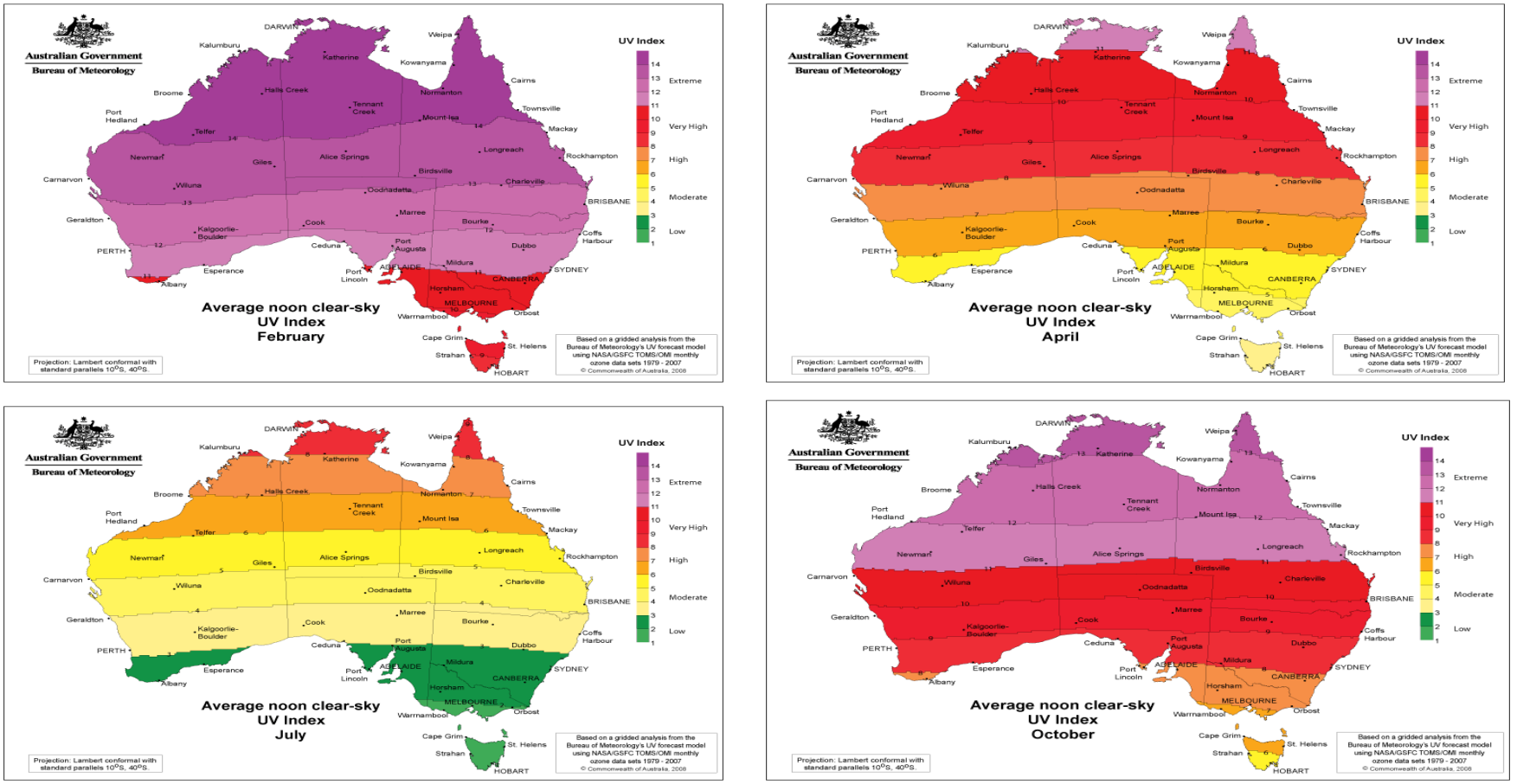


Figure 3.1. Average noon clear sky UV index of February (summer), April (autumn), July (winter) and October (spring) of Australia. Melbourne's UV index varies from low to very high (http://www.bom.gov.au/).

3.2. Materials and Methods

3.2.1. Study species and leaf extraction

Leaves from fully grown trees representative of five genera of Myrtaceae (*Angophora floribunda*, *Callistemon salignus*, *Corymbia ficifolia*, *Eucalyptus camaldulensis* and *Syzygium smithii*) growing around the campus of La Trobe University, Melbourne, Australia was harvested in the second week of February (summer), April (autumn), July (winter) and October (spring) of 2017-18. In this study, leaf nodal position was used as a proxy for relative leaf age. Leaf node was noted before collection: younger leaf arose from a node close to the end of branchlet (i.e., node 1) while the older leaf arose from a more proximal node (i.e., node 8). Five replication of leaf samples were collected from both nodal positions for each plant species, and they were collected in all four seasons. Leaves were collected from relatively younger trees reachable from the ground and the partially shaded conditions (pers. obs). The reflectance of leaves was measured before they were freeze-dried in preparation for mass spectrometric analysis. The freeze-dried leaves were finely ground to ≤ 0.25 mm using a ball mill (Retsch MM400, Germany) at 30 Hz. 20 mg of finely ground leaf powder was weighted in duplicate into 2 mL microtubes, and 1mL of 80:20 MeOH-H_2O (v/v) was added to each sample, and subsequently mixed with a vortex mixer for two minutes and sonicated for 10 minutes (Unisonics, Australia). Samples were then centrifuged for 10 minutes at 15,000 rpm at room temperature (Eppendorf 5415D bench centrifuge, Hamburg, Germany). The extract was transferred to a clean labelled microtube. 1mL of the extract was transferred to HPLC vials for liquid chromatography-mass spectrometry (LC-MS) analysis, and a further 5µL of each extract were pooled to create a quality control (QC) sample to be analysed at the same time.

3.2.2. Chromatographic and mass spectrometric analysis

The quantification of phenolic compounds was performed using an ultra-high-performance liquid chromatographic system (UPLC, UHPLC+ focused, Thermo Scientific™, Waltham, MA, USA) combined with a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific™, Waltham, MA, USA) and a diode array detector. Data was collected in negative ion mode, scanning a mass range m/z of 100–1500. Negative ionisation mode was preferred over positive ionisation mode for all the phenolic compounds subgroups studied because of increased sensitivity, clearer fragmentation patterns and less extensive fragmentations. The samples were injected into the mass spectrometry randomly using the "RAND" function in Microsoft Excel, with blank and QC samples being injected every tenth sample. The MS/MS analyses were carried out in automatic fragmentation mode, where the three most intense mass peaks were fragmented. The MS analyses that include prediction of chemical formula and exact mass calculation were performed by using Thermo Xcalibur Qual Browser software version 3.0.63 (Thermo Scientific, USA. Nitrogen gas was used as the sheath, auxiliary and sweep gas, and the spray voltage was set at 3,600 V. The capillary temperature was set to 300°C, with the S-lens RF level set at 64 and an auxiliary gas heater temperature of 310°C. A Hypersil GOLD C18 column (150×2.1mm, 1.9μm, Thermo Scientific, USA) was used with the column compartment temperature set to 30°C, and a flow rate of 0.3mL/min throughout data acquisition. The mobile phase consisted of (A) 0.1% formic acid and (B) acetonitrile. The flow rate of the mobile phase was maintained at 0.3mL/min. A linear gradient was used beginning with 2% of B and reaching 100% of B at 15 min, then kept steady at 100% of B until 18 minutes and recovered to the initial condition where it was held for two min. The quality control (QC) and blank were run at the interval of every 10^{th} sample.

3.2.3. Data processing

The raw files from Xcalibur were imported to Genedata Expressionist Refiner MS version 12.0, Basel, Switzerland (https://www.genedata.com/). Various filters such as RT structure removal, chemical noise subtraction, peak detection, isotope clustering, adduct detection, singleton filter, and signal clustering were used to reduce noise in the data. Further, QC was used for samples normalisation to minimise and correct for the batch variation. The generated data matrix was exported and visualised and analysed in GeneData Analyst™ 12.0.6 software (Genedata AG, Basel, Switzerland). GeneData Analyst was used for further integration and interpretation of results by using various statistical applications. The samples were annotated based on their genera, age, and seasons.

3.2.4. Leaf reflectance measurements

An Ocean Optics Jaz spectrometer from Ocean Optics (www.OceanOptics.eu) was used to record leaf colour, which is equipped with a pulsed xenon light source and reflection probe of 400 μm. A polytetrafluoroethylene white standard (WS^{-1}, Ocean Optics) was used to calibrate the spectrophotometer. The irradiance of natural illumination was measured from the adaxial area of the leaves inside the lab during midday. Reflectance spectra were divided into UV: 300-400 nm, blue: 400-500 nm, green: 500-600 and red: 600-700 nm wavelengths. Colours of leaves from various seasons were represented in a two-dimensional colour space using the segment classification method described in Endler (1990). Plot chroma, hue and brightness were calculated to study the influence of seasons on the chromatic component of leaf colours as described by Farnier & Steinbauer (2016). The leaves were then stored in envelopes and immediately freeze-dried and used later for further mass spectrometric analysis.

3.2.5. Statistical analyses and identification of compounds

The data from Genedata were further exported to MATLAB R2019b. Principal component analysis, partial least squares discriminant analysis (PLS-DA), and multilevel PLS-DA analyses were carried out in MATLAB using the PLS Toolbox 8.6.2.39. The dataset composed of the varying metabolites was used to perform partial least squares–discriminant analysis (PLS–DA) comparing the samples between their seasons and leaf age. Firstly, the dataset was $\log_{10}$ transformed and auto-scaled. The (variable importance in projection) VIP score was used, which measures how much important is each variable for creating the discrimination model and is calculated as a weighted sum of squares of the partial least square loadings, where the weights are based on the amount of y-variance explained in each dimension. The partial least squares discriminant analysis classification models were fitted to the plant metabolites for each time point separately to examine if there were metabolic differences between the two groups. The PLS-DA models were fitted and cross-validated using venetian blinds w/ 10 splits and 1 sample per split, autoscale was used in pre-processing, and p-values ≤ 0.05 were considered significant. The most important metabolites in the discrimination were further tentatively identified based on their chemical structure, molecular formula and molecular mass and various published literature. These compounds were further compared with various web-based resources such as Chemspider (version 2021.0.6.0), mzCloud (https://www.mzcloud.org/), RIKEN tandem mass spectral database (ReSpect version 2013.11.11), and Human Metabolome Database (HMDB version 4.0). Leaf colour data for the various season were analysed using MANOVA followed by Tukey's *post-hoc* tests for multiple comparisons.

3.3. Results

3.3.1. Leaf reflectance

MANOVA results showed statistically significant differences in leaf colours with season in *Corymbia ficifolia* ($F_{9,34}$ = 80.78, P<0.001, Wilks' λ = 0.001), *Syzygium smithii* ($F_{9,34}$ = 459.683, P<0.001, Wilks' λ = 0.0001), *Angophora floribunda* ($F_{9,34}$ = 77.26, P<0.001, Wilks' λ = 0.001), *Callistemon salignus* ($F_{9,47.5}$ = 116.367,P < 0.001, Wilks'λ = 0.0001) and *Eucalyptus camaldulensis* ($F_{9,34}$ = 153.86, P < 0.001, Wilks' λ = 0.001). The spectra of leaves between various seasons for individual species were calculated and the colour space used to study influence seasons on the chromatic component of leaf colours (Farnier & Steinbauer, 2016). Our results showed that *Corymbia* leaves reflected strongly across the entire spectrum, and particularly winter leaves had strong reflectance on the green-red spectrum. *Eucalyptus* leaves had redder hues in summer and reflected strongly on the red spectrum, whereas winter leaves reflected strongly in the green spectrum. *Angophora* leaves reflected across the entire spectrum, and summer leaves reflected strongly in the red-green spectrum. *Callistemon* leaves reflected in the red spectrum on autumn and summer leaves reflected in reddish green. *Syzygium* reflected across the entire spectrum, particularly autumn reflected in the red spectrum and winter reflected the greenish-red spectrum (Figure 3.2). The results were further compared with the chemistry of seasonal leaves.

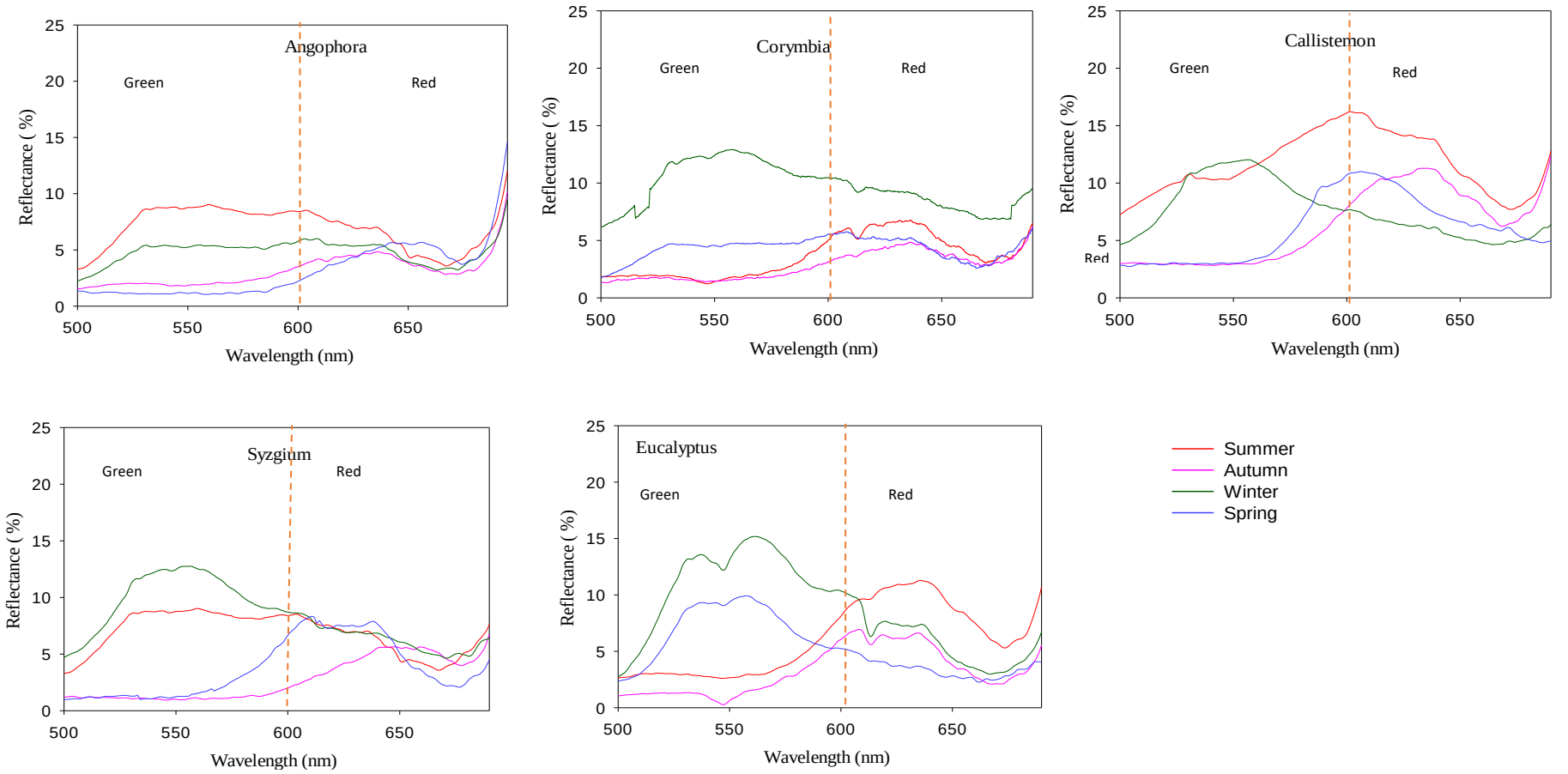


Figure 3.2. Change in reflectance in *Angophora floribunda, Callistemon salignus, Corymbia ficifolia, Syzygium smithii and Eucalyptus camaldulensis* leaves by season. The data is from averaged spectra from multiple scans. The red vertical line separates the green and red-light wavelength.

3.3.2. Seasonal variation in phenolics

3.3.2.1. Winter leaves

PLS-DA model based on winter versus the rest of the seasons showed significant variation between the two groups (Figure 3.3). Top VIPs discriminating between two groups were selected, and out of those five were identified as phenolic compounds, one was identified as a fatty acid group, and another two compounds could not be identified (Table 3.1). My data suggests that the concentration of various hydrolysable tannins such as di-HHDP-galloyl glucose, digalloyl glucose, tetragalloyl glucose and galloyl glucose all decreased in the winter season compared to the rest of the seasons, whereas the concentration of dihydroxybenzoic acid increased in the winter season compared to season. Also, the relative concentration of HHDP-glucose decreased in all other species except in *Angophora* (Figure 3.4).

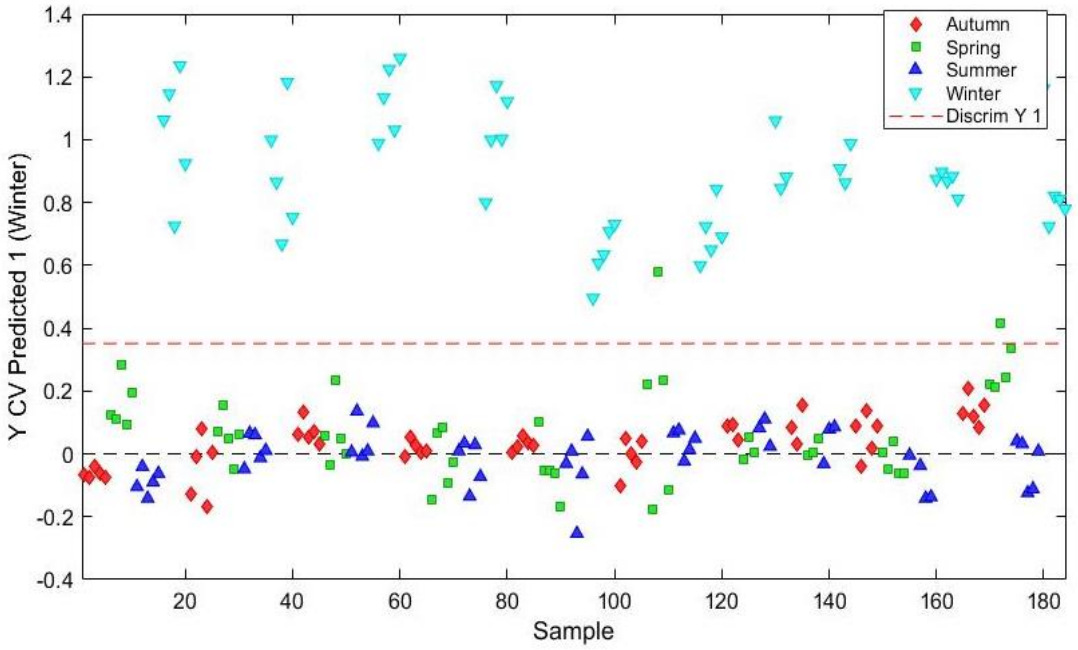


Figure 3.3. Loading plots from PLS-DA models of metabolic profiles of winter leaves *versus* summer, spring, and autumn leaves of all the species combined.

Table 3.1. Top statistically significant metabolites between winter *versus* summer, spring, and autumn leaves. These features are tentatively identified based on online databases and literature (*P*-value ≤0.001).

m/z	Rt(min)	Fragments	Tentative identification
483.0779	4.15	331.0701, 271.05, 169.0135, 125.0236	digalloylglucose
787.0984	4.63	635.0905, 465.0701, 300.9908, 169.0137	tetragalloylglucose
481.0617	2.11	421.0401, 300.9997, 275.0204	HHDP-glucose
935.0795	5.52	633.0748, 300.9995, 169.0135	di-HHDP-galloylglucose
331.0672	3.13	271.0466, 169.0137, 151.0031, 125.0231	galloylglucose
153.0193	3.46	109.0285,123.0442	dihydroxybenzoic acid
331.2494	8.23	313.2379, 59.0133	fatty acid
245.9472	2.34	~	~
315.8847	1.51	~	~

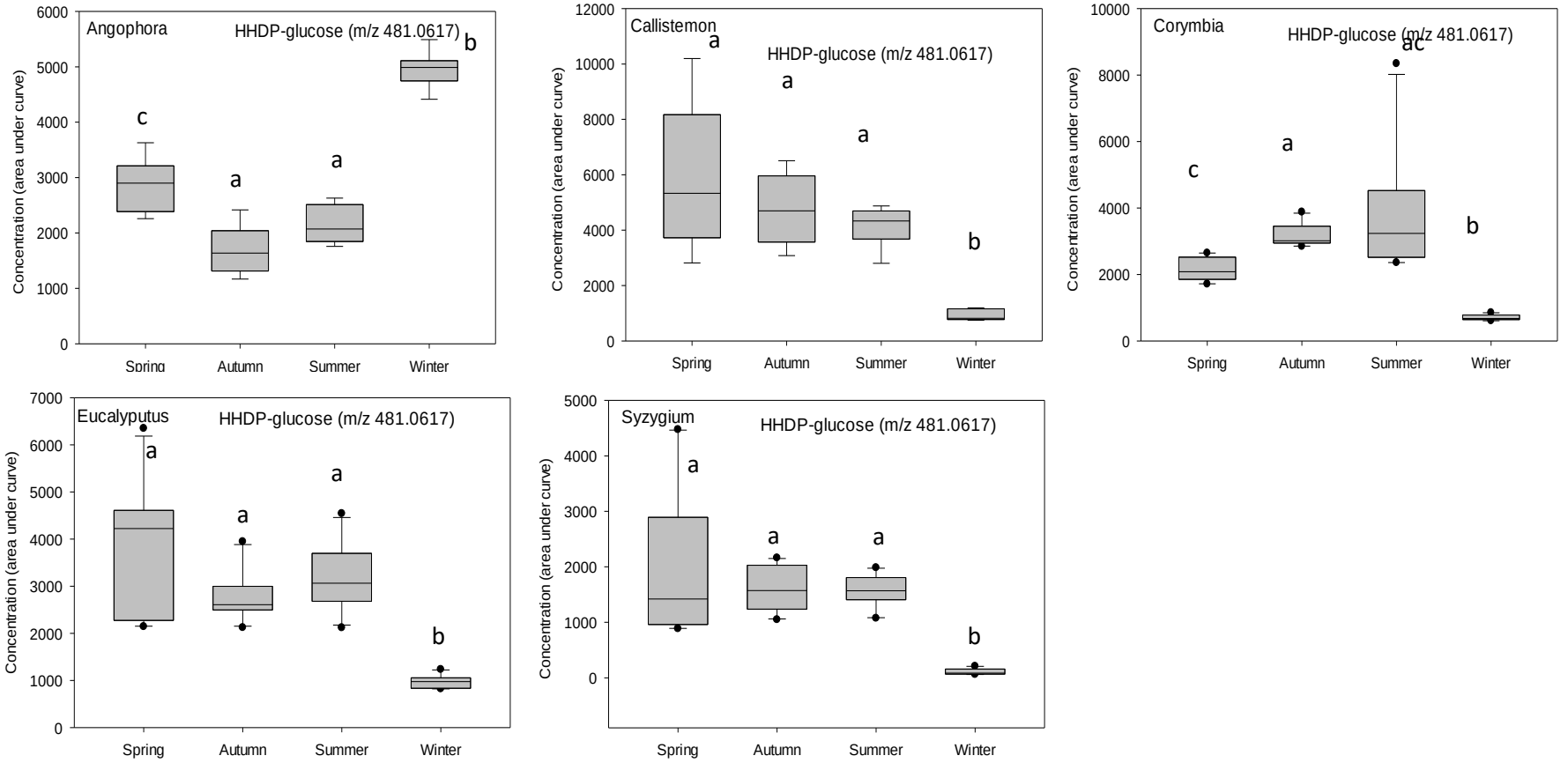


Figure 3.4a. Box plot showing top statistically significant phenolic compound from Table 3.1 in individual species of the plant between winter *versus* summer, spring, autumn season leaves. In each box plot, the horizontal line that is crossing the box is the median, lower and upper quartiles are at the bottom and top of the box respectively and the whiskers are the maximum and minimum values. The clusters represented with the same letter code are not significantly different (Tukey multiple comparisons of means, $P<0.05$).

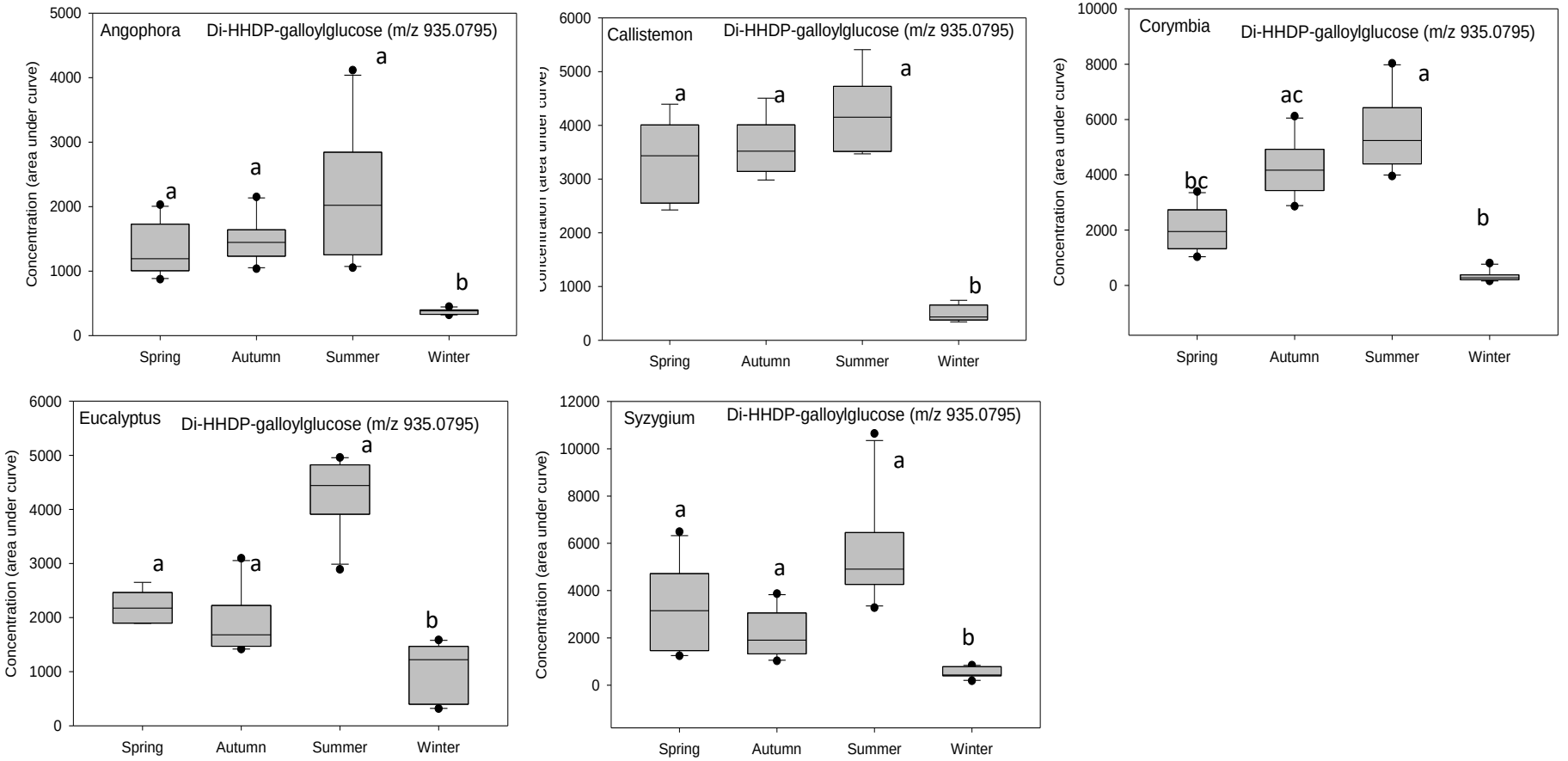


Figure 3.4b. Box plot showing top statistically significant phenolic compounds from Table 3.1 in individual species of the plant between winter *versus* summer, spring, autumn season leaves. See the explanation of the figure in the caption for Figure 3.4a.

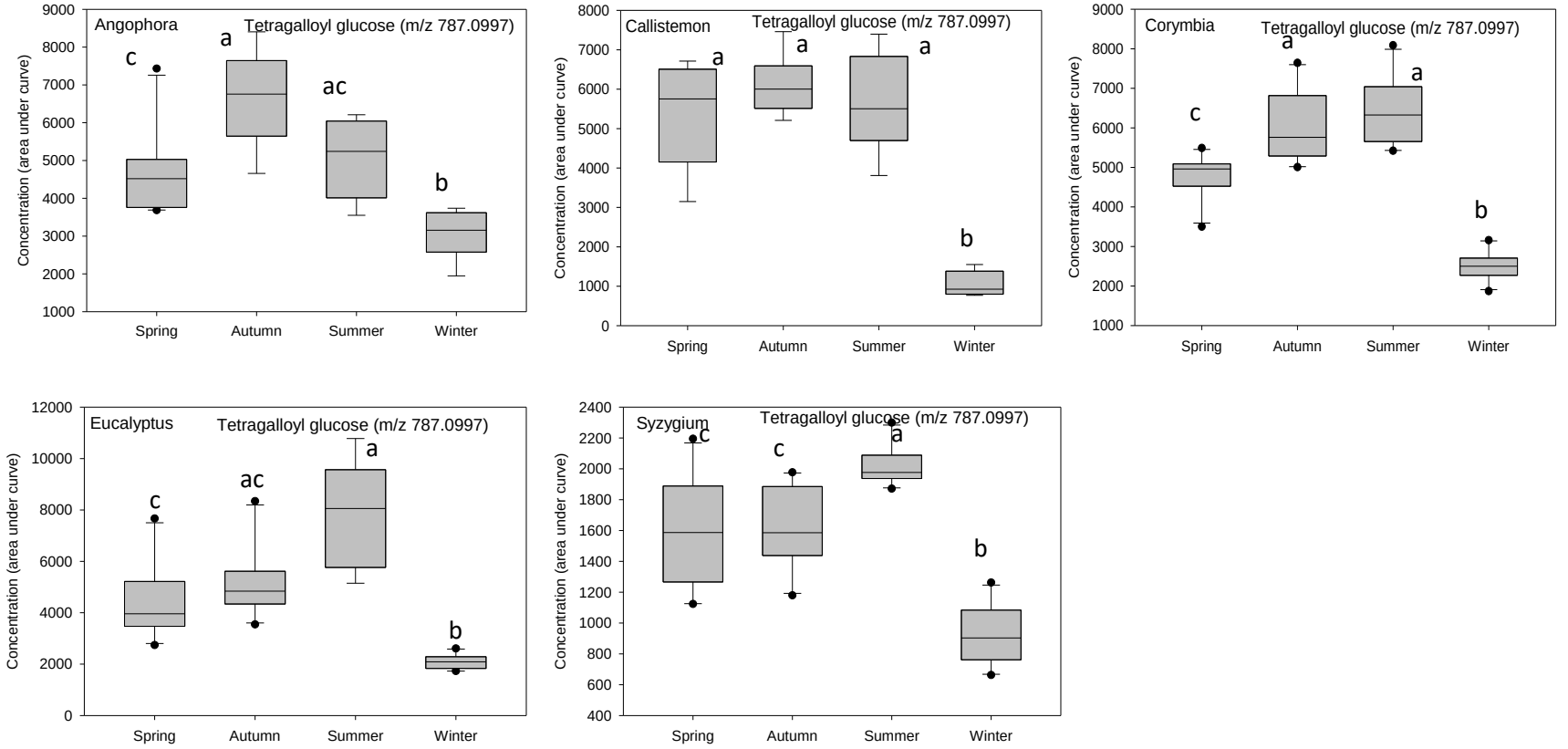


Figure 3.4c. Box plot showing top statistically significant phenolic compounds from Table 3.1 in individual species of the plant between winter *versus* summer, spring, autumn season leaves. See the explanation of the figure in the caption for Figure 3.4a.

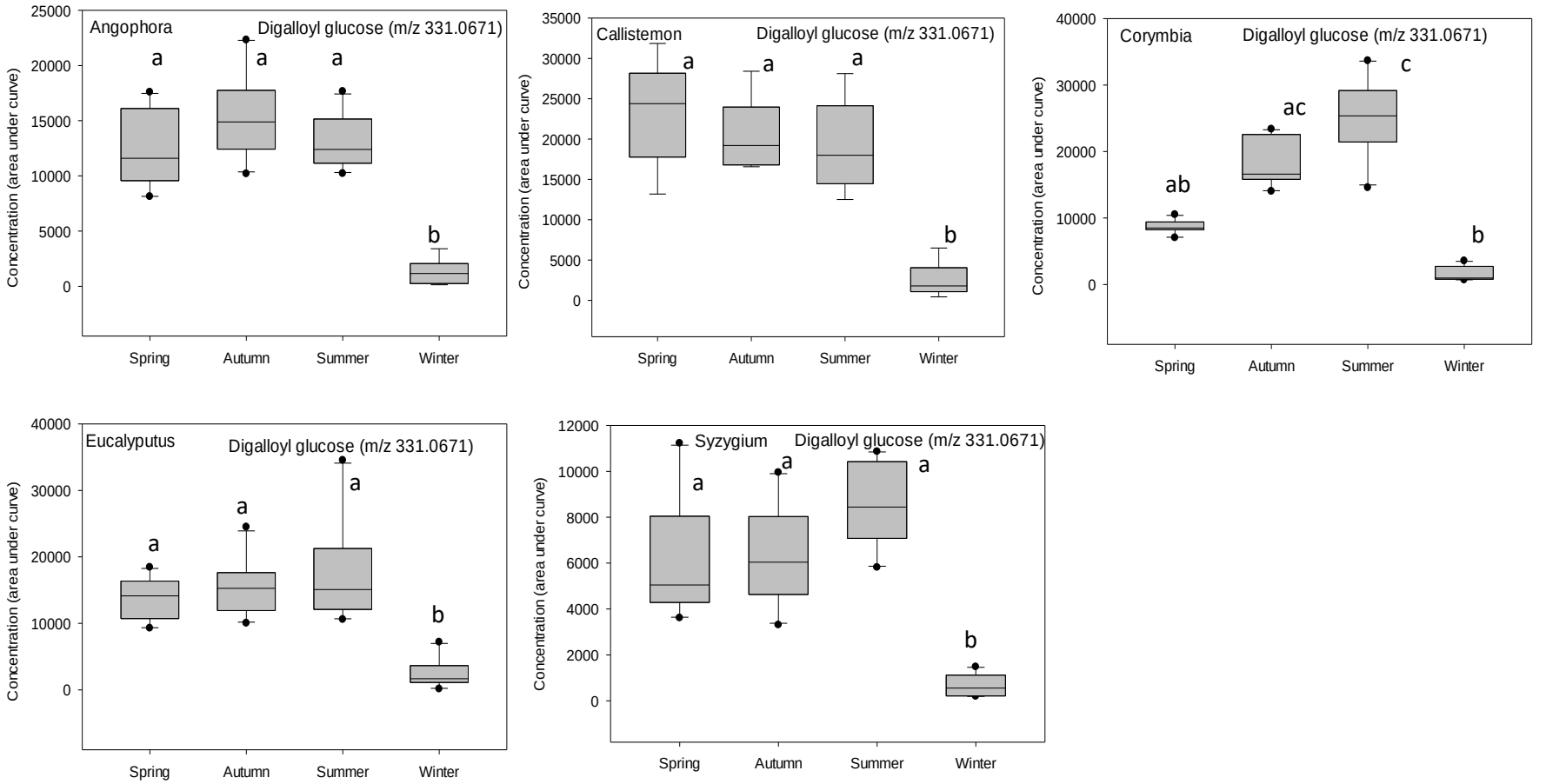


Figure 3.4d. Box plot showing top statistically significant phenolic compounds from Table 3.1 in individual species of the plant between winter *versus* summer, spring, autumn season leaves. See the explanation of the figure in the caption for Figure 3.4a.

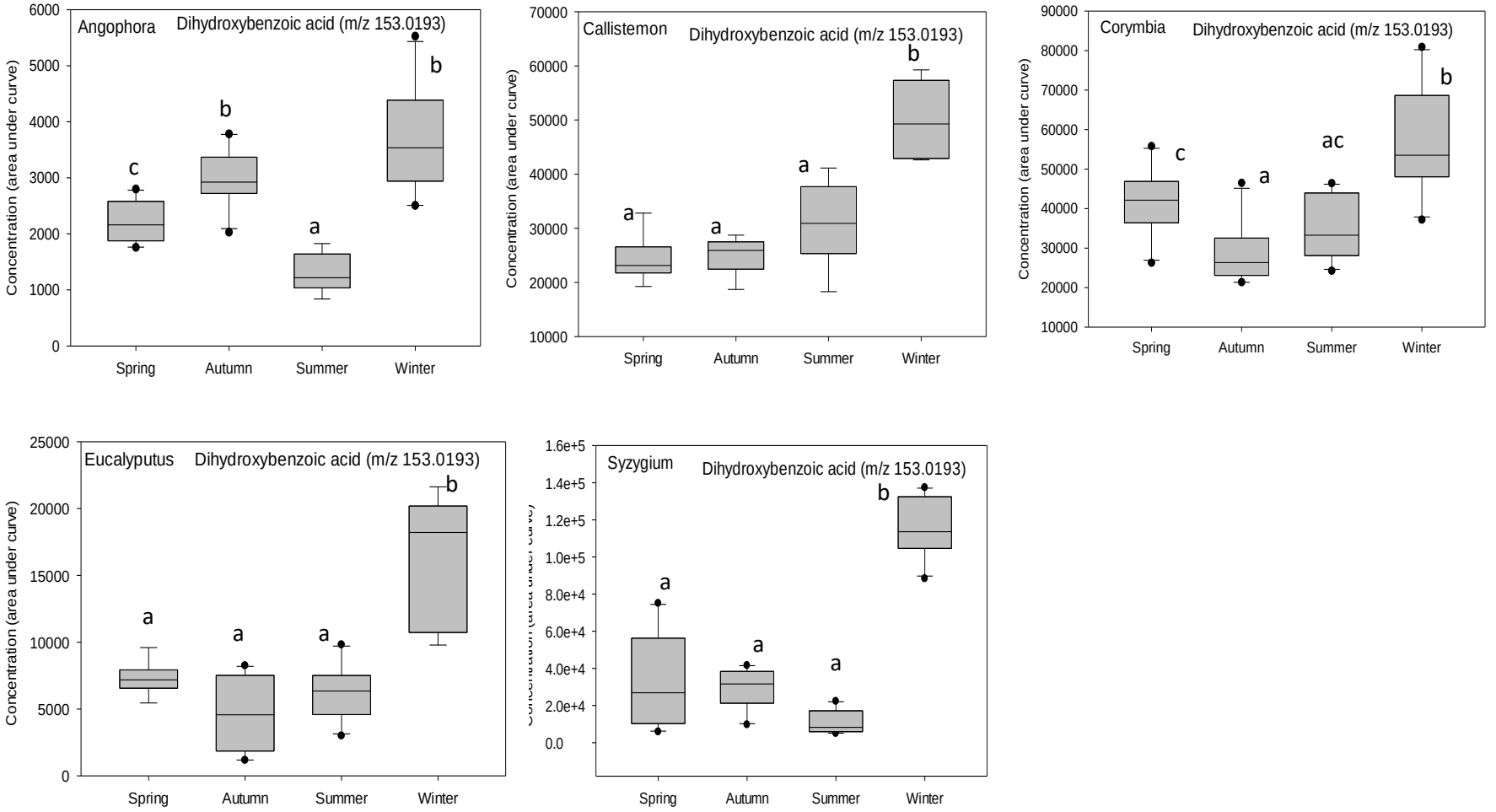


Figure 3.4e. Box plot showing top statistically significant phenolic compounds from Table 3.1 in individual species of the plant between winter *versus* summer, spring, autumn season leaves. See the explanation of the figure in the caption for Figure 3.4a.

3.3.2.2. Summer leaves

PLS-DA model based on summer vs rest of seasons leaves showed significant variation between two groups (Figure 3.5). Top VIPs between these two groups were selected, and out of those five were identified as phenolic compounds, one was identified as benzoic acid ester, and another three compounds could not be identified (Table 3.2). My data suggested that the relative concentration of various flavonoid glycoside such as flavonoid-7-O-glucuronide, flavonoid-3-O-glycoside increased during the summer season compared to the rest of the seasons, whereas the concentration of gallic acid and trihydroxybenzene decreased during the summer season compared to other seasons (Figure 3.6).

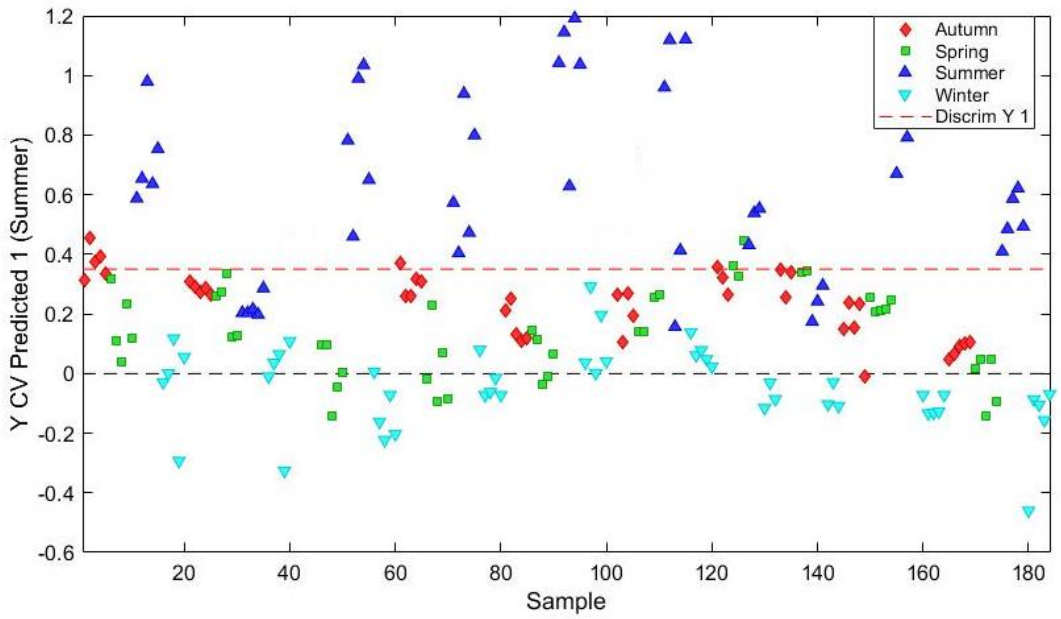


Figure 3.5. Loading plots from PLS-DA models for metabolic profiles between summer *versus* winter, spring and autumn leaves for all the species combined.

Table 3.2. Top statistically significant metabolites between summer *versus* winter, spring and autumn leaves. These features are tentatively identified based on online databases and literature.

m/z	Rt(min)	Fragments	Tentative identification
491.0842	5.86	315.05182, 299.0192, 163.03947	flavonoid-7-O-glucuronide
535.0725	5.02	313.0348, 359.0403	flavonoid-3-O-glycoside
477.1032	5.91	299.0192,161.0450,315.0515, 313.0348	flavonoid glycoside
169.01343	2.66	125.02354	gallic acid
125.0234	3.06	97.0281, 69.034	trihydroxybenzene
221.0808	9.22	149.0964, 121.0287, 71.0503, 69.0347	benzoic acid ester
425.1819	9.39	~	unknown
447.2502	12.35	~	unknown
647.2131	7.93	~	unknown

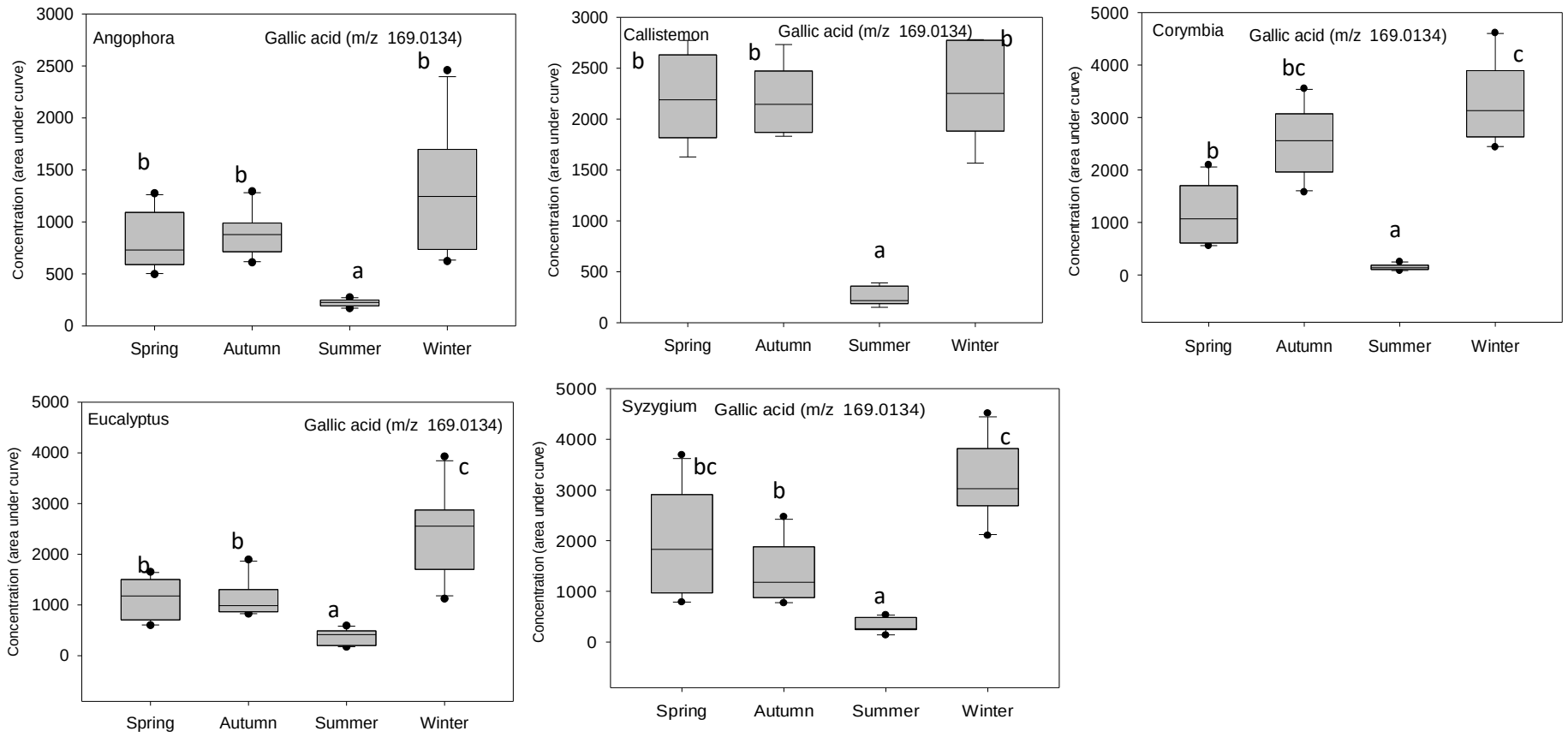


Figure 3.6a. Box plot showing top statistically significant phenolic compounds from Table 3.2 in individual species of the plant between summer *versus* winter, spring, autumn seasons leaves. In each box plot, the horizontal line that is crossing the box is the median, lower and upper quartiles are at the bottom and top of the box respectively and the whiskers are the maximum and minimum values. The clusters represented with same letter code are not significantly different (Tukey multiple comparisons of means, $P<0.05$).

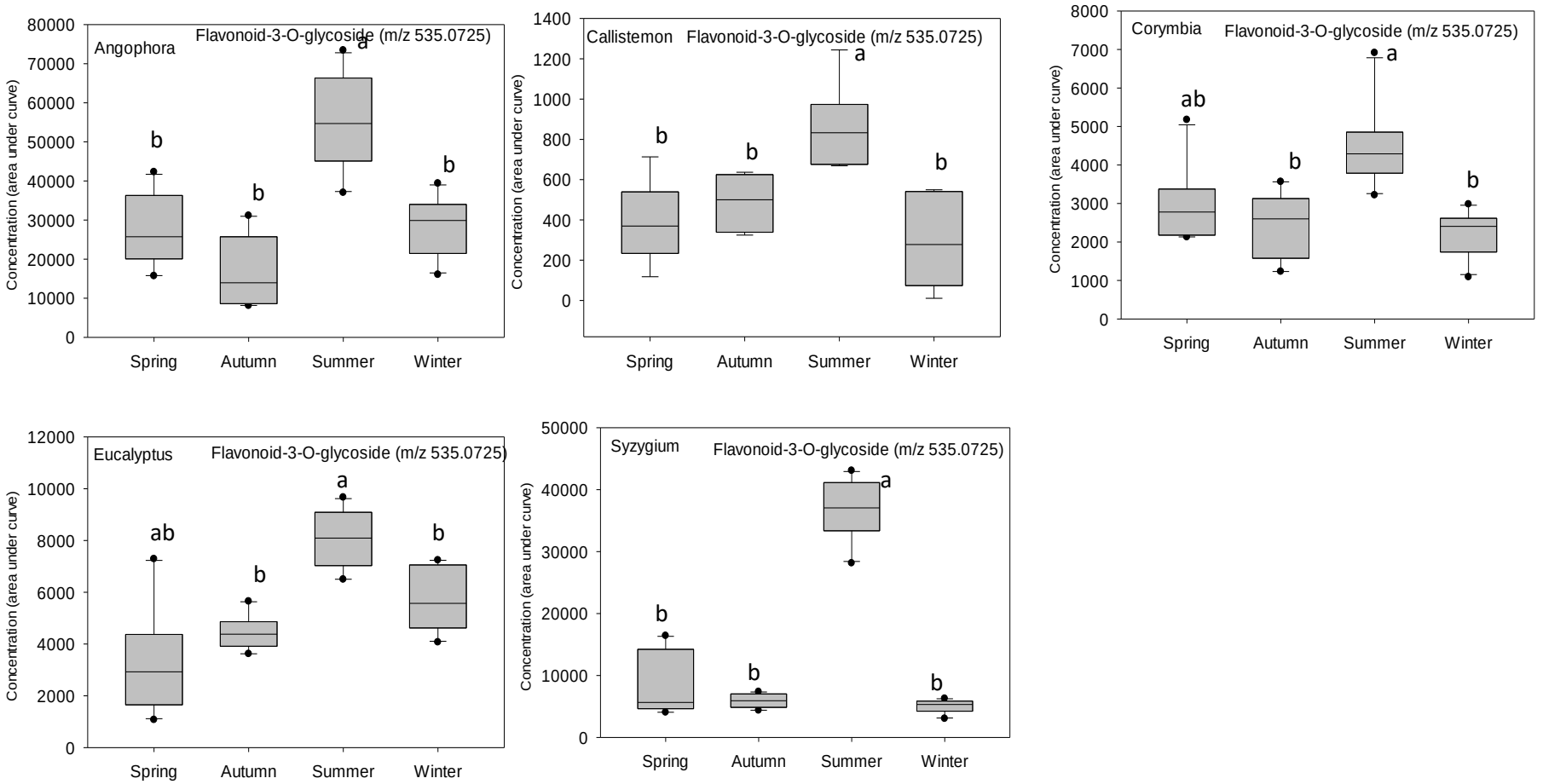


Figure 3.6b. Box plot showing top statistically significant phenolic compounds from Table 3.2 in individual species of the plant between summer *versus* winter, spring, autumn season leaves. See the explanation of the figure in the caption for Figure 3.6a.

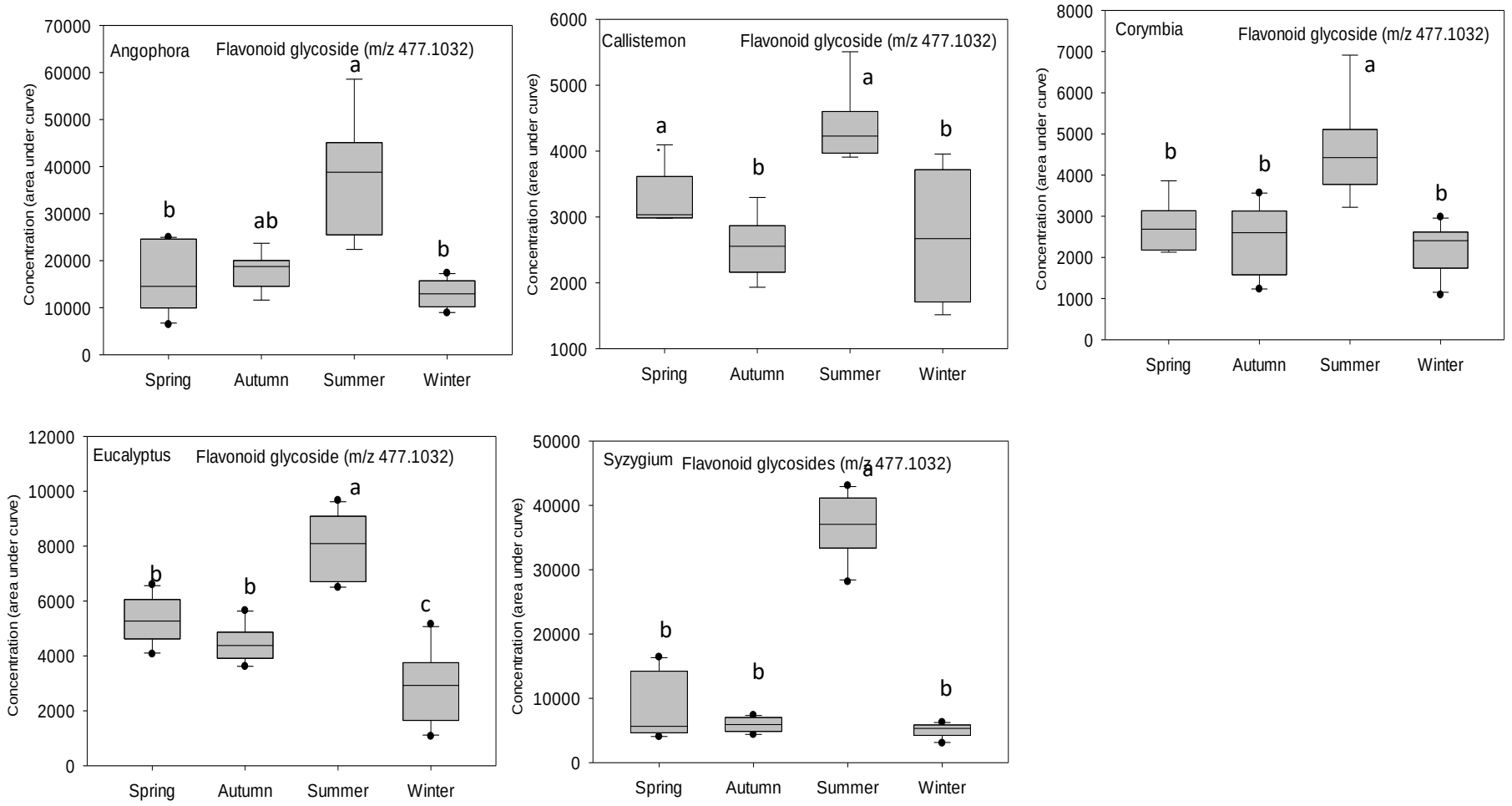


Figure 3.6c. Box plot showing top statistically significant phenolic compounds from Table 3.2 in individual species of the plant between summer *versus* winter, spring, autumn season leaves. See the explanation of the figure in the caption for Figure 3.6a.

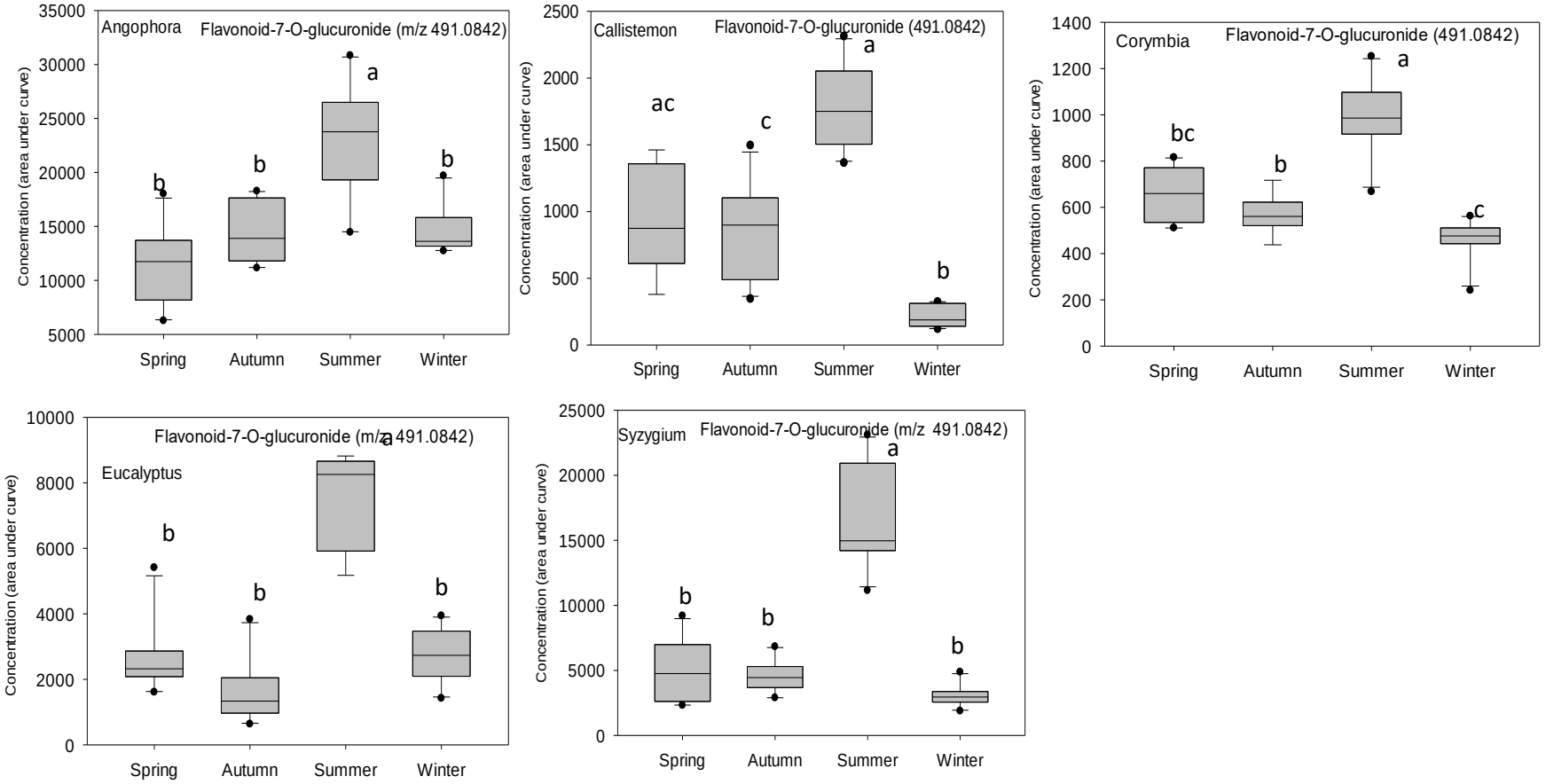


Figure 3.6d. Box plot showing top statistically significant phenolic compounds from Table 3.2 in individual species of the plant between summer *versus* winter, spring, autumn season leaves. See the explanation of the figure in the caption for Figure 3.6a.

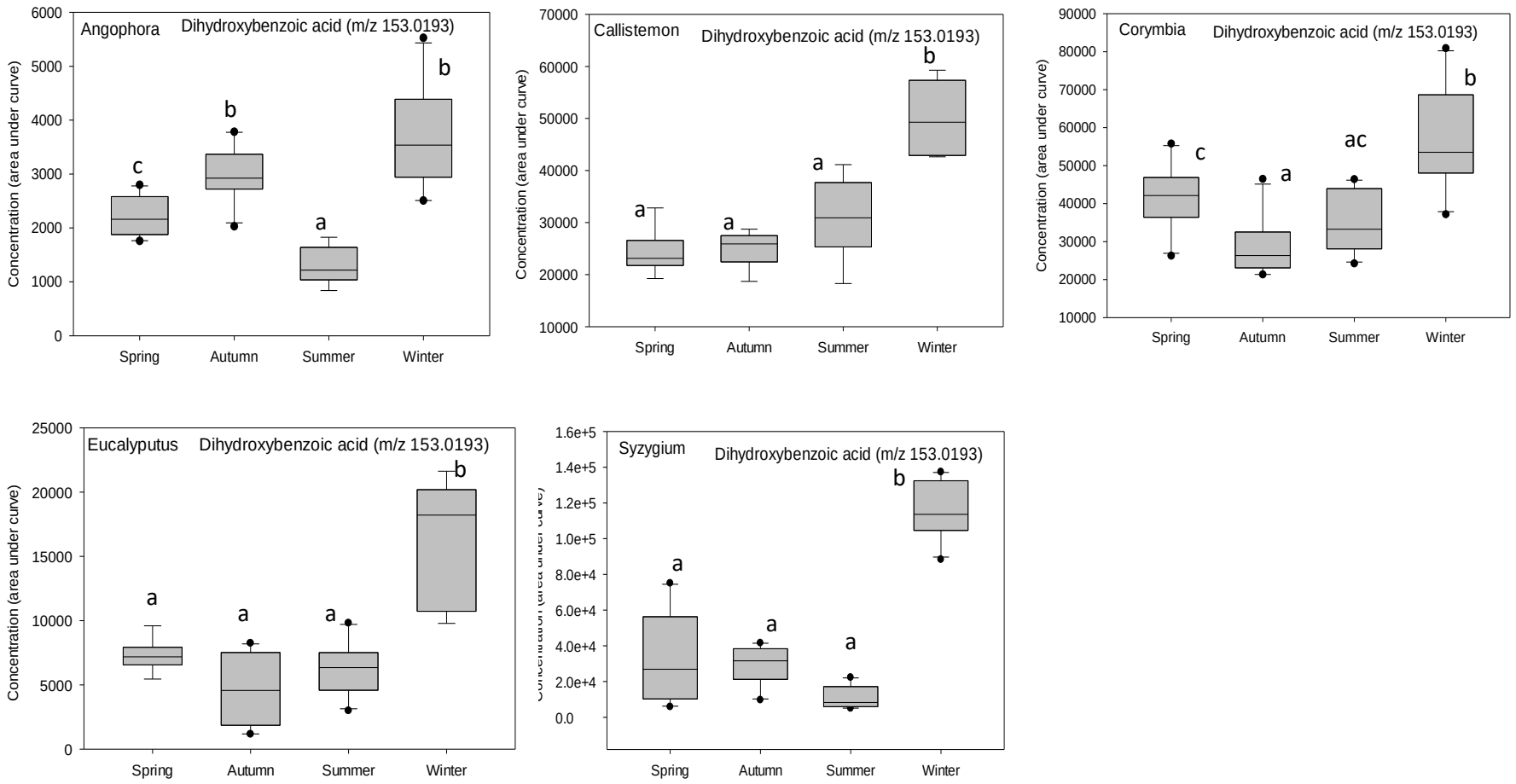


Figure 3.6e. Box plot showing top statistically significant phenolic compounds from Table 3.2 in individual species of the plant between summer *versus* winter, spring, autumn season leaves. See the explanation of the figure in the caption for Figure 3.6a.

3.3.2.3 Autumn leaves

PLS-DA model based on autumn *versus* rest of seasons leaves showed significant variation between two groups (Figure 3.7). Top VIPs between these two groups were selected and out of those four were identified as phenolic compounds, one was identified as triterpenoid (possibly 3-trans-Caffeoyltormentic acid with matching fragments of m/z 469.3312, m/z 433.3533 and m/z 161.0243), and another two compounds could not be identified (Table 3.3). My data suggest that there was a significant decrease in the concentration of catechin derivative (m/z 591.1531) in autumn leaves in all four of the genera except *Eucalyptus*, which shows a significant decrease of catechin derivative in autumn leaves compared to summer and winter, but no significant difference between spring and autumn leaves. Similarly, there was a significant decrease of catechin gallate (m/z 441.0837) in autumn leaves of all other genera except *Eucalyptus*, which showed a substantial difference between autumn, winter, and spring leaves but not between autumn and summer leaves (Figure 3.8).

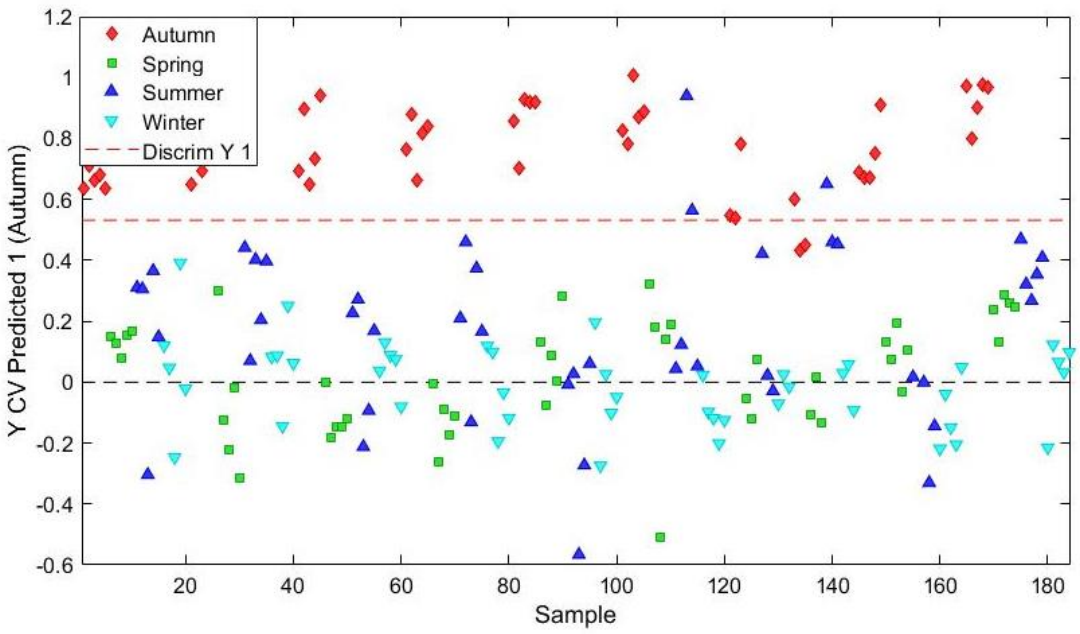


Figure 3.7. Loading plots from PLS-DA models for metabolic profiles between autumn vs winter, spring, summer season leaves for all the species combined.

Similarly, there was a significant increase in the concentration of flavonoid derivative (m/z 341.1038) in autumn leaves in four of the genera except *Callistemon* leaves, which shows a substantial increase of flavonoid derivative in autumn leaves compared to spring and winter but no significant difference between summer and autumn leaves. Also, there was a significant increase in concentration of flavonoid glycoside (m/z 507.1146) in autumn leaves all other genera except *Syzygium* leaves which shows a significant difference between autumn, winter and spring leaves but not significant difference between autumn and summer leaves.

Table 3.3. Top statistically significant metabolites between autumn *versus* winter, spring and summer leaves. These features are tentatively identified based on online databases and literature.

m/z	**Rt(min)**	**Fragments**	**Tentative identification**
591.1531	5.45	289.0724, 301.0724	catechin derivative
507.1146	5.85	299.0566, 125.0236	flavonoid glycoside
441.0837	5.65	289.0724, 245.0854, 169.0192, 125.0236	catechin gallate
341.1038	3.61	341.1026, 311.0918, 299.0553	flavonoid derivative
649.3741	10.81	469.3312,433.3533, 161.0243	triterpenoid
646.3448	8.34	~	unknown
450.2823	9.35	~	unknown

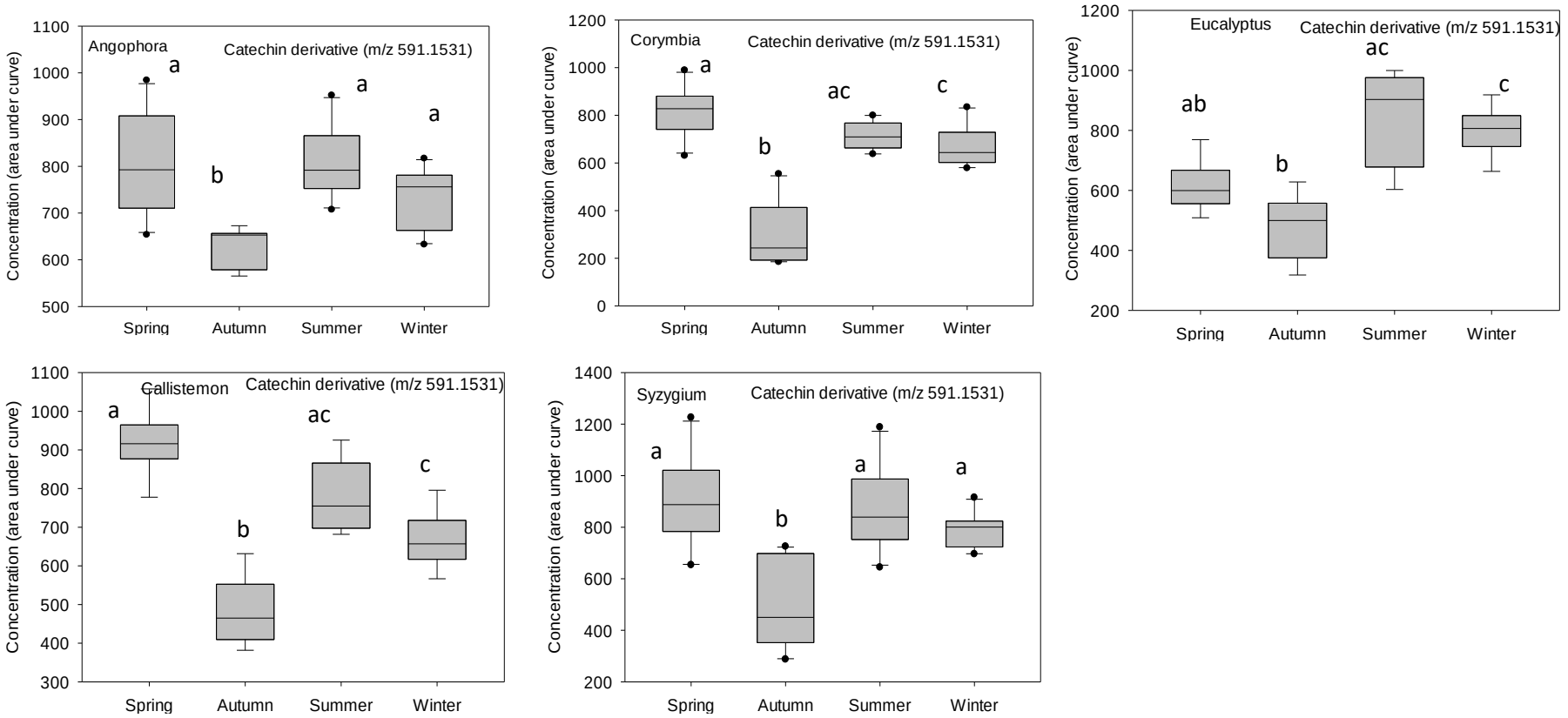


Figure 3.8a. Box plot showing top statistically significant phenolic compound from Table 3.3 in individual species of the plant between autumn *versus* summer, winter and spring leaves. In each box plot, the horizontal line that is crossing the box is the median, lower and upper quartiles are at the bottom and top of the box, respectively, and the whiskers are the maximum and minimum values. The clusters represented with the same letter code are not significantly different (Tukey multiple comparisons of means, $P<0.05$).

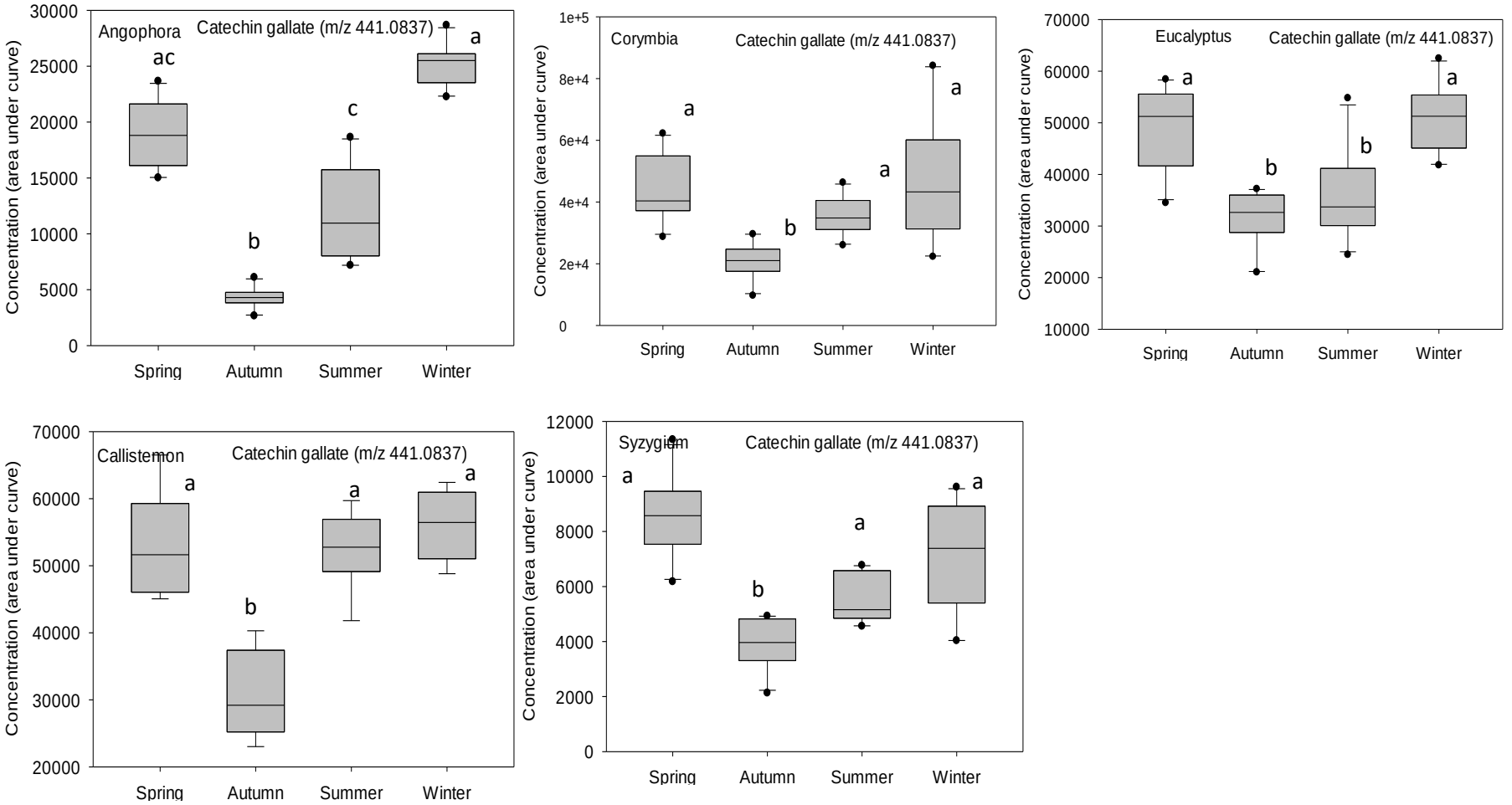


Figure 3.8b. Box plot showing top statistically significant phenolic compounds from Table 3.3 in individual species of the plant between autumn *versus* summer, winter and spring season leaves. See explanation of figure in the caption for Figure 3.8a.

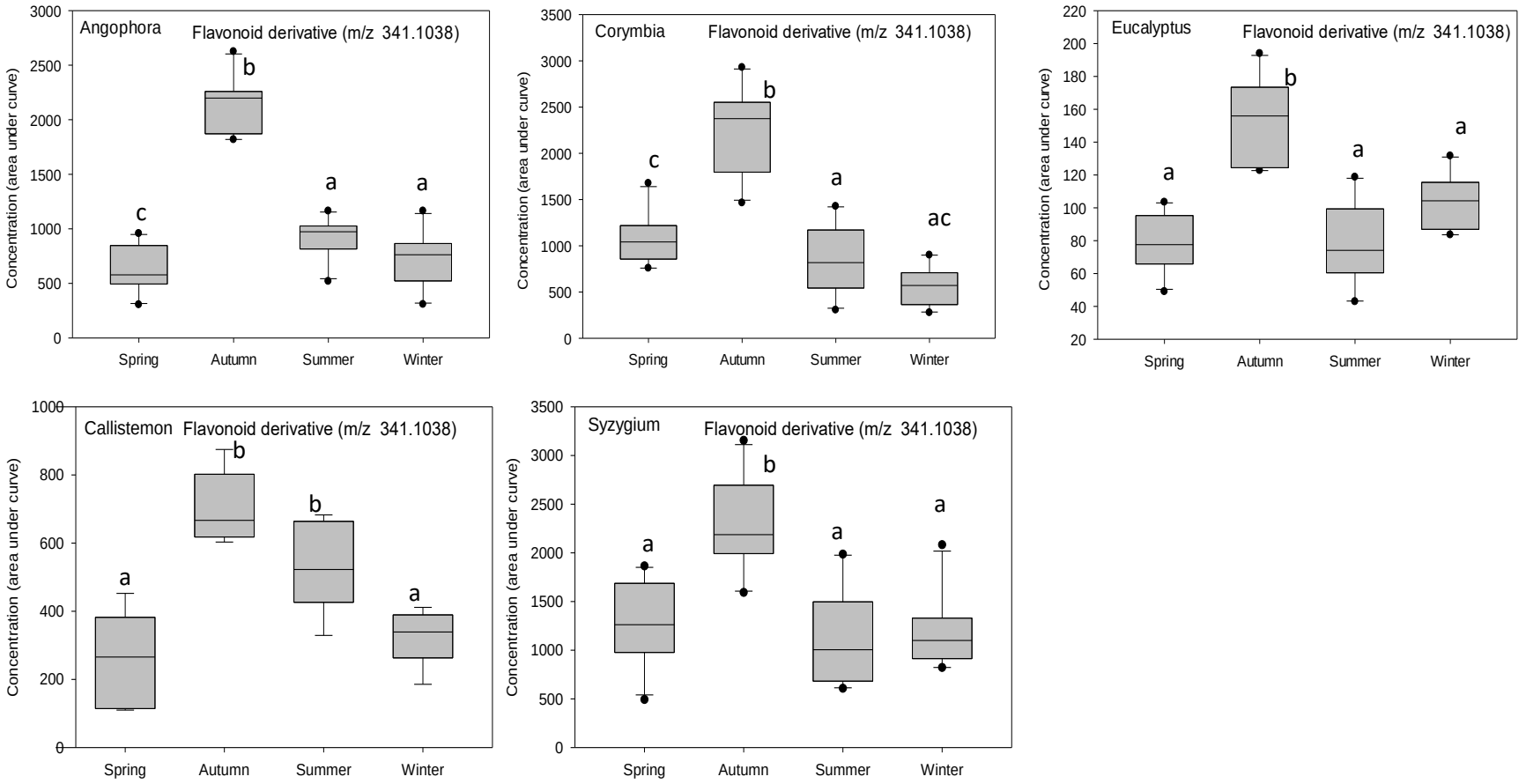


Figure 3.8c. Box plot showing top statistically significant phenolic compounds from Table 3.3 in individual species of the plant between autumn *versus* summer, winter and spring season leaves. See explanation of figure in the caption for Figure 3.8a.

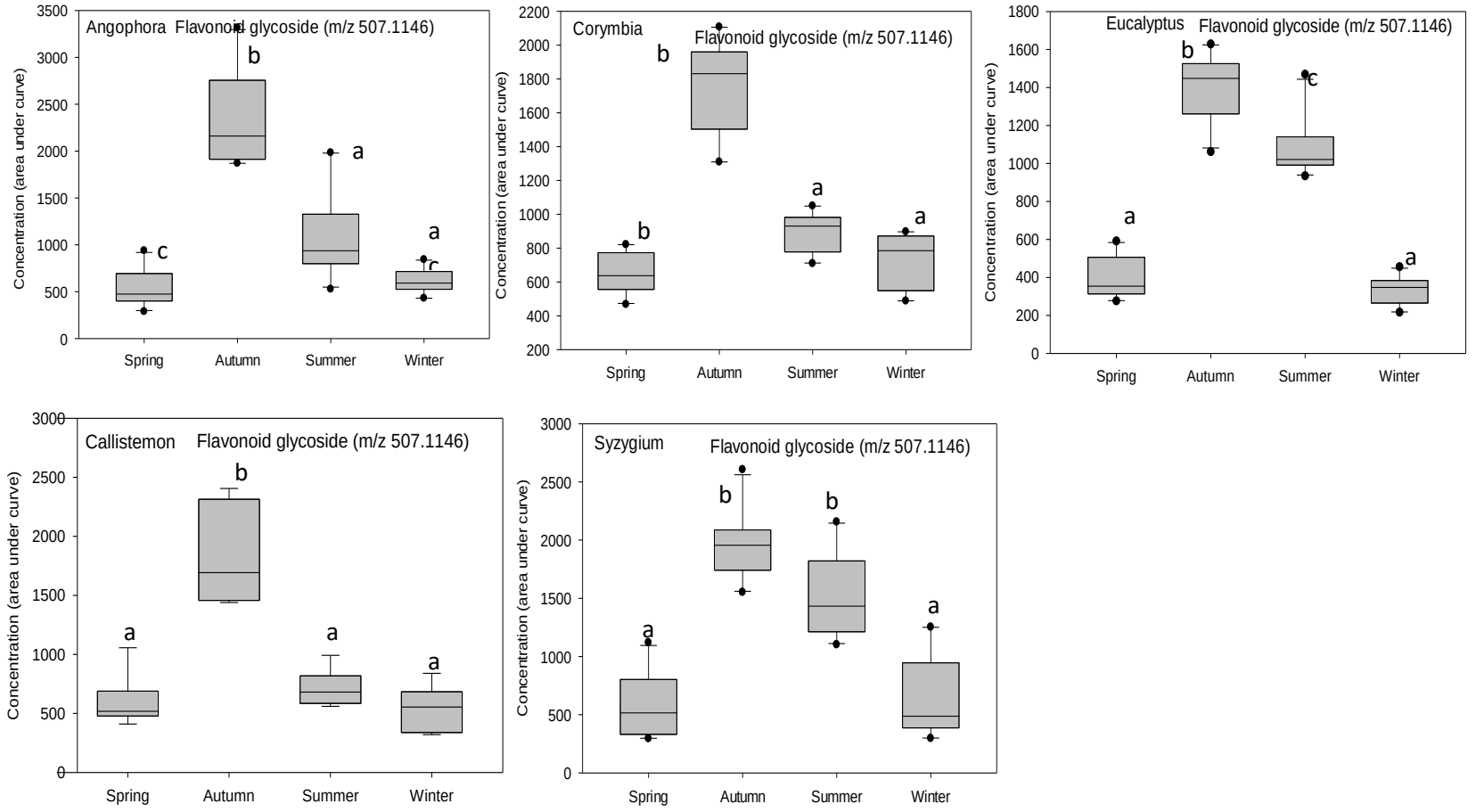


Figure 3.8d. Box plot showing top statistically significant phenolic compounds from Table 3.3 in individual species of the plant between autumn *versus* summer, winter and spring season leaves. See explanation of figure in the caption for Figure 3.8a**.**

3.3.2.4. Spring leaves

PLS-DA model based on spring vs rest of seasons leaves showed significant variation between two groups (Figure 3.9). Top VIPs between these two groups were selected, and out of those three compounds were identified as phenolic compounds, two were identified as a fatty acid group, and one was identified as hexose, and three were unidentified (Table 3.4). There is a significant increase in the concentration of catechin derivative (m/z 739.1907) in spring leaves in four of the genera except in *Angophora* leaves, which showed a significant increase of catechin derivative in spring leaves compared to autumn and winter, but no significant difference between summer and spring leaves. Similarly, there was a significant increase in the concentration of vescalagin I or castalagin I (m/z 466.0291) in autumn leaves all other genera except *Eucalyptus* and *Callistemon* leaves, which showed a significant difference between autumn, winter and spring leaves but not significant difference between spring and summer leaves (Figure 3.10).

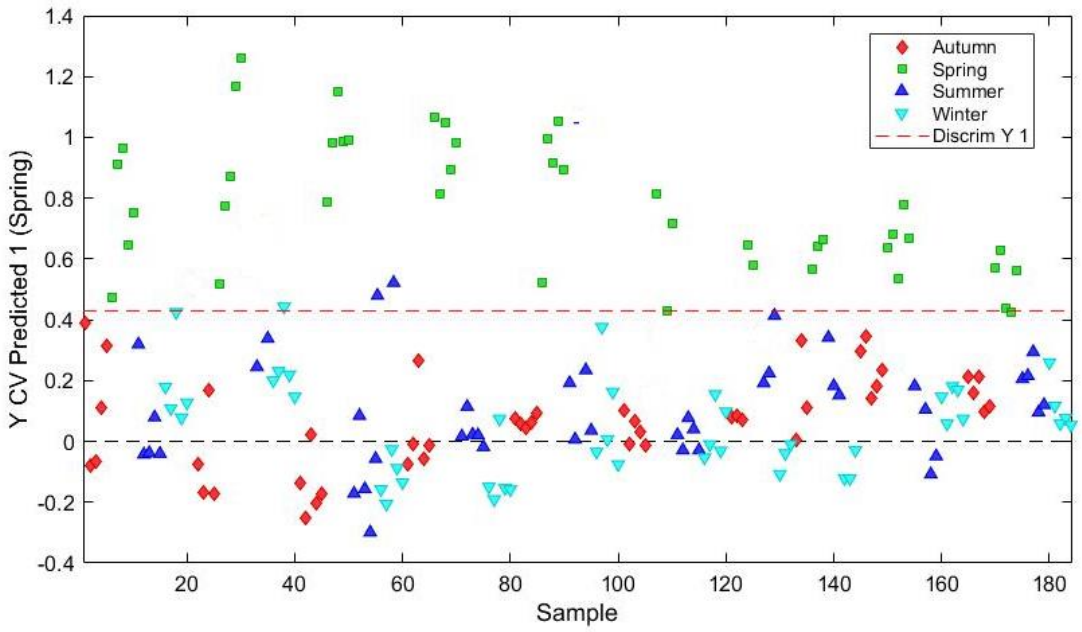


Figure 3.9. Loading plots from PLS-DA models for metabolic profiles between spring versus autumn, summer, winter leaves for all the species combined.

Table 3.4. Top statistically significant metabolites between spring *versus* winter, autumn, and summer leaves. These features are tentatively identified based on online databases and literature.

m/z	Rt(min)	Fragments	Tentative identification
739.1907	3.48	289.0724, 161.0238, 125.0235, 407.0779	catechin derivative
466.0291	3.39	300.9996,275.0204, 169.0138, 450.9951, 125.0235	vescalagin I/ castalagin I
466.0291	4.46	450.9951, 300.9996, 275.0204, 169.0138, 125.0235,	vescalagin II/ castalagin II
179.0552	2.03	149.0455, 161.0455	hexose
395.1926	8.05	131.034, 149.0451, 293.0873	fatty acyl glycosides
285.2078	9.12	267.1972, 241.2180	fatty acyl derivative
587.3139	~	~	unknown
256.9373	~	~	unknown
214.9488	~	~	unknown

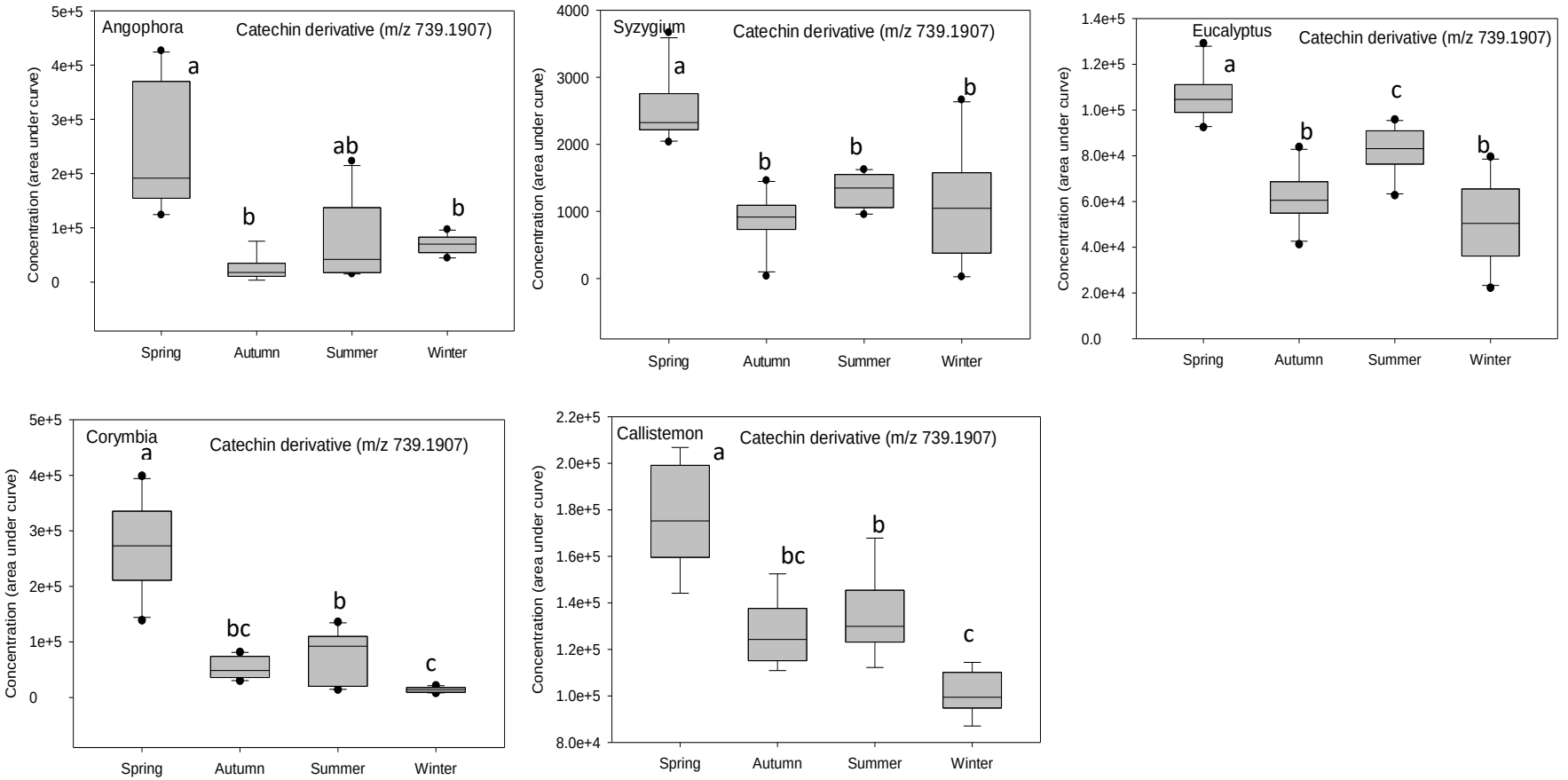


Figure 3.10a. Box plot showing top statistically significant phenolic compounds from Table 3.4 in individual species of the plant between spring *versus* summer, autumn and winter leaves. In each box plot, the horizontal line that is crossing the box is the median, lower and upper quartiles are at the bottom and top of the box, respectively, and the whiskers are the maximum and minimum values. The clusters represented with the same letter code are not significantly different (Tukey multiple comparisons of means, $P<0.05$).

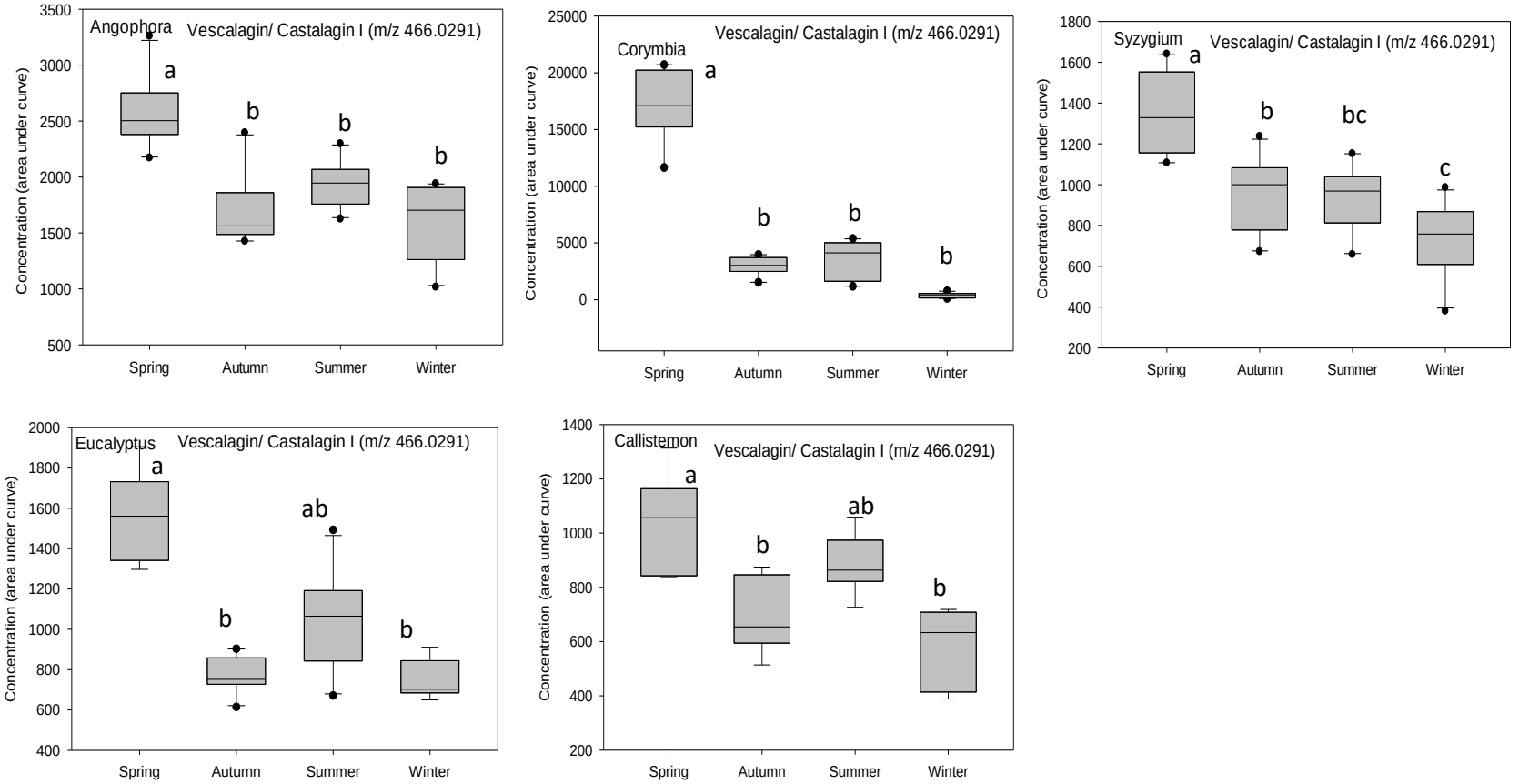


Figure 3.10b. Box plot showing top statistically significant phenolic compounds from Table 3.4 in individual species of the plant between spring *versus* summer, autumn and winter leaves. See explanation of figures in the caption for Figure 3.10a.

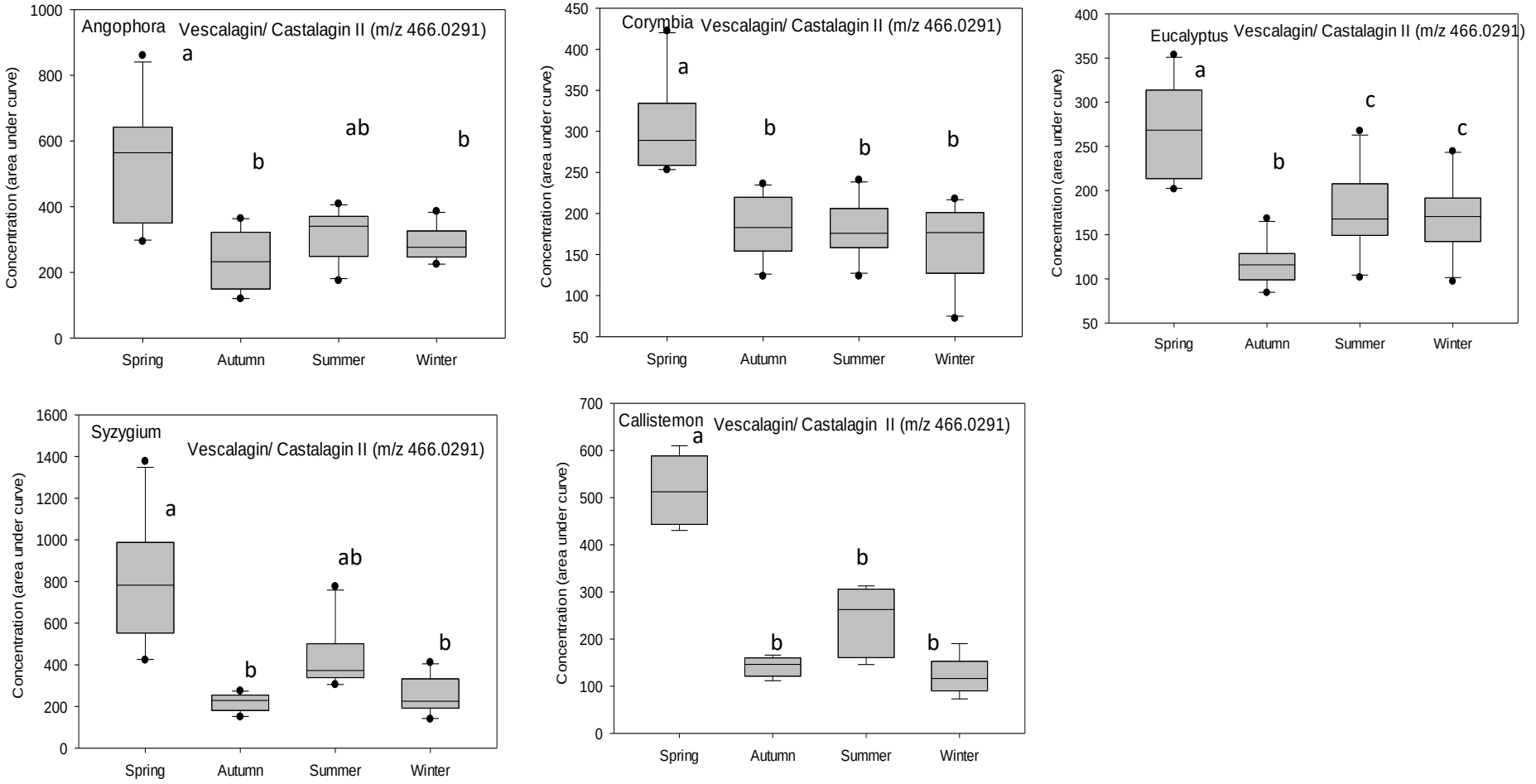


Figure 3.10c. Box plot showing top statistically significant phenolic compounds from Table 3.4 in individual species of the plant between spring *versus* summer, autumn and winter leaves. See explanation of figures in the caption for Figure 3.10a.

3.3.3. Effect of leaf age

PCA analysis did not show any clear trend or grouping between younger and older leaves. However, employing PLS-DA and removing the subject variation in the data showed significant variation between the two-age group (Figure 3.11). The top ten variable importance projections (VIPs) between younger and older leaves were selected, and out of those, six were identified as phenolic compounds, one was identified as a fatty acid group, and another three compounds could not be identified (Table 3.5). My data suggest that various hydrolysable tannins such as di-HHDP-galloylglucose and tetragalloyl glucose were in higher relative concentration in younger leaves compared to in older leaves. Also, various phenolic hexoside, ellagitannin dimer, ellagic acid hexoside were in higher relative concentrations in younger leaves compared to older leaves (Figure 3.12).

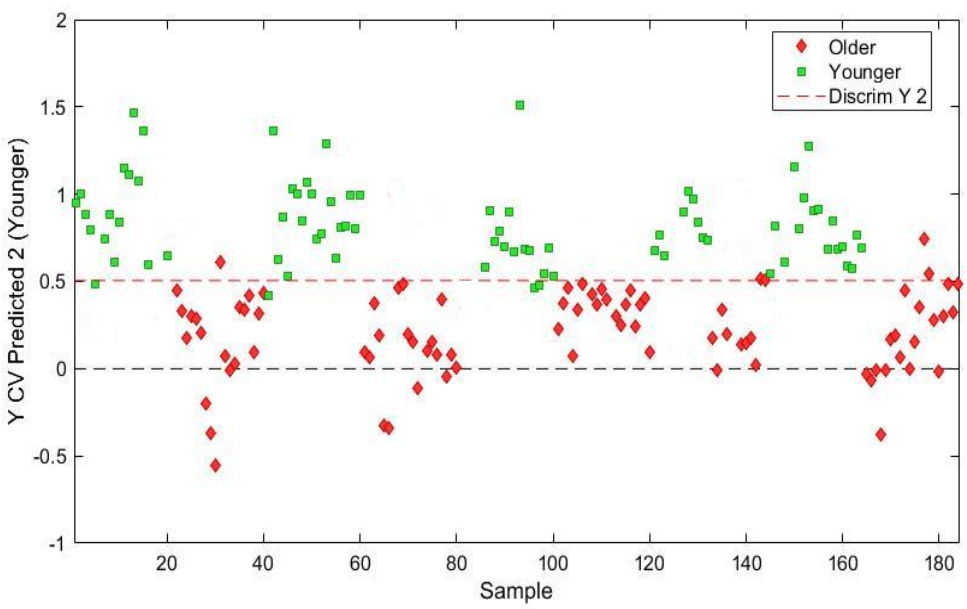


Figure 3.11. Loading plots from PLS-DA model for metabolic profiles between younger and older leaves for all the species combined.

Table 3.5. Top statistically significant features between young and old leaves. These features are tentatively identified based on online databases and literature

m/z	Rt(min)	Fragments	Tentative identification
371.0993	3.69	311.0772, 249.0619, 121.0286	phenolic hexoside
393.0472	5.22	169.0137, 125.0284, 449.0720	tetragalloyl glucose
935.0795	5.74	375.0354, 169.0137, 125.0284	di-HHDP-galloylglucose
612.0769	4.63	935.0741, 633.0730, 300.9998, 169.0146	ellagitannin dimer
463.0528	4.46	300.9998	ellagic acid hexoside
521.1337	6.44	169.0134, 313.060, 300.9996, 125.0284	galloyl hexose derivative
161.0448	2.51	143.0344, 117.0552, 59.0133	hydroxy fatty acid
653.2292	7.29	~	unknown
886.5539	10.52	~	unknown
709.3819	7.17	~	unknown

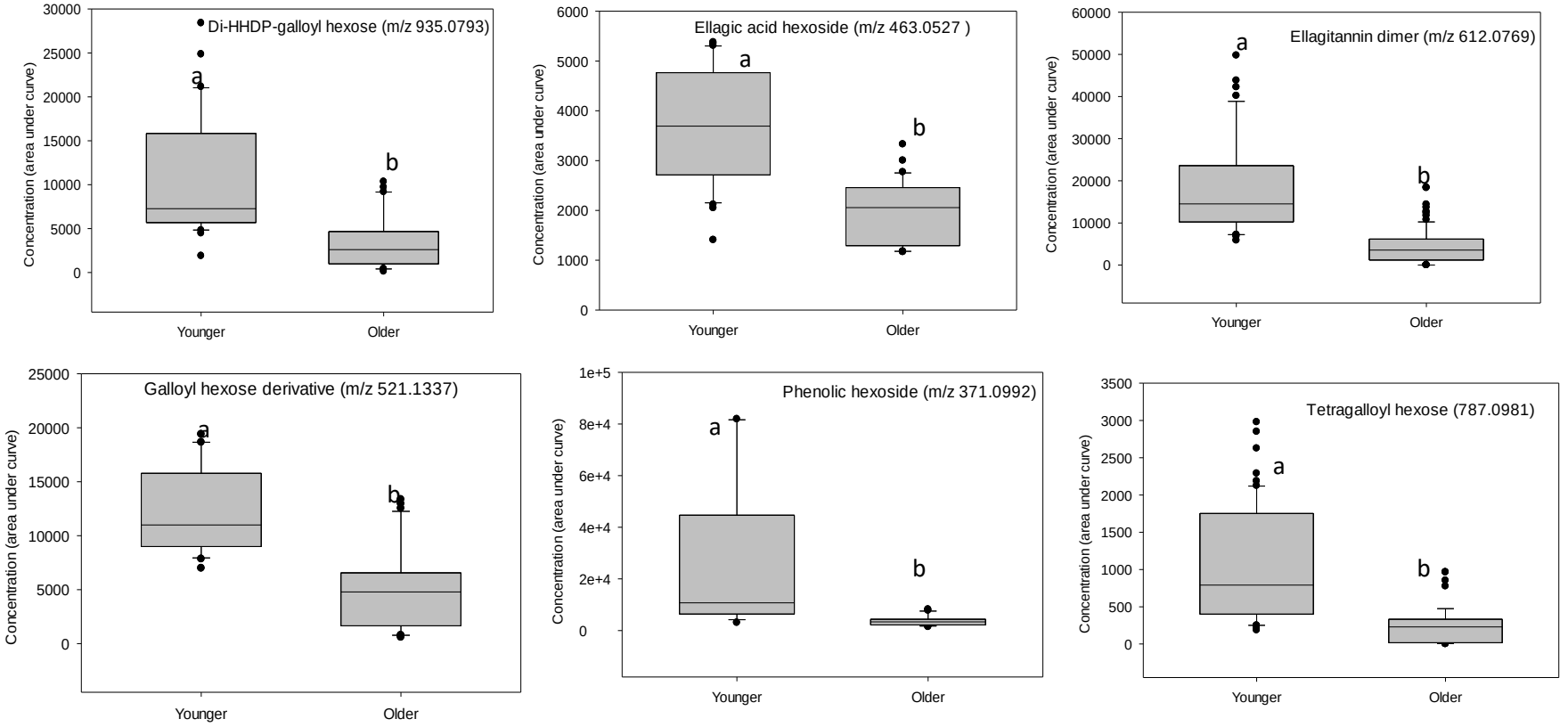


Figure 3.12. Box plot showing top statistically significant phenolic compounds based on the age of leaves. In each box plot, the horizontal line that is crossing the box is the median, lower and upper quartiles are at the bottom and top of the box, respectively, and the whiskers are the maximum and minimum values. The clusters represented with the same letter code are not significantly different (Tukey multiple comparisons of means, $P<0.05$).

3.4. Discussion

My study provides broader insights into commonalities and differences in seasonal fluctuations of phenolic chemistry, leaf reflectance and among leaves at different nodal positions of five species representative belonging to the Myrtaceae. It further differentiates seasonal leaves based on changes in individual foliar phenolic compounds and based on relative leaf age. As such, it is an attempt to make such broad comparisons between various species representative belonging to Myrtaceae as most of the previous studies relate to genus *Eucalyptus*. My study of representative genera within family Myrtaceae has shown both qualitative and quantitative variation in tannins and correlations possibly reliant on several classes of phenolic compounds.

3.4.1. Leaf reflectance

My leaf reflectance data shows that the summer leaves reflected across the red-green or red spectrum region, and most of the winter leaves reflected strongly towards strong green or red-green region. Summer *Eucalyptus* and *Callistemon* particularly reflected strongly in the red spectrum. Leaf colour depends on various pigments such as phenolic compounds, chlorophylls, xanthophylls, carotenoids, and cuticular waxes, and they are key factors modifying reflectance spectra (Carter & Knapp, 2001; Baltzer & Thomas, 2005). My chemistry data show an increased concentration of various flavonoid glycosides (flavonoid-3-O-glycoside flavonoid-7-O-glucuronide) in summer leaves and various hydrolysable tannins in winter leaves. Flavonoid glycosides are known to increase in high UV and low temperatures, and along with anthocyanins, these flavonoid glycosides are also responsible for the red colouration of leaves (Close *et al.*, 2003; Groenbaek *et al.*, 2019). According to coevolution theory, the leaf redness signals herbivores about the presence of low nitrogen content and high phenolic compounds (e.g., flavones, flavonols and tannins), which are generally unattractive and feeding deterrents to herbivores (Archetti, 2000; Chittka & Döring, 2007). However, studies in herbivores such as aphids

have shown that colour preferences cannot be generalised, and they can be species-specific. For example, preference of *Anoeconeossa bundoorensis* and *Glycaspis brimblecombei* to red colour stimuli whereas preference of *Ctenarytaina eucalypti* and *Ctenarytaina bipartita* to green and yellow stimuli. The choice of red leaves by these aphids could be because young leaves are not associated with leaf abscission (Farnier *et al.*, 2014). On the other hand, the photoprotection hypothesis suggests that the red leaves have higher flavonoids, such as anthocyanins which protect them from the excess of light (Close & Beadle, 2003). For example, a recent study proposed that anthocyanins reflect co-evolutionary interactions with aphids deterring herbivores as well as protecting young *Eucalyptus* leaves from high irradiance (Farnier & Steinbauer, 2016). Therefore, the red colouration of my summer leaves might be due to the presence of various flavonoids to protect the leaves against high irradiance and also might offer protection against herbivores.

3.4.2. Seasonal variation in phenolics

Studies have shown that the concentration of phenolic compounds in leaves can vary with an immediate environmental condition such as photoperiod, light intensity such as a cloudy or sunny day or week and temperature (Yao *et al.*, 2005; Covelo & Gallardo, 2011). However, various studies also have shown a strong variation of phenolic compounds with the seasons (Macauley & Fox, 1980; Riipi *et al.*, 2004; Gori *et al*., 2020), and the extent of this seasonal variation can highly rely on the above-described factors. My data shows that the hydrolysable tannins were in lower concentration during winter but not during other seasons. The lower concentration of hydrolysable tannins in winter might be explained by lower herbivory pressure in winter as most herbivory pressure is in summer and spring and the wetter environment (Lowman, 1985; Lowman & Heatwole, 1992; Aide, 1993; Mazía *et al*., 2012) and also it might also be explained as investment of plant resources on the production of other group phenolic compounds because phenolic

compounds share the common precursors and intermediates such as condensed tannins (Santos-Sánchez *et al.,* 2019). Therefore, although the low concentration of hydrolysable tannins in winter can be attributed to low solar radiation and herbivory, whether this correlation has any causative basis should be further examined. Phenolics compounds also play an important role in photoprotection and yet still be present in greater concentrations in plants in colder areas that are exposed to less intensive radiation despite having longer day lengths in summer. It is found that elevation, most likely acting as a proxy for cold temperatures, explained most variation in concentrations of total phenolics and FPCs in *Eucalyptus microcorys* (Moore *et al*., 2004). My data did not suggest any influence of photoinhibition on increased concentrations of total phenolics and FPCs with colder temperatures as described above in *Eucalyptus microcorys.*

I also found that the autumn leaves had a higher concentration of various flavonoid glycosides, especially gallated catechins. Catechin glycosides are known for their bitterness and astringency and are reactive oxygen species scavengers (Bernatoniene & Kopustinskiene, 2018). The major role of catechins is to protect the leaves from UV damage, and their production is highly affected by photosynthesis (Wei *et al.,* 2011). I also found the concentrations of various flavonoids glycosides were higher in summer leaves and various hydrolysable tannins (castalagin) and catechin in spring leaves. Solar radiation and herbivory pressure are very high in spring and summer in Australia (Lowman, 1985; Lowman & Heatwole, 1992). Both castalagin (Abid *et al.,* 2017) and flavonoid glycosides are known for their antioxidant activity, and primarily flavonoids are known to accumulate to a greater extent in response to UV_B radiation hence known for their active role in the protection of plants against oxidative damage caused by shorter wavelengths, especially UV_B (Close & McArthur, 2002; Agati and Tattini, 2010). Also, the high concentration of flavonoid glycosides in the epidermal layer of leaves helps to reduce the risk of generation of reactive oxygen species (ROS) by decreasing the penetration of UV radiation to leaves

(Burchard *et al.*, 2000). Therefore, it can be hypothesised that higher irradiation during summer might initiate common oxidative signal components, ultimately up-regulating flavonoid biosynthesis.

3.4.3. Effect of leaf age

My data shows a higher concentration of various hydrolysable tannins such as di-HHDP-galloylglucose and tetragalloyl glucose in younger leaves of myrtle species than in older leaves. My finding supports a study by Salminen & Karonen (2011), who found that younger leaves were richer in hydrolysable tannins than are older leaves. The decrease in concentrations of hydrolysable tannins in older leaves might be due to an increase in non-extractable phenolic compounds and/or due to the rise in polymerisation into higher molecular weight phenolics (Covelo and Gallardo, 2004). Also, as leaves age, the proportion of structural carbohydrates such as cellulose and lignin, fibrous polymers increase, thereby diluting the concentration of other plant specialised metabolites and might be one of the mechanisms describing the change in concentration of phenolic compounds (Kitajima *et al*., 2016; Li *et al*., 2016). Further, younger leaves have a higher contribution to the fitness of the plant; hence, younger leaves are generally highly protected than older leaves (Bielczynski *et al.*, 2017; Keith and Mitchell-Olds, 2017). These younger leaves have less mechanical protection than older leaves against herbivores and depend more on chemical protection from the plant (Hsu and Harris, 2010). The presence of various hydrolysable tannins such as gallotannins are known to play an essential role in defending leaves from insect herbivores by inhibiting digestive enzymes and precipitating proteins in the gut of insect herbivores (Barbehenn & Constabel, 2011). However, high concentrations of these metabolites do not necessarily lead to lower consumption of leaves and in some cases, it might just be the opposite (Ossipov, 2001); the herbivore may consume more leaves to compensate for the effect of this factor (Kause *et al.*, 1999). A study on the relationship between herbivory and leaf age showed that mostly invertebrate

herbivores generally preferred young leaves while mammalian herbivores showed the opposite pattern (Boege & Marquis, 2005). Therefore, these functional strategies and their trade-off with growth and defence may vary with the leaf age, and tree phenology might have a strong influence on the relationships between secondary metabolites and the herbivore (Valladares, 2018). Various hydrolysable tannins such as ellagitannins (ETs) were also found in higher concentrations in my younger leaves. ETs are known for their high structural diversity and wide distribution in plants (Era *et al.*, 2020). Although ETs are weak protein precipitants, they are known to be the most oxidatively active tannins acting as effective pro-oxidant in insect midgut (Salminen and Karonen, 2011). Thus, the accumulation of ETs in younger leaves suggests an active role as pro-oxidants (Barbehenn and Constabel, 2011; Summers & Felton, 1994). Although chlorophylls, xanthophylls, carotenoids, along with anthocyanins are known major pigments for the colour production of leaves, the redness of young leaves can be further supported by the presence of hydrolysable tannins, which are known to indirectly support photoprotection by preventing anthocyanin degradation at the abaxial layer, increasing the pigment stability and helping to maintain red leaves (Luo *et al*., 2019).

Therefore, my study suggests that leaf age and environmental factors have a substantial impact on the concentration of leaf phenolics. It also indicates that the biological activity of phenolic compounds in leaves is a complex trait relying on several classes and individual phenolic compounds, various conditions, and developmental stages. I demonstrated diversified and changeable responses of phenolic compounds in plant leaves. Plants rely on more than one line of defence; therefore, further research involving similar groups of phenolic compounds and including various secondary metabolites should be conducted to understand the relationship between phenolic and other plant specialised compounds and trade-offs between these compounds functioning at different times of the season.

3.5.References

Agati, G., & Tattini, M. (2010). Multiple functional roles of flavonoids in photoprotection: Letters. *New Phytologist*, *186*(4), 786–793.

Aide, T. M. (1993). Patterns of Leaf Development and Herbivory in a Tropical Understory Community. *Ecology*, *74*(2), 455–466. JSTOR.

Amorati, R., & Valgimigli, L. (2015). Advantages and limitations of common testing methods for antioxidants. *Free Radical Research*, *49*(5), 633–649.

Amthor, J. S. (2010). From sunlight to phytomass: On the potential efficiency of converting solar radiation to phyto-energy. *The New Phytologist*, *188*(4), 939–959.

Aoussar, N., Rhallabi, N., Ait Mhand, R., Manzali, R., Bouksaim, M., Douira, A., & Mellouki, F. (2020). Seasonal variation of antioxidant activity and phenolic content of *Pseudevernia furfuracea*, *Evernia prunastri* and *Ramalina farinacea* from Morocco. *Journal of the Saudi Society of Agricultural Sciences*, *19*(1), 1–6.

Appel, H. M., Govenor, H. L., D'Ascenzo, M., Siska, E., & Schultz, J. C. (2001). Limitations of Folin assays of foliar phenolics in ecological studies. *Journal of Chemical Ecology*, *27*(4), 761–778.

Archetti, M. (2000). The origin of autumn colours by coevolution. *Journal of Theoretical Biology*, *205*(4), 625–630.

Baltzer, J. L., & Thomas, S. C. (2005). Leaf optical responses to light and soil nutrient availability in temperate deciduous trees. *American Journal of Botany*, *92*(2), 214–223.

Barbehenn, R. V., & Peter Constabel, C. (2011). Tannins in plant–herbivore interactions. *Phytochemistry*, *72*(13), 1551–1565.

Barton, K. E., & Koricheva, J. (2010). The ontogeny of plant defence and herbivory: Characterising general patterns using meta-analysis. *The American Naturalist*, *175*(4), 481–493.

Bernatoniene, J., & Kopustinskiene, D. M. (2018). The role of catechins in cellular responses to oxidative stress. *Molecules: A Journal of Synthetic Chemistry and Natural Product Chemistry, 23*(4).

Bhattacharya, A., Sood, P., & Citovsky, V. (2010). The roles of plant phenolics in defence and communication during *Agrobacterium* and Rhizobium infection. *Molecular Plant Pathology, 11*(5), 705–719.

Bielczynski, L. W., Łącki, M. K., Hoefnagels, I., Gambin, A., & Croce, R. (2017). Leaf and plant age affect photosynthetic performance and photoprotective capacity. *Plant Physiology*, *175*(4), 1634–1648.

Blainski, A., Lopes, G. C., & De Mello, J. C. P. (2013). Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium Brasiliense* L. *Molecules*, *18*(6), 6852–6865.

Bornman, J. F., Barnes, P. W., Robinson, S. A., Ballaré, C. L., Flint, S. D., & Caldwell, M. M. (2015). Solar ultraviolet radiation and ozone depletion-driven climate change: Effects on terrestrial ecosystems. *Photochemical & Photobiological Sciences: Official Journal of the European Photochemistry Association and the European Society for Photobiology*, *14*(1), 88–107.

Burchard, P., Bilger, W., & Weissenböck, G. (2000). Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV_A and UV_B radiation in developing rye primary leaves as assessed by ultraviolet-induced chlorophyll fluorescence measurements. *Plant, Cell & Environment, 23*(12), 1373–1380.

Carter, G. A., & Knapp, A. K. (2001). Leaf optical properties in higher plants: Linking spectral characteristics to stress and chlorophyll concentration. *American Journal of Botany*, *88*(4), 677–684.

Chittka, L., & Döring, T. F. (2007). Are autumn foliage colors red signals to aphids? *PLoS Biology, 5*(8).

Close, D. C., & Beadle, C. L. (2003). Alternate energy dissipation? Phenolic metabolites and the xanthophyll cycle. *Journal of Plant Physiology*, *160*(4), 431–434.

Close, D. C., Davies, N. W., & Beadle, C. L. (2001). Temporal variation of tannins (galloylglucoses), flavonols and anthocyanins in leaves of *Eucalyptus nitens* seedlings: Implications for light attenuation and antioxidant activities. *Functional Plant Biology*, *28*(4), 269–278.

Close, D. C., & McArthur, C. (2002). Rethinking the role of many plant phenolics: Protection from photodamage not herbivores? *Oikos*, *99*(1), 166–172. Jstor.

Covelo, F., & Gallardo, A. (2004). Green and senescent leaf phenolics showed spatial autocorrelation in a *Quercus robur* population in northwestern Spain. *Plant and Soil*, *259*(1/2), 267–276.

Ellnain-Wojtaszek, M., Kruczyński, Z., & Kasprzak, J. (2001). Analysis of the content of flavonoids, phenolic acids as well as free radicals from *Ginkgo biloba* L. leaves during the vegetative cycle. *Acta Poloniae Pharmaceutica*, *58*(3), 205–209.

Endler, J. A. (1990). On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society*, *41*(4), 315–352.

Era, M., Matsuo, Y., Saito, Y., & Tanaka, T. (2020). Production of ellagitannin hexahydroxydiphenoyl ester by spontaneous reduction of dehydrohexa-hydroxydiphenoyl ester. *Molecules*, *25*(5), 1051.

Ercisli, S., Orhan, E., Ozdemir, O., Sengul, M., & Gungor, N. (2008). Seasonal variation of total phenolic, antioxidant activity, plant nutritional elements, and fatty acids in tea leaves (*Camellia sinensis* var. *sinensis*) grown in Turkey. *Pharmaceutical Biology*, *46*(10–11), 683–687.

Farnier, K., Dyer, A. G., & Steinbauer, M. J. (2014). Related but not alike: Not all Hemiptera are attracted to yellow. *Frontiers in Ecology and Evolution*, *2*.

Farnier, K., & Steinbauer, M. J. (2016). Elevated anthocyanins protect young *Eucalyptus* leaves from high irradiance but also indicate foliar nutritional quality to visually attuned psyllids. *Ecological Entomology*, *41*(2), 168–181.

Gori, A., Nascimento, L. B., Ferrini, F., Centritto, M., & Brunetti, C. (2020). Seasonal and diurnal variation in leaf phenolics of three medicinal Mediterranean wild species: what is the best harvesting moment to obtain the richest and the most antioxidant extracts. *Molecules (Basel, Switzerland)*, *25*(4).

Groenbaek, M., Tybirk, E., Neugart, S., Sundekilde, U. K., Schreiner, M., & Kristensen, H. L. (2019). Flavonoid glycosides and hydroxycinnamic acid derivatives in baby leaf rapeseed from white and yellow flowering cultivars with repeated harvest in a 2-years field study. *Frontiers in Plant Science*, *10*.

Hussain, A. I., Anwar, F., Hussain Sherazi, S. T., & Przybylski, R. (2008). Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. *Food Chemistry*, *108*(3), 986–995.

Hoch, W. A., Zeldin, E. L., & McCown, B. H. (2001). Physiological significance of anthocyanins during autumnal leaf senescence. *Tree Physiology*, *21*(1), 1–8.

Hsu, E., & Harris, S. (2010). Plants, health and healing: *On the Interface of Ethnobotany and Medical Anthropology*. Berghahn Books.

Kabtni, S., Sdouga, D., Bettaib Rebey, I., Save, M., Trifi-Farah, N., Fauconnier, M.-L., & Marghali, S. (2020). Influence of climate variation on phenolic composition and antioxidant capacity of *Medicago minima* populations. *Scientific Reports*, *10*(1), 8293.

Kalili, K. M., & Villiers, A. de. (2011). Recent developments in the HPLC separation of phenolic compounds. *Journal of Separation Science*, *34*(8), 854–876.

Kause, A., Ossipov, V., Haukioja, E., Lempa, K., Hanhimäki, S., & Ossipova, S. (1999). Multiplicity of biochemical factors determining the quality of growing birch leaves. *Oecologia*, *120*(1), 102–112.

Keith, R. A., & Mitchell-Olds, T. (2017). Testing the optimal defence hypothesis in nature: Variation for glucosinolate profiles within plants. *Plos one*, *12*(7), e0180971.

Keski-Saari, S., Pusenius, J., & Julkunen-Tiitto, R. (2005). Phenolic compounds in seedlings of *Betula pubescens* and *B. pendula* are affected by enhanced UV_B radiation and different nitrogen regimens during early ontogeny. *Global Change Biology*, *11*(7), 1180–1194.

Kitajima, K., Wright, S. J., & Westbrook, J. W. (2016). Leaf cellulose density as the key determinant of inter- and intra-specific variation in leaf fracture toughness in a species-rich tropical forest. *Interface Focus*, *6*(3), 2015-100.

Koricheva, J., & Barton, K. E. (2012, April). Temporal changes in plant secondary metabolite production. The ecology of plant secondary metabolites: From genes to global processes.

Kotilainen, T., Tegelberg, R., Julkunen-Tiitto, R., Lindfors, A., O'Hara, R. B., & Aphalo, P. J. (2010). Seasonal fluctuations in leaf phenolic composition under UV manipulations reflect contrasting strategies of alder and birch trees. *Physiologia Plantarum*, *140*(3), 297–309.

Krizek, D. T. (2004). Influence of PAR and UV_A in determining plant sensitivity and photomorphogenic responses to UV_B radiation. *Photochemistry and Photobiology*, *79*(4), 307–315.

Krizek, D. T., Mirecki, R. M., & Britz, S. J. (1997). Inhibitory effects of ambient levels of solar UV_A and UV_B radiation on growth of cucumber. *Physiologia Plantarum*, *100*(4), 886–893.

Lester, G. E., Lewers, K. S., Medina, M. B., & Saftner, R. A. (2012). Comparative analysis of strawberry total phenolics via Fast Blue BB vs. Folin–Ciocalteu: Assay interference by ascorbic acid. *Journal of Food Composition and Analysis*, *27*(1), 102–107.

Li, N., He, N., Yu, G., Wang, Q., & Sun, J. (2016). Leaf non-structural carbohydrates regulated by plant functional groups and climate: Evidence from a tropical to cold-temperate forest transect. *Ecological Indicators*, *62*, 22–31.

Lima, A. L. da S., Zanella, F., Schiavinato, M. A., & Haddad, C. R. B. (2006). Nitrogenous compounds, phenolic compounds and morphological aspects of leaves: Comparison of deciduous and semideciduous arboreal legumes. *Scientia Agricola*, *63*(1), 40–45.

Loney, P. E., McArthur, C., Potts, B. M., & Jordan, G. J. (2006). How does ontogeny in a *Eucalyptus* species affect patterns of herbivory by Brushtail Possums? *Functional Ecology*, *20*(6), 982–988.

Lowman, M. D., & Heatwole, H. (1992). Spatial and temporal variability in defoliation of Australian Eucalypts. *Ecology*, *73*(1), 129–142.

Luo, H., Li, W., Zhang, X., Deng, S., Xu, Q., Hou, T., Pang, X., Zhang, Z., & Zhang, X. (2019). In planta high levels of hydrolysable tannins inhibit peroxidase mediated anthocyanin degradation and maintain abaxially red leaves of *Excoecaria cochinchinensis*. *BMC Plant Biology*, *19*(1), 315.

Macauley, B. J., & Fox, L. R. (1980). Variation in total phenols and condensed tannins in *Eucalyptus*: Leaf phenology and insect grazing. *Australian Journal of Ecology*, *5*(1), 31–35.

Marsh, K. J., Kulheim, C., Blomberg, S. P., Thornhill, A. H., Miller, J. T., Wallis, I. R., Nicolle, D., Salminen, J.-P., & Foley, W. J. (2017). Genus-wide variation in foliar phenolics in Eucalypts. *Phytochemistry*, *144*, 197–207.

Mattila, H., Mishra, K. B., Kuusisto, I., Mishra, A., Novotná, K., Šebela, D., & Tyystjärvi, E. (2020). Effects of low temperature on photoinhibition and singlet oxygen production in four natural accessions of *Arabidopsis*. *Planta*, *252*(2), 19.

Mazía, N., Chaneton, E. J., Dellacanonica, C., Dipaolo, L., & Kitzberger, T. (2012). Seasonal patterns of herbivory, leaf traits and productivity consumption in dry and wet Patagonian forests. *Ecological Entomology*, *37*(3), 193–203.

Min, K., Freeman, C., Kang, H., & Choi, S.-U. (2015). The regulation by phenolic compounds of soil organic matter dynamics under a changing environment. *BioMed Research International*, *2015*.

Moore, B. D., Wallis, I. R., Palá-Paúl, J., Brophy, J. J., Willis, R. H., & Foley, W. J. (2004). Antiherbivore chemistry of *Eucalyptus*—Cues and deterrents for marsupial folivores. *Journal of Chemical Ecology*, *30*(9), 1743–1769

Moore, B. D., Wallis, I. R., Wood, J. T., & Foley, W. J. (2004). Foliar nutrition, site quality, and temperature influence foliar chemistry of tallowwood (*Eucalyptus Microcorys*). *Ecological Monographs*, *74*(4), 553–568.

Mota, I., Rodrigues Pinto, P. C., Novo, C., Sousa, G., Guerreiro, O., Guerra, Â. R., Duarte, M. F., & Rodrigues, A. E. (2012). Extraction of phenolic compounds from *Eucalyptus globulus* bark: Process optimisation and screening for biological activity. *Industrial & Engineering Chemistry Research*, *51*(20), 6991–7000.

Morales, L. O., Tegelberg, R., Brosché, M., Keinänen, M., Lindfors, A., & Aphalo, P. J. (2010). Effects of solar UV_A and UV_B radiation on gene expression and phenolic accumulation in *Betula pendula* leaves. *Tree Physiology*, *30*(7), 923–934.

Nurmi, K., Ossipov, V., Haukioja, E., & Pihlaja, K. (1996). Variation of total phenolic content and individual low-molecular-weight phenolics in the foliage of mountain birch trees (*Betula pubescens* ssp.*tortuosa*). *Journal of Chemical Ecology*, *22*(11), 2023–2040.

Ossipov, V., Haukioja, E., Ossipova, S., Hanhimäki, S., & Pihlaja, K. (2001). Phenolic and phenolic-related factors as determinants of suitability of mountain birch leaves to an herbivorous insect. *Biochemical Systematics and Ecology*, *29*(3), 223–240.

Petridis, A., Therios, I., Samouris, G., Koundouras, S., & Giannakoula, A. (2012). Effect of water deficit on leaf phenolic composition, gas exchange, oxidative damage and antioxidant activity of four Greek olive (*Olea europaea* L.) cultivars. *Plant Physiology and Biochemistry: PPB*, *60*, 1–11.

Pichersky, E., & Lewinsohn, E. (2011). Convergent evolution in plant specialised metabolism. *Annual Review of Plant Biology*, *62*(1), 549–566.

Ramakrishna, A., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, *6*(11), 1720–1731.

Rehman, F., Khan, F. A., & Badruddin, S. M. A. (2012). Role of phenolics in plant defence against insect herbivory. In L. D. Khemani, M. M. Srivastava, & S. Srivastava (Eds.), *Chemistry of Phytopotentials: Health, Energy and Environmental Perspectives* (pp. 309–313). Springer.

Reynertson, K. A., Yang, H., Jiang, B., Basile, M. J., & Kennelly, E. J. (2008). Quantitative analysis of antiradical phenolic constituents from fourteen edible Myrtaceae fruits. *Food Chemistry*, *109*(4), 883–890.

Ribeiro, D. A., de Macêdo, D. G., Boligon, A. A., Menezes, I. R. A., de Almeida Souza, M. M., & da Costa, J. G. M. (2019). Influence of seasonality on the phenolic composition of *Secondatia floribunda* A.DC (Apocynaceae) during its phenological cycle. *Acta Physiologiae Plantarum*, *41*(12), 185.

Riipi, M., Haukioja, E., Lempa, K., Ossipov, V., Ossipova, S., & Pihlaja, K. (2004). Ranking of individual mountain birch trees in terms of leaf chemistry: Seasonal and annual variation. *Chemoecology*, *14*(1), 31–43.

Roy, C. R., Gies, H. P., & Toomey, S. (1995). The solar UV radiation environment: Measurement techniques and results. *Journal of Photochemistry and Photobiology B: Biology*, *31*(1), 21–27.

Salminen, J. P., Ossipov, V., Haukioja, E., & Pihlaja, K. (2001). Seasonal variation in the content of hydrolysable tannins in leaves of *Betula pubescens*. *Phytochemistry*, *57*(1), 15–22.

Salminen, J.-P., Roslin, T., Karonen, M., Sinkkonen, J., Pihlaja, K., & Pulkkinen, P. (2004). Seasonal variation in the content of hydrolysable tannins, flavonoid glycosides, and proanthocyanidins in oak leaves. *Journal of Chemical Ecology*, *30*(9), 1693–1711.

Salminen, J.-P., & Karonen, M. (2011). Chemical ecology of tannins and other phenolics: We need a change in approach. *Functional Ecology*, *25*(2), 325–338.

Salminen, J.-P., Karonen, M., & Sinkkonen, J. (2011). Chemical ecology of tannins: Recent developments in tannin chemistry reveal new structures and structure-activity patterns. *Chemistry-A European Journal*, *17*(10), 2806–2816.

Santos, R. M., Oliveira, M. S., Ferri, P. H., & Santos, S. C. (2011). Seasonal variation in the phenol content of *Eugenia uniflora* L. leaves. Revista Brasileira de Plantas Medicinais, 13(1), 85–89.

Shi, B., Zhang, W., Li, X., & Pan, X. (2017). Seasonal variations of phenolic profiles and antioxidant activity of walnut (*Juglans sigillata* Dode) green husks. *International Journal of Food Properties*, *20*(sup3), S2635–S2646.

Siatka, T., & Kašparová, M. (2010). Seasonal variation in total phenolic and flavonoid contents and DPPH scavenging activity of *Bellis perennis* L. flowers. *Molecules (Basel, Switzerland)*, *15*(12), 9450–9461.

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defence mechanism in plants under stressful conditions [Review Article]. *Journal of Botany.*

Stapleton, A. E., & Walbot, V. (1994). Flavonoids can protect maise DNA from the induction of ultraviolet radiation damage. *Plant Physiology*, 105(3), 881–889.

Stark, S., Julkunen-Tiitto, R., Holappa, E., Mikkola, K., & Nikula, A. (2008). Concentrations of foliar quercetin in natural populations of white birch (*Betula pubescens*) Increase with latitude. *Journal of Chemical Ecology*, *34*(11), 1382–1391.

Summers, C. B., & Felton, G. W. (1994). Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): Potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochemistry and Molecular Biology*, *24*(9), 943–953.

Taylor GS (1997) Effect of plant compounds on the population dynamics of the lerp insect, *Cardiaspina albitextura* Taylor (Psylloidea: Spondyliaspididae) on eucalypts. In: Raman A (ed) Ecology and evolution of plant-feeding insects in natural and man-made environments. Backhuys, Leiden, pp 37–57

Tian, L.-W., Xu, M., Li, Y., Li, X.-Y., Wang, D., Zhu, H.-T., Yang, C.-R., & Zhang, Y.-J. (2012). Phenolic compounds from the branches of *Eucalyptus maideni*. *Chemistry & Biodiversity*, *9*(1), 123–130.

Tuominen, A., & Salminen, J.-P. (2017). Hydrolysable tannins, flavonol glycosides, and phenolic acids show seasonal and ontogenic variation in *Geranium sylvaticum*. *Journal of Agricultural and Food Chemistry*, *65*(31), 6387–6403.

Valifard, M., Mohsenzadeh, S., Kholdebarin, B., & Rowshan, V. (2014). Effects of salt stress on volatile compounds, total phenolic content and antioxidant activities of *Salvia mirzayanii*. *South African Journal of Botany*, *93*, 92–97.

Veberic, R., Colaric, M., & Stampar, F. (2008). Phenolic acids and flavonoids of fig fruit (*Ficus carica* L.) in the northern Mediterranean region. *Food Chemistry*, *106*(1), 153–157.

Valladares, F. J. (2018). Ecological significance of inherent variation in relative growth rate and its components. *Functional Plant Ecology*. *67-100.*

Vlasveld, C., O'Leary, B., Udovicic, F., Burd, M., Vlasveld, C., O'Leary, B., Udovicic, F., & Burd, M. (2018). Leaf heteroblasty in eucalypts: Biogeographic evidence of ecological function. *Australian Journal of Botany*, *66*(3), 191–201.

Wam, H. K., Stolter, C., & Nybakken, L. (2017). Compositional changes in foliage phenolics with plant age, a natural experiment in boreal forests. *Journal of Chemical Ecology*, *43*(9), 920–928.

Waterman, P. G. (1992). Roles for secondary metabolites in plants. *Ciba Foundation Symposium*, *171*, 255–269; discussion 269-275.

Way, M. L., Jones, J. E., Nichols, D. S., Dambergs, R. G., & Swarts, N. D. (2020). A Comparison of laboratory analysis methods for total phenolic content of cider. *Beverages*, *6*(3), 55.

Wei, K., Wang, L., Zhou, J., He, W., Zeng, J., Jiang, Y., & Cheng, H. (2011). Catechin contents in tea (*Camellia sinensis*) as affected by cultivar and environment and their relation to chlorophyll contents. *Food Chemistry*, *125*(1), 44–48.

Yao, L., Caffin, N., D'Arcy, B., Jiang, Y., Shi, J., Singanusong, R., Liu, X., Datta, N., Kakuda, Y., & Xu, Y. (2005). Seasonal variations of phenolic compounds in Australia-grown tea (*Camellia sinensis*). *Journal of Agricultural and Food Chemistry*, *53*(16), 6477–6483.

Zlatić, N., Jakovljević, D., & Stanković, M. (2019). Temporal, plant part, and interpopulation variability of secondary metabolites and antioxidant activity of *Inula helenium* L. *Plants*, *8*(6), 179.

Chapter IV

UV_A stress and the expression of phenolic compounds by *Eucalyptus camaldulensis* ssp. *camaldulensis*: Influence of provenance and leaf age

4.1. Introduction

Plants endure numerous abiotic and biotic stressors during their lifetime by altering their physiology and biochemistry, which consequently influences the metabolome of the plants (Ghosh *et al.,* 2017; Pascual *et al.,* 2017). A diverse array of plant specialised metabolites (PSMs) such as phenolic compounds, alkaloids, terpenoids and sulphur-containing compounds can be synthesised by plants in response to internal regulatory processes (Guerriero *et al.,* 2018). Identification of these individual metabolites mediating tolerance at the physiological, biochemical, or molecular level is essential to understand changes in the environment and acclimatory response to these changes by the plant (Bechtold & Field, 2018). Since metabolomics best represents the molecular phenotype, non-targeted metabolomics has emerged as a powerful tool to investigate the metabolomic response to various environmental stressors (Alonso *et al.,* 2015; Pascual *et al.,* 2017).

Plant phenolic compounds are one of the major groups of plant specialised metabolites, and they comprise at least one aromatic ring and one or more hydroxyl groups (Tsao, 2010). These organic compounds are known to have varied roles in plants, including defence against biotic and abiotic stressors such as herbivory and UV and associated oxidative stress, regulation of seed germination and plant growth (Bhattacharya *et al.,* 2010; Ghiassi Tarzi *et al.,* 2012; Calzada *et al.,* 2019). These phenolic compounds are ubiquitous throughout the plant kingdom, from the earliest known plants such as green algae to later diverged angiosperms (Ruhfel *et al.,* 2014). Phenolic compounds were previously hypothesised to vary in response to resource availability and plant defence theory. Resource availability theory suggests that fast-growing and species growing in the resource-rich environment have lower amounts of constitutive phenolic compounds compared to slow-growing species and species growing in resource-poor environments, and plant defence theory suggests that the defensive needs of plants have influenced the

evolution of phenolic compounds (Coley *et al.,* 1985, Herms & Mattson, 1992, Koricheva *et al.,* 2004; Barton & Koricheva, 2010). However, broad distribution of phenolic compounds within the plant kingdom and increasing evidence suggests that oxidative pressure from photodamage might be another critical factor causing variation of phenolic compounds in the plant (within an individual plant species and/or between species), and herbivory may have exerted little selective pressure compared to photoprotection (Close & McArthur, 2002; Wang *et al.,* 2019).

Plants synthesise various phenolic compounds, and the production of these compounds are determined by various genetic and physiological influences (Joubert *et al.,* 2008; Bunning *et al.,* 2010). The expression of these compounds can be shaped by allocation and opportunity mediated in response to environmental influences (Coley *et al.,* 1985; Sagers & Coley, 1995). There are two fundamentals but contrasting theories behind the ontogenetic variation of expression of PSMs in plants (Borzak *et al.,* 2015). The growth and differentiation balance hypothesis focus on intrinsic factors limiting the production of PSMs in young plants, and the optimal defence theory focuses more on extrinsic factors leading to high levels of PSMs in young plants (Pavia *et al.,* 2002; Stamp, 2004; Massad *et al.,* 2012). A meta-analysis of 116 literature that included 153 plant species from 36 plant families showed that PSM such as phenolic compounds to be highest during the seedling stage in woody plants. In contrast, herbs showed a significant increase in secondary metabolites across the entire ontogenetic trajectory (Barton & Koricheva, 2010). Another study suggested that young and unexpanded leaves, in general, have higher nutrient concentrations and phenolic concentrations, particularly gallotannins, terpenes, flavonoids and total phenolics, than fully expanded mature leaves (Barton *et al*., 2019).

Foliar phenolics were found to be highest in young leaves and lowest in the adult tree of *Eucalyptus froggattii* Blakely (Goodger *et al.,* 2013), two species of *Zea mays* L. (Hichem

et al., 2009), and in *Quercus variabilis* Blume (Wang *et al.,* 2016). In contrast, other studies have found increased phenolic concentration with leaf age associated with low foliar phenolics in young leaves and high foliar phenolics in old leaves (Elger *et al.,* 2009; Abdallah *et al.,* 2013; Blum-Silva *et al.,* 2015). Further, higher phenolic concentrations were reported in the leaves of adult trees from natural populations of three *Eucalyptus* species (*E. polyanthemos* subsp. *vestita, E. yarraensis* Maiden &. Cambage and *E. cladocalyx* var. *nana* F. Muell.) compared with glasshouse-grown seedlings (Goodger *et al.,* 2006). The relationship between ontogeny and the composition and levels of plant phenolics might be complicated by the fact that individual phenolic compounds may have different responses to leaf age. For example, it was shown that low molecular weight phenolic compounds decreased, and higher molecular weight phenolic compounds such as tannins concentration increased with age in *Betula pubescens* Ehrh. (Wam *et al.,* 2017). In a recent study on 525 eucalypt species, it was shown that total phenolic concentration rarely correlates with their biological activity, and further characterisation of individual phenolic compounds is needed to provide detailed insight into phenolic compounds and their activity (Marsh *et al.,* 2019). Overall, it is difficult to generalise the relationship between ontogeny and foliar phenolic compounds due to the limited research, a small number of plant species, the broad distribution of plant phenolics, and conflicting results in the literature (Goodger *et al.,* 2013).

Various abiotic stress such as UV radiation and drought are known to be potential elicitor agents of the synthesis of specialised metabolites in plants (Mao *et al.,* 2019). Almost 95% of UV_A and 5% of UV_B radiation penetrates the Earth's atmosphere to the surface. Although UV_A is less damaging than UV_B, it can penetrate deeper and is more abundant (>20 times radiant energy than UV_B) and hence can cause significant damage to plant tissue and DNA (Gill & Tuteja, 2010). The lower cloud cover over Oceania and Antarctica

compared to the rest of the world has significantly increased the flux of UV_A on the surface (Mao *et al.,* 2019). Therefore, plants at these latitudes are exposed to higher $UV_{A,}$ with UV flux increasing by approximately 1–2% per 1° latitude towards the equator (Sullivan, 2005; Ballaré *et al.,* 2011; Bornman *et al.,* 2015). Although plants are exposed 10-100 times more UV_A photons than UV_B, most studies and comprehensive reviews are focused on the effects of UV_B radiation on phenolic composition and abundance (Caldwell *et al.,* 1998; Conner & Neumeier, 2002; Kakani *et al.,* 2003). Hence further research is required to understand the impact of UV_A radiation on plant tissue and the response of plant phenolics to this abiotic stressor. Various phenolic compounds have different absorption maxima and are known to absorb harmful UV at various wavelengths without impacting photosynthetically active radiation (Boulet *et al.,* 2017). For example, hydroxycinnamic acid has an absorption maxima at 310−332 nm, flavones at 250−270 and 330−350 nm and flavonols at 250−270 nm and 350−390 nm (Aleixandre-Tudo *et al.,* 2017). There have been very few contrasting studies on plant phenolic response to UV_A radiation. A study by Li & Kubota (2009) found an increase in the concentration of anthocyanin in *Lactuca sativa* (L.) under enhanced $UV_{A,}$ and another similar study by Iwai (2010) found that UV_A enhanced the content of phenolics in *Perilla frutescens* (L). Similarly, another study by Bantis (2016) also reported the increased concentration of phenolic compounds in two basil, *Ocimum basilicum* (L). with supplemental UV_A LED lights. However, another study by Tsormpatsidis (2008) did not find any significant effects of UV_A on the content of phenolic compounds in lettuce, *Lactuca sativa* (L.). Further, a study on *Eucalyptus nitens* seedlings showed no significant effect of enhanced UV_A on total phenolic concentration (Close *et al.,* 2007). These various contrasting reports are because of diverse experimental approaches, varying UV_A spectrum and dosage used during the experiment (Koutchma,

2019). However, modern LED lights can emit light at a specific wavelength or fixed spectrum and energy and solve most of these experimental issues.

Most of the earlier published literature concerning foliar phenolic compounds mostly evoke a defensive role against herbivores. But these same phenolic compounds have also received attention for their possible role in protecting plants from the abiotic threat of photodamage caused by ROS (Close and McArthur, 2002). Further, the effect of age on leaf phenolics is also unclear. Therefore, I was interested to understand the effect of elevated UV_A and the influence of leaf age on the production of individual foliar phenolic compounds in plants. It has long been assumed that intraspecific differences in phenolic compounds and other specialised metabolites are a result of phenotypic plasticity of various chemotypes in response to the local biotic and abiotic environment (Agrawal, 2011; Woods *et al.,* 2012). The underlying assumption is that there might be different selection pressures (due to differences in various factors such as rainfall and UV light) that have resulted in evolutionary changes in plant metabolites. I was interested in investigating whether the origin of a plant affects the concentrations of metabolites expressed under different conditions. This study used five chemotypes of *Eucalyptus camaldulensis* subspecies *camaldulensis,* distributed across a wide latitudinal range and occur across a UV_A gradient. The native range of this *Eucalyptus camaldulensis* spans five degrees of latitude. Consequently, different populations could exhibit divergent responses to a range of environmental pressures and are hypothesised to have evolved under these ecological pressures such as solar radiation, temperature, and rainfall. Specific aims were (1) to identify intraspecific variation in phenolic composition between provenances and consider correlations with latitude and rainfall; (2) to assess the responses of the provenances to high and low UV_A treatments and (3) investigate the influence of leaf age on phenolic composition.

4.2. Materials and Methods

4.2.1. Study species and sites

Seed of *Eucalyptus camaldulensis* ssp. *camaldulensis* was purchased from the Australian Tree Seed Centre (ATSC), Canberra. Seedlots were selected based on latitude to represent populations spanning the entire geographic range of the subspecies. The parent trees of these seedlots span locations varying in rainfall from 200 to 600 mm per annum (Table 4.1 and Figure 4.1).

Table 4.1. *Eucalyptus camaldulensis* ssp. *camaldulensis* ATSC seedlot number with their chemotype code, GPS coordinates, location, and average annual rainfall.

Seedlot number	Code	GPS coordinates	Location	Average annual rainfall
20440	G_1	-31.46S, 143.65E	30 km ENE of Wilcannia (NSW)	264
20561	G_2	-35.75S, 141.96E	Lake Albacutya (VIC)	363
20437	G_3	-32.30S, 138.45E	Boolcunda Creek (SA)	306
20430	G_4	-33.85S, 148.70E	Cowra (NSW)	598
20429	G_5	-33.10S, 147.15E	Condobolin (NSW)	421

Seeds were raised inside the glasshouse using Scotts Osmocote seed & cutting premium potting mix for a month, and transferred to 12 cm pots (0.97 litres) in a temperature-controlled glasshouse at the Agriculture Reserve at La Trobe University, Melbourne, Australia. Plants were grown in a native plant potting mix and given 200 mL of water every other day. Six plant replicates for every five chemotypes (a total of thirty plants per treatment for all four treatments) were grown, and a 50 cm distance was maintained between the LED lights and the tops of the plants. The branches were bent and repositioned using bonsai wire, and the frames with LED lights were adjusted to keep a constant distance between plants and the lights. The experiment was conducted in the controlled glasshouse conditions with an optimal temperature of 21°C and 18/6 hours of

light/darkness. The plants were rotated 2-3 times per week so that all plants were equally illuminated.

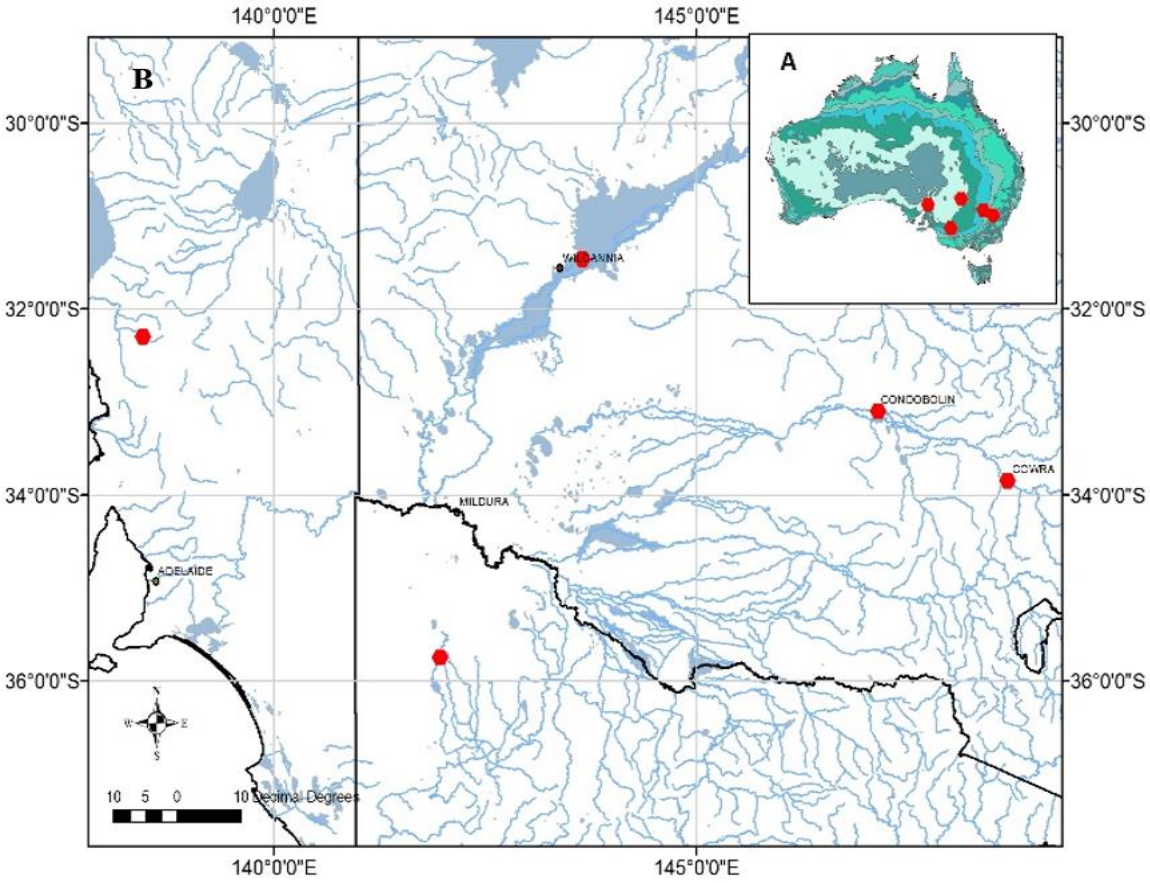


Figure 4.1. (A) Map of Australia showing the average annual rainfall contours based on data for the period 1961 to 1990 and red dots showing ATSC seed collection locations; (B) major rivers and tributaries around seed collection locations.

4.2.2. Glasshouse setup and leaf sampling

This study was conducted under glasshouse and ambient conditions (plants potted outside the glasshouse in natural condition). The potted saplings of all five chemotypes were exposed to artificial UV_A LED lights (i.e. (1) high (2) low UV treatments and (3) control treatment without UV_A) under glasshouse conditions and (4) ambient (natural) light conditions outside the glasshouse. The two latter treatments represent a procedural control and a natural reference, respectively. Six plant replicates per chemotype were used,

making it 30 plants per treatment and a total of 120 plants. Transparent UV absorbing plastic film (SUN 5 Pro) that blocks the solar UV radiation purchased from Folien-Vertriebs GmbH, Dernbach, Germany, was used to cover the top of the three $1m^3$ experimental enclosures inside the glasshouse. The spectral properties of the film for UV_A and UV_B transmittance were measured by an ALMEMO 2390-5 data logger, equipped with UV_A and UV_B sensors (Ahlborn Mess und Regelungstechnik GmbH, Holzkirchen, Germany). All the sidewalls of the experimental enclosure were covered by black plastic so that only UV filtered global radiation light could reach inside from the top of the enclosure. LED lights were fitted with adjustable chains for maintaining relevant height as needed (Figure 4.2).

To investigate the effect of leaf age on phenolic composition, branches and petioles were tagged using coloured cable ties to identify leaves according to age. Tagged leaves from a single branch were harvested after (1) three months, (2) six months and (3) one year of UV_A treatment. Harvested leaves were transferred to a paper envelope and freeze-dried immediately using a Christ Alpha 1-4 LSC freeze drier and stored in the dark until further analysis.

Figure 4.2. Experimental setup showing *Eucalyptus camaldulensis* treated with high and low UV_A in the glasshouse. The top of the compartment was isolated from the greenhouse compartment by UV-absorbing plastic film and black plastic surrounded the plants on four sides to ensure plants were only exposed to the light from the LED lights. Six plant replicates per chemotype were used, making it 30 plants per treatment and a total of 120 plants.

4.2.3. LED light setup

UV_A emitting LED lights at the maximum wavelength of 370 nm and photosynthetically active radiation (PAR) emitting LED lights at the wavelength of 400 nm were purchased from Shenzhen Vanq Technology Co., Ltd, Guangdong Sheng, China. Two types of UV_A LED lights were purchased: LED lights with (i) radiation of 1800 [kJ/m²] UV_A per day simulating high UV in mid-summer (high UV light), and lights with (ii) radiation of 800

[kJ/m²] UV_A per day simulating winter annual UV_A (low UV light). The LED lights were purchased based on average annual midsummer and winter radiation data for Yallambie, Victoria, Australia (37°726'S, 145°104'E) and were provided by the Australian Radiation Protection and Nuclear Safety Agency (ARPANSA). The spectral irradiance of the LED lights was measured in ARPANSA, Yallambie, Victoria and was found to be suitable for simulating high summer UV and winter UV when placed 50 cm away from the plant. Two chambers in the glasshouse were fitted with high and low UV_A-LED lights in combination with PAR-LED lights, while the control chamber was equipped with only PAR-LED lights, and the same LED lights were used throughout the study.

4.2.4. Leaf extraction

Freeze-dried leaves were finely ground to ≤ 0.25 mm using a ball mill (Retsch MM400, Germany) at 30 Hz. 20mg of finely ground leaf powder was weighted in duplicate into 2 mL microtubes, and 1mL of 80:20 MeOH-H_2O (v/v) was added to each sample, and subsequently mixed with a vortex mixer for two minutes and sonicated for 10 minutes (Unisonics, Australia). Samples were then centrifuged for 10 minutes at 15,000 rpm at room temperature (Eppendorf 5415D bench centrifuge, Hamburg, Germany). The extract was transferred to a clean labelled microtube. 1 mL of the extract was transferred to HPLC vials for liquid chromatography-mass spectrometry (LC-MS) analysis, and a further 5µL of each extract were pooled to create a quality control (QC) sample to be analysed at the same time.

4.2.5. Chromatographic and mass spectrometric analysis

The identification of phenolic compounds was performed by an ultra-high-performance liquid chromatographic system (UPLC, UHPLC+ focused, Thermo Scientific™, Waltham, MA, USA) combined with a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific™, Waltham, MA, USA). Data was collected in negative

ion mode, scanning a mass range m/z of 100–1500. Negative ion mode was preferred over the positive for phenolic compounds analysis for all the subgroups because of its sensitivity, and clearer fragmentation patterns and less extensive fragmentation. Nitrogen gas was used as the sheath and auxiliary and sweep gas, and the spray voltage was set at 3,600 V. The capillary temperature was set to 300°C, with the S-lens RF level set at 64 and an auxiliary gas heater temperature of 310°C. A Hypersil GOLD C_{18} column (150×2.1mm, 1.9µm, Thermo Scientific, USA) was used with the column compartment temperature set to 30°C, and a flow rate was maintained at 0.3mL/min throughout data acquisition. The mobile phase consisted of (A) 0.1% formic acid and (B) acetonitrile. A linear gradient was used beginning with 2% of B and reaching 100% of B at 15 min, then kept steady at 100% of B until 18 min and then returned to an initial condition where it was held for 2 min. The MS/MS analyses were carried out by automatic fragmentation, where the three most intense mass peaks were fragmented. The mass spectrometric (MS) analysis, including the prediction of chemical formula and exact mass calculation, was performed by using Thermo Xcalibur Qual Browser software version 3.0.63 (Thermo Scientific, USA). The samples were injected into the mass spectrometry randomly (order organised by using the "RAND" function in Microsoft Excel), with blank and QC samples being injected every 10^{th} sample. Details of compound identification are described in Chapter II.

4.2.6. Data processing

The raw files from Xcalibur were imported to Genedata Expressionist Refiner MS version 12.0, Basel, Switzerland (https://www.genedata.com/). The noise from the data was removed using filters toolsRT structure removal, chemical noise subtraction, peak detection, isotope clustering, adduct detection, singleton filter, and signal clustering. Further, QC was used for samples normalisation to minimise and correct for the batch

variation. The volume of the cluster generated data from Genedata matrix was exported and visualised, and analysed in Genedata Analyst™ 12.0.6 software (Genedata AG, Basel, Switzerland). Genedata Analyst was used for further integration and interpretation of results by using various statistical applications. The samples were annotated based on their chemotypes (G_1-G_5), age of leaves three months, six months and twelve months old), and UV treatments (i.e. ambient, glasshouse, control, high UV light, low UV light).

4.2.7. Data analyses

Principal Components Analysis (PCA) was used to visualise metabolite variation between sample groups and treatments. A 2-Groups test which uses Student's *t*-test in Genedata was used to compare features between two treatments and K-Groups analysis that uses ANOVA was used to compare more than two treatments. The highly significant values were ranked based on low *P*-values (α) and higher effect size after Bonferroni correction (at $\alpha = 0.05$). To perform a Bonferroni correction, the critical *P*-values were divided by the number of features, and hence only the most statistically significant features were selected from the volcano plot in Genedata Analyst and these features were tentatively identified by using online mass data bank, standards, and MS/MS data. The data were tested for normal distribution by skewness and kurtosis test (*P* value between -1.96 and +1.96) (Mardia, 1970). The non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) was applied for statistically significant differences between the two groups ($P < 0.05$). Then the data were analysed using a mixed model, with random effects for chemotype. The data were analysed using a mixed model, with random effects for chemotype and fixed term for light treatment and an interaction latitude × light. The SPSS Statistical Software (version 25; SPSS Inc., Chicago, IL, USA) was used to perform all statistical tests.

4.3. Results

4.3.1. Effect of provenance on phenolic composition

A PCA plot showed variation between various chemotypes of *Eucalyptus camaldulensis* (G_1-G_5) across both PCs. The variation was 3.80% across PC 1 and 3.0% across PC 2. G_1 and G_2 showed the greatest separation in PC 1, and G_2 and G_5 showed the greatest separation in PC 2. Further, K-groups analysis (ANOVA) was used in Genedata analyst to identify differently expressed metabolites between chemotypes. Five metabolites from this analysis that were differentially expressed had high loadings on these PCs (Table 4.2).

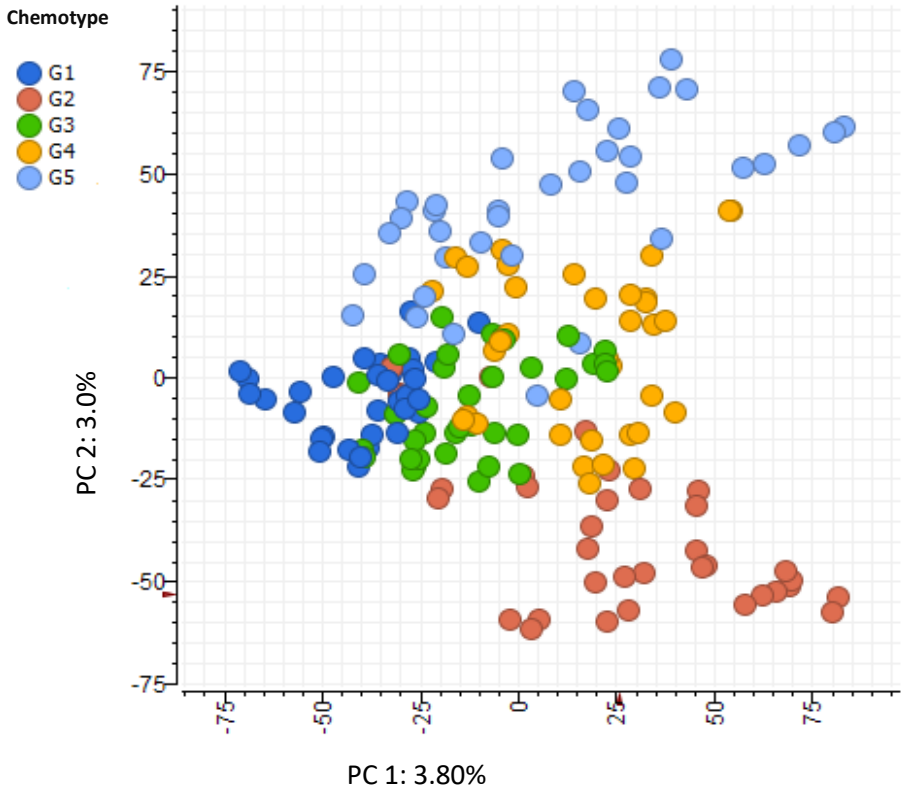


Figure 4.3. PCA analysis of metabolite profiling data. Principal components analysis of various chemotypes of *Eucalyptus camaldulensis* ssp. *camaldulensis*. Each point represents a metabolite profile of a biological replicate.

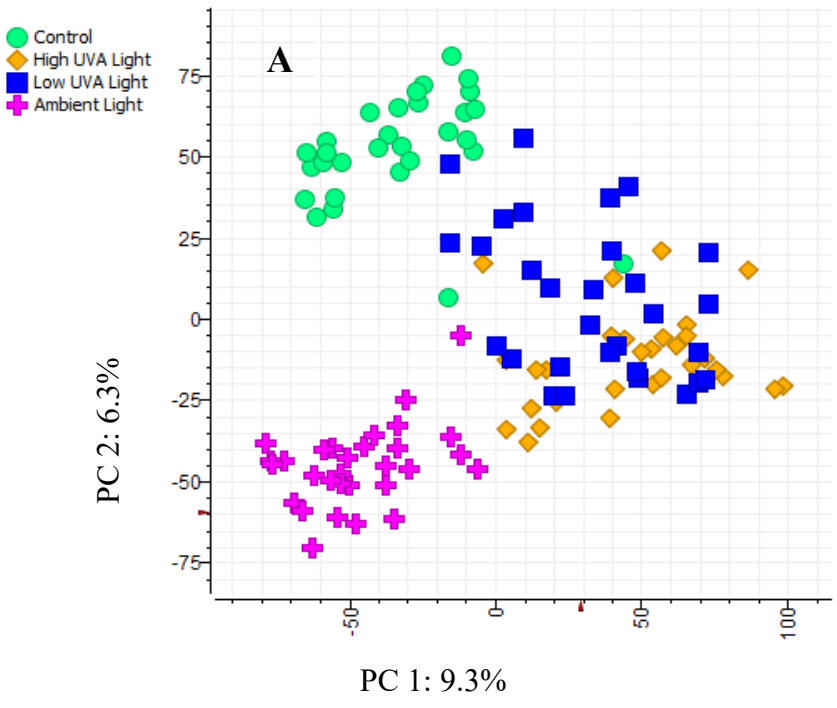
Four out of the five most significant compounds that were tentatively identified were pentagalloyl glucose (m/z 939.1107), galloyl glucose (m/z 331.0669), quercetin derivative (m/z 433.0408), HHDP-galloyl glucose (m/z 633.0735) and unknown compound (m/z 337.2052). Petagalloyl glucose was found to be highest in concentration in G_1 and lowest in G_2. Similarly, the quercetin derivative was found to be highest in G_1 and lowest in G_4. I found a correlation between pentagalloyl glucose and quercetin with both light and chemotype (Table 4.2). Also, there was a correlation between HHDP galloyl glucose and light. Similarly, there was also a correlation between pentagalloyl glucose, quercetin and unknown compounds with latitude and light. Also, there was a correlation between pentagalloyl glucose and quercetin with rainfall.

Table 4.2. Statistical results of mixed model analyses of phenolic compounds as affected by UV light

Compounds	**Interaction**	**d.f.**	***F***	***P***
pentagalloyl glucose	light*chemotype	101	13.86	< 0.01
quercetin	chemotype	15.01	19.51	<0.01
quercetin	light*chemotype	104	6.21	<0.01
galloyl glucose	light*chemotype	–	–	n.s.
unknown compound	light*chemotype	–	–	n.s.
HHDP galloyl glucose	light	12.99	4.17	<0.05
pentagalloyl glucose	rainfall	108	15.26	<0.01
quercetin	rainfall	111	79.35	<0.01
galloyl glucose	rainfall	–	–	n.s.
unknown compound	rainfall	–	–	n.s.
HHDP galloyl glucose	rainfall	–	–	n.s.

4.3.2. Effect of UV_A light

The PCA plot showed a clear separation between the light treatments in *Eucalyptus camaldulensis* (Figure 4.4A). High and low light samples cluster away from the control and ambient light samples on PC 1. Remodelling data with just high and low light samples on PCA shows a clear separation between high and low light samples (Figure 4.4B). K-group analysis (ANOVA) in Genedata analyst was used to identify the most significant compounds. The variation was 5.9 % across PC 1 and 4.4% across PC 2 in figure 4.4B. This variation was caused by 20 top compounds were selected based on their effect size and are listed below (Table 4.3). The selected features were targeted by an additional MS/MS tandem mass spectrometry for further fragmentation and were tentatively identified by using fragmentation patterns and online databases and the literature. A total of 17 compounds were identified as phenolic compounds. Various hydrolysable tannins (both ellagitannins and gallotannins) were found in higher concentrations in leaves treated with high UV_A light (Figure 4.5 and Table 4.3).



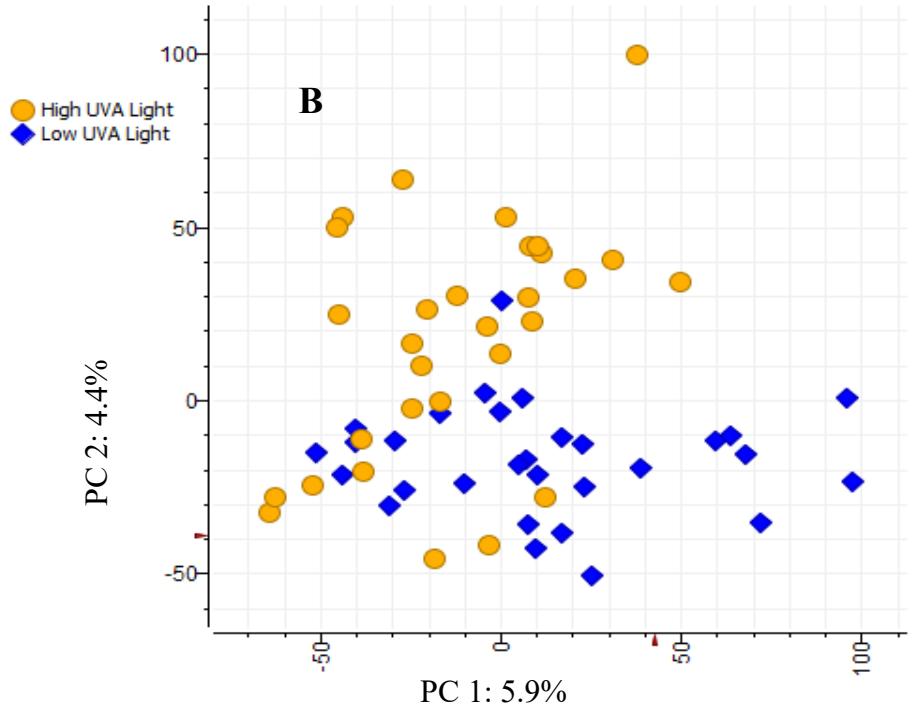


Figure 4.4. PCA plot between (A) high UV_A, low UV_A, controlled (glasshouse) and ambient conditions, and (B) PCA plot between high UV_A and low UV_A treatments.

Table 4.3. Top statistically significant compounds that are arranged according to their response to high UV_A light (*P*-value ≤ 0.001).

m/z	RT(mins)	Fragments	Tentative identification
1091.1214	5.85	939.1103, 769.0907, 617.0801, 465.0781, 169.0132	hexagalloylglucose I
1091.1214	5.94	939.1103, 769.0907, 617.0801, 465.0781, 169.0132	hexagalloylglucose II
481.0617	2.06	421.0407, 300.9986, 275.0194	HHDP-glucose
1417.1458	3.82	765.0638, 633.0735, 300.9983,275.0201	ellagitannin dimer
545.0568	5.91	939.1103, 769.0907, 617.0801, 465.0781, 169.0132	hexagalloylglucose
785.0843	5.42	633.0736, 300.9991, 275.0198, 249.0399, 169.0133	HHDP-digalloylglucose
104.0131	5.47	~	unknown
331.0669	5.8	271.0459, 169.0132, 151.0012, 125.0234	galloyl glucose
783.0686	3.37	481.0619, 300.9984, 275.0196	diHHDP glucose
787.0984	4.63	635.0892, 465.0701, 300.9982, 249.0401, 169.0131	tetragalloyl glucose
183.0287	5.26	168.0053, 124.0153	methylgallate
433.1725	5.21	~	unknown
785.0843	4.04	633.0736, 300.9991, 275.0198, 249.0399, 169.0133	HHDP-digalloylglucose
633.0735	5.45	463.0533, 300.9985,275.0201, 169.0135	HHDP-galloyl glucose
185.0377	4.15	~	unknown
124.0147	4.17	~	unknown
859.0662	4.33	300.9985,275.0201, 169.0135	ellagitannin

860.0802	4.34	300.9985,275.0201, 169.0135	ellagitannin
787.0984	4.41	635.0892, 465.0701, 300.9982, 249.0401, 169.0131	tetragalloylglucose
483.0775	4.13	331.0666, 271.0457, 169.0132, 125.0232	digalloylglucose

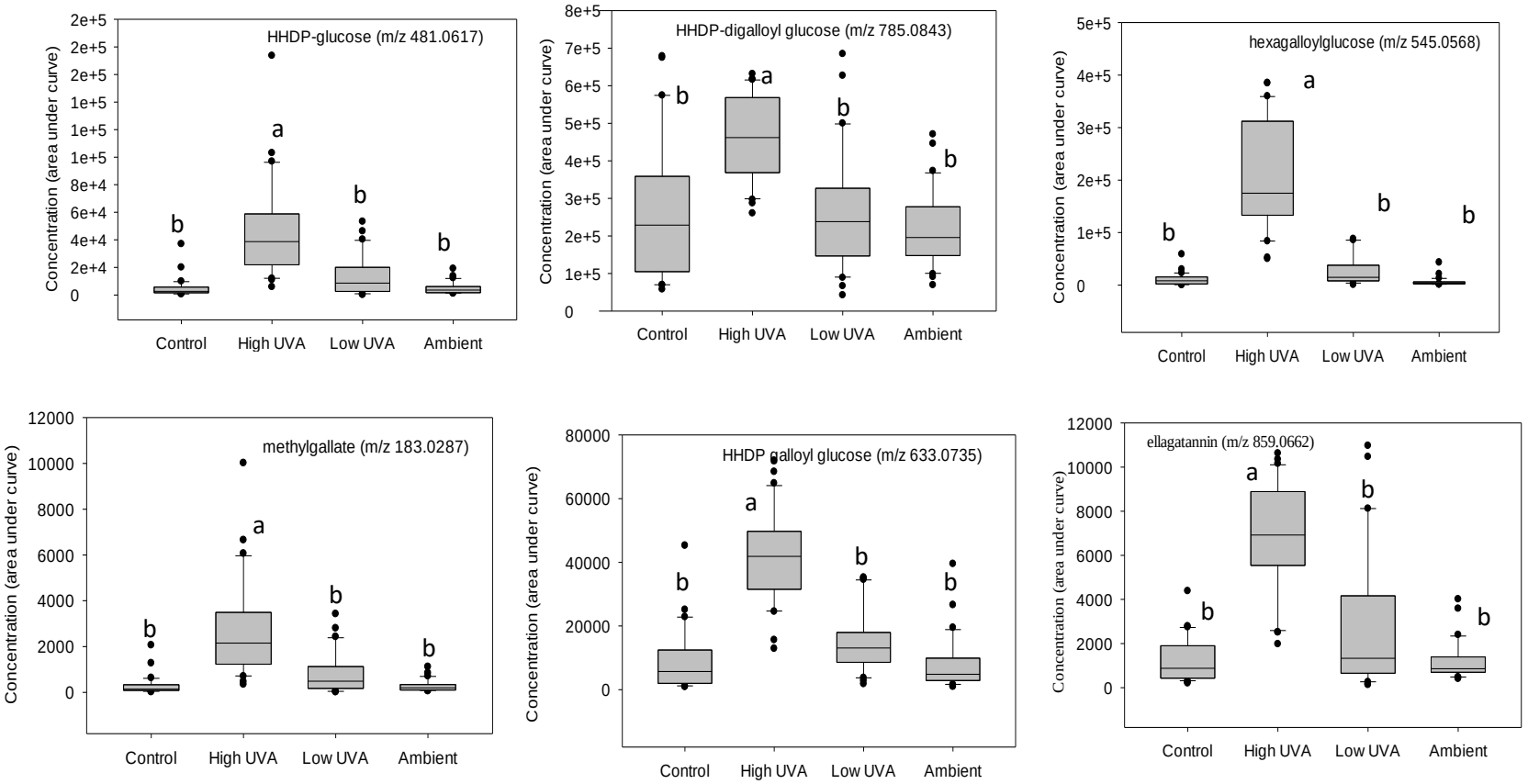


Figure 4.5a. Box plots showing top statistically significant phenolic compounds influenced by various levels of UV_A light treatment. In each box plot, the horizontal line that is crossing the box is the median, lower and upper quartiles are at the bottom and top of the box, respectively and the whiskers are the maximum and minimum values. The clusters represented with the same letter code are not significantly different (Tukey multiple comparisons of means, $P < 0.05$).

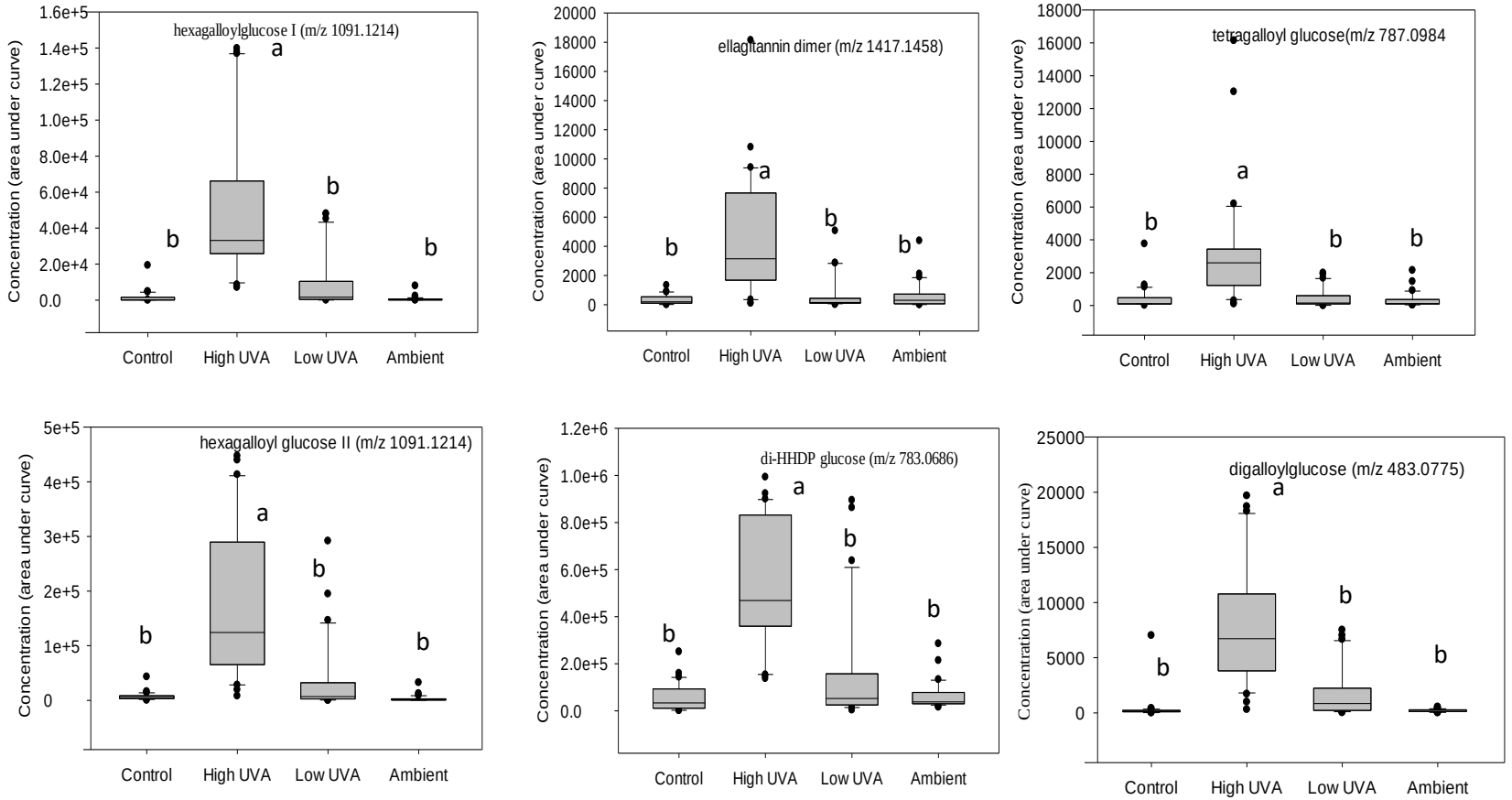


Figure 4.5b. Box plots showing top statistically significant phenolic compounds influenced by various levels of UV_A light treatment. See explanation of figure in the caption for Figure 4.5a.

4.3.3. Effect of leaf age

Leaf age was the most influential factor in the expression of compounds as can be seen in PCA plot Figure 4.6. Leaves were collected after three months, six months and one year from the plants. Leaves sampled at six months and one year overlapped in the PCA plot, so they were combined and labelled as ‘older leaves’ and three months old leaves were labelled as younger leaves. Based on fold change and *P*-value ≤ 0.01, 14 top compounds were deemed significantly different between the two classes. Pedunculagin, catechin, kaempferol galloyl glucoses were found to be more abundant in older leaves. Similarly, hydrolysable tannins and quercetin were more abundant in younger leaves (Table 4.4 and Figure 4.7).

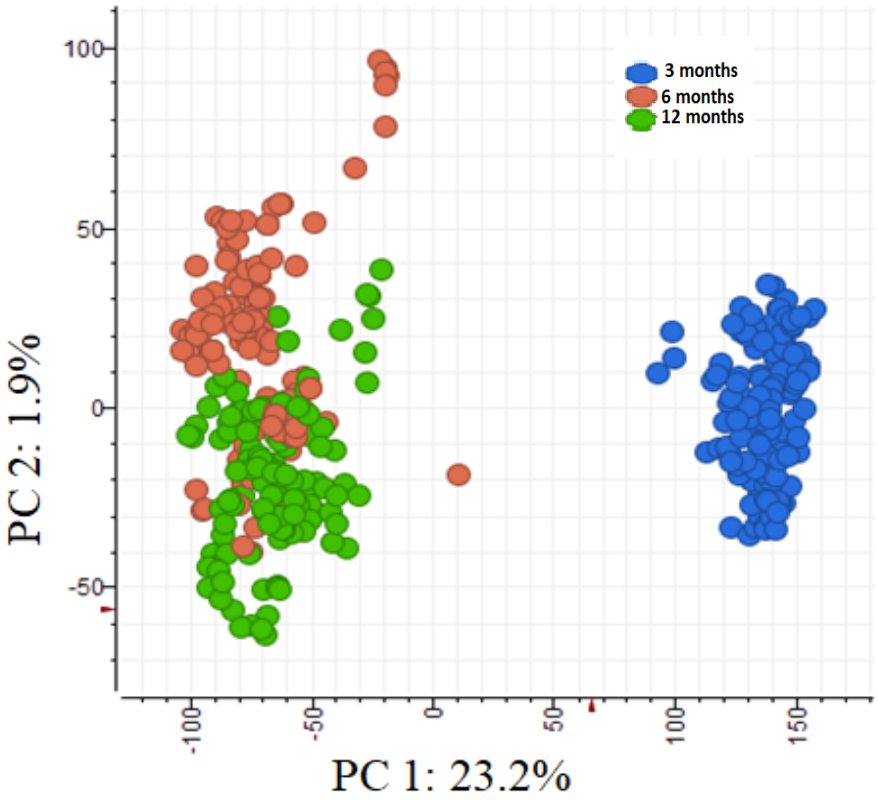


Figure 4.6. PCA plot between three months, six months, and one-year-old leaves. Responses of individual compounds are given in Table 4.4. Leaves sampled at six months and one year were combined in one group and called ‘older leaves’, and three months old leaves were called younger leaves.

Table 4.4. Top statistically significant features between younger and older leaves (*P*-value ≤ 0.001).

m/z	RT (min)	Tentative identification	Younger leaves	Older leaves
383.1865	15.33	unknown		↑
433.0464	5.26	ellagic acid glycoside	↑	
271.082	3.65	glycosylated hydroquinone		↑
463.0511	4.72	ellagic acid hexoside	↑	
433.0462	5.27	ellagic acid glycoside	↑	
599.1039	5.49	kaempferol derivative		↑
315.0734	3.98	phenolic glycoside		↑
197.0812	3.65	unknown	↑	
499.1602	14.21	unknown		↑
783.0693	3.30	pedunculagin		↑
461.0720	5.85	flavonoid glycoside	↑	
935.0795	5.51	di-HHDP galloylglucose	↑	
789.4441	16.02	unknown		↑
471.2746	13.32	unknown	↑	
399.1814	15.01	unknown		↑
609.1459	5.10	quercetin derivative	↑	

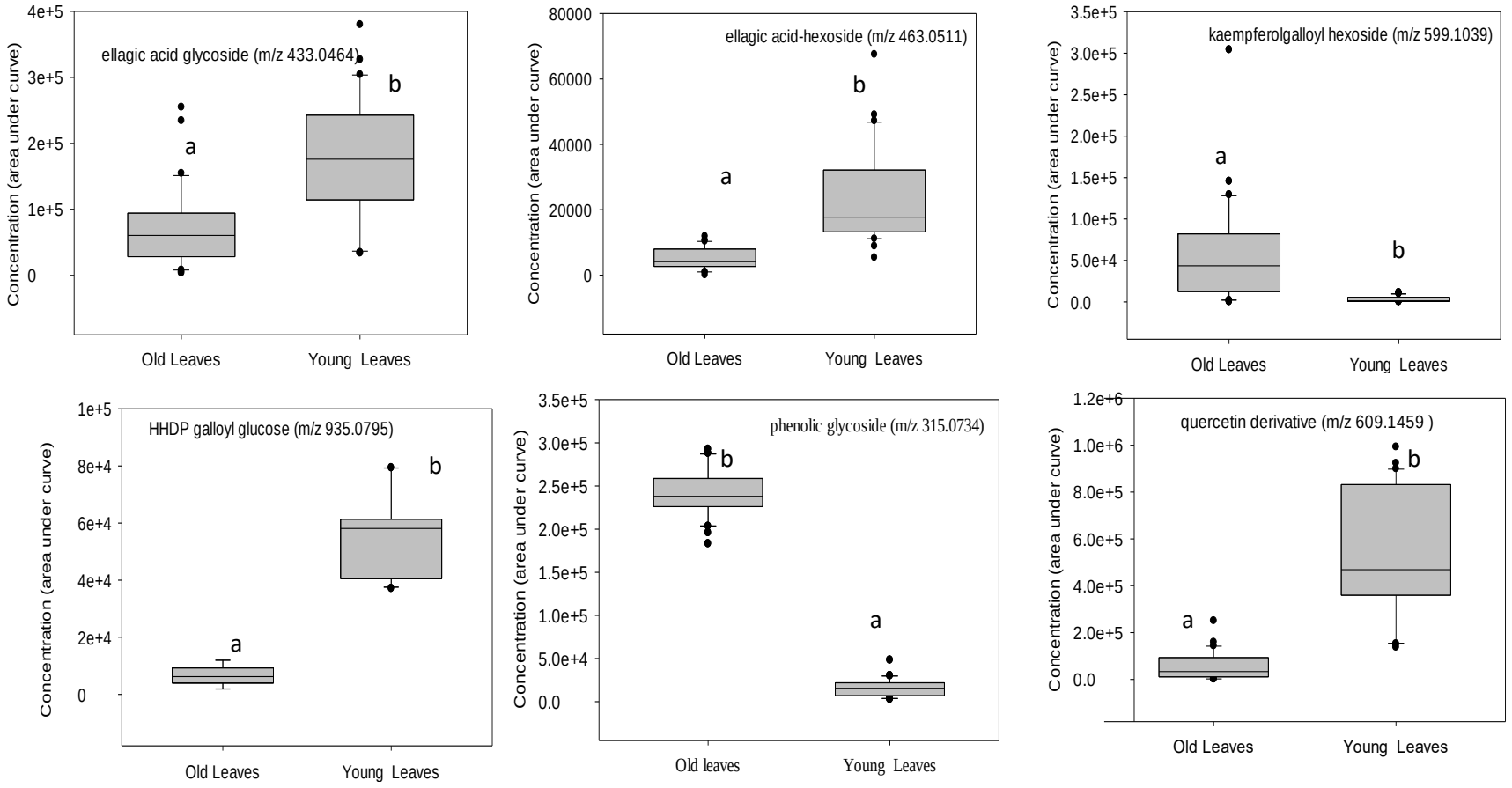


Figure 4.7. Box plot showing top statistically significant phenolic compounds influenced by leaf age. In each box plot, the horizontal line crossing the box is the median, lower and upper quartiles are at the bottom and top of the box, respectively, and the whiskers are the maximum and minimum values. The clusters represented with the same letter code are not significantly different (Tukey multiple comparisons of means, $P<0.05$).

4.4. Discussion

4.4.1. Effect of provenance on phenolic composition

The provenance for the five chemotypes of *Eucalyptus camaldulensis* in our study varies by nearly 5° in latitude, which might result in an increase of 5-10% of UV towards the equator. The GLM analysis shows that light × chemotype interactions were the most significant factor explaining the variation in concentration of pentagalloyl glucose, and both chemotype and light × chemotype interactions were the most significant factor for variation of quercetin. My result shows that the concentration of pentagalloyl glucose and quercetin was higher in lower latitudes suggesting their roles in photoprotection. Pentagalloyl glucose has a high antioxidant potential due to the presence of more galloyl groups and might have an active role in photoprotection against photodamage (Tian *et al*., 2009). My finding aligns with research that indicates that pentagalloylglucose is an effective 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenger, and some experiments have even shown that pentagalloylglucose has higher free radical scavenging properties than vitamin C (Abdelwahed *et al.,* 2007; Zhang *et al.,* 2009). Also, various studies suggest an enhanced biosynthesis of quercetin under UV_B stress suggesting its active role in photoprotection (Ryan *et al.,* 2002; Agati & Tattini, 2010; Shourie *et al*., 2014). Also, quercetin and its glycosides in the chloroplast are known for their role as an effective singlet oxygen quencher which is generated by excess blue light (Agati *et al*., 2007).

My data also shows that rainfall is the most important factor explaining differences variation in the data in the pentagalloyl glucose and quercetin. My results also showed a higher concentration of quercetin and pentagalloyl glucose in lower average rainfall areas. Various investigations have shown plant flavonols such as quercetin and anthocyanins having strong radical scavenging activity and contributing to the mitigation of oxidative

and drought stress, and these compounds are elevated under drought (Hernández *et al*., 2004; Nakabayashi *et al.*, 2014). Drought stress is also known to enhance quantitative and qualitative improvement of various phenolic acids, tannins, formylated phloroglucinol compound and flavonoids as reported in *Arabidopsis* (Nakabayashi *et al.* 2014), *Amaranthus* (Sarker & Oba, 2018) and *Eucalyptus* (McKiernan *et al*., 2016) by hence allowing the plants to grow in semi-arid and dry areas (Tharayil *et al.*, 2011; Sarker & Oba, 2018).

4.4.2. Effect of UV_A

My result shows an increase in the concentration of various phenolic acids, hydrolysable tannins and flavonoids during high UV_A treatment, suggesting an active photoprotection role of these phenolics against photodamage by high UV light. My result was supported by a study of *Eucalyptus nitens*, which showed an increased concentration of galloylglucoses and flavonoids in summer, which suggested the role of galloylglucoses that might quench free radical and support plant's photochemical efficiency (Close *et al*., 2001). Similar patterns were observed in barberry (*Mahonia repens*) and Victorian plum tree (*Prunus domesticata* var. *Victoria*) (Hillis and Swain 1959; Grace *et al.* 1998). In addition, research on *Eucalyptus nitens* seedlings found twice the level of galloylglucoses (di-, tri-, tetra- and penta-galloylglucose) in the sun adapted foliage compared to shade-adapted foliage (Close *et al.* 2001). Various high molecular tannins (hydrolysable and condensed) have shown up to 15-20 times effective quenching of peroxyl radicals than simple polyphenols (Hagerman *et al.*, 1998). The high antioxidant properties of these tannins can be achieved by a high degree of polymerisation of many phenolic hydroxyl groups, which contribute to quenching efficacy (Ariga & Hamano, 1990; Hodnick *et al.*, 1998).

Glasshouse treatments differ from natural light in many ways, not necessarily reflecting exact natural light conditions and its exact effects is hard to predict. Studies have shown the occurrence of intumescences leading to tissue necrotisation and cellular collapse, and deposition of phenolic compounds in the absence of natural light for photoprotection (Pinkard *et al.*, 2006). Also, light conditions have been associated with strong differences in oil gland development (James & Bell, 2000), including in *E. camaldulensis* and leaf thickness, and it can be expected that this might strongly influence PSM concentrations and greatly reduce the photosynthetic capacity (James & Bell, 1995). I did not observe any physical changes to leaves, such as epidermal blistering or curling, as has been reported by other authors (Pinkard *et al.*, 2006).

4.4.3. Effect of leaf age

Eucalyptus camaldulensis leaves exhibited contrasting phenolic composition between younger and older leaf ages. The younger leaves (up to three months old) exhibited a higher abundance of smaller phenolic acid, flavonoids, quercetin, hydrolysable tannins (both galloyl glucose and ellagitannins) and older leaves (six months and one year old) exhibited a higher abundance of glycosylated hydroquinone, kaempferol galloyl hexoside and pedunculagin. There was significant variation of phenolic compounds between three months and six months and minor variation between 6 months and 12 months which implies that phenolic compounds might go drastic changes up to 6 months in *Eucalyptus* leaves, and leaf age might be a major factor for these changes. The optimal defence theory predicts that plants focus their defence on tissues that are of high fitness value (Pavia *et al.*, 2002). Therefore, since young leaves are more vulnerable to herbivory, they are generally highly protected than older leaves (Gherlenda *et al.*, 2016). This might be the reason for the accumulation of highly bioactive ellagitannins in my young leaves. My findings also support the study by Salminen (2004), which states that young leaves are

richer in hydrolysable tannins and flavonoid glycosides than old leaves. Various hydrolysable tannins, especially ellagitannins, act as prooxidants, as their reaction products are known to cause oxidative stress in insect digestive tracts by producing ROS (e.g., semiquinone radicals) in various insects, highlighting their roles in defence against herbivory (Barbehenn *et al.,* 2006; Barbehenn & Constabel, 2011). Various research has shown consumption of these tannins can cause adverse reactions due to phenolic oxidation in the highly alkaline midgut of some species of caterpillar (Barbehenn *et al.,* 2006; Barbehenn & Constabel, 2011).

The photosynthetic system is not fully functional in the young leaves of many plants (Bielczynski *et al.,* 2017). Various studies show the presence of tannins, formylated phloroglucinol compounds in the epidermal layer and flavonoids within the subdermal secretory cavities in various *Eucalyptus* (Goodger *et al*., 2016; Migacz *et al.*, 2018; Santos *et al*., 2019) and 44 species of Myrtaceae (Cardoso *et al.,* 2009). This accumulation of tannins in the upper and lower epidermis of leaves may help to protect the leaves from insect attack and from the excess of photosynthetic capacity, which can lead to cellular damage caused by reactive oxygen species produced under excessive light conditions (Bertamini & Nedunchezhian, 2003; Luo *et al.,* 2019). Hydrolysable tannins are also known to indirectly support photoprotection by preventing anthocyanin degradation at the abaxial layer, increasing the pigment stability and helping to maintain red leaves (Luo *et al.,* 2019). Further, low hydrolysable tannins in older leaves compared to younger leaves might be due to compositional shift from low molecular weight phenolics to condensed tannins (*Betula pubescens* Ehr.) (Wam *et al.,* 2017). Although tannins are known to be located in multiple locations, condensed tannins are especially located in vacuoles (Fleurat-Lessard *et al*., 2016). My findings have shown that the composition of some phenolic compounds varies with (i) provenance, (ii) UV_A exposure and (iii) leaf age (Table

4.5). I have shown that leaf phenolics change dramatically sometime between 3 and 6 months of age and then may remain constant. This has never been shown before and has significant ecological consequences. A higher concentration of phenolic compounds (e.g., galloyl glucose, phenolic acids, quercetin and ellagitannins) was found in leaves treated with high UV_A and in young leaves, highlighting their role in photoprotection and defence. Further, young leaves have a higher concentration of ellagitannin, so they could be considered to be heavily defended against insect herbivores. There was also an increase of various hydrolysable tannins, flavonoid in high UV_A treated leaves, suggesting their active role in UV_A protection.

Table 4.5. Summary of various phenolic compounds and the effect of UV_A on in leaves from my glasshouse experiment, their variation with leave age. ↑ indicates increased the relative concentration of the most significant compounds that were found with high UV_A treatment.

Compounds	**Response to high UV_A**	**Leaf age**
galloyl glucoses	↑	younger
ellagitannins	↑	younger
quercetin	↑	younger
flavonoids	↑	younger
phenolic acids	↑	younger

Therefore, from my results, it may be concluded that physiological signalling associated with UV stress up-regulates biosynthesis of various individual phenolic compounds, and these phenolic compounds might have been evolved for the protection of plants against oxidative damage caused by high UV light.

4.5. References

Abdallah, S. B., Rabhi, M., Harbaoui, F., Zar-kalai, F., Lachâal, M., & Karray-Bouraoui, N. (2013). Distribution of phenolic compounds and antioxidant activity between young and old leaves of *Carthamus tinctorius* L. and their induction by salt stress. *Acta Physiologiae Plantarum*, *35*(4), 1161–1169.

Agrawal, A. A. (2011). Current trends in the evolutionary ecology of plant defence. *Functional Ecology*, *25*(2), 420–432.

Ahanger, M. A., Gul, F., Ahmad, P., & Akram, N. A. (2018). Chapter 3—Environmental stresses and metabolomics—Deciphering the role of stress responsive metabolites. In P. Ahmad, M. A. Ahanger, V. P. Singh, D. K. Tripathi, P. Alam, & M. N. Alyemeni (Eds.), *Plant Metabolites and Regulation Under Environmental Stress* (pp. 53–67). Academic Press.

Agati, G., & Tattini, M. (2010). Multiple functional roles of flavonoids in photoprotection: *New Phytologist*, *186*(4), 786–793.

Aleixandre-Tudo, J. L., Buica, A., Nieuwoudt, H., Aleixandre, J. L., & du Toit, W. (2017). Spectrophotometric analysis of phenolic compounds in grapes and wines. *Journal of Agricultural and Food Chemistry*, *65*(20), 4009–4026.

Alonso, A., Marsal, S., & Julià, A. (2015). Analytical methods in untargeted metabolomics: State of the art in 2015. *Frontiers in Bioengineering and Biotechnology*, *3*.

Ariga, T., & Hamano, M. (1990). Radical scavenging action and its mode in procyanidins B-1 and B-3 from azuki beans to peroxyl radicals. *Agricultural and Biological Chemistry*, *54*(10), 2499–2504

Ballaré, C. L., Caldwell, M. M., Flint, S. D., Robinson, S. A., & Bornman, J. F. (2011). Effects of solar ultraviolet radiation on terrestrial ecosystems. Patterns, mechanisms, and interactions with climate change. *Photochemical & Photobiological Sciences: Official Journal of the European Photochemistry Association and the European Society for Photobiology*, *10*(2), 226–241.

Balyan, U., & Sarkar, B. (2017). Aqueous extraction kinetics of phenolic compounds from jamun (*Syzygium cumini* L.) seeds. *International Journal of Food Properties*, *20*(2), 372–389.

Bantis, F., Ouzounis, T., & Radoglou, K. (2016). Artificial LED lighting enhances growth characteristics and total phenolic content of *Ocimum basilicum*, but variably affects transplant success. *Scientia Horticulturae*, *198*, 277–283.

Barbehenn, R. V., Jones, C. P., Hagerman, A. E., Karonen, M., & Salminen, J.-P. (2006). Ellagitannins have greater oxidative activities than condensed tannins and galloyl glucoses at high pH: Potential impact on caterpillars. *Journal of Chemical Ecology*, *32*(10), 2253–2267.

Barbehenn, R. V., & Peter Constabel, C. (2011). Tannins in plant-herbivore interactions. *Phytochemistry*, *72*(13), 1551–1565.

Barton, K. E., & Koricheva, J. (2010). The ontogeny of plant defence and herbivory: Characterising general patterns using meta-analysis. *The American Naturalist*, *175*(4), 481–493.

Barton, K. E., Edwards, K. F., & Koricheva, J. (2019). Shifts in woody plant defence syndromes during leaf development. *Functional Ecology*, *33*(11), 2095–2104.

Bechtold, U., & Field, B. (2018). Molecular mechanisms controlling plant growth during abiotic stress. *Journal of Experimental Botany*, *69*(11), 2753–2758.

Bertamini, M., & Nedunchezhian, N. (2003). Photoinhibition of photosynthesis in mature and young leaves of grapevine (*Vitis vinifera* L.). *Plant Science*, *164*(4), 635–644.

Bhattacharya, A., Sood, P., & Citovsky, V. (2010). The roles of plant phenolics in defence and communication during *Agrobacterium* and Rhizobium infection. *Molecular Plant Pathology*, *11*(5), 705–719.

Bielczynski, L. W., Łącki, M. K., Hoefnagels, I., Gambin, A., & Croce, R. (2017). Leaf and plant age affect photosynthetic performance and photoprotective capacity. *Plant Physiology*, *175*(4), 1634–1648.

Blum-Silva, C. H., Chaves, V. C., Schenkel, E. P., Coelho, G. C., & Reginatto, F. H. (2015). The influence of leaf age on methylxanthines, total phenolic content, and free radical scavenging capacity of *Ilex paraguariensis* aqueous extracts. *Revista Brasileira de Farmacognosia*, *25*(1), 1–6.

Bornman, J. F., Barnes, P. W., Robinson, S. A., Ballaré, C. L., Flint, S. D., & Caldwell, M. M. (2015). Solar ultraviolet radiation and ozone depletion-driven climate change: Effects on terrestrial ecosystems. *Photochemical & Photobiological Sciences: Official Journal of the European Photochemistry Association and the European Society for Photobiology*, *14*(1), 88–107.

Borzak, C. L., Potts, B. M., Davies, N. W., & O'Reilly-Wapstra, J. M. (2015). Population divergence in the ontogenetic trajectories of foliar terpenes of a *Eucalyptus* species. *Annals of Botany*, *115*(1), 159–170.

Boulet, J.-C., Ducasse, M.-A., & Cheynier, V. (2017). Ultraviolet spectroscopy study of phenolic substances and other major compounds in red wines: Relationship between astringency and the concentration of phenolic substances: UV spectroscopy of red wine components. *Australian Journal of Grape and Wine Research*, *23*(2), 193–199.

Bunning, M. L., Kendall, P. A., Stone, M. B., Stonaker, F. H., & Stushnoff, C. (2010). Effects of seasonal variation on sensory properties and total phenolic content of 5 lettuce cultivars. *Journal of Food Science*, *75*(3), S156-161.

Caldwell, M. M., Björn, L. O., Bornman, J. F., Flint, S. D., Kulandaivelu, G., Teramura, A. H., & Tevini, M. (1998). Effects of increased solar ultraviolet radiation on terrestrial ecosystems. *Journal of Photochemistry and Photobiology B: Biology*, *46*(1), 40–52.

Close, D. C., McArthur, C., Hagerman, A. E., Davies, N. W., & Beadle, C. L. (2007). Phenolic acclimation to ultraviolet-A irradiation in *Eucalyptus nitens* seedlings raised across a nutrient environment gradient. *Photosynthetica*, *45*(1), 36–42.

Close, D. C., & McArthur, C. (2002). Rethinking the role of many plant phenolics: Protection from photodamage not herbivores? *Oikos*, *99*(1), 166–172. JSTOR.

Coley, P. D., Bryant, J. P., & Chapin, F. S. (1985). Resource availability and plant antiherbivore defence. *Science (New York, N.Y.)*, *230*(4728), 895–899.

Conner, J. K., & Neumeier, R. (2002). The effects of UV_B radiation and intraspecific competition on growth, pollination success, and lifetime female fitness in *Phacelia campanularia* and *P. purshii* (Hydrophyllaceae). *American Journal of Botany*, *89*(1), 103–110.

Datta, J., & Lal, N. (2012). Temporal and spatial changes in phenolic compounds in response to Fusarium wilt in chickpea and pigeon pea. *Cellular and Molecular Biology (Noisy-Le-Grand, France)*, *58*(1), 96–102.

de Lima-Saraiva, S. R. G., Oliveira, F. G. da S., Junior, R. G. de O., Araújo, C. de S., de Oliveira, A. P., Pacheco, A. G. M., Rolim, L. A., de Amorim, E. L. C., César, F. C. S., & Almeida, J. R. G. da S. (2017). Chemical analysis and evaluation of

antioxidant, antimicrobial, and photoprotective activities of *Schinopsis brasiliensis* Engl. (Anacardiaceae). *The Scientific World Journal*, *2017*.

Dillon, S., McEvoy, R., Baldwin, D. S., Rees, G. N., Parsons, Y., & Southerton, S. (2014). Characterisation of adaptive genetic diversity in environmentally contrasted populations of *Eucalyptus camaldulensis* Dehnh. (River Red Gum). *PLOS ONE*, *9*(8), e103515.

Elger, A., Lemoine, D. G., Fenner, M., & Hanley, M. E. (2009). Plant ontogeny and chemical defence: Older seedlings are better defended. *Oikos*, *118*(5), 767–773.

Fleurat-Lessard, P., Béré, E., Lallemand, M., Dédaldéchamp, F., & Roblin, G. (2016). Co-occurrence of tannin and tannin-less vacuoles in sensitive plants. *Protoplasma*, *253*(3), 821–834.

Gherlenda, A. N., Moore, B. D., Haigh, A. M., Johnson, S. N., & Riegler, M. (2016). Insect herbivory in a mature *Eucalyptus* woodland canopy depends on leaf phenology but not CO_2 enrichment. *BMC Ecology*, *16*(1), 47.

Ghiassi Tarzi, B., Gharachorloo, M., Baharinia, M., & Mortazavi, S. A. (2012). The Effect of germination on phenolic content and antioxidant activity of chickpea. *Iranian Journal of Pharmaceutical Research: IJPR*, *11*(4), 1137–1143.

Ghosh, D., Lin, Q., Xu, J., & Hellmann, H. A. (2017). Editorial: How plants deal with stress: Exploration through proteome investigation. *Frontiers in Plant Science*, *8*.

Gill, Sarvajeet S., Anjum, N. A., Gill, R., Jha, M., & Tuteja, N. (2015). DNA damage and repair in plants under ultraviolet and ionising radiations. *The Scientific World , 2015*, 250158.

Gill, Sarvajeet Singh, & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry: PPB*, *48*(12), 909–930.

Goodger, J. Q. D., Gleadow, R. M., & Woodrow, I. E. (2006). Growth cost and ontogenetic expression patterns of defence in cyanogenic *Eucalyptus* spp. *Trees*, *20*(6), 757–765.

Goodger, J. Q. D., Heskes, A. M., & Woodrow, I. E. (2013). Contrasting ontogenetic trajectories for phenolic and terpenoid defences in *Eucalyptus froggattii*. *Annals of Botany*, *112*(4), 651–659.

Goodger, J. Q. D., Seneratne, S. L., Nicolle, D., & Woodrow, I. E. (2016). Foliar essential oil glands of *Eucalyptus* (Myrtaceae) Are a rich source of flavonoids and related non-volatile constituents. *PLOS ONE*, *11*(3), e0151432.

Gourlay, G., & Constabel, C. P. (2019). Condensed tannins are inducible antioxidants and protect hybrid poplar against oxidative stress. *Tree Physiology*, *39*(3), 345–355.

Guerriero, G., Berni, R., Muñoz-Sanchez, J. A., Apone, F., Abdel-Salam, E. M., Qahtan, A. A., Alatar, A. A., Cantini, C., Cai, G., Hausman, J.-F., Siddiqui, K. S., Hernández-Sotomayor, S. M. T., & Faisal, M. (2018). Production of plant secondary metabolites: Examples, tips and suggestions for biotechnologists. *Genes*, *9*(6).

Hagerman, A. E., Riedl, K. M., Jones, G. A., Sovik, K. N., Ritchard, N. T., Hartzfeld, P. W., & Riechel, T. L. (1998). High molecular weight plant polyphenolics (tannins) as biological antioxidants. *Journal of Agricultural and Food Chemistry*, *46*(5), 1887–1892.

Herms, D. A., & Mattson, W. J. (1992). The Dilemma of Plants: To grow or defend. *The Quarterly Review of Biology*, *67*(3), 283–335. Jstor.

Hichem, H., Mounir, D., & Naceur, E. A. (2009). Differential responses of two maise (*Zea mays* L.) varieties to salt stress: Changes on polyphenols composition of foliage and oxidative damages. *Industrial Crops and Products*, *30*(1), 144–151.

Hillis, W. E. (1966). Polyphenols in the leaves of *Eucalyptus*: A chemotaxonomic survey—I.: Introduction and a study of the series globulares. *Phytochemistry*, *5*(6), 1075–1090.

Hodnick, W. F., Ahmad, S., & Pardini, R. S. (1998). Induction of oxidative stress by redox active flavonoids. In J. A. Manthey & B. S. Buslig (Eds.), *Flavonoids in the Living System* (pp. 131–150). Springer US.

Isah, T. (2019). Stress and defence responses in plant secondary metabolites production. *Biological Research*, *52*(1), 39.

Iwai, M., Ohta, M., Tsuchiya, H., & Suzuki, T. (2010). Enhanced accumulation of caffeic acid, rosmarinic acid and luteolin-glucoside in red perilla cultivated under red diode laser and blue LED illumination followed by UV_A irradiation. *Journal of Functional Foods*, *2*(1), 66–70.

Jaakola, L., & Hohtola, A. (2010). Effect of latitude on flavonoid biosynthesis in plants. *Plant, Cell & Environment*, *33*(8), 1239–1247.

James, S. A., & Bell, D. T. (1995). Morphology and anatomy of leaves of *Eucalyptus camaldulensis* clones: Variation between geographically separated locations. *Australian Journal of Botany*, *43*(4), 415–433.

James, S. A., & Bell, D. T. (2000). Influence of light availability on leaf structure and growth of two *Eucalyptus globulus* ssp. *globulus* provenances. *Tree Physiology*, *20*(15), 1007–1018.

Joubert, E., Richards, E. S., Merwe, J. D. V. der, De Beer, D., Manley, M., & Gelderblom, W. C. A. (2008). Effect of species variation and processing on phenolic

composition and in vitro antioxidant activity of aqueous extracts of Cyclopia spp. (Honeybush Tea). *Journal of Agricultural and Food Chemistry*, *56*(3), 954–963.

Kakani, V. G., Reddy, K. R., Zhao, D., & Sailaja, K. (2003). Field crop responses to UV_B radiation: A review. *Agricultural and Forest Meteorology*, *120*(1), 191–218.

Keith, R. A., & Mitchell-Olds, T. (2017). Testing the optimal defence hypothesis in nature: Variation for glucosinolate profiles within plants. *PLOS ONE*, *12*(7), e0180971.

Koricheva, J., Nykänen, H., & Gianoli, E. (2004). Meta-analysis of trade-offs among plant antiherbivore defences: Are plants jacks-of-all-trades, masters of all? *The American Naturalist*, *163*(4), E64-75.

Koutchma, T. (2019). Ultraviolet LED technology for food applications*:* From farms to kitchens. Academic Press.

Kulkarni, M., Soolanayakanahally, R., Ogawa, S., Uga, Y., Selvaraj, M. G., & Kagale, S. (2017). Drought response in wheat: Key genes and regulatory mechanisms controlling root system architecture and transpiration efficiency. *Frontiers in Chemistry*, *5*.

Li, Q., & Kubota, C. (2009). Effects of supplemental light quality on growth and phytochemicals of baby leaf lettuce. *Environmental and Experimental Botany*, *67*(1), 59–64.

Luo, H., Li, W., Zhang, X., Deng, S., Xu, Q., Hou, T., Pang, X., Zhang, Z., & Zhang, X. (2019). In planta high levels of hydrolysable tannins inhibit peroxidase mediated anthocyanin degradation and maintain abaxially red leaves of *Excoecaria cochinchinensis*. *BMC Plant Biology*, *19*(1), 315.

Mao, K., Yuan, Z., Zuo, Z., Xu, T., Shen, X., & Gao, C. (2019). Changes in global cloud cover based on remote sensing data from 2003 to 2012. *Chinese Geographical Science*, *29*(2), 306–315.

Marsh, K. J., Wallis, I. R., Kulheim, C., Clark, R., Nicolle, D., Foley, W. J., & Salminen, J.-P. (2019). New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. *The New Phytologist*, *225*(1), 488–498.

Massad, T. J., Dyer, L. A., & C, G. V. (2012). Costs of defence and a test of the carbon-nutrient balance and growth-differentiation balance hypotheses for two co-occurring classes of plant defence. *PLOS ONE*, *7*(10), e47554.

Migacz, I. P., Raeski, P. A., Almeida, V. P. de, Raman, V., Nisgoski, S., Muniz, G. I. B. de, Farago, P. V., Khan, I. A., & Budel, J. M. (2018). Comparative leaf morpho-anatomy of six species of *Eucalyptus* cultivated in Brazil. *Revista Brasileira de Farmacognosia*, *28*(3), 273–281.

Moreira, X., Castagneyrol, B., Abdala-Roberts, L., Berny-Mier y Teran, J. C., Timmermans, B. G. H., Bruun, H. H., Covelo, F., Glauser, G., Rasmann, S., & Tack, A. J. M. (2018). Latitudinal variation in plant chemical defences drives latitudinal patterns of leaf herbivory. *Ecography*, *41*(7), 1124–1134.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), 270–278.

Pascual, J., Cañal, M. J., Escandón, M., Meijón, M., Weckwerth, W., & Valledor, L. (2017). Integrated physiological, proteomic, and metabolomic analysis of UV stress responses and adaptation mechanisms in *Pinus radiata. Molecular & Cellular Proteomics: MCP*, *16*(3), 485–501.

Pavia, H., Toth, G. B., & Åberg, P. (2002). Optimal Défense Theory: Elasticity analysis as a tool to predict intraplant variation in defences. *Ecology*, *83*(4), 891–897. Jstor.

Pinkard, E., Gill, W., & Mohammed, C. (2006). Physiology and anatomy of lenticel-like structures on leaves of *Eucalyptus nitens* and *Eucalyptus globulus* seedlings. *Tree Physiology*, *26*(8), 989–999.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, *20*(7), 933–956.

Rodríguez-Calzada, T., Qian, M., Strid, Å., Neugart, S., Schreiner, M., Torres-Pacheco, I., & Guevara-González, R. G. (2019). Effect of UV_B radiation on morphology, phenolic compound production, gene expression, and subsequent drought stress responses in chilli pepper (*Capsicum annuum* L.). *Plant Physiology and Biochemistry*, *134*, 94–102.

Ruhfel, B. R., Gitzendanner, M. A., Soltis, P. S., Soltis, D. E., & Burleigh, J. G. (2014). From algae to angiosperms–inferring the phylogeny of green plants (Viridiplantae) from 360 plastid genomes. *BMC Evolutionary Biology*, *14*(1), 23.

Ryan, K. G., Swinny, E. E., Markham, K. R., & Winefield, C. (2002). Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. *Phytochemistry*, *59*(1), 23–32.

Sagers, C. L., & Coley, P. D. (1995). Benefits and costs of defense in a neotropical shrub. *Ecology*, *76*(6), 1835–1843.

Salminen, J.-P., Roslin, T., Karonen, M., Sinkkonen, J., Pihlaja, K., & Pulkkinen, P. (2004). Seasonal variation in the content of hydrolysable tannins, flavonoid glycosides, and proanthocyanidins in oak leaves. *Journal of Chemical Ecology*, *30*(9), 1693–1711.

Santos, B. M. dos, Zibrandtsen, J. F. S., Gunbilig, D., Sørensen, M., Cozzi, F., Boughton, B. A., Heskes, A. M., & Neilson, E. H. J. (2019). Quantification and localisation of formylated phloroglucinol compounds (FPCs) in *Eucalyptus* species. *Frontiers in Plant Science*, *10*.

Sarker, U., & Oba, S. (2018). Drought stress enhances nutritional and bioactive compounds, phenolic acids and antioxidant capacity of *Amaranthus* leafy vegetable. *BMC Plant Biology*, *18*.

Stamp, N. (2004). Can the growth differentiation balance hypothesis be tested rigorously? *Oikos*, *107*(2), 439–448.

Sullivan, J. H. (2005). Possible impacts of changes in UV_B radiation on North American trees and forests. *Environmental Pollution*, *137*(3), 380–389.

Tharayil, N., Suseela, V., Triebwasser, D. J., Preston, C. M., Gerard, P. D., & Dukes, J. S. (2011). Changes in the structural composition and reactivity of Acer rubrum leaf litter tannins exposed to warming and altered precipitation: Climatic stress-induced tannins are more reactive. *New Phytologist*, *191*(1), 132–145.

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients*, *2*(12), 1231–1246.

Wam, H. K., Stolter, C., & Nybakken, L. (2017). Compositional changes in foliage phenolics with plant age, a natural experiment in boreal forests. *Journal of Chemical Ecology*, *43*(9), 920–928.

Wang, S., Alseekh, S., Fernie, A. R., & Luo, J. (2019). The structure and function of major plant metabolite modifications. *Molecular Plant*, *12*(7), 899–919.

Wang, X.-F., Liu, J.-F., Gao, W.-Q., Deng, Y.-P., Ni, Y.-Y., Xiao, Y.-H., Kang, F.-F., Wang, Q., Lei, J.-P., & Jiang, Z.-P. (2016). Défense pattern of Chinese cork oak

across latitudinal gradients: Influences of ontogeny, herbivory, climate and soil nutrients. *Scientific Reports*, *6*(1), 27269.

Woods, E. C., Hastings, A. P., Turley, N. E., Heard, S. B., & Agrawal, A. A. (2012). Adaptive geographical clines in the growth and defence of a native plant. *Ecological Monographs*, *82*(2), 149–168.

Chapter V

Changes in phenolic compounds of *Eucalyptus camaldulensis* affected by the senescence-inducing psyllid *Cardiaspina albitextura*

5.1. Introduction

Plant phenolic compounds have long been considered as classical defensive compounds protecting plants from herbivores (War *et al*., 2012). However, there is increasing evidence suggesting that oxidative pressure from photodamage might be another important factor causing variation of phenolic compounds in the plant (within an individual plant species and/or between species), and herbivory may have exerted little selective pressure compared to photoprotection (Close & McArthur, 2002). Plant secondary metabolites can evolve in response to multiple selective forces and hence serve their function as defensive compounds (Agrawal *et al*., 2012; Züst *et al*., 2012), photoprotective roles (Solovchenko & Merzlyak, 2008), or both roles (Moore *et al*., 2014).

Plant phenolic compounds have been associated with passive and active defence by an array of responses using constitutive and induced phenolic substances. Constitutive PSMs are always present in the plant and used mainly for the prevention of diseases or infections while induced defences are produced at the right time, concentration, and location to be effective in herbivore resistance only after an individual has been damaged to reduce further damage (Tahvanainen *et al.,* 1985; Hammerschmidt & Schultz, 1996; Lin *et al*., 2016). Plant maximises its fitness by balancing between constitutive and induced defences based on pressure from herbivory and available resources. Under low herbivore pressure, plants are known to invest more in constitutive defence; however, under high herbivore pressure, plants deploy various induced defences (Bixenmann *et al.*, 2016). Therefore, induced defences are considered a cost-saving strategy allowing plants to only invest in defence when there is the presence of herbivores (Agrawal, 2000). Phenolic compounds induced responses have been described in various studies, including induction of various phenolic compounds in *Epirrita autumnata* due to larval feeding (Kaitaniemi *et al*., 1998), induction of cinnamic acid and coumaric acid by biting and chewing insects in *Gossypium hirsutum* (Dixit *et al*., 2017) and induction of various tannins in *Eucalyptus globulus* by

the larva of *Mnesampela privata* (Rapley *et al*., 2007). Similarly, a feeding study on a lerp forming psyllid, *Glycaspis brimblecombei* Moore, on *Eucalyptus camaldulensis* did not show any observable symptoms of premature senescence but increased concentration of phenolic compounds such as quercetin glucoside and pentagalloylglucose (Patton *et al*., 2018). Another study on the galling herbivore *Leptocybe invasa* Fisher & La Salle on *Eucalyptus camaldulensis* showed higher quercetin and kaempferol derivatives in the outer part of gall and higher HHDP tannins in inner gall, signalling photoprotection and plant defence, respectively (Isaias *et al*., 2018).

Phenolic compounds exhibit great structural diversity, embodying a variety of functions in plant-herbivore interactions. Tannins are among the most abundant phenolic compounds and are known to defend leaves against insect herbivores by deterrence and toxicity (Barbehenn *et al.,* 2006; Barbehenn and Constabel, 2011). Tannins function as anti-herbivore agents by acting as prooxidants causing oxidative stress to the insect herbivores and damaging gut cellular components and nutrients (Barbehenn & Constabel, 2011; Salminen, 2014). Also, the protein precipitating ability of tannins in neutral and acidic solutions contributes to important defence functions (Marsh et al., 2020). In addition to tannins, another major phenolic group, flavonoids, were found to be feeding deterrents, cytotoxic and interact with different enzymes through complexation. For example, luteolin, quercetin 3-rhamnoside and myricetin 3-rhamnoside have shown deterrent and mortality in insects such as aphids (Mallikarjuna *et al.,* 2004; Sosa *et al.,* 2004). Various formylated phloroglucinol compounds (FPCs) that are widely found across eucalypt species are known to play an important role in defence against herbivory and mediating animal-plant interactions such as feeding preferences of marsupial folivores, such as koalas and possums (DeGabriel *et al.*, 2010; Marsh *et al.*, 2019).

My study species *Eucalyptus camaldulensis* Dehnh., River Red Gum, is an extremely widespread eucalypt, occurring across a wide and discontinuous geographic range in

Australia spanning a wide range of latitudes (McDonald *et al.,* 2009). *Eucalyptus* is heteroblastic and can produce morphologically distinct juvenile, transitional and adult leaves, and the relative abundance of classes of the phenolic compound can vary markedly depending upon the ontogenetic stage (O'Reilly-Wapstra *et al.*, 2007).

My study herbivore species, White Lace Lerp psyllid, *Cardiaspina albitextura* Taylor (Psylloidea: Spondyliaspididae), is one representative of arguably the most important genus of Australian Psylloidea. In Australia, *Cardiaspina* psyllid nymphs are renowned for their capacity to cause severe defoliation and damage to eucalypts leaves and are considered contributors to eucalypt dieback (Steinbauer *et al.*, 2014, 2018). Various aphids and psyllids which are known as senescence feeding herbivores are evolved to feed older leaves that contain higher soluble amino acids. They accelerate the rate of leaf senescence and enhance the nitrogen content in their food (White, 2015). Feeding on leaves by nymphs shifts the balance of photosynthetic pigments leading to declining chlorophyll content and increasing screening pigments like anthocyanins causing reddening of leaves and predisposing affected eucalypt leaves to photodamage (Steinbauer *et al.*, 2014). This reddening, however, might not adversely affect nymphal survival and might be the response to the protection of leaves against photodamage (Close and Beadle, 2003; Steinbauer *et al.*, 2018). *C. albitextura* also shows a preference for older leaves of *E. camaldulensis* for oviposition and feeding sites (Morgan and Taylor, 1988).

This chapter aimed to document changes in foliar phenolics of *E. camaldulensis* affected by a senescence-inducing species of *Cardiaspina* psyllid. The damaged leaves (including canopy and understorey leaves) were studied to investigate changes in foliar phenolics in response to the photodamage-like symptoms such as leaf reddening and chlorotic lesions induced by these small sucking insects.

5.2. Materials and Methods

5.2.1. Study species and sites

The study used twelve trees, including six mature remnant trees having canopy leaves and six saplings having understorey leaves. *Cardiaspina albitextura* infested leaves were collected from a remnant eucalypt woodland known as Fotheringham Nature Reserve, Dandenong, Victoria (37°58'35.28" S, 145°11'40.04" E) in June 2017. Relative leaf age was designated based on leaf node and distance from the terminal end of the branchlet (all branchlets were missing an apical bud). Leaf node was used to classify leaves; younger leaves arose from a node close to the end of branchlet (e.g., nodes 1 to 3) while the older leaves arose from a more distal node (e.g., nodes 10 to 13). Leaves were harvested from each tree and transported to the lab on ice. In the lab, total psyllids numbers (lerps + psyllids) were counted and where leaves were first cleaned with 70% ethanol and freeze-dried in envelopes. My leaf collection showed that among canopy leaves, 26.4% of leaves were of adult morphology, 8.3% were transitional, and 65.3% were juvenile, and among understorey leaves, 2.8% of leaves were of adult morphology, 0% were transitional, and 97.2% were juvenile.

5.2.2. Leaf extraction

Freeze-dried leaves were finely ground to ≤ 0.25 mm using a ball mill (Retsch MM400, Germany) at 30 Hz. 20mg of finely ground leaf powder was weighted in duplicate into 2 mL microtubes, and 1 mL of 80:20 MeOH-H_2O (v/v) was added to each sample, and subsequently mixed using a vortex for two minutes and then sonicated for 10 minutes (Unisonics, Australia). Samples were then centrifuged for 10 minutes at 15,000 rpm at room temperature (Eppendorf 5415D bench centrifuge, Hamburg, Germany). The extract was transferred to a clean labelled microtube. 1 mL of the extract was transferred to HPLC vials for liquid chromatography-mass spectrometry (LC-MS) analysis, and a further 5 μL

of each extract were pooled to create a quality control (QC) sample to be analysed at the same time.

5.2.3. Chromatographic and mass spectrometric analysis

Identification of phenolic compounds was performed by an ultra-high-performance liquid chromatographic system (UPLC, UHPLC+ focused, Thermo Scientific™, Waltham, MA, USA) combined with a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific™, Waltham, MA, USA) and a diode array detector. Data were collected in negative ion mode, scanning a mass range m/z of 100–1500. Negative ion mode was preferred over positive for phenolic compounds analysis for all the subgroups because of its sensitivity, and clearer fragmentation patterns and less extensive fragmentation. The nitrogen gas was used as the sheath and auxiliary and sweep gas, and the spray voltage was set at 3,600 V. The capillary temperature was set to 300°C, with the S-lens RF level set at 64 and an auxiliary gas heater temperature of 310°C. A Hypersil GOLD C_{18} column (150×2.1 mm, 1.9 μm, Thermo Scientific, USA) was used with the column compartment temperature set to 30°C, and a flow rate was maintained at 0.3mL/min throughout data acquisition. The mobile phase consists of (A) 0.1% formic acid and (B) acetonitrile. A linear gradient was used beginning with 2% of B and reaching 100% of B at 15 mins, then kept steady at 100% of B until 18 minutes, and then returned to an initial condition where it was held for 2 min. The MS/MS analyses were carried out by automatic fragmentation, where the three most intense mass peaks were fragmented. The mass spectrometric (MS) analysis, including the prediction of chemical formula and exact mass calculation, was performed by using Thermo Xcalibur Qual Browser software version 3.0.63 (Thermo Scientific, USA). The samples were injected into the mass spectrometry randomly (order organised by using the "RAND" function in Microsoft Excel), with blank and QC samples being injected every 10^{th} sample.

5.2.4. Data processing

The raw files from X-calibur were imported to Genedata Expressionist Refiner MS version 12.0, Basel, Switzerland (https://www.Genedata.com/). Various filters such as RT structure removal, chemical noise subtraction, peak detection, isotope clustering, adduct detection and singleton filter were used. Further, quality control data was used for sample normalisation to minimise and correct for any batch variation. The generated data matrix of features (consisting of peak identifier, m/z value, peak volume, and retention time) was exported, visualised, and analysed in Genedata Analyst™ 12.0.6 software (Genedata AG, Basel, Switzerland). The samples were annotated based on the number of psyllids, age of leaves (i.e., younger and older) and position of leaves (i.e., understorey and canopy).

5.2.5. Data analyses

Principal Components Analysis (PCA) (an unsupervised method) and partial least squares (PLS) analysis (a supervised method) was used to visualise metabolite variation between sample groups and treatments. A two-group test in Genedata was used to compare features between two treatments, and K-groups analysis that uses ANOVA was used to compare more than two treatments. The most significant values were ranked based on low *P*-values (α) and higher effect size after Bonferroni correction (at $\alpha = 0.05$). To perform a Bonferroni correction, the critical *P*-values were divided by the number of features (in this case, 8400 features), and hence only the most statistically significant features were selected from the volcano plot in Genedata Analyst. These features were tentatively identified by using an online mass data bank, standards, and MS/MS data as per Chapter 2. A multivariate linear regression model for continuous explanatory variables (number of total psyllids) was used in Genedata to consider the effect of the number of psyllids on changes in phenolics.

The data were transformed using a square root transformation and tested for normality using skewness and kurtosis test (*P* value between -1.96 and +1.96) (Mardia, 1970). The

non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) was applied for statistically significant differences between the two groups ($P < 0.05$). The data were analysed using a generalised linear model (GLM) with random effects for individual trees. Psyllids abundance was treated as a covariate to avoid performing two sets of analyses. Tree size (Canopy *versus* Understorey) was a statistically significant term in the preliminary analyses, therefore, treated data for each type separately by splitting the data imported from Excel. SPSS Statistical Software (version 25; SPSS Inc., Chicago, IL, USA) was used to perform all statistical tests.

5.3. Results

5.3.1. Effect of tree type (understorey and canopy)

The principal component analysis visualises the grouping based on the tree type, which showed a clear separation between understorey and canopy leaves (Figure 5.1). PC 1 showed 13.3% of variation captured by principal components separation, and PC 2 showed 2.4% of variation captured by principal components separation. Based on fold change and using a *P*-value < 0.001, features were deemed significantly different between the two classes were selected using by using a two-group test in Genedata Analyst. These features were identified as galloyl glucose derivative, benzyl galloyl glucose, hydrophenyl propanoic acid and phenyl propanoic acid derivative (Table 5.1). Galloyl glucose derivatives strongly influence PC 2, while phenyl propanoic acid strongly influences PC 1. Various galloyl glucose derivatives were found to be higher in concentration in understorey trees, and phenyl propanoic acids were found to be higher in canopy leaves (Figure 5.2).

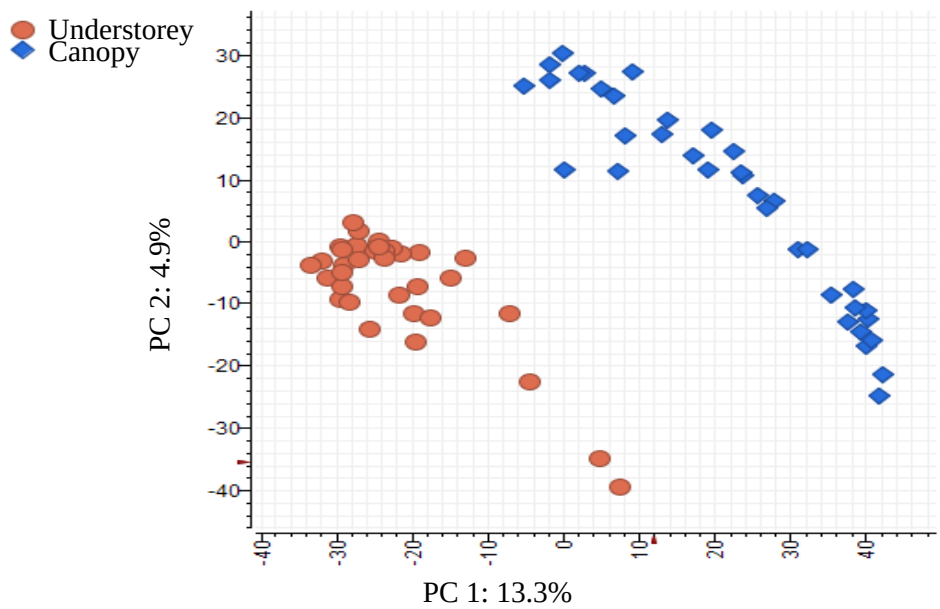


Figure 5.1. The PCA plot of *Eucalyptus camaldulensis* leaves based on tree types. Each point represents a metabolite profile of an individual leaf.

Table 5.1. Major compounds identified by the two-group test for tree types between canopy and understorey trees (*P*-value ≤0.001).

$[M - H]^-$	Rt(mins)	Major Fragments	Tentative identification
507.114	4.1	313.0615, 300.9984, 271.0455, 169.0129	galloyl glucose derivative
421.1143	5.74	313.0615, 169.0129, 125.0225	galloyl glucose derivative
165.0548	6.15	147.0446, 121.0653	hydroxyphenyl propanoic acid
327.1083	4.47	147.0446, 121.0653	phenyl propanoic acid derivative
633.3801	12.94	~	unknown

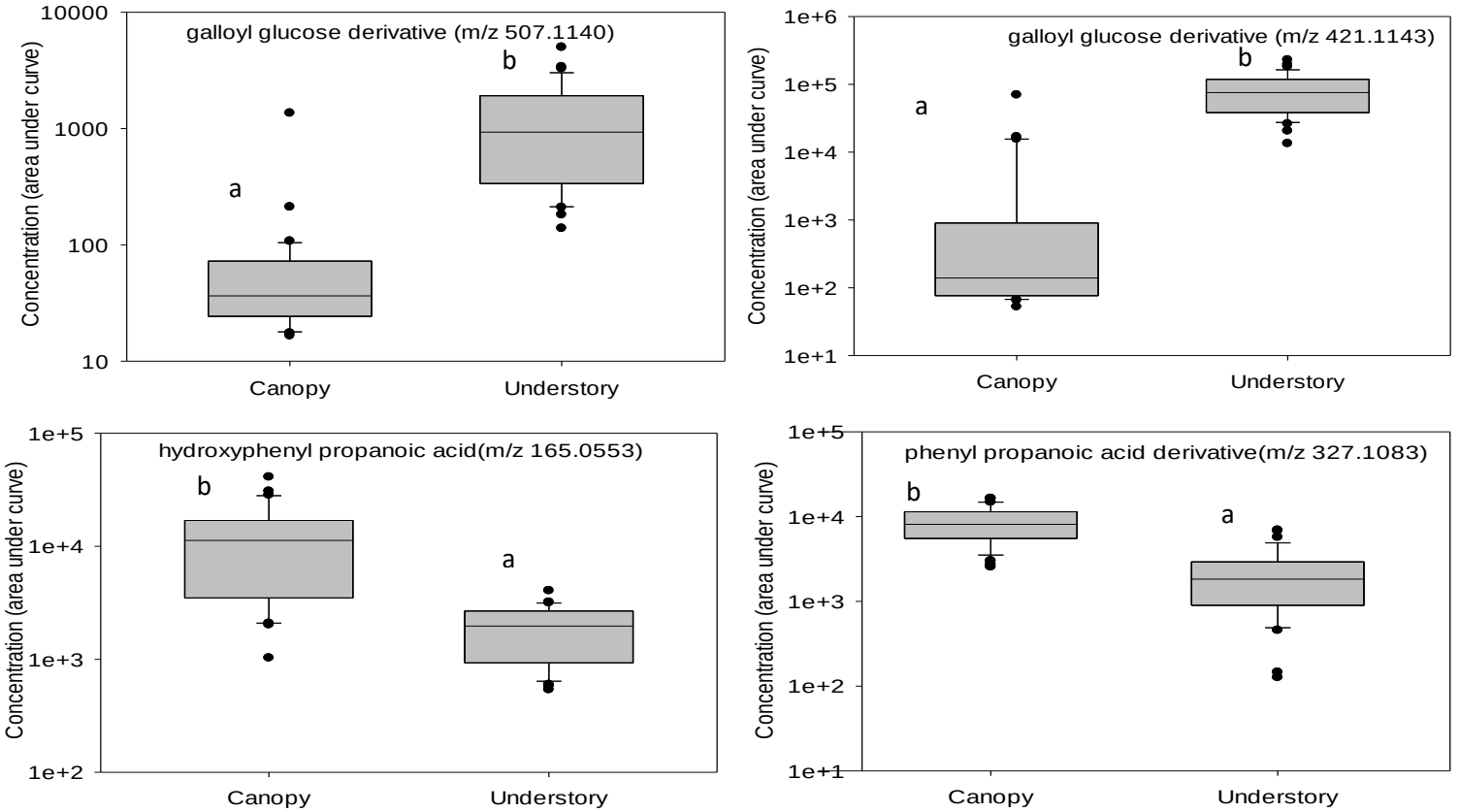


Figure 5.2. Box plots showing top statistically significant phenolic compounds between canopy and understorey leaves. In each box plot, the horizontal line that is crossing the box is the median, lower and upper quartiles are at the bottom and top of the box, respectively, and the whiskers are the maximum and minimum values.

5.3.2. Effect of psyllids

A PCA plot revealed that PC 1 explained 62.8% of the variation in the data, and PC 2 explained 12.4% of the variation (Figure 5.3). A multivariate linear regression model identified the features that were deemed significantly different at *P*-value < 0.001. Out of six features, four were identified as phenolic compounds (Table 5.2). Statistical results of generalised linear model analyses (Table 5.3) reveal that for all four phenolic compounds, the tree is the most important factor explaining differences variation in the data for understorey trees. Only in the case of canopy trees, the density of psyllids had a statistically significant effect on the concentrations of the compounds considered (*P*-value < 0.05). Also, both leaf age and tree id were significant factors for variation of kaempferol glycoside in understorey trees.

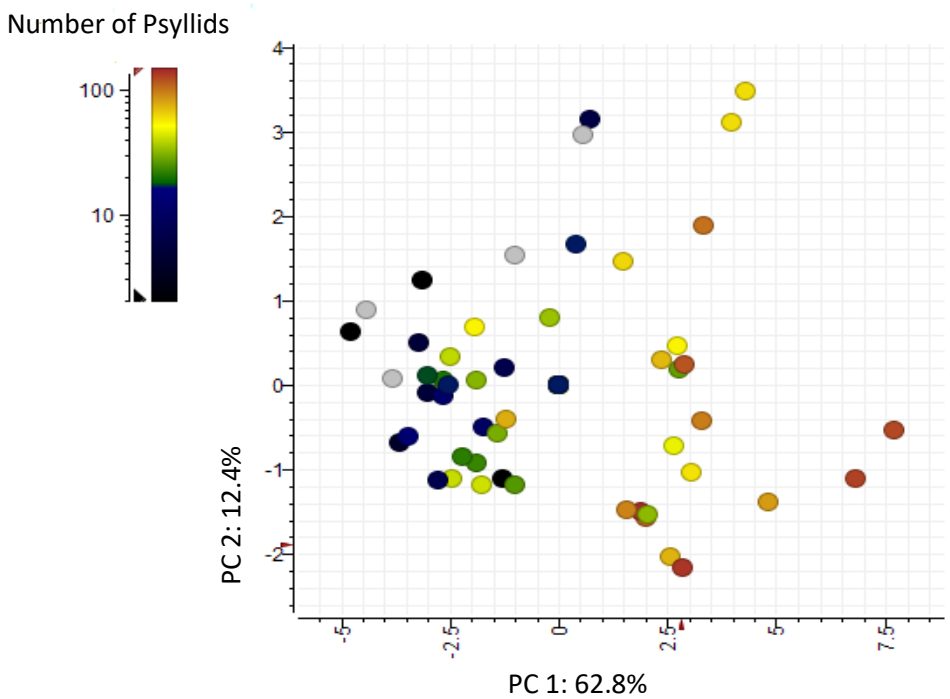


Figure 5.3. PCA plot of *Eucalyptus camaldulensis* leaves based on the number of psyllids on each leaf. Each point represents the metabolite profile of an individual leaf.

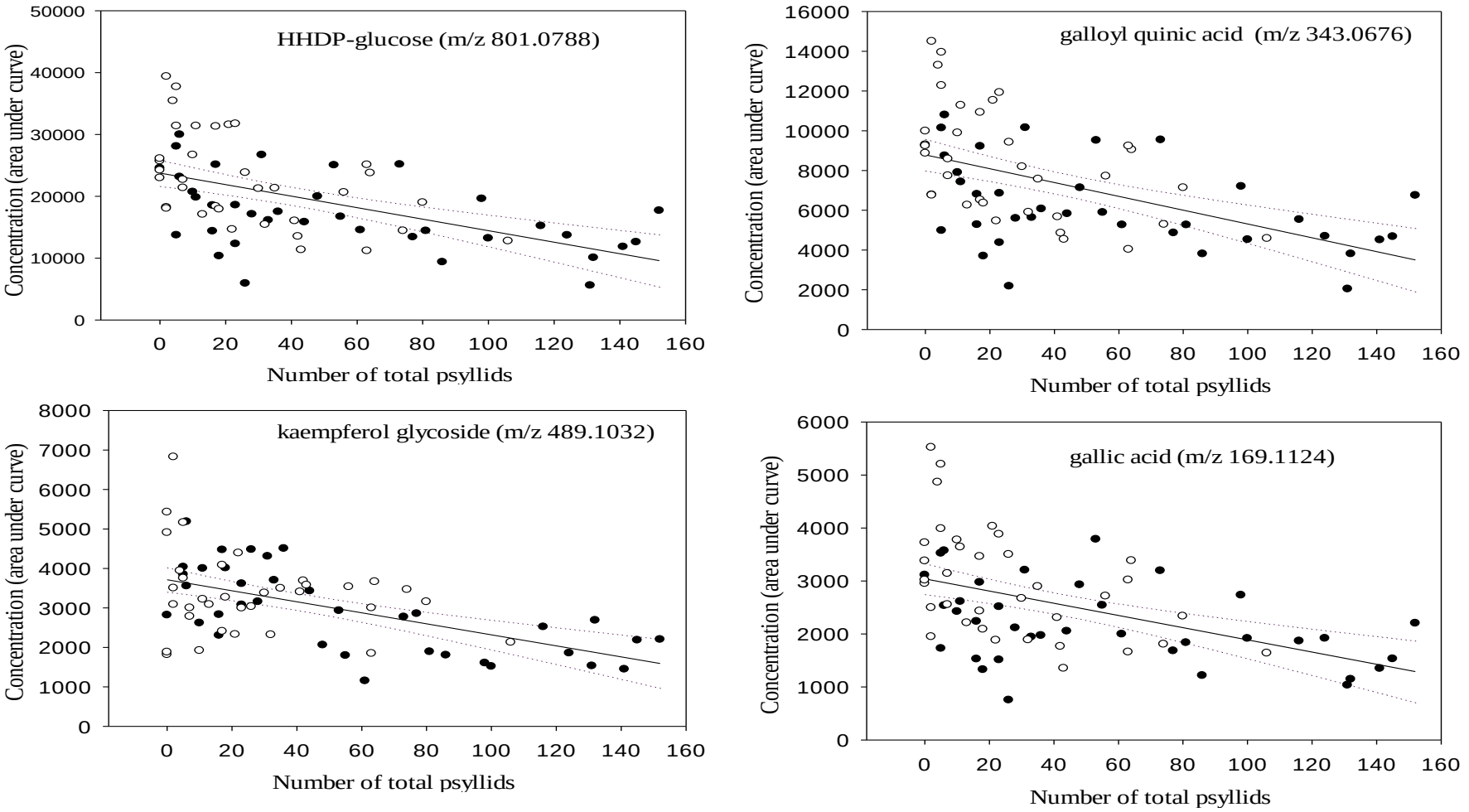


Figure 5.4. Regressions showing the relationship between various phenolic compounds and total psyllids. Regression lines are with 95% confidence intervals. Symbols: filled circle (●) = canopy leaves, empty circle (○) = understorey leaves.

Table 5.2. The most significant compounds identified from the linear regression model.

$[M-H]^-$	Rt(mins)	Major Fragments	Tentative identification
801.0788	3.01	633.07, 300.99,275.0234, 169.0129	HHDP-glucose derivative
489.1032	6.52	447.09622, 429.0822, 285.0399	kaempferol glycoside
169.0143	2.68	125.0231	gallic acid
343.0676	2.86	191.0551, 169.0132	galloyl quinic acid
187.0970	6.46	169.0864, 125.0962	succinic acid derivative
277.0211	7.74	~	unknown

Table 5.3. Statistical results of mixed model analyses of the compounds identified in PCA plot.

Tree size and compound	Source	*d.f.*	*F*	P
Understory				
HHDP-glucose	Intercept	1	308.534	<0.010
	Error	14.668		
	Total psyllids (covariate)	1	1.492	0.235
	Error	22		
	Tree	5	8.124	**<0.010**
	Error	8.239		
	Relative leaf age	1	0.105	0.754
	Error	7.931		
	Relative leaf age* tree	5	0.385	0.854
	Error	22		
Galloyl quinic acid				
	Intercept	1	309.077	<0.010
	Error	15.344		
	Total psyllids (covariate)	1	1.508	0.232
	Error	22		
	Tree	5	7.757	**<0.010**
	Error	8.350		
	Relative leaf age	1	0.041	0.846
	Error	8.032		
	Relative leaf age* tree	5	0.373	0.862
	Error	22		
Gallic acid				
	Intercept	1	278.839	<0.010
	Error	15.354		
	Total psyllids (covariate)	1	0.907	0.351
	Error	22		
	Tree	5	5.221	**0.026**
	Error	6.994		
	Relative leaf age	1	0.692	0.434
	Error	6.807		
	Relative leaf age* tree	5	0.609	0.694
	Error	22		
Kaempferol glycoside				
	Intercept	1	250.443	<0.010
	Error	15.72		
	Total psyllids (covariate)	1	0.000	0.983
	Error	22		
	Tree	5	9.530	**<0.010**
	Error	9.774		
	Relative leaf age	1	7.227	0.024
	Error	9.320		
	Relative leaf age* tree	5	0.267	0.926
	Error	22		
Canopy				

HHDP-glucose	Intercept	1	310.241	<0.010
	Error	20.149		
	Total psyllids (covariate)	1	6.20	**0.02**
	Error	23		
	Tree	5	8.12	0.19
	Error	6.25		
	Relative leaf age	1	0.0	0.99
	Error	5.37		
	Relative leaf age* tree	5	1.15	0.36
	Error	23		
Galloyl quinic acid				
	Intercept	1	288.958	<0.010
	Error	19.539		
	Total psyllids (covariate)	1	4.998	**0.035**
	Error	23		
	Tree	5	2.058	0.200
	Error	6.176		
	Relative leaf age	1	0.046	0.838
	Error	5.351		
	Relative leaf age* tree	5	1.224	0.330
	Error	23		
Gallic acid				
	Intercept	1	287.782	<0.010
	Error	15.587		
	Total psyllids (covariate)	1	6.845	**<0.010**
	Error	23		
	Tree	5	2.799	0.119
	Error	6.101		
	Relative leaf age	1	0.044	0.841
	Error	5.330		
	Relative leaf age* tree	5	1.299	0.299
	Error	23		
Kaempferol glycoside				
	Intercept	1	442.708	<0.010
	Error		9.753	
	Total psyllids (covariate)	1	18.429	**<0.010**
	Error	23		
	Tree	5	2.382	0.172
	Error	5.396		
	Relative leaf age	1	0.120	0.743
	Error	5.120		
	Relative leaf age* tree	5	3.544	**0.016**
	Error	23		

5.3.3. Effect of leaf age

Principal component analysis was used to visualise the grouping based on their age (Figure 5.5). PC 1 showed 50.8% of variation captured by principal components separation, and PC 2 showed 27.9% of variation captured by principal components separation (Figure 5.5). Based on fold change and *P*-value ≤ 0.001, features deemed significantly different between the two classes (younger and older leaves) were selected using the two-group test in Genedata Analyst. These features were identified as monogalloyl glucose, trigalloyl glucose, two ellagitannins and a fatty acid ester (Table 5.4). Galloyl glucose derivatives strongly influence PC 2, while phenyl propanoic acid strongly influences PC 1. Trigalloyl glucose derivatives strongly influence PC 2, while HHDP galloyl glucose and di-HHDP galloyl glucose strongly influence PC 1.

There was an increased concentration of monogalloyl glucose, di-HHDP-glucose and HHDP glucose in younger leaves; however, trigalloyl glucose was higher in concentration in older leaves (Figure 5.6).

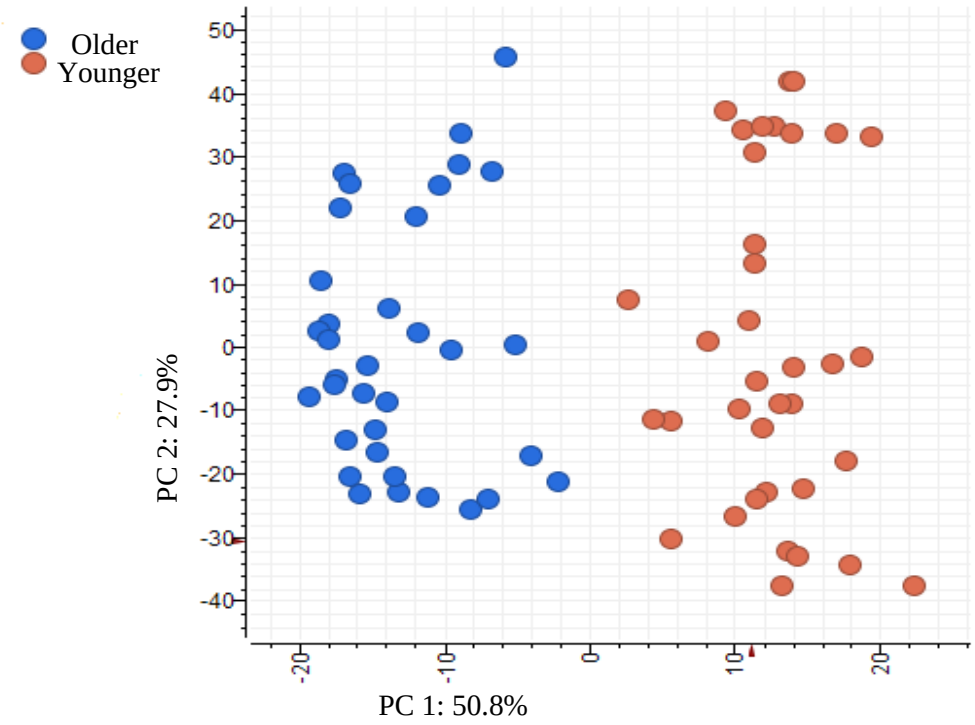


Figure 5.5. PCA plot of *Eucalyptus camaldulensis* based on the relative age of leaves. Each point represents a metabolite profile of an individual leaf.

Table 5.4. Major compounds identified from the two-group tests comparing younger and older leaves.

$[M-H]^-$	**Rt(mins)**	**Major Fragments**	**Tentative identification**
633.0735	3.71	300.9996, 275.0235, 169.0129	HHDP galloyl glucose
331.0669	3.14	271.0459, 169.0132, 151.0012, 125.0234	mono galloyl glucose
635.0892	4.43	465.0669, 313.0615, 300.9984, 271.0455, 169.0129	trigalloyl glucose
783.0679	3.39	481.0619, 300.9984, 275.0196	di-HHDP-glucose
345.2286	8.65	199.1704, 181.1598, 145.0508	fatty acid esters

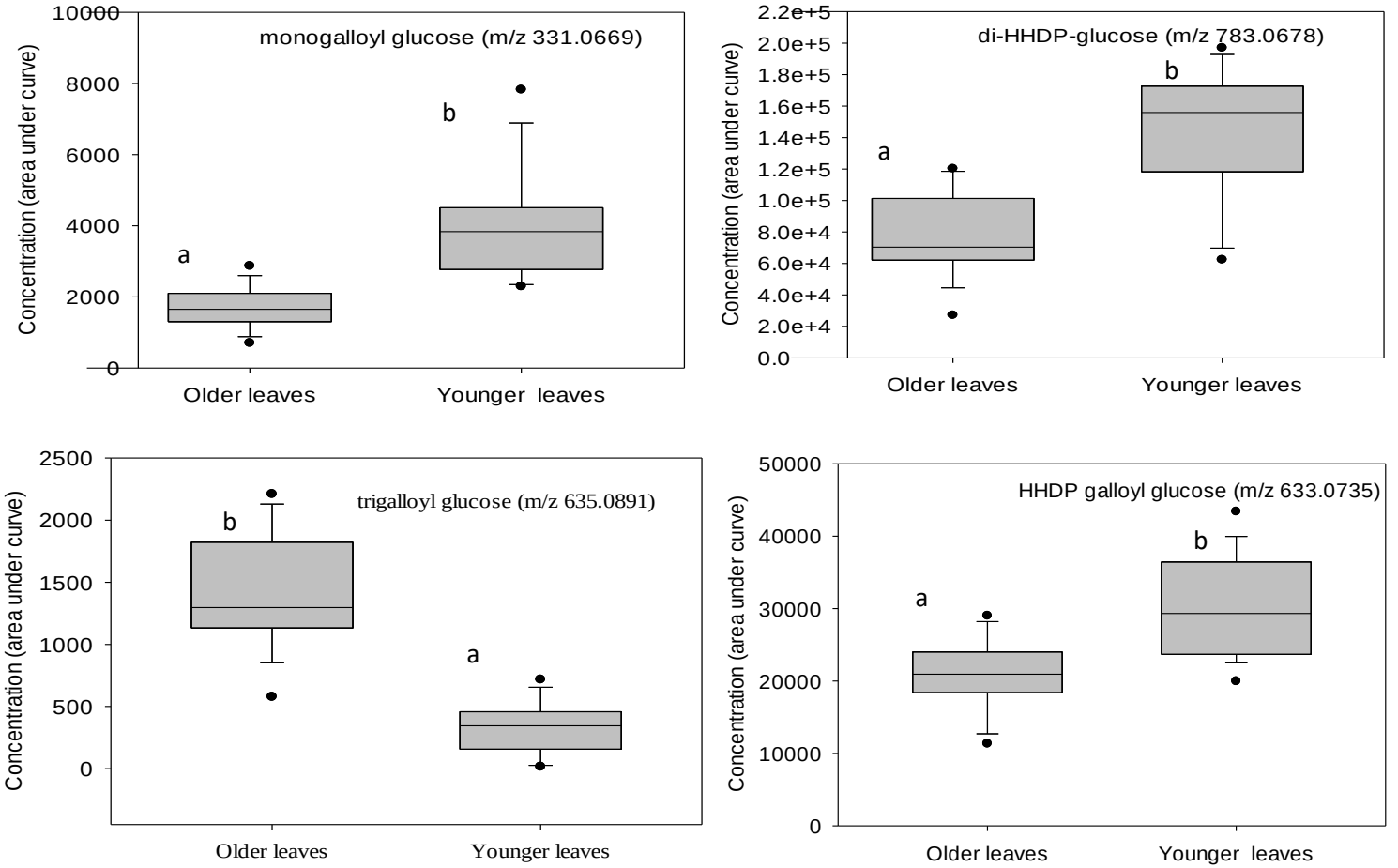


Figure 5.6. Box plots showing the relative concentration of top statistically significant phenolic compounds between older and younger leaves. In each box plot, the horizontal line crossing the box is the median, lower and upper quartiles are at the bottom and top of the box, respectively, and the whiskers are the maximum and minimum values.

5.4. Discussion

This study documents and quantify changes in foliar phenolics of *E. camaldulensis* affected by a senescence-inducing species of *Cardiaspina* psyllid. I showed that the concentration of phenolic compounds in the leaves significantly vary with tree type or position of leaves, herbivory and various stages of growth and development. My study also showed that the effect of phenolic compounds on insect herbivory is a very complex trait and depend on the type of herbivore, nutritional context, and phenology. Also, there was a negative relationship between psyllid abundance and the concentration of various selected phenolic compounds.

5.4.1. Effect of tree type (understorey and canopy)

The natural light gaps by treefalls can cause significant variation in the amount of sunlight that plants receive and can affect resources available to plants and hence play an important role in forest dynamics (Dominy *et al.,* 2003). Variation in light and growing conditions of canopy and understorey plants may therefore affect populations of insect herbivores. Factors such as solar radiation can modify foliage nutritional quality and influence the feeding behaviour and oviposition patterns of insect herbivores (Corff & Marquis, 1999). My understorey leaves were found to have higher concentrations of galloylglucose derivatives, and canopy leaves had higher concentrations of propanoic acid derivatives. Understorey leaves receive less direct solar radiation compared to canopy leaves. Such resource limitation of light may favour low photosynthesis and slow growth and more significant investment in leaf defence by the production of phenolics compounds. Hence according to the resource availability hypothesis, these understorey leaves are predicted to invest more in various tannins, and leaf toughness and herbivory are expected to be lower in these leaves (Dominy *et al.,* 2003). My result supports this hypothesis as we have fewer psyllids on understorey leaves and a higher concentration of galloylglucoses.

My result was further supported by the study by Dudt & Shure (1994), who showed that slow-growing and shade-tolerant plants species have higher phenolic levels and experience higher herbivory than fast-growing shade-intolerant species. Although the concentration of phenolic compounds in my studies has been shown to affect the herbivore density, studies have shown that herbivore performance on the leaves cannot be determined by phenolic contents alone as they can be affected by various other factors such as light, leaf age and toughness, nutrient, and nitrogen content (Zhang *et al.*, 2009).

5.4.2. Effect of psyllids

For all four phenolic compounds, the number of psyllids was the most significant factor explaining differences in the data for canopy trees. In contrast, the tree size was a significant factor for the variation of all four phenolic compounds for understorey trees. There was a negative correlation between the concentration of various phenolic compounds (i.e., HHDP-glucose, galloylquinic acid derivatives, kaempferol glycoside, gallic acid) and the number of psyllids on canopy leaves, i.e., a lower number of psyllids were found on leaves with high hydrolysable tannins and flavonoids. Foliar concentrations of phenolic compounds and other carbon-based nutrients typically increases with increasing sunlight in canopy leaves compared to understorey leaves (Nichols-Orians, 1991). The non-preference and/or deterrent of these leaves with high phenolic compounds might be explained by the prooxidant activity of hydrolysable tannins (Barbehenn and Constabel, 2011; Salminen and Karonen, 2011). My study also showed that psyllids were negatively correlated with foliar flavonoids. These flavonoids are also known to have cytotoxic properties (Boussahel, 2015), and are known to have feeding deterrents against many insect pests (Panche *et al.*, 2016). The GLM analyses showed that covariate (total psyllid) is only significant for the canopy leaves and not the understorey leaves, which differs from the results of my linear regression figures. This might be because the linear regressions only consider one possible predictor variable on the concentration of the plant

phenolic compounds, but there can be numerous explanations for the differences in the abundance of psyllids on canopy and understory trees, and we only measured on the aspect plant phenolics. Also, one of the explanations for the variation of phenolic compounds based on the tree size in the understorey could be due to spatial distribution of plants resulting in the variation of the availability of the light (Covelo & Gallardo, 2004). These correlations between foliar phenolics concentration and light availability have been observed in various previous studies (Nichols-Orians, 1991; Shure and Wilson, 1993). Further, relative leaf age does not seem to be an important factor, possibly because all leaves are likely to have been >6 months old despite the node of harvest.

5.4.3. Effect of leaf age

Various hydrolysable tannins such as HHDP-glucose, galloyl glucose were found in higher concentrations in younger leaves compared to the older leaves (Figure 5.6). This finding also supports the study by Salminen (2004), who reported that young leaves are richer in hydrolysable tannins than older leaves. The production of a higher concentration of hydrolysable tannins in younger leaves might help them to defend better against intense selective pressure such as herbivory. Also, various hydrolysable tannins are known to prevent the degradation of anthocyanin and increase the pigment stability leading to maintenance of the redness of young leaves of some species (Luo *et al.,* 2019). Another possible explanation of low hydrolysable tannins in older leaves compared to younger leaves might be the compositional shift from low molecular weight phenolics to condensed tannins in older leaves (Wam *et al.,* 2017).

5.5. References

Agrawal, A. A., Hastings, A. P., Johnson, M. T. J., Maron, J. L., & Salminen, J.-P. (2012). Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science (New York, N.Y.)*, *338*(6103), 113–116.

Barbehenn, R. V., Jones, C. P., Hagerman, A. E., Karonen, M., & Salminen, J.-P. (2006). Ellagitannins have greater oxidative activities than condensed tannins and galloyl glucoses at high pH: Potential impact on caterpillars. *Journal of Chemical Ecology*, *32*(10), 2253–2267.

Barbehenn, R. V., & Peter Constabel, C. (2011). Tannins in plant–herbivore interactions. *Phytochemistry*, *72*(13), 1551–1565.

Boussahel, S., Speciale, A., Dahamna, S., Amar, Y., Bonaccorsi, I., Cacciola, F., Cimino, F., Donato, P., Ferlazzo, G., Harzallah, D., & Cristani, M. (2015). Flavonoid profile, antioxidant and cytotoxic activity of different extracts from Algerian *Rhamnus alaternus* L. bark. *Pharmacognosy Magazine*, *11*(Suppl 1), S102–S109.

Close, D. C., Davies, N. W., & Beadle, C. L. (2001). Temporal variation of tannins (galloylglucoses), flavonols and anthocyanins in leaves of *Eucalyptus nitens* seedlings: Implications for light attenuation and antioxidant activities. *Functional Plant Biology*, *28*(4), 269–278.

Close, D. C., & Beadle, C. L. (2003). Alternate energy dissipation. Phenolic metabolites and the xanthophyll cycle. *Journal of Plant Physiology*, *160*(4), 431–434.

Close, D. C., & McArthur, C. (2002). Rethinking the role of many plant phenolics: Protection from photodamage not herbivores? *Oikos*, *99*(1), 166–172.

Corff, J. L., & Marquis, R. J. (1999). Differences between understorey and canopy in herbivore community composition and leaf quality for two oak species in Missouri. *Ecological Entomology*, *24*(1), 46–58.

DeGabriel, J. L., Moore, B. D., Marsh, K. J., & Foley, W. J. (2010). The effect of plant secondary metabolites on the interplay between the internal and external environments of marsupial folivores. *Chemoecology*, *20*(2), 97–108.

Dudt, J. F., & Shure, D. J. (1994). The Influence of Light and Nutrients on Foliar Phenolics and Insect Herbivory. *Ecology*, *75*(1), 86–98.

Dominy, N. J., Lucas, P. W., & Wright, S. J. (2003). Mechanics and chemistry of rain forest leaves: Canopy and understorey compared. *Journal of Experimental Botany*, *54*(390), 2007–2014.

Gherlenda, A. N., Moore, B. D., Haigh, A. M., Johnson, S. N., & Riegler, M. (2016). Insect herbivory in a mature *Eucalyptus* woodland canopy depends on leaf phenology but not CO_2 enrichment. *BMC Ecology*, *16*(1), 47.

Hammerschmidt, R., & Schultz, J. C. (1996). Multiple defenses and signals in plant defense against pathogens and herbivores. In J. T. Romeo, J. A. Saunders, & P. Barbosa (Eds.), *Phytochemical Diversity and Redundancy in Ecological Interactions* (pp. 121–154). Springer US.

Hunter, M. D., & Schultz, J. C. (1995). Fertilisation mitigates chemical induction and herbivore responses within damaged oak trees. *Ecology*, *76*(4), 1226–1232.

Lawler, I. R., Foley, W. J., Eschler, B. M., Pass, D. M., & Handasyde, K. (1998). Intraspecific variation in *Eucalyptus* secondary metabolites determines food intake by folivorous marsupials. *Oecologia*, *116*(1–2), 160–169.

Liu, T., Li, Z., Li, R., Cui, Y., Zhao, Y., & Yu, Z. (2019). Composition analysis and antioxidant activities of the *Rhus typhina* L. stem. *Journal of Pharmaceutical Analysis*,*9*(5), 332–338.

Lowman, M. D. (1985). Temporal and spatial variability in insect grazing of the canopies of five Australian rainforest tree species. *Australian Journal of Ecology*, *10*(1), 7–24.

Luo, H., Li, W., Zhang, X., Deng, S., Xu, Q., Hou, T., Pang, X., Zhang, Z., & Zhang, X. (2019). In planta high levels of hydrolysable tannins inhibit peroxidase mediated anthocyanin degradation and maintain abaxially red leaves of *Excoecaria Cochinchinensis*. *BMC Plant Biology*, *19*(1), 315.

Mallikarjuna, N., Kranthi, K. R., Jadhav, D. R., Kranthi, S., & Chandra, S. (2004). Influence of foliar chemical compounds on the development of *Spodoptera litura* (Fab.) in interspecific derivatives of groundnut. *Journal of Applied Entomology*, *128*(5), 321–328.

Marsh, K. J., Wallis, I. R., Kulheim, C., Clark, R., Nicolle, D., Foley, W. J., & Salminen, J.-P. (2019). New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. *The New Phytologist*, *225*(1), 488–498.

McDonald, M. W., Brooker, M. I. H., & Butcher, P. A. (2009). A taxonomic revision of *Eucalyptus camaldulensis* (Myrtaceae). *Australian Systematic Botany*, *22*(4), 257–285.

Moore, B. D., Andrew, R. L., Külheim, C., & Foley, W. J. (2014). Explaining intraspecific diversity in plant secondary metabolites in an ecological context. *New Phytologist*, *201*(3), 733–750.

Moore, B. D., Wallis, I. R., Wood, J. T., & Foley, W. J. (2004). Foliar nutrition, site quality, and temperature influence foliar chemistry of tallowwood (*Eucalyptus microcorys*). *Ecological Monographs*, *74*(4), 553–568.

Morgan, F. D., & Taylor, G. S. (1988). The White Lace Lerp in Southeastern Australia. In A. A. Berryman (Ed.), *Dynamics of Forest Insect Populations: Patterns, Causes, Implications* (pp. 129–140). Springer US.

O'Reilly-Wapstra, J. M., Humphreys, J. R., & Potts, B. M. (2007). Stability of genetic-based defensive chemistry across life stages in a *Eucalyptus* species. *Journal of Chemical Ecology*, *33*(10), 1876–1884.

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: An overview. *Journal of Nutritional Science*, *5*, e47.

Salminen, J.-P. (2014). The chemistry and chemical ecology of ellagitannins in plant–insect interactions: From underestimated molecules to bioactive plant Constituents. In *Recent Advances in Polyphenol Research* (pp. 83–113). John Wiley & Sons, Ltd.

Salminen, J.-P., & Karonen, M. (2011). Chemical ecology of tannins and other phenolics: We need a change in approach: Chemical ecology of tannins. *Functional Ecology*, *25*(2), 325–338.

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defence mechanism in plants under stressful conditions. *Journal of Botany*, *2012*, 1–26.

Solovchenko, A. E., & Merzlyak, M. N. (2008). Screening of visible and UV radiation as a photoprotective mechanism in plants. *Russian Journal of Plant Physiology*, *55*(6), 719-737.

Sosa, T., Chaves, N., Alias, J. C., Escudero, J. C., Henao, F., & Gutiérrez-Merino, C. (2004). Inhibition of mouth skeletal muscle relaxation by flavonoids of *Cistus ladanifer* L.: A plant defence mechanism against herbivores. *Journal of Chemical Ecology*, *30*(6), 1087–1101.

Steinbauer, M. J., Burns, A. E., Hall, A., Riegler, M., & Taylor, G. S. (2014). Nutritional enhancement of leaves by a psyllid through senescence-like processes: Insect manipulation or plant defence? *Oecologia*, *176*(4), 1061–1074.

Steinbauer, Martin J, Salminen, J.-P., & Watson, S. J. (2018). Yellow, red, dead: The nutritional consequences for *Cardiaspina densitexta* (Hemiptera: Aphalaridae) nymphs of inducing senescence in old *Eucalyptus fasciculosa* leaves. *Austral Entomology*, *57*(2), 265–278.

Tahvanainen, J., Helle, E., Julkunen-Tiitto, R., & Lavola, A. (1985). Phenolic compounds of willow bark as deterrents against feeding by mountain hare. *Oecologia*, *65*(3), 319–323.

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., & Sharma, H. C. (2012). Mechanisms of plant defence against insect herbivores. *Plant Signaling & Behavior*, *7*(10), 1306–1320.

White, T. C. R. (2003). Nutrient retranslocation hypothesis: A subset of the flush-feeding/senescence-feeding hypothesis. *Oikos*, *103*(1), 217–217.

Chapter VI

Synthesis

6.1. Introduction

Plant phenolic compounds were long considered classical defence compounds against herbivores; therefore, most published literature concerning foliar phenolic compounds evoke a defensive role against herbivores (vertebrate especially but possibly less so invertebrate herbivores) (Close and McArthur, 2002; Salminen and Karonen, 2011). However, these same phenolic compounds have recently received attention for their possible role in protecting plants from abiotic stress, such as photodamage caused by reactive oxygen species (Close and McArthur, 2002). Most of the published literature relating to eucalypts uses total phenolic content to consider the biotic and abiotic effects of phenolics in plants and on herbivores, and Folin-Ciocalteu (FC) method is generally used for the estimation of total phenolic content (Blainski *et al*., 2013; Way *et al.*, 2020). However, qualitative and quantitative determination of phenolic compounds by FC method is hampered by their structural complexity and diversity and does not necessarily correlate with biological or antioxidant activity (Appel *et al.*, 2001; Lester *et al.*, 2012; Amorati & Valgimigli, 2015; Way *et al*., 2020).

Tannins are one of the most abundant phenolic compounds in the family Myrtaceae, especially among eucalypts (Macauley and Fox, 1980; Yoshida *et al*., 2010). Hydrolysable tannins are the most abundant tannin subgroup (i.e., 64 ± 28% of hydrolysable tannins out of total tannins), and ellagitannins are the dominant hydrolysable tannins, with some having concentrations > 100 mg g^{-1} of dry weight in eucalypts (Marsh *et al*., 2017). The protein precipitation capacity (PPC) of tannins was traditionally thought to be responsible for their anti-herbivore activity. Hence, this was used as a functional measure to estimate tannin activity (Salminen and Karonen, 2011). Since condensed tannins and gallotannins were known to precipitate proteins, they were given much importance over ellagitannins. However, newer findings show that ellagitannins have much higher *in vitro* prooxidant

activities causing oxidative stress and damaging insect gut lumen than gallic acid derivatives, gallotannins or proanthocyanidins (Barbehenn and Constabel, 2011; Salminen, 2014). These findings provide a different explanation than traditional theory based on PPC because the prooxidant activity of tannin is reversely proportional to protein precipitation capacity and *vice versa*. On the other hand, ellagitannins also have active antioxidant properties within plant cells, which in this context of oxidative stress might become a "double-edged sword" (Mullen *et al*., 2002; Castañeda-Arriaga *et al*., 2018).

Plant polyphenolic compounds, including ellagitannins and gallotannins, can even vary between individual trees grown side by side and can dramatically change with season and module age, and this is true for most plant traits. Also, each species has a unique tannin composition; therefore, tannin-herbivore interactions in one species of plant cannot be readily extrapolated to explain tannin-herbivore interactions in other species and also may rarely correlate with herbivory feeding responses (Marsh *et al.*, 2020). The matter is further complicated by the varied tolerance level of different groups of tannins by different herbivores (insect and mammalian) (Barbehenn and Constabel, 2011).

My study of representative genera within family Myrtaceae has shown both qualitative and quantitative variation in tannins and correlations possibly reliant on several classes of phenolic compounds. I used an untargeted metabolomics-based approach to investigate contrasting functions of phenolic compounds in five genera of myrtaceous plants. My objectives were to (a) identify and document phenolic compounds expressed by these species (Chapter II), (b) document and quantify the influence of seasonal and ontogenetic factors on phenolic composition (Chapter III), (c) investigate the role of elevated UV_A on the expression of foliar phenolic compounds in five genotypes of *Eucalyptus camaldulensis* (Chapter IV) and (d) document and quantify changes in foliar phenolics of *E. camaldulensis* affected by a senescence-inducing species of *Cardiaspina* psyllid

(Chapter V). Time permitting, I would have liked to investigate the relationship between oxidative activity, tolerance of photodamage and insect herbivory. The summary and conclusion of my finding are shown in my experimental and empirical investigations chapter below.

6.2.1. Chapter II

Identification of known and unknown foliar phenolic compounds in five species of Myrtaceae (*Angophora floribunda, Callistemon salignus, Corymbia ficifolia, Eucalyptus camaldule*nsis and *Syzygium smithii*)

I characterised 48 phenolic compounds, mainly comprising hydrolysable tannins and flavonoids and phenolic acids. These hydrolysable tannins are synthesised from galloyl glucoses and pentagalloyl glucose serves as the precursor molecule for both higher-molecular-weight ellagitannins and gallotannins. Further, several phenolic compounds were shared among taxa. The antioxidant and prooxidant activities of these phenolic compounds, as estimated from the published literature, are given in Table 6.1 and Table 6.2. These data relate directly to my identifications of these compounds and their structures which is why I include these tables here. However, their biological activities have relevance to all my chapters, so I present a synthesis of their importance to my studies later in the chapter.

Table 6.1. *In vitro* antioxidant activities of phenolic compounds identified during my research. Parameters adapted from Fukumoto & Mazza (2000).

Phenolic compound	Group	Antioxidant activity (µM of compound added)	Chapter
gallic acid	phenolic acids	1500-2000	II to V
hydroxybenzoic acid	phenolic acids	>4000	II and IV
syringic acid	phenolic acids	>4000	II
ellagic acid	phenolic acids	>4000	II and IV
caffeic acid	phenolic acids	500-1000	II
chlorogenic acid	phenolic acids	1000-1500	II
myricetin	flavonols	500-1000	II
quercetin	flavonols	200-300	II and IV
rutin	flavonols	500-1000	II
kaempferol	flavonols	>4000	II
(+)-catechin	flavanols	500-1000	II and IV
(-)-epicatechin	flavanols	500-1000	II and IV

Antioxidant activity of various phenolic compounds was defined as the concentration of compound need to be added to reach 0% malonaldehyde of the control.

Table 6.2 shows the *in vitro* oxidative activities of ellagitannins measured using a 96-well plate reader with modification reported in Barbehenn *et al.* (2006) at high pH (pH 9-12, which is similar to the gut of lepidopteran insects), although published studies estimating the oxidative activity of tannins are limited (Moilanen & Salminen, 2008). The oxidative activity of ellagitannins is the highest among the tannins, i.e., condensed tannins < galloylglucoses < ellagitannins (Barbehenn *et al.*, 2006). Ellagitannins are the most dominant phenolic compounds in eucalypt (Marsh *et al*., 2017), and they were the most influenced (both biotic and abiotic) phenolic compounds in our study.

Table 6.2. Identities and *in vitro* oxidative activities of ellagitannins that were identified during my research. Calculations and parameters are based on Moilanen & Salminen (2008).

Ellagitannin	Oxidative activity (10^{-3} abs / s / mM)	Chapter
HHDP-di-galloylglucose	3.3 ± 0.2	II to V
di-HHDP galloylglucose	3.4 ± 0.2	II to V
di-HHDP-glucose	5.0 ± 0.2	II to V
HHDP galloyl glucose	4.9 ± 0.2	II to V
vescalagin	10.2 ± 0.5	II and IIII
castalagin	13.8 ± 0.3	II and IIII
HHDP-tri-galloylglucose	8.4 ± 0.1	II to V

6.2.2. Chapter III

Seasonal and ontogenetic changes in leaf reflectance and phenolic compounds in five species of Myrtaceae (*Angophora floribunda, Callistemon salignus, Corymbia ficifolia* and *Eucalyptus camaldule*nsis and *Syzygium smithii*)

My findings revealed an increase in the concentration of three flavonoid glycosides during summer and a decrease in six hydrolysable tannins during winter. Flavonoid glycosides are characterised by an ortho-dihydroxy B-ring substitution and have high antioxidant properties. As a consequence, these compounds can quench reactive oxygen species (Agati and Tattini, 2010). Therefore, my findings suggested that an increase in flavonoid glycosides could be quenching free radicals when there is increased exposure to UV, i.e., during summer. However, a similar study showed an increased concentration of various galloylglucoses and flavonoids in winter if the plants are grown in a chilling cold environment due to cold-induced photoinhibition (Close, 2001). My results did not show any cold-induced photoinhibition signs because the temperature did not drop to an extreme in the winter. Further, the decrease in the concentration of various hydrolysable tannins in

my winter leaves could be explained by the diversion of plant resources in the production of condensed tannins in winter that share common precursors (Santos-Sánchez *et al.*, 2019).

I also reported higher concentrations of some hydrolysable tannins in younger leaves. This might be because younger leaves are more susceptible to photodamage and are more valuable than older leaves, requiring more protection (Zhang *et al*., 2016). I also found significant variation in the metabolites of older leaves compared to younger leaves which might be due to the formation of complex metabolites such as condensed tannins and terpenoids in older leaves (Salminen *et al.*, 2004; Salminen and Karonen, 2011; Goodger *et al.*, 2013). These contrasting trajectories between younger and older leaves may result from the plant maintaining the optimal balance between the resources required to produce and store these metabolites and resources available for the production of these metabolites and throughout the plant ontogeny (Goodger *et al.*, 2013).

6.2.3. Chapter IV

UV_A stress and the expression of phenolic compounds by *Eucalyptus camaldulensis* ssp. *camaldulensis*: influences of provenances and leaf age

I showed that five provenances of *Eucalyptus camaldulensis* ssp. *camaldulensis* exhibited significant quantitative differences in their metabolomes which suggests that prolonged exposure to variable environmental conditions (e.g., UV, temperature, and moisture available) has shaped presumed genetic differentiation to express the differential synthesis of phenolic metabolites when grown under experimental conditions.

My data showed a strong correlation between pentagalloyl glucose and quercetin with light and rainfall across a latitudinal gradient. Since various studies suggest that pentagalloyl glucose and quercetin have high antioxidant potential (Tian *et al*., 2009,

Torres-León *et al.*, 2017), they might be directly involved in free radical scavenging properties in the leaves in the presence of an excessive amount of UV light and during the drought stress. My result also showed a significant variation of metabolites from three-month-old leaves to six-month-old leaves. However, this variation slowed down after six months to twelve months. My study also showed a higher concentration of various hydrolysable tannins in younger leaves compared to older leaves. This decrease in concentrations of hydrolysable tannins in older leaves might be due to an increase in non-extractable phenolic compounds and/or due to an increase in polymerisation into higher molecular weight phenolics (Covelo and Gallardo, 2004). Further, it can also be due to plants allocating more of their resources for the production of carbohydrates and fibrous polymers (Kitajima *et al*., 2016; Li *et al*., 2016). Although my study was conducted on only one subspecies of *E. camaldulensis*, I expect my findings should have substantial relevance to the other subspecies of *Eucalyptus,* which span similar latitudinal gradients (McDonald *et al*., 2009).

6.2.4. Chapter V

Changes in phenolic compounds of *Eucalyptus camaldulensis* affected by the senescence-inducing psyllid *Cardiaspina albitextura*

My research showed a negative correlation between the numbers of psyllid on the canopy leaves and the concentrations of several phenolic compounds, i.e., a lower number of psyllids were found on leaves with high hydrolysable tannins and flavonoids. A similar result was shown by the galling herbivore *Leptocybe invasa* Fisher & La Salle on *Eucalyptus camaldulensis* where higher quercetin and kaempferol derivatives were found in the outer part of gall and higher HHDP tannins in inner gall, signalling photoprotection and plant defence, respectively (Isaias *et al*., 2018). I also found that psyllids numbers

were greatly influenced by the tree type (canopy and understorey). My analyses showed that the variation in phenolic compound concentrations was explained by tree type than by abundance of psyllids or relative leaf age for understory trees. *Cardiaspina* psyllids are known to hasten the onset of leaf senescence, shifting the balance of photosynthetic pigments leading to declining chlorophyll content and increasing screening pigments like anthocyanins causing reddening of leaves. This reddening, however, might not adversely affect nymphal survival and might be the response to the protection of leaves against photodamage (Steinbauer *et al.*, 2018). Therefore, I conclude that, although a higher concentration of hydrolysable tannins might negatively correlate with the number of psyllid nymphs on leaves, psyllids may not have influenced the concentration of phenolic compounds in leaves.

6.3. Summary

My findings suggest that UV_A influence the expression of different phenolic compounds, i.e., increased concentrations of various hydrolysable tannins by high UV_A and of various flavonoid glycosides in a period of higher solar radiation in summer and spring. Further, various low molecular phenolic acids and flavonoids were present in all genera, and these phenolic compounds are known for their high antioxidant activity (Table 6.1). The position and degree of hydroxylation play a key role in determining the antioxidant activity of flavonoids (Kumar and Pandey, 2013). Although there are various *in vitro* studies for antioxidant properties of flavonoids using purely chemical methods, there is still uncertainty concerning their functions *in vivo*.

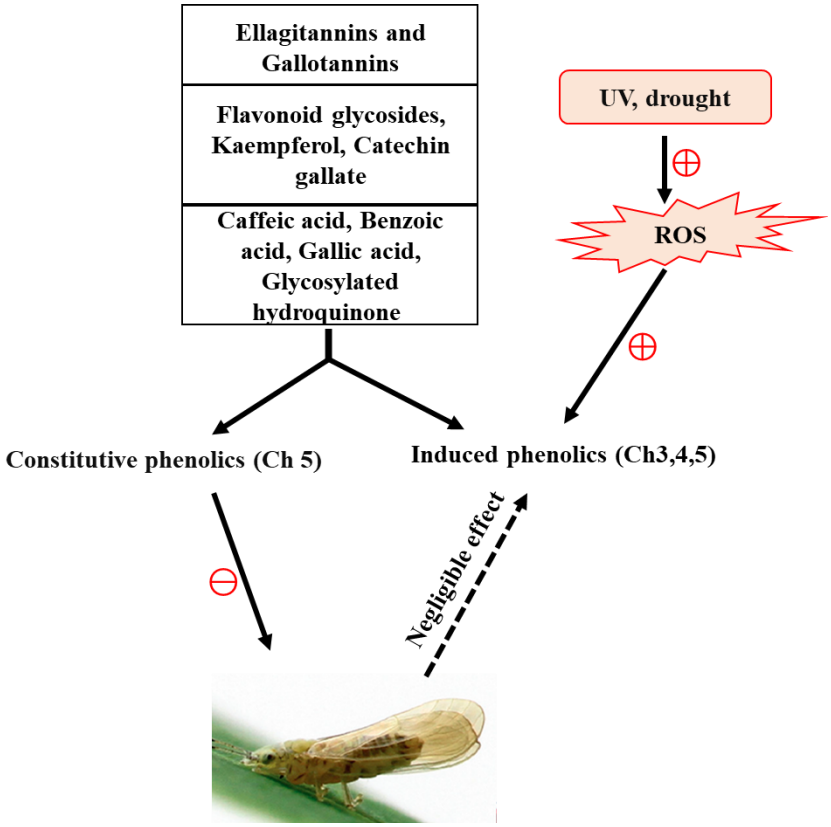


Figure 6.1. Conceptual diagram illustrating relative influences of endogenous and exogenous factors on foliar phenolic compounds in Myrtaceae. Solid arrows represent major effects leading to an increase in phenolic concentrations, and the dotted arrow represents negligible or no effect. There was a negative relationship between psyllid abundance and the concentration of phenolics. Symbols indicate positive (⊕) or negative (⊖) effects.

Similarly, looking at tannin subgroups, their degree of polymerisation and a higher number of hydroxyl groups can help explain their variation in oxidative activity and protein precipitation capacity (Hagerman *et al.*, 1999). Various major hydrolysable tannins, especially ellagitannins tannins such as HHDP-glucose, di-HHDP-glucose, HHDP galloyl glucose, HHDP-di-galloylglucose and gallotannnins such as galloyl glucose, digalloyl glucose, trigalloyl glucose, tetragalloyl glucose and pentagalloyl glucose were found to be present in all five species of Myrtaceae. Results from Chapters II and III and IV showed that, although species had comparable compositions, the

concentrations of individual phenolics varied between species and with leaf ontogeny, which presumably can be traced back to their evolutionary history of exposure to contrasting environmental factors such as UV light. Various hydrolysable tannins also varied with leaf age and seasons in my glasshouse experiment. The increase in the concentration of ellagitannins and galloylglucose during high UV_A light treatment in the glasshouse and their high antioxidative properties (Table 6.1 and Table 6.2) suggest their active role in the photoprotection of the leaves. Even if some tannins may not have an active role in photoprotection, some can indirectly support photoprotection by preventing anthocyanin degradation by increasing pigment stability and helping to maintain red colouration (Luo *et al*., 2019).

The ellagitannins identified during my research differs greatly in oxidative activity. HHDP-di-galloylglucose has the lowest activity, and castalagin has the highest (Table 6.2). These differences are explained by their structural differences, i.e., the number of -COOH groups, number of sugar groups, number of HHDP groups and molecular weight (Salminen, 2014). I suggest that future researchers should estimate these parameters from the structure of the compounds they identified to understand better the tannin oxidation hypothesis in relation to plant-herbivore interactions.

6.4. Concluding remarks

My research has demonstrated that the biological activity of phenolic compounds is a complex phenomenon affected by the class of phenolic compounds and the structure of individual compounds interacting with a range of external factors. Further, evolutionary reasons for the observed variation in phenolic compounds are largely unknown, but my study supports the photoprotection theory of plant phenolics, and it might be the key driving selective agent. My argument is supported by the presence of identical groups of phenolic compounds (mostly similar tannins, phenolic acids, and flavonoids) across all

five Myrtaceae species. Conservation of plant polyphenolics across taxa suggests a common function. Therefore, it may be concluded that physiological signalling associated with oxidative stress increases the biosynthesis of some phenolic compounds, and phenolic compounds have evolved, at least in part, for the protection of plants against stresses such as UV. I also found evidence suggesting that a concentration of specific groups of plant phenolic compounds may negatively affect psyllid abundance.

6.5 References

Agati, G., Tattini, M., (2010). Multiple functional roles of flavonoids in photoprotection: Letters. New Phytol. 186, 786–793.

Amorati, R., & Valgimigli, L. (2015). Advantages and limitations of common testing methods for antioxidants. *Free Radical Research*, *49*(5), 633–649.

Appel, H. M., Govenor, H. L., D'Ascenzo, M., Siska, E., & Schultz, J. C. (2001). Limitations of Folin assays of foliar phenolics in ecological studies. *Journal of Chemical Ecology*, *27*(4), 761–778.

Barbehenn, R. V., Jones, C. P., Hagerman, A. E., Karonen, M., & Salminen, J.-P. (2006). Ellagitannins have greater oxidative activities than condensed tannins and galloyl glucoses at high pH: Potential impact on caterpillars. *Journal of Chemical Ecology*, *32*(10), 2253–2267.

Barbehenn, R.V., Peter Constabel, C., (2011). Tannins in plant–herbivore interactions. Phytochemistry, Plant-Insect Interactions 72, 1551–1565.

Blainski, A., Lopes, G. C., & De Mello, J. C. P. (2013). Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium Brasiliense* L. *Molecules*, *18*(6), 6852–6865.

Castañeda-Arriaga, R., Pérez-González, A., Reina, M., Alvarez-Idaboy, J. R., & Galano, A. (2018). Comprehensive investigation of the antioxidant and prooxidant effects of phenolic compounds: A double-edged sword in the context of oxidative stress. *The Journal of Physical Chemistry B*, *122*(23), 6198–6214.

Close, D.C., McArthur, C., (2002). Rethinking the role of many plant phenolics: protection from photodamage not herbivores. *Oikos* 99, 166–172. JSTOR.

Dai, J., Mumper, R.J. (2010). Plant Phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules 15*, 7313–7352.

Fensham, R. J. (1994). Phytophagous insect-woody sprout interactions in tropical eucalypt forest. I. Insect herbivory. *Australian Journal of Ecology*, *19*(2), 178–188.

Fukumoto, L. R., & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of Agricultural and Food Chemistry*, *48*(8), 3597–3604.

Goodger, J. Q. D., Heskes, A. M., & Woodrow, I. E. (2013). Contrasting ontogenetic trajectories for phenolic and terpenoid defences in *Eucalyptus froggattii*. *Annals of Botany*, *112*(4), 651–659.

Hagerman, A.E., Riedl, K.M., Rice, R.E., (1999) Tannins as biological antioxidants, in: Gross, G.G., Hemingway, R.W., Yoshida, T., Branham, S.J. (Eds.), Plant polyphenols 2: Chemistry, biology, pharmacology, ecology, basic life sciences. *Springer* US, Boston, MA, pp. 495–505.

Isaias, R. M. dos S., Ferreira, B. G., Alvarenga, D. R. de, Barbosa, L. R., Salminen, J.-P., & Steinbauer, M. J. (2018). Functional compartmentalisation of nutrients and phenolics in the tissues of galls induced by *Leptocybe invasa* (Hymenoptera: Eulophidae) on *Eucalyptus camaldulensis* (Myrtaceae). *Austral Entomology*, *57*(2), 238–246.

Kitajima, K., Wright, S. J., & Westbrook, J. W. (2016). Leaf cellulose density as the key determinant of inter- and intra-specific variation in leaf fracture toughness in a species-rich tropical forest. *Interface Focus*, *6*(3), 20150100.

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: an overview. *The Scientific World Journal*, Article ID 162750, *2013*

Lester, G. E., Lewers, K. S., Medina, M. B., & Saftner, R. A. (2012). Comparative analysis of strawberry total phenolics via Fast Blue BB vs. Folin–Ciocalteu:

Assay interference by ascorbic acid. *Journal of Food Composition and Analysis*, *27*(1), 102–107.

McDonald, M. W., Brooker, M. I. H., & Butcher, P. A. (2009). A taxonomic revision of *Eucalyptus camaldulensis* (Myrtaceae). *Australian Systematic Botany*, *22*(4), 257–285.

Moilanen, J., Salminen, J.-P. (2008). Ecologically neglected tannins and their biologically relevant activity: chemical structures of plant ellagitannins reveal their in vitro oxidative activity at high pH. *Chemoecology* 18, 73–83.

Macauley, B. J., & Fox, L. R. (1980). Variation in total phenols and condensed tannins in *Eucalyptus*: Leaf phenology and insect grazing. *Australian Journal of Ecology*, *5*(1), 31–35.

Marsh, K. J., Wallis, I. R., Kulheim, C., Clark, R., Nicolle, D., Foley, W. J., & Salminen, J. (2020). New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. *New Phytologist*, *225*(1), 488–498.

Marsh, K. J., Kulheim, C., Blomberg, S. P., Thornhill, A. H., Miller, J. T., Wallis, I. R., Nicolle, D., Salminen, J.-P., & Foley, W. J. (2017). Genus-wide variation in foliar polyphenolics in eucalypts. *Phytochemistry*, *144*, 197–207.

Mullen, W., McGinn, J., Lean, M. E. J., MacLean, M. R., Gardner, P., Duthie, G. G., Yokota, T., & Crozier, A. (2002). Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. *Journal of Agricultural and Food Chemistry*, *50*(18), 5191–5196.

Nakabayashi, R., Mori, T., & Saito, K. (2014). Alternation of flavonoid accumulation under drought stress in *Arabidopsis thaliana*. *Plant Signaling & Behavior*, *9*(8), e29518.

Ohmart, C. P., Stewart, L. G., & Thomas, J. R. (1983). Phytophagous insect communities in the canopies of three *Eucalyptus* Forest types in south-eastern Australia. *Australian Journal of Ecology*, *8*(4), 395–403.

Ossipov, V., Haukioja, E., Ossipova, S., Hanhimäki, S., Pihlaja, K. (2001). Phenolic and phenolic-related factors as determinants of the suitability of mountain birch leaves to an herbivorous insect. *Biochem. Syst. Ecol.* 29, 223–240.

Pratt, D.E., Hudson (1990) Natural antioxidants not exploited commercially: Hudson, B. J. F. (Ed.), *Food Antioxidants*, Elsevier Applied Food Science Series. Springer Netherlands, Dordrecht, pp. 171–191.

Recher, H. F., Majer, J. D., & Ganesh, S. (1996). Seasonality of canopy invertebrate communities in eucalypt forests of eastern and western Australia. *Australian Journal of Ecology*, *21*(1), 64–80.

Rice-Evans, C.A., Miller, N.J., Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical. Biol. Med*. 20, 933–956.

Salminen, J.-P., Karonen, M., (2011) Chemical ecology of tannins and other phenolics: we need a change in approach. *Funct. Ecol.* 25, 325–338.

Salminen, J.-P., Roslin, T., Karonen, M., Sinkkonen, J., Pihlaja, K., Pulkkinen, P (2004). Seasonal variation in the content of hydrolysable tannins, flavonoid glycosides, and proanthocyanidins in oak leaves. J. Chem. Ecol. 30, 1693–1711.

Salminen, J.-P. (2014). The Chemistry and chemical ecology of ellagitannins in plant–insect interactions: from underestimated molecules to bioactive plant

constituents. *Recent Advances in Polyphenol Research* (pp. 83–113). John Wiley & Sons, Ltd.

Santos-Sánchez, N.F., Salas-Coronado, R., Hernández-Carlos, B., Villanueva-Cañongo, C. (2019). Shikimic acid pathway in biosynthesis of phenolic compounds. *Plant Physiol. Asp. Phenolic Compd*.

Steinbauer, M. J., Salminen, J.-P., & Watson, S. J. (2018). Yellow, red, dead: The nutritional consequences for *Cardiaspina densitexta* (Hemiptera: Aphalaridae) nymphs of inducing senescence in old *Eucalyptus fasciculosa* leaves. *Austral Entomology*, *57*(2), 265–278.

Torres-León, C., Ventura-Sobrevilla, J., Serna-Cock, L., Ascacio-Valdés, J. A., Contreras-Esquivel, J., & Aguilar, C. N. (2017). Pentagalloylglucose (PGG): A valuable phenolic compound with functional properties. *Journal of Functional Foods*, *37*, 176–189.

Wotherspoon, K. P. (1998). Patterns of phytophagous insect herbivory and abundance on juvenile regrowth of *Eucalyptus* subgenera coexisting in southeastern Tasmania. *Australian Journal of Ecology*, *23*(5), 430–442.

Zhang, T.-J., Chow, W. S., Liu, X.-T., Zhang, P., Liu, N., & Peng, C.-L. (2016). A magic red coat on the surface of young leaves: Anthocyanins distributed in trichome layer protect *Castanopsis fissa* leaves from photoinhibition. *Tree Physiology*, *36*(10), 1296–1306.

Zinsmeister, H. D., Becker, H., & Eicher, T. (1991). Bryophytes, a source of biologically active, naturally occurring material. *Angewandte Chemie International Edition in English*, *30*(2), 130–147.