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Non-volatile secondary metabolites in foliar oil glands of *Eucalyptus* species

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Abstract

Plants synthesise a vast range of secondary metabolites that are stored in specialised cells or organs. The presence of sub-dermal glands rich in volatile terpene essential oils is characteristic of the trees of the genus *Eucalyptus* (Myrtaceae). Recent studies showed that non-volatile compounds (NVCs), particularly monoterpene acid glucose esters (MAGEs), co-occur with volatile components in *Eucalyptus* foliar oil glands. The principal aim of this thesis is to characterise MAGEs and other non-volatiles localised to *Eucalyptus* oil glands and to explore their relationships to the co-housed oil components.

Glandular extracts from a range of *Eucalyptus* species belonging to the two major subgenera, *Symphyomyrtus* and *Eucalyptus*, were investigated in Chapter 2. Non-volatiles were extracted from enzymatically isolated glands and analysed using high-performance liquid chromatography (HPLC) and mass spectrometry (LC-MS). MAGEs were identified based largely on diagnostic mass spectral fragmentation patterns. MAGEs were the dominant NVC in *Symphyomyrtus* species. In contrast, the sampled subgenus *Eucalyptus* species lacked MAGEs, but were rich in phenolics. Volatile oil components were also analysed using gas chromatography with flame ionisation detection and mass spectrometry (GC-FID and GC-MS). Monoterpenes and sesquiterpenes were identified and quantified for each species. In addition, cell suspensions of *E. polybractea* were successfully established from leaf-derived callus as a potential tool to investigate the biosynthesis of MAGEs.

Glandular extracts from subgenus *Eucalyptus* species rich in non-volatiles containing phenolic moieties were further analysed in Chapter 3. A suite of unsubstituted B-ring flavanones was identified as the dominant glandular NVCs. In addition, flavones, flavanone-*O*-glucosides, flavanone- β -triketone conjugates, triketone heterodimers and chromone-*C*-glucosides were also identified. Flavanones were quantified and species-specific variations were observed. This chapter also showed that flavanones are exclusively localised to the glands rather than present throughout leaf tissues. A positive correlation was observed between some monoterpenes and sesquiterpenes with total flavanones and particularly with pinostrobin. Interestingly, β -triketones were also found in the volatile extracts of glands from *E. suberea* and *E. brevistylis*.

The presence of glandular β -triketones was further explored in Chapter 4. A major discovery of this thesis was the occurrence of two gland types in *E. brevistylis* that differ in their colour and importantly, in their metabolic contents. ‘Sesquiterpene glands’, which are translucent-white in appearance, contained sesquiterpene alcohols. ‘Triketone glands’, which are golden-brown in appearance, contained mostly the β -triketone conglomerone and sesquiterpene hydrocarbon caryophyllenes in lower abundance. None of the glands contained the NVCs identified from the other species in this thesis. The results were consistent in trees from a natural population of *E. brevistylis* and in glasshouse-grown seedlings and saplings. In addition, ‘Triketone glands’ seem to develop earlier than ‘sesquiterpene glands’ in leaf ontogeny. This is the first identification of such metabolic differentiation of embedded glands from any tree species.

The work presented in this thesis revealed many novel aspects of oil glands, particularly in relation to their non-volatile and volatile constituents and how they are related to one other. Overall, the findings of this thesis contribute significantly to the knowledge on *Eucalyptus* foliar oil gland chemistry and biology.

Declaration

This is to certify that:

- This thesis comprises only my original work towards my PhD, except where otherwise stated in the Preface
- Due acknowledgement has been made to all other material used
- This thesis is fewer than 80,000 words in length excluding tables, figures, references and appendices.

Samiddhi Lankani Senaratne

February 2020

Preface

Aspects of this thesis have been included in articles published by the Plant Physiology research group investigating the chemistry and biology of *Eucalyptus* foliar glands. I have been listed as co-first author on two of these and second author on the third. The publications are:

1. Goodger, JQD[‡], **Senaratne, SL[‡]**, Nicolle, D, Woodrow, IE 2016. Foliar essential oil glands of *Eucalyptus* subgenus *Eucalyptus* (Myrtaceae) are a rich source of flavonoids and related non-volatile constituents. PLoS ONE 11(3): e0151432 (pp 1-18).

[‡]*These authors contributed equally to the work*

2. Goodger JQD[‡], **Senaratne SL[‡]**, Nicolle D, Woodrow IE 2018. Differential metabolic specialisation of foliar oil glands in *Eucalyptus brevistylis* Brooker (Myrtaceae). Tree Physiology 38: 1451.

[‡]*These authors contributed equally to the work*

3. Goodger JQD, **Senaratne SL**, van der Peet P, Williams SJ, Nicolle D, Woodrow IE 2019. *Eucalyptus* subgenus *Eucalyptus* (Myrtaceae) trees are abundant sources of medicinal pinocembrin and related methylated flavanones. Industrial Crops & Products 131: 166-172.

The chapters included in this thesis cover many of the results presented in the publications, but they are re-packaged with less of a pharmaceutical industry focus and include additional findings and discussion of different aspects. I was the principal contributor to, and primary author of, all chapters presented in this thesis. My supervisors have been recognised for their invaluable contribution to all chapters and all other contributors are mentioned in the acknowledgements.

The mass spectrometry was carried out in the Metabolomics Australia, the University of Melbourne. Alice Ngyen provided LC-MS technical support and Daniel Dias and Nirupama Jayasinghe provided GC-MS technical support. The NMR experiment and data analysis presented in Chapter 3 were carried out by Phillip van der Peet and Spencer Williams at the Bio21 Institute, the University of Melbourne. SEM

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This research would not have been possible without the generous financial support from the Holsworth Wildlife Research Endowment (managed by Equity Trustees), which provided funding for three years for laboratory and field work. It is greatly appreciated. Also, I am grateful for the School of Botany Foundation, for the scholarship that provided me financial support to present research work at an international conference. Melbourne Research scholarship (MRS) followed by the Australian Postgraduate Award (APA) funded me throughout my period of study.

Besides my supervisors, there are people who supported me in many ways. I thank Dr. Dean Nicolle and the Currency Creek Arboretum for providing the leaf materials for this study. I also thank Daniel Dias, Nirupama Jayasinghe, Alice Ngyen, Siriya Natera and Adrian Lutz and all the members of Metabolomics Australia for assisting with LC-MS and GC-MS runs. I would also like to thank Spencer Williams, Phillip van der Peet and their team at Bio21 for helping with NMR. Thank you, Simon Crawford, for assisting me in electron microscopy. I thank the entire team of the School of BioSciences for all the support and friendship given throughout the past years.

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Chinthaka, I would not have made my journey this far without your support and encouragement. Your love and unconditional trust keep me doing what I want in my life. Thank you for all your support. I must remind our little son, Minthaka. It was a time that you needed me the most. I started this when you were very little, and now you have grown. Your sacrifices, though you do not understand what they are, it means a lot to me.

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List of abbreviations

ANOVA	Analysis of variance
DAS	Days after sowing
DMAPP	Dimethylallyl diphosphate
DW	Dry weight
ESI	Electro-spray ionisation
FPP	Farnesyl pyrophosphate
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionisation detection
GC-MS	Gas chromatography-mass spectroscopy
GGPP	Geranylgeranyl diphosphate
GTs	Glandular trichomes
HPLC	High performance liquid chromatography
IPP	Isopentenyl diphosphate
LA	Leaf area
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectroscopy
LDW	Leaf dry weight
<i>m/z</i>	Mass to charge ratio
MAGEs	Monoterpene acid glucose esters
MS	Mass spectrometry
NAA	Naphthaleneacetic acid
NMR	Nuclear magnetic resonance
PDA	Photo diode array
PSMs	Plant secondary metabolites
RT	Retention time
TDZ	Thidiazuron
THC	Tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
UV	Ultraviolet
WA	Western Australia

Chapter 1

Metabolomes in plant secretory structures

1.1. Introduction

Plant secondary metabolites (PSMs) are a group of chemical compounds involved in functions other than plant primary metabolism. These compounds, which are also referred to as phytochemicals, are often found in low abundance and usually stored in dedicated cells or organs (Pichersky and Gang 2000). A vast range of PSMs have been identified and extracted from both vegetative and reproductive tissues of many plants. They are highly diversified in structure and have been classified into major groups such as terpenoids and phenolics based on different arrangements of carbon, hydrogen and oxygen atoms, and alkaloids that also contain nitrogen (Acamovic and Brooker 2005; Croteau *et al.* 2000; Wink 2004). PSMs are generally considered important in plant defence mechanisms such as in anti-herbivory, anti-microbial and oxidative stress tolerance activities. PSMs can also help plants compete against other plants for nutrients and light (Acamovic and Brooker 2005). In addition to their important roles in plants, PSMs are commercially attractive compounds due to their vast range of biological activities. Some of these metabolites have been used by humans for millennia for various purposes such as in traditional medicine, fragrances and colouring in cosmetics, as natural pesticides and as food flavours (Croteau *et al.* 2000; Harborne 2001).

Toxic PSMs can have deleterious effects on the synthesising plants themselves and so are sequestered in specialised cells or organs, referred to as secretory structures. Such compartmentalisation has been identified as a mechanism of resistance to auto-toxic compounds (Sirikantaramas *et al.* 2007; Wittstock and Gershenzon 2002). For example, there is evidence that early and intermediate stages of biosynthesis of some PSMs occur in tissues like stems, leaves, flowers and roots, while the later stages, where more toxic end products are synthesized, are limited to the specialised secretory structures (Beaudoin and Facchini 2014; De Luca and Laflamme 2001). Moreover, in the biosynthesis of some PSMs, the intermediate steps can be observed in the cell cytoplasm

while the toxic end products are produced and housed in compartments like vacuoles (Roze *et al.* 2011). Since the exposure of these toxic end-products can cause damage to other plant tissues, they may finally be translocated to the lumen of specialised secretory structures where they can accumulate at levels high enough to be effective as defence chemicals (Knight *et al.* 2002).

This chapter reviews present knowledge on the diversity of secretory structures in plants and the classes of compounds localised within them. The review will include the glandular secretory structures in plants that synthesise and store PSMs. This information will provide background to the research presented in this thesis which focuses on the metabolome of secretory structures in species of the genus *Eucalyptus*.

1.2. Plant secretory structures

Based on the substances secreted, two major groups of secretory structures are found throughout the plant kingdom (Fahn 1979; Fahn 1988). The first group stores or eliminates unmodified or slightly modified substances translocated by vascular tissues. For example, hydathodes, salt glands and nectaries eliminate water, salt and phloem sap, respectively. In contrast, the second group, glandular secretory structures, synthesise PSMs in their epithelial cells before storing them within dedicated cells or extracellular lumina within the structure. This group is highly structurally diversified, varying from single-cell idioblasts to multicellular glandular secretory complexes such as resin ducts, laticifers, trichomes, sub-dermal ducts and oil glands/cavities. Such secretory structures occur commonly in leaves, fruits and stems, but may also be present in roots. Some of these structures, like trichomes, are located superficially on the surface of the plant and thereby may act as a first line of defence. In contrast, laticifers, resin ducts and oil glands are embedded in nature, submerging PSMs within plant tissues.

Another special type of secretory tissue, named osmophores, has been observed in flowers of some plant families (Sazima *et al.* 1993; Stern *et al.* 1987). This is a specialised secretory epithelium located in the adaxial region of a sac formed near the proximal portion of the floral lip mostly in Orchidaceae family. They emit volatile compounds by a cuticular diffusion process or sometimes via the intercellular spaces and stomatal openings (de Melo *et al.* 2010). This type of secretory structure differs from the

aforementioned structures by not appearing to store the synthesized substances within an extracellular cavity.

Presence of a specific type of secretory structures seems to be characteristic of particular plant families and has long been used as an important trait in taxonomic classification. For example, resin ducts are a characteristic feature in conifers, but glandular trichomes have not been reported from them (Lange and Turner 2013). Many plant families have several types of secretory structures; for example, fruits in Anacardiaceae possess both secretory ducts and idioblasts (Augusta Dantas Tolke *et al.* 2017). Leguminosae plants also contain several types of secretory structures, with the developing flower inflorescence containing phenolic-accumulating cells, mucilage cells, oil glands and secretory trichomes (De Barros *et al.* 2017).

1.2.1. Excretory idioblasts

Cells which differ in their form, size, contents, and wall structure from neighbouring homogenous tissue elements are commonly termed ‘idioblasts’ (Foster 1956). A broad range of cells fit into this category including some cell types that do not contain any PSMs. Nonetheless, excretory idioblasts are cells that do contain PSMs. They are found in a vast range of organisms in the plant kingdom from bryophytes through to angiosperms, both in terrestrial as well as aquatic plants (Hara *et al.* 2015). A wide variety of excretory idioblasts have been identified based on the chemical nature of their contents such as oil cells, mucilage cells, tannin idioblasts, lithocysts, myrosin cells, crystal idioblasts and latex cells or laticifers (Foster 1956).

Despite idioblasts being single cells, they still display a surprising amount of variation in structure. Many excretory idioblasts are spherical in form, particularly those found in families Magnoliaceae and Winteraceae. In contrast, elongated tubular and branched types are present in Cruciferae plants (e.g. myrosin cells; Foster 1956). The cell wall of idioblast cells appear to be composed of three distinct layers: a thick shell-like non-cellulotic envelope, a transparent hydrophilic zone and a hydrophobic core (Tretyn *et al.* 1996). PSMs are stored in either the cell vacuole or cytoplasm (Roshchina and Roshchina 2012). In oil cells - idioblasts that secrete oil - the oil accumulates in the centre of the cell as an oil drop that has been described as an oil sac (**Figure 1.1**; Cummings and Schroeder 1942; Scott *et al.* 1963).

Excretory idioblasts can be found in a large range of plant families. Among them oil cells appear to be the most widely distributed, having been reported from more than 20 families including Araceae, Aristolochiaceae, Calucanthaceae, Lauraceae, Magnoliaceae and Rutaceae (Roshchina and Roshchina 2012). A well-known example of oil cells are those present in the fruit of avocado (*Persea americana*), but they can also be found in leaves, seed, and roots of the species (Platt and Thomson 1992).

Despite the vast distribution of idioblasts in plants, relatively little is known about their biochemistry at the cellular level – largely due to the difficulty of extracting compounds from scattered single cells embedded in tissues. Nevertheless, phenolics, tannins, terpenoids and alkaloids have all been identified from idioblasts of different plant families (Roshchina and Roshchina 2012). In addition, mineral calcium oxalate containing idioblasts are one of the most commonly occurring type; however, these oxalates generally are considered a cellular by-product rather than a PSM *per se* (Franceschi and Horner 1980), although such stringent definitions can be contentious. Some scientists accept oxalates as useful PSMs, which are translocated from storage to required parts of plants (Osuji and Ndukwu 2005; Osuji 2013). A recent study by Muliya *et al.* (2018) showed that the same species can contain different idioblastic cells that produce different compound groups. Alkaloids, phenols and terpenoids were found in different cell types in *Tetracera scandens* (Muliya *et al.* 2018). In contrast, in some species complex mixtures of compounds are found in the same secretory cell. For example, secretory idioblasts in *Piper umbellatum* (Piperaceae) leaves accumulate phenolic compounds, alkaloids, and lipids in their vacuoles (Marinho *et al.* 2011).

1.2.2. Laticifers

Laticifers are specialised cells that produce and store milky suspensions comprised of many PSMs. They are found in more than 20 plant families such as Apocynaceae, Asclepiadaceae, Asteraceae, Euphorbiaceae, Moraceae and Osaceae (Foster 1956; Hagel *et al.* 2008). Two main types of laticifers can be recognised in plants (Pickard 2008). Non-articulated laticifers are single elongated cells that can be branched or unbranched (**Figure 1.1**). Being single cells, these laticifers are occasionally considered as a type of idioblast in the literature. Non-articulated laticifers are often found only in primary plant vascular tissues, and have rarely been identified in and around secondary vascular tissues (Najmaddin and Khalid 2018). In contrast, articulated laticifers are multi-cellular structures comprised of piles of elongated cells that can

extend for a considerable distance throughout plant tissues, as in *Hevea brasiliensis* (rubber plant). The walls separating the cells become perforated or are completely absent to facilitate the smooth flow of substances within the laticifer. Articulated laticifers can be found in all vegetative organs and may arise both in primary and secondary plant tissues (Najmaddin and Khalid 2018).

The PSM contents of laticifers are commonly termed ‘latex’ and are mostly milky in appearance, although latex can appear yellow, orange, red, brown, or colourless. The appearance of latex can differ between plants depending on their constituents and may even vary in different parts of a single plant. Latex is often exuded in response to damage and its colour can change after it has been exuded by chemical processes such as oxidation (Najmaddin and Khalid 2018). In general, latex contains terpenoids, cardiac glycosides, alkaloids, lignans, vindolinenoids and tannins. In many species, it performs a defensive role protecting against herbivory and inferring a level of disease resistance. Latex of some species is important as a commercial product such as the latex of *Hevea* that still plays a major role in the global economy as a source of natural rubber (Dussourd and Eisner 1987).

1.2.3. Glandular trichomes

Trichomes are epidermal appendages of protodermal origin found on surfaces of some plants. Given their external localisation and relative ease of separation from other tissues, trichomes have been extensively studied compared to the other secretory structures. These investigations have moved beyond simply identifying PSM contents to advanced biosynthetic and enzymological studies and even the modification of trichome development by genetic manipulation (Wagner 1991). The broader term ‘trichomes’ includes both glandular and non-glandular trichomes, which differ in their ability to produce and store PSMs. Non-glandular trichomes are simple hair-like structures found on the surface of some plants and do not contain appreciable quantities of PSMs, but may still perform a mechanical defence role. In contrast, glandular trichomes (GTs) or glandular hairs are secretory structures specialised to synthesize, store and/or secrete PSMs; hence, only this type will be further considered in this review (Aziz *et al.* 2005; Schnetzler *et al.* 2017).

GTs are highly diversified in structure compared to the other secretory structures, with three main structural types found in plants. The three types are shield-shaped peltate trichomes, biseriate trichomes and capitate trichomes that end with a distinct, compact

head. A fourth type of finger-shaped trichomes called digitiform trichomes is much rarer and are only known from Lamiaceae species (Jia *et al.* 2013; Liu *et al.* 2018). Some species only contain a single trichome type; for example, all trichomes are of capitate type in *Dorstenia cayapia*, *Maclura tinctoria* and *Sorocea bonplandii* (Moraceae; Schnetzler *et al.* 2017). In contrast, *Artocarpus heterophyllus* from the same family contains both capitate and peltate types (Schnetzler *et al.* 2017). All three major trichome types share some basic structural features such as a unicellular or multicellular head of different shape and size, and a unicellular or multicellular peduncle (**Figure 1.1**). The number of secretory cells (4 or 8 cells), peduncle size and head cell number vary in different species. Capitate trichomes are described as more numerous, but comparatively smaller in size compared to the other types (De Barros *et al.* 2017; Liu *et al.* 2018; Schnetzler *et al.* 2017). They accumulate metabolic products in the space between the gland cell walls and cuticle which is termed the ‘oil sac’ or ‘subcuticular space’ and acts as a compartment that is virtually outside the plant body (**Figure 1.1**).

The majority of studies on GTs have been conducted on plants from the Asteraceae and Lamiaceae families; however, GTs are found in a range of other plant families such as Cannabinaceae, Compositae, Cucurbitaceae, Fabaceae, Geraniaceae, Labiatae and Solanaceae (Fahn 2000; Lange and Turner 2013). Many Lamiaceae species possess both peltate and capitate trichomes. Examples include GTs in peppermint (*Mentha x piperita*), basil (*Ocimum basilicum*) and *Salvia aurea* leaves where both trichome types occur (Gang *et al.* 2001; Gershenzon *et al.* 1992; Serrato Valenti 1997; Turner *et al.* 2000a). Trichomes are found in both vegetative and reproductive organs; for example, species in family Moraceae and Leguminosae have secretory trichomes on the leaf blade, petiole and stem and also on the flowers and peduncle of the inflorescence (De Barros *et al.* 2017; Schnetzler *et al.* 2017). The shrub species *Tetracera scandens* (Dilleniaceae) also displays GTs on the stem (Muliyah *et al.* 2018).

GTs can accumulate a variety of PSMs including essential oils and resins that can contain polysaccharides, terpenoids, lipids, acyl sugars, phenolics, flavonoids and alkaloids (McCaskill and Croteau 1999). They are considered highly active cell factories due to their high metabolic productivity. Previous evidence suggests that a vast variation occurs among the constituents in each trichome type in different species. When both capitate and peltate trichomes occur in the same species, differences between their constituents and/or secretions suggest that different metabolic pathways occur in each type. For example, in *Lavandula pinnata* (Lamiaceae) capitate trichomes contain both

lipophilic and polysaccharide substances while peltate trichomes only secrete lipophilic substances (Huang *et al.* 2008). Similarly, different types of trichomes have been observed in tomato (*Solanum*) where one type produces acyl sugars and the other type produces terpenoids (Balcke *et al.* 2017).

1.2.4. Resin ducts/secretory canals

Resin ducts are elongated, internal secretory structures embedded in the primary and secondary phloem of stems and leaves (Milani *et al.* 2012; Palermo *et al.* 2018). They are most commonly known from the Family Pinaceae, but have also been identified from other families such as Leguminosae, Burseraceae and Anacardiaceae (Joel and Fahn 1980; Murthy *et al.* 2016). The distribution and number of ducts is a significant morphological trait long used in taxonomic studies within the family Pinaceae (Wu and Hu 1997).

Resin ducts can be simple sac-like structures named resin blisters or may be much more complex resin-filled canals. Resin canals can be interconnected in a three-dimensional reticulate system that is distributed in the cortex of stems and throughout the leaves. Examples of resin ducts include resin blisters in *Abies* spp. and the complex resin duct system in *Picea* and *Pinus* spp. (Fahn 1988). The ducts are lined by thin walled un lignified secretory epithelium surrounding an intercellular space (**Figure 1.1**). Resin ducts can be formed at different ontogenic stages such as early seedling growth in conifers, and may also develop in later stages of ontogeny as a result of wounding and exposure to chemicals (Hanes 1927; Hudgins and Franceschi 2004).

Resin ducts are so named because they accumulate resinous substances. The PSMs in the resin matrix include phenolic compounds, alkaloids and mucopolysaccharides (Reis *et al.* 2016). Resin ducts in stems and needles of conifers are also filled with a mixture of volatile oil and together with resinous substances this is termed oleoresin. This heterogenous mixture has helped conifers to be highly adaptable to terrestrial environments (Abbott *et al.* 2010; McCaskill and Croteau 1999). Oleoresin contains a monoterpene and sesquiterpene-rich volatile fraction and a semi volatile diterpenoid-rich rosin fraction. Herbivore or pathogen attacks can induce the synthesis and secretion of oleoresin (Franceschi *et al.* 2005). Specialisation of metabolism in epithelial cells of resin ducts have been observed in conifers (Turner *et al.* 2019).

1.2.5. Oil glands

Oil glands are multi-cellular embedded structures found in almost all parts of some plants including apical shoots, leaves, floral tissues and even woody tissues like bark and roots. They also occur in secondary phloem of some species where they arise from phloem parenchyma and rays (Bohte and Drinnan 2011; Chattaway 1955; Eyles *et al.* 2004; Machado *et al.* 2017). They are characteristic of some plant families like Myrtaceae, Rutaceae, Leguminosae and Rubiaceae (De Barros *et al.* 2017; Machado *et al.* 2017; Vieira *et al.*).

An oil gland is composed of a large extracellular cavity surrounded by outer secretory epithelial cells (**Figure 1.1**; Carr and Carr 1970). The structure of oil glands has been explored in great detail in some species such as members of the Rutaceae family (Machado *et al.* 2017). The mature epithelial cells are flattened with very thin walls and contain large oil bodies, leucoplasts surrounded by endoplasmic reticulum (ER), and mitochondria (Machado *et al.* 2017). The epithelium is generally multi-layered; however, the glands that were reported from Leguminosae species have a uniseriate epithelium (single cell layer) while biseriate epithelium (two cell layers) have also been reported, albeit rarely (Milani *et al.* 2012). A parenchyma sheath has been observed in anatomical studies of some species of family Leguminosae (De Barros *et al.* 2017). The outermost cell-layer may have thick cell walls, at least in *Citrus*, and may act as a protective sheath (Bosabalidis and Tsekos 1982). However, then the question arises – how are the contents released to the surrounding environment?

Oil gland contents are comprised mostly of volatile oil components and sometimes resinous non-volatiles. As denoted by its name, oil glands were initially thought to house exclusively volatile oil components. Recent studies on *Eucalyptus* and *Leptospermum* species have debunked this theory and revealed that they also contain non-volatile components (Goodger *et al.* 2009; Killeen *et al.* 2015). Resinous non-volatiles have also been detected in the glands from the Leguminosae species *Copaifera trapezifolia* (Milani *et al.* 2012). The contents are thought to be synthesised by the secretory cells and stored in the extracellular cavity.

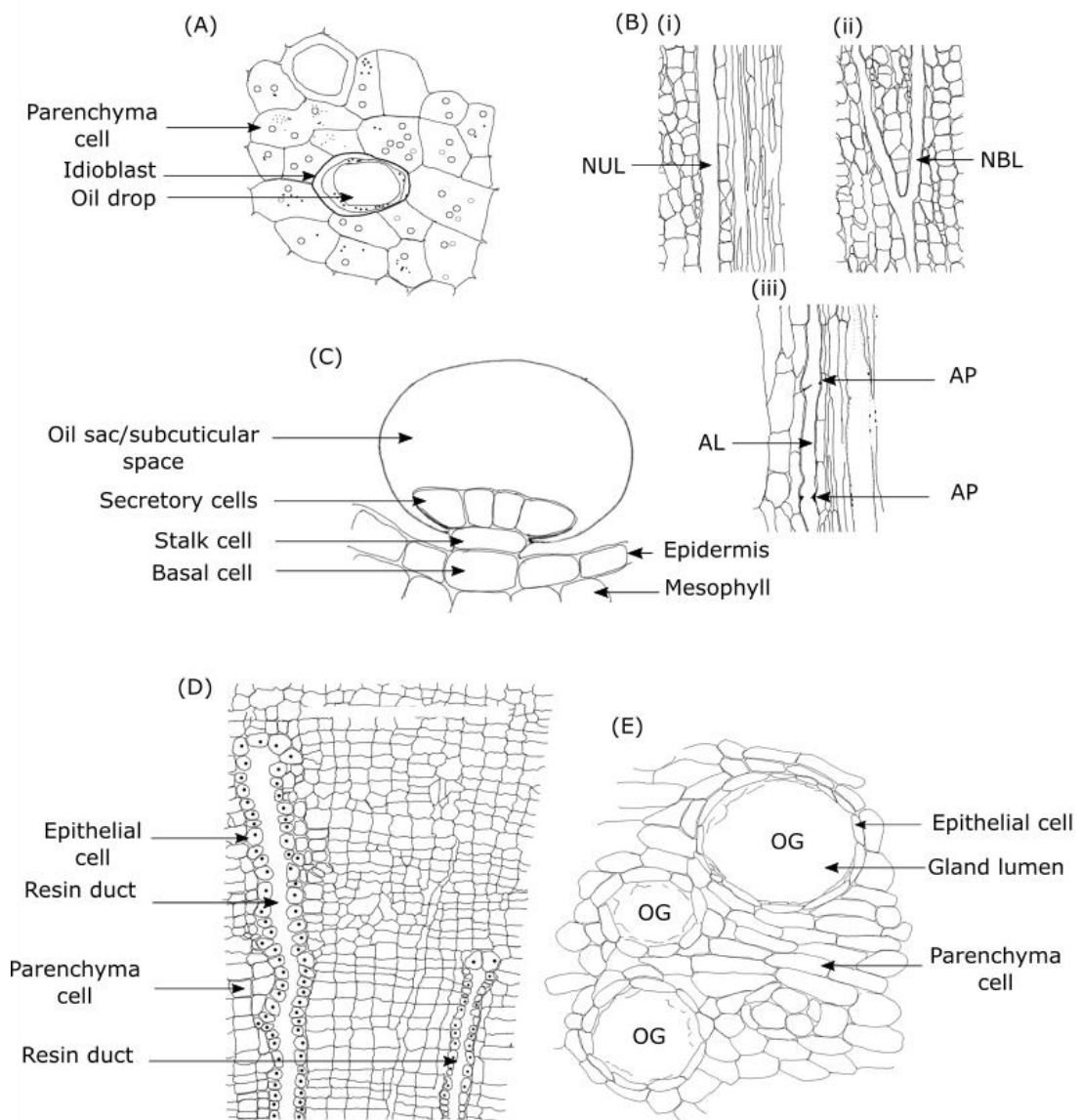


Figure 1.1: Schematic diagram of different types of specialised glandular secretory structures in plants. (A) Idioblast (B) Laticifers (i) Non-articulated-unbranched laticifer (NUL), (ii) Non-articulated branched laticifer (NBL), (iii) Articulated laticifer (AL). The articulation points (AP) between individual laticifer cells can be seen. (C) Glandular trichome (D) Resin ducts and (E) Oil glands (OG; Chattaway 1955; Cummings and Schroeder 1942; Franceschi *et al.* 2005; Hagel *et al.* 2008; Turner *et al.* 2000b).

1.2.6. The ultrastructure of secretory cells

The ultrastructure of the glandular secretory cells has been studied to a great extent, particularly in GTs. The cells secreting lipophilic substances commonly have plastids that contain osmiophilic material and are usually surrounded by ER. They also have numerous mitochondria (Fahn 2000). Some major steps of terpenoid biosynthesis occur in cellular plastids and it can be argued that all lipophilic PSMs are synthesised mainly in plastids. For example, lipophilic material has been found in both mitochondria and plastids in trichomes of two species of *Fagonia* (Fahn and Shimony 1998). Moreover, the structure of the secretory cell plastid has characteristics that are associated with terpene biosynthesis. When the sequestered oil contains high amounts of monoterpenes the leucoplasts in secretory cells completely lack ribosomes and thylakoids. In contrast, when there is low amounts of monoterpenes, thylakoids, ribosomes and tubular networks exist associated with the plastids (Fahn 2000).

1.2.7. Formation of secretory structures

The developmental formation of secretory structures in plants can be either schizogenous or lysigenous in origin. Debates exist around which particular process is responsible for the origin of secretory structures. The processes have been described in detail in only a limited number of species, mostly relating to oil gland formation in Rutaceae species. Resin ducts are schizogenously formed where localized cell separation following the breakdown of cell wall components creates spaces (Ishizaki 2015). However, an early study by McNair (1918) showed that the secretory canals of *Rhus diversiloba* (Anacardiaceae) mainly develop schizogenously, with lysigeny occurring at a later stage, suggesting a schizogenous origin. A classical concept regarding the formation of oil glands has been described in detail as a schizogenous origin by Carr and Carr (1970). Similarly, Lersten (1986) supports the theory of schizogenous development of oil glands in *Lysimachia nummularia* where the gland cavity forms from an enlarged apoplastic space. However, an earlier study (Chattaway 1955) described glands in petioles, leaves and stems of *Eucalyptus* (Myrtaceae) as lysigenous in origin. In this process, an intercellular space is created by spatially specified cell death to leave a space for PSM storage (Ishizaki 2015). Knight *et al.* (2001) suggested that early stages of gland cavity formation in *Citrus sinensis* fruit involve schizogeny, later Bennici and Tani (2004) and Chen and Wu (2010) further developed the theory and showed that the oil glands originate schizolysigenously. In this process, the cavity is formed by an initial

schizogenous event followed by lysis of the cells (Chen and Wu 2010). Schyzolysigenous development of glands has also been described in the Rutaceae family where oil production begins before lumen formation (Machado *et al.* 2017).

Some plants produce secretory structures, particularly resin ducts and glands, as a result of injury, which are then referred to as traumatic ducts or glands. They are more common in the Pinaceae family while some genera such as *Abies*, *Cedrus*, *Tsuga* and *Pseudolarix* produce resin ducts only as a result of injury (Fahn *et al.* 1979; Wimmer and Grabner 1997). These structures can be seen in the secondary xylem of the plants. Injuries such as insect attack, fungal elicitation and mechanical wounding can induce traumatic duct development (Martin *et al.* 2002). In addition, some chemicals also can induce traumatic duct initiation, but this seems to vary depending on the type of the chemical signalling molecule applied. For example, in conifers application of methyl jasmonate and ethylene induced the formation of traumatic ducts, but application of methyl salicylate did not (Hudgins and Franceschi 2004). Traumatic oil glands formation in response to wounding has also been detected in the phloem of *Eucalyptus globulus* (Eyles *et al.* 2004).

1.3. PSMs in secretory structures

Despite the large number of PSMs known to date, only a limited number is known to be exactly localised to secretory structures. Most compounds are assumed to be present in these structures, without proven scientific evidence about their localisation. The lack of this knowledge is mainly due to the difficulty of isolating secretory structures for extraction, particularly those that are embedded in other tissues. It is clear that PSMs are also present in tissues other than specialised secretory structures. For example, some hydrophobic metabolites are stored in plant cell vacuoles while some lipophilic PSMs are stored in the cuticle (Acamovic and Brooker 2005). There is evidence for the presence of phenolic compounds in the epidermis and hypodermis of some species of Fabaceae, and the vascular bundle sheath in leaves of species from Crassulaceae and Myrtaceae families (Marilia and Diego 2008). Therefore, it is important to identify the specific compounds stored in secretory structures.

In general, terpenoids, phenolics, alkaloids and diterpene resin acids are the compound groups that have been definitively identified within specific glandular

secretory structures. This section describes the different classes of PSMs, both volatile and non-volatile, that have been identified to date from specialised secretory structures.

1.3.1. Phenolic compounds

Phenolic compounds are one of the most abundant PSMs found in the plant kingdom with more than 10,000 compounds currently known. They contain one or more phenol groups (-OH group) attached to an aromatic benzene ring. Plant phenolics include structurally different groups such as phenolic acids, flavonoids, tannins, stilbenes and lignans (Dai and Mumper 2010). Phenolics are responsible for the red, blue and purple colours of most fruits and vegetables and are also important in human health (Cheynier 2012). They have been identified from different tissue-extracts of many plants such as whole leaf extracts. Some studies have identified phenolics from different secretory structures mainly from trichomes; however, compared to the enormous number of phenolics only relatively few have proven to be localised in secretory structures. Simple phenolic acids, which are either derivatives of benzoic or cinnamic acids, have been identified from secretory structures using their blue fluorescence under UV irradiation (**Table 1.1**). Examples include common cinnamic acid derivatives, mainly *p*-coumaric acid, with ferulic and caffeic acids in lower abundance, from idioblast cells referred to as ‘giant oil cells’ or ‘cotyledonary bodies’ of a *Pharbitis* cultivar (Tretyn *et al.* 1996). Other than simple phenolic acids, flavonoids, cannabinoids and polyketides are the other major classes that have been identified from secretory structures and are described in detail in this section.

Phenolic biosynthesis occurs via two pathways - the shikimate/phenylpropanoid pathway and the malonic acid pathway (**Figure 1.2**). The synthesis of most plant phenolics occurs in the chloroplast via the shikimate pathway. It directly provides phenylpropanoids such as cinnamic acid and *p*-coumaric acid. The malonic acid pathway has less significance in seed plants (Marilia and Diego 2008). In the shikimate pathway, simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway, such as erythrose-4-phosphate and phosphoenolpyruvic acid (PEP), undergo enzyme catalytic reactions and produce aromatic amino acids particularly phenylalanine. They act as the substrate for the phenylpropanoid pathway and flavonoid pathways that synthesize flavonoids and isoflavonoids (Cheynier *et al.* 2013; Santos-Sánchez *et al.* 2019). Herrmann (1995) and Bentley and Haslam (1990) describe the shikimate pathway in great detail.

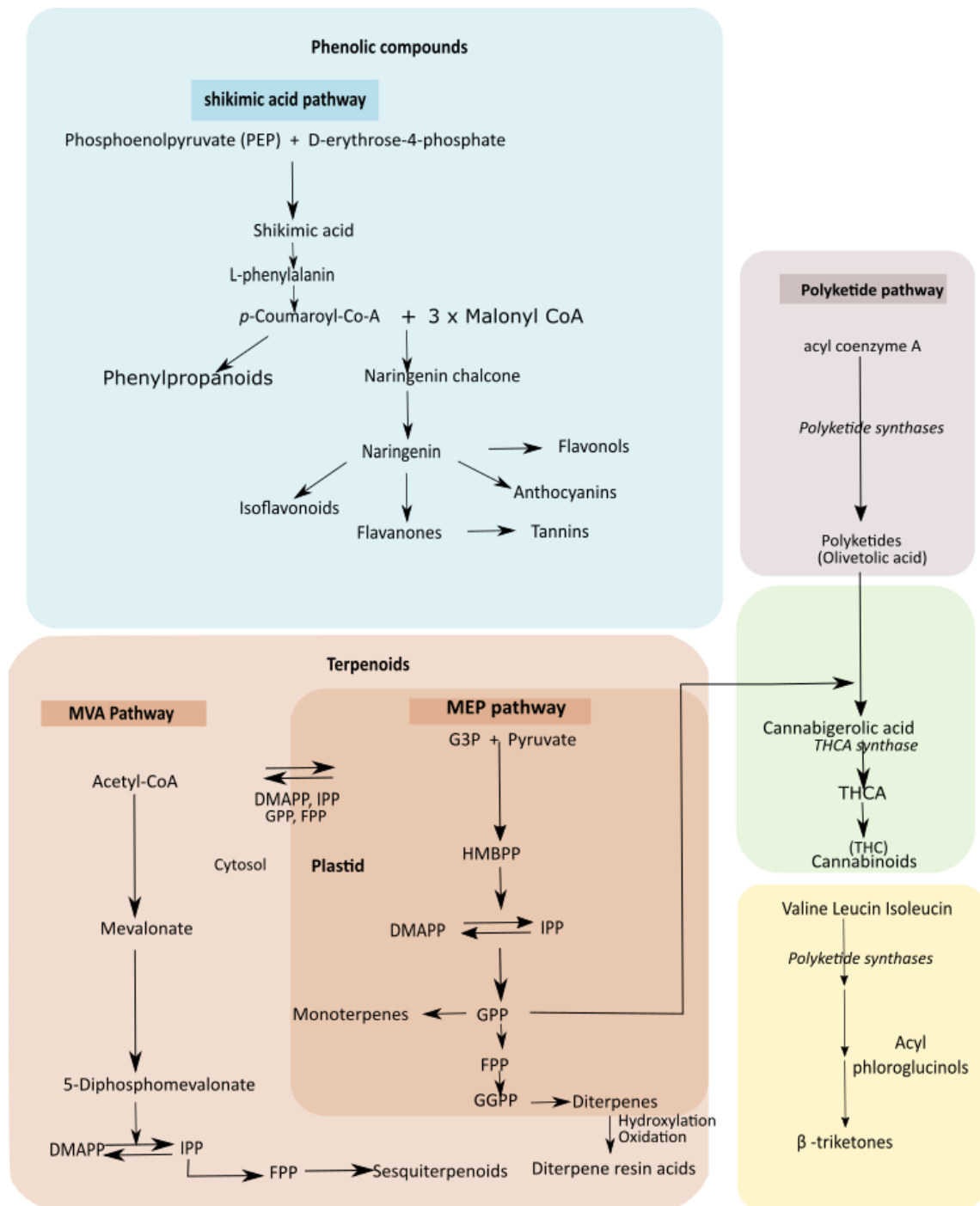
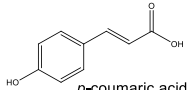
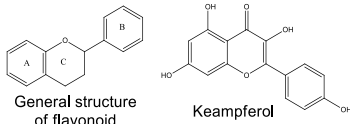
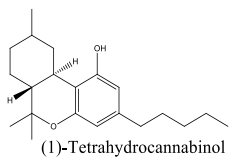
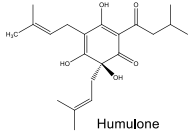
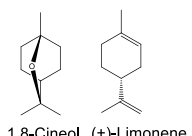
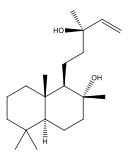
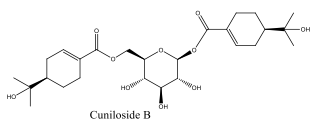
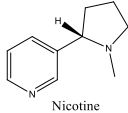
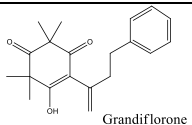
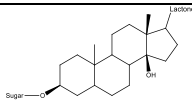


Figure 1.2: Biosynthesis pathways of major groups of secondary metabolites in the plant secretory cell.. Abbreviations IPP: isopentenyl diphosphate, DMAPP: Dimethylallyl diphosphate, FPP: farnesyl pyrophosphate, GGPP: Geranylgeranyl diphosphate, THCA: tetrahydrocannabinolic acid, THC: Tetrahydrocannabinol (Andre *et al.* 2016; Bentley and Haslam 1990; Cheynier *et al.* 2013; Killeen *et al.* 2015; Vranová *et al.* 2013).

Table 1.1: Examples for different compound groups isolated from plant secretory structures and their structures.

Secondary metabolite	Structure	Example	Reference
Phenolic compounds			
Phenolic acids	 <i>p</i> -coumaric acid	Idioblasts in <i>Pharbitis</i> cultivar (Japanese morning glory)	(Tretyn <i>et al.</i> 1996)
Flavonoids	 General structure of flavonoid Keampferol	Capitate GTs in <i>Lychnophora ericoides</i> (Asteraceae)	(GobboNeto <i>et al.</i> 2008).
Cannabinoids	 (1)-Tetrahydrocannabinol	GTs in <i>Cannabis sativa</i>	(Andre <i>et al.</i> 2016)
Polyketides	 Humulone	GTs of hop plants (<i>Humulus lupulus</i>)	(Wang <i>et al.</i> 2008)
Terpenoids			
Monoterpenes and Sesquiterpenes	 1,8-Cineol (+)-Limonene	Oil glands of <i>Eucalyptus polybractea</i>	(King <i>et al.</i> 2006)
Diterpenes	 Sclareol	Capitate GTs in <i>Salvia sclarea</i> (Lamiaceae)	(Schmiderer <i>et al.</i> 2008)
Monoterpene acid glucose ester	 Cunilioside B	Oil glands of <i>Eucalyptus</i>	(Goodger <i>et al.</i> 2009)
Alkaloids	 Nicotine	<i>Papaver somniferum</i> latex	(Hagel <i>et al.</i> 2008)
Triketones	 Grandiflorone	Oil glands of <i>Leptospermum scoparium</i> and <i>L. morrisonii</i>	(Killeen <i>et al.</i> 2015)
Cardiac glycosides	 Sugar Lactone	Laticifers in <i>Asclepias humistrata</i> (Asclepiadaceae)	(Zalucki <i>et al.</i> 2001)

i. Flavonoids

Flavonoids are a structurally diverse group of compounds widely distributed in the plant kingdom with over 6000 structures identified (Austin and Noel 2003; Iwashina 2000). The basic flavonoid structure is the flavan nucleus, containing 15 carbon atoms, consisting of two C6 phenyl aromatic rings (A and B rings) and a third C3 ring that contains a single oxygen atom (C ring; **Table 1.1**). Flavonoids are categorised into six subgroups based on the oxidation state of the central C ring, i.e., the flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (Dai and Mumper 2010). Hydroxylation, methylation, methyl and/or glycosyl substitution and also occasional additions of groups such as aromatic and aliphatic acids, sulphate, prenyl, methylenedioxy or isoprenyl to the flavonoid nucleus makes variations in basic flavonoid structure resulting in the vast number of flavonoids found in nature.

Some flavonoids have proven to be localised within the secretory structures of plants with the majority of these found in trichomes. The flavonoid categories identified from secretory structures include free lipophilic flavonoid aglycones, flavonols, flavones, unsubstituted B-ring flavanones and C-methylated flavonoids. Although flavonoids are present in both trichome types, they have mostly been identified from peltate trichomes. For example, free lipophilic flavonoid aglycones have been found in peltate trichomes of mint (Lamiaceae) and flavone aglycones thymusin, thymonin, cirsimaritin and genkwanin have been isolated from the extracts of peltate trichomes in oregano hybrid (Bosabalidis *et al.* 1998; Voirin *et al.* 1993). Flavone luteolin and flavonol kaempferol was identified from the trichome extracts of Fabaceae species alfalfa (Aziz *et al.* 2005). Capitulate GTs of *Lychnophora ericoides* (Asteraceae) produce a suite of flavonoids with no oxidation at B ring: chrysin, pinocembrin, pinostrobin, pinobanksin and 3-O-acetylpinobanksin (GobboNeto *et al.* 2008). *Tetrascera scandens* (Tetrascera) glandular trichomes showed evidence for flavonoids, however, the specific compound is unknown since the identification is histochemical based, with the compound tentatively confirmed by the appearance of yellow colour after aluminium trichloride was added to the sample (Muliyah *et al.* 2018). Flavonoids were also present in capitate trichomes of *Cucurbita pepo* var. *styriaca* (Kolb and Muller 2004). Despite many flavonoids being identified from within trichomes, only a few have been localised to embedded oil glands in any plant species. Pinocembrin, an unsubstituted B-ring flavanone, is one such example and was isolated from the oil gland cavities of four *Eucalyptus* species (Heskes *et al.* 2012b). Similarly, C-methylated flavonoids were

identified from *Leptospermum scoparium* and *L. morrisonii* using Raman spectroscopy (Killeen *et al.* 2015).

Flavonoid biosynthesis initiates from phenylalanine and acetyl CoA to form coumaryl CoA (**Figure 1.2**). Then chalcone synthase, a type III polyketide synthase catalyses condensation of 4-coumaryl-CoA with three molecules of malonyl-CoA and form a chalcone. Different flavonoids with related structures such as flavanones, flavones, flavonol are produced by different biosynthetic pathways originating from the chalcone. These biosynthetic steps are catalysed by enzymes located on the cytoplasmic side of endoplasmic reticulum (Burbulis and Winkel-Shirley 1999).

ii. Cannabinoids

Cannabinoids are a special group of terpeno-phenolic compounds differing in the structure of their terpene moiety and/or the length of the prenyl side chain attached to the phenolic portion (Pacifico *et al.* 2008). Approximately 70 cannabinoids are known to date and they have a wide range of pharmaceutical effects in humans. The majority of them are produced in *Cannabis sativa* (marijuana; Cannabaceae). They have also been detected in *Radula* and *Helichrysum* genera, however, *Cannabis* remain the major genus in which cannabinoids are found (Andre *et al.* 2016). One of the best known cannabinoids, (1)-tetrahydrocannabinol (THC), accumulates in GTs that are largely found in female flowers and also in most aerial parts of the plants, with lower quantities found in seeds, roots and pollen (Andre *et al.* 2016; Sirikantaramas *et al.* 2005).

The biosynthesis pathway of the most known cannabinoid, (1)-tetrahydrocannabinol, has been described in detail (**Figure 1.2**). The precursors of cannabinoid biosynthesis originate from two distinct biosynthetic pathways. They are the polyketide pathway, giving rise to olivetolic acid and the plastidal MEP pathway, leading to GPP (Andre *et al.* 2016). Cannabinoid biosynthesis begins with condensation of geranyl pyrophosphate with olivetolic acid (a polyketide) catalysed by the enzyme geranylpyrophosphate:olivatolate geranyltransferase. The intermediate product cannabigerolic acid then produces tetrahydrocannabinolic acid (THCA) in the presence of THCA synthase. THCA is then decarboxylated to form (1)-tetrahydrocannabinol via a non-enzymatic reaction (Sirikantaramas *et al.* 2005).

iii. Polyketides

Polyketides are PSMs synthesised from repetitive condensation reactions that link small carbon precursors, typically 2- and 3-carbon acyl groups (Pfeifer and Khosla 2001). Polyketides have been identified from GTs, in particular, the GTs of hop plants (*Humulus lupulus*) produce the α - and β -acid polyketides humulone and lupulone, that have long been used as the bittering agents in beer (Wang *et al.* 2008).

Polyketides are biosynthesised from acyl coenzyme A (CoA) precursors by polyketide synthases (Jez *et al.* 2000; Lussier *et al.* 2012). Polyketides in plants are synthesized by type III polyketide synthases by the condensation of acetyl units with a CoA-linked precursor molecule. Various precursor substrates and different reaction types such as cyclization, hydroxylation, glycosylation and methylation results in a vast structural diversity in polyketides (Lussier *et al.* 2012).

iv. Other phenolic compounds

In addition to the above major phenolic groups, some other phenolic compounds have also been identified from secretory structures, albeit generally in lower abundance. For example, tannins accumulate in laticifers, idioblasts, and both peltate and capitate trichomes in species from the families Musaceae, Adoxaceae and Lamiaceae (Corsi and Bottega 1999; Fahn 1979; Zobel 1986). In addition, two long-chain phenolic lipids, anacardic acid and cardanols, have been identified from the lumen of the secretory pockets of *Ginkgo biloba* (Ginkgoaceae) stem (Cartayrade *et al.* 1990). It is noteworthy that *Ginkgo biloba* is the only living species in the Division Ginkgophyta and have a very restricted geographical distribution (Cartayrade *et al.* 1990; Royer *et al.* 2003). Moreover, the phenylpropenes eugenol and chavicol, the simplest forms of phenylpropanoids, have also been identified from basil GTs (Gang *et al.* 2001).

Additional indirect evidence for the presence of phenolic compounds in GTs is available based on histochemical tests. For example, idioblasts apparently secreting phenolic compounds have been tentatively identified from the ground tissue in stems of *Cyperus papyrus* L., and in leaves of *Crassula multicava*, *Forsteronia australis*, *F. glabrescens*, *Pinus elliottii*, *Sapium glandulatum*, *Zornia latifolia* and *Z. reticulata* (Marilia and Diego 2008). Similarly, the idioblasts in leaves of the aquatic monocotyledonous plant *Egeria densa* produced a light blue autofluorescence when irradiated with UV light indicating the presence of a compound group likely to be

phenolics (Hara *et al.* 2015). Similarly, histochemical staining suggests idioblasts containing phenolics are found in sepals, petals, stamens and the carpel of flowers in Leguminosae inflorescences (De Barros *et al.* 2017). Furthermore, secretory idioblasts in *Piper umbellatum* (Piperaceae) leaves appear to accumulate phenolic compounds (Marinho *et al.* 2011). Such histochemistry is reasonably accurate at identifying broad compound classes, but further studies are needed to confirm the structural identity of the specific phenolics accumulating in these secretory structures.

1.3.2. Terpenoids

Terpenoids are the most abundant group of PSMs with a great structural diversity. The basic skeleton of terpenoids is made up of five-carbon isoprene units and they are classified based on the number of units attached together. Terpenes containing a single isoprene unit (C_5) are named hemiterpenes, those with two units (C_{10}) are named monoterpenes, three units (C_{15}) are named sesquiterpenes, four units (C_{20}) are named diterpenes and so on, with compounds containing up to thousands of units named polyterpenes. The basic terpene structure can be modified further by plants, and other structural groups attached to form different terpenoid compounds. To date, over 15,000 terpenoid structures have been discovered in plants with many more yet to be structural elucidated (Gershenzon and Croteau 2018). While the vast majority of them are known from whole tissue extracts like leaves, some scientific evidence is present for the localisation of them to specialised secretory structures. For example, both volatile and non-volatile forms of terpenoids are stored in oil cell idioblasts, resin ducts, GTs and oil glands.

Terpene biosynthesis in plants has been well characterised. The two major pathways involved are the mevalonic acid pathway (MVA) and the methylerythritol phosphate (MEP) pathway. The MVA pathway occurs in cytosol, peroxisomes and endoplasmic reticulum while the MEP pathway occurs in plastids (Lichtenthaler *et al.* 1997; Rohmer 1999). A diverse array of monoterpene synthases are involved in terpene synthesis in plants (Kellogg and Poulter 1997). The product of the mevalonate pathway, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) synthesised from acetyl-CoA, are called isoprene units, and are the basic building blocks of terpenoid formation. IPP and DMAPP are synthesized from pyruvate with glyceraldehyde 3-phosphate in the MEP pathway. Isoprene units then undergo enzyme catalysed condensation reactions and fuse together in head-to-head or head-to-tail to

form pyrophosphates, which then give rise to different terpene groups. Monoterpenes are produced from geranyl pyrophosphate (GPP; C₁₀), sesquiterpenes and triterpenes are produced from farnesyl pyrophosphate (FPP; C₁₅) while diterpenes, tetraterpenes and polyterpenes are produced from geranylgeranyl pyrophosphate (GGPP; C₂₀) (Kellogg and Poulter 1997; Liang 2002). Kellogg and Poulter (1997) describe the isoprenoid biosynthetic pathway in detail. Compounds from other chemical classes can be incorporated with terpene moieties to form more complex terpenoid derivatives such as monoterpene acid glucose esters and monoterpene indole alkaloids. Sesquiterpenes, triterpenes, and polyterpenes are produced in the cytosol and endoplasmic reticulum (ER) while monoterpenes, diterpenes, tetraterpenes seem to be exclusively produced in plastids (Croteau *et al.* 2000).

i. Monoterpenes and sesquiterpenes

Monoterpenes and sesquiterpenes are volatile lipophilic terpenoids that together form the essential oil found in many plant species. They are relatively common constituents in oil cells, resin ducts, GTs and oil glands. For example, resin ducts in *Pinus* and *Bursera* spp. are filled with terpene rich oleoresin that contains an array of monoterpenes such as camphene, cineole, limonene, phellandrene and α - and β -pinene (Becerra 1990; Zulak and Bohlmann 2010). There is much evidence confirming mono- and sesquiterpenes are localised to GTs. Both peltate and capitate types contain the two compound groups in different species. Examples include the monoterpenes in peltate glands of mint (*Mentha x piperita*) leaves (Voirin *et al.* 1993), (-)-carvone from spearmint (*Mentha spicata*) leaves (Gershenzon *et al.* 1989), (-)-pulegone, (+)-menthone and (+)-limonene from peltate glands of *Schizonepeta tenuifolia* (Liu *et al.* 2018) and monoterpenes in capitate GTs in *Salvia sclarea* (Schmiderer *et al.* 2008). In some species monoterpenes are stored in one type of GTs whereas sesquiterpenes are stored in another type of GTs. For example, in *Salvia sclarea* the monoterpenes linalool and linalyl acetate are housed in capitate glands in petal surfaces (along with diterpenes) whereas sesquiterpenes are located in peltate glands (Schmiderer *et al.* 2008). Sesquiterpene lactones, in which one of the methyl groups of the isopropyl group of the sesquiterpene is oxidized to a lactone group, have been detected in idioblast oil cells in *Liriodendron tulipifera* (Magnoliaceae) leaves (Mariani *et al.* 1989). Volatile mono- and sesquiterpenes have been the focus of many biochemical investigations and have gained more attention compared to the other volatile or non-volatile PSMs, and therefore there

is much evidence available for their presence in different secretory structures in the literature. This attention is likely a consequence of the economic importance of many volatile terpenes e.g. menthol and the relative ease by which GTs can be isolated from plant tissues for study.

ii. Diterpenes and diterpene resin acids

Diterpenes occur mostly in the form of resin acids, which are 20-carbon, bi- or tricyclic carboxylic acids (Keeling and Bohlmann 2006). Diterpenes and diterpene resin acids are found in conifer resin ducts in Pinaceae and also in GTs of some plant families such as Lamiaceae and Cistaceae. For example, the fragrant diterpene sclareol, was found in the capitate GTs in petal surfaces of *Salvia sclarea* (Schmiderer *et al.* 2008). In tobacco, the diterpenes, α - and β -4,8,13-divatriene-1,3-diol have been shown to be synthesised in GTs (Keene and Wagner 1985). Similarly, Falara *et al.* (2008) reports the presence of diterpenes in GTs of *Cistus criticus* (Cistaceae). Labdane-type diterpene marrubiin was detected in the cells of GTs in leaves of *Marrubium vulgare* (Lamiaceae) (Piccoli and Bottini 2008). Diterpene resin acids are found in resin ducts of *Picea glauca* (Abbott *et al.* 2010). In addition to resin acids, minor amounts of diterpene aldehydes and diterpene alcohols occur in conifers (Abbott *et al.* 2010).

Cyclic diterpene resin acids are synthesised from the common acyclic biosynthetic precursor GGPP that is derived in the MEP pathway (**Figure 1.2**; Lichtenthaler 1999). First, the diterpene synthase enzymes convert the 20-carbon substrate to diterpenes. These diterpenes are hydroxylated and then oxidised by enzymes to form diterpene acids (Keeling and Bohlmann 2006; Lichtenthaler 1999). Research suggests that cytochrome P450 monooxygenases (CYP450) play a role in the modifications of diterpenes to diterpene acids (Geisler *et al.* 2016; Keeling and Bohlmann 2006).

iii. Monoterpene acid glucose esters

Monoterpene acid glucose esters (MAGEs) are non-volatile terpenoids in which their terpene group is esterified to a glucose molecule. The monoterpenoids found commonly in MAGEs are oleuropeic and/or menthialfolic acid. In the majority of MAGEs, the acid group is neutralised via esterification at the primary hydroxyl position of the glucopyranose ring. However, in some compounds the esterification occurs at the anomeric carbon of glucose molecule; for example, in globulisin B, cuniloside A and B and 1-O-[(S)-oleuropeyl]- β -D-glucopyranose (Goodger *et al.* 2009; Hasegawa *et al.* 2008; Manns and Hartmann 1994). Either one or both of these aliphatic acids are found as aglycones in a MAGE. The distribution and the structural diversity of MAGEs have been described by Goodger and Woodrow (2011).

MAGEs are found co-localised with volatile oils in *Eucalyptus* oil glands (Goodger and Woodrow 2011). Some of the MAGEs have been identified directly from the oil glands of eucalypts. The two MAGEs, cuniloside B and froggattiside A, were the non-volatile PSMs identified from embedded glands of any plant species for the first time. The two compounds were isolated from the foliage oil glands of *E. polybractea*, *E. froggattii* and *E. globulus* using an enzyme digestion protocol (Goodger *et al.* 2009). Following that, eighteen MAGEs including putative structures were identified from oil glands of 19 *Eucalyptus* species. Cuniloside B, froggattiside A and two unknown MAGEs were found in the gland of *Melaleuca armillaris*, a species also from family Myrtaceae (Heskes *et al.* 2012b). MAGEs are also known from other plant families such as Euphorbiaceae, but their exact localisation in secretory structures outside of Myrtaceae is unknown (Meng *et al.* 2010).

The biosynthesis of MAGEs is not yet clearly described. The two monoterpene acids oleuropeic acid and menthialfolic acid are likely to be derived from the monoterpenes α -terpineol and linalool, respectively (Goodger and Woodrow 2011). The reactions that convert monoterpenes to corresponding monoterpene acids are likely catalysed by cytochrome P-450 enzymes (Chadha and Madyastha 1984). Despite limited evidence from biotransformation studies (Renganathan and Madyastha 1983), there is a crucial lack of knowledge on the process of esterification of monoterpene acid to glucopyranose in MAGEs in plant tissues. Glucotransferase enzymes are likely to be involved in the process similar to those that produce O-glycosides (Ikan 1999). Nonetheless, there is no knowledge on the specific glucotransferases involved in the esterification of monoterpene acids to glucopyranose in MAGEs.

1.3.3. Alkaloids

Alkaloids are low molecular-weight cyclic compounds containing nitrogen in a negative oxidation state and are derived mostly from amino acids (Ziegler and Facchini 2008). They are a highly diversified group in plants and are estimated to be composed of some 12,000 different compounds. A remarkably large number of these compounds have been used as therapeutics, stimulants, relaxants, psychedelics, euphorants and poisons by humans for millennia. The major groups include terpene indole alkaloids that contain an indole moiety attached to an isoprene unit, benzyloquinoline alkaloids, purine alkaloids, pyrrolidine alkaloids, piperidine alkaloids, pyrrolizidine alkaloids, tropane alkaloids, aminated terpenes and polyketide alkaloids (Ziegler and Facchini 2008).

Alkaloids have been identified from almost all types of plant secretory structures. The first alkaloid identified was from opium poppy latex (*Papaver somniferum*; Papaveraceae) and later the alkaloids in opium latex were identified as the benzyloquinoline alkaloids morphine, codeine and thebaine (Hagel *et al.* 2008). At least five different types of alkaloids are present in the latex of different plants. These include monoterpene indole alkaloids, pyrrolidine alkaloids and piperidine alkaloids. For example, idioblasts in the parenchymatic and epidermal tissues of *Camptotheca acuminata* (Nyssaceae) accumulate crystals of camptothecin, a monoterpene indole alkaloid, in their vacuoles (Pasqua *et al.* 2009). Camptothecin has also been found in GTs on both the stem and leaf of the same species (Pasqua *et al.* 2009). St-Pierre *et al.* (1999) reports another monoterpenoid indole alkaloid vindoline in idioblasts and laticifers in *Catharanthus roseus* (Apocynaceae). Pyrrolidine alkaloids such as 1,4-dideoxy-1,4-imino-D-arabinitol are present in mulberry (*Morus* spp.) latex (Hirayama *et al.* 2007), and the piperidine alkaloid lobeline accumulates in the latex of *Lobelia* spp. (Dussourd 2003). Alkaloids are also present in GT secretions of most *Nicotiana* species. Nicotine, a well-known addictive alkaloid, has been identified from GTs of many *Nicotiana* species where it is the major alkaloid constituent **Table 1.1**). The alkaloids anabasine and nornicotine have also been found in GT secretions of *N. benthamiana* and *N. glauca* (Thurston *et al.* 1966). Histochemical evidence also suggests oil glands can contain alkaloids in their lumen. For example, the glands in *Metrodorea nigra* (Rutaceae) show black staining when treated with Lugol's reagent indicating the likely presence of alkaloids (Machado *et al.* 2017).

1.3.4. Triketones

β -Triketones are a rare group of PSMs that have four methyl substituents on a six-membered carbon ring and an acyl side chain. They can be derived from either a phloroglucinol ring, a resorcinol ring or a cyclopentanedione ring (Hellyer 1968; van Klink *et al.* 1999). Leptospermone, grandiflorone and flavesone are the mostly commonly found triketones in plants, but interestingly they have only been found in members of the family Myrtaceae. Triketones have been localised to the oil glands of *Leptospermum* species using Raman microscopy. Specifically, the triketone grandiflorone was shown to be present in the leaf oil glands of *L. scoparium* and *L. morrisonii* using this technique (Killeen *et al.* 2015).

There is a paucity of knowledge on the biosynthesis of triketones. Killeen *et al.* (2015) briefly describes the biosynthesis pathway of major triketones. Acyl phloroglucinols are intermediates in the biosynthetic pathway where polyketide synthases and reductases are involved in the synthesis of grandiflorone, leptospermone, isoleptospermone and flavesone.

1.3.5. Other PSMs

Apart from the aforementioned groups of PSMs found commonly in secretory structures of many plant families, some other compounds have also been reported from certain families less frequently. Despite not being commonly found, these compounds can still be accumulated at high abundances in some plant species, for example, acyl sugars and cardiac glycosides. An acyl sugar is an aliphatic acyl group esterified to the hydroxyl groups of glucose or sucrose. Acyl groups vary in their length and backbone structure (Arrendale *et al.* 1990; Kroumova *et al.* 2016). Acyl sugars are a highly abundant group of PSMs found in the trichomes of Solanaceae species, and have been shown to perform an important defensive role against insect herbivores (Slocombe *et al.* 2008; Weinhold and Baldwin 2011). Cardiac glycosides are compounds consisting of a cardenolide or bufadienolide aglycone linked to one or more sugar molecules. These compounds may be toxic at high doses, but are also used at therapeutics levels to treat heart disorders (Akinmoladun *et al.* 2014). The compounds are found in the sap in laticifers in some plant families, and are particularly well known from Asclepiadaceae species such as *Asclepias humistrata* (Zalucki *et al.* 2001).

1.3.6. Biosynthesis of PSMs in secretory structures

Biochemical evidence suggests that the biosynthesis of PSMs takes place fully or partially within the same specialised secretory structures in which these compounds accumulate. In particular, a number of publications on family Lamiaceae show that PSM biosynthesis in GTs occurs in the secretory cells located at the apex of the trichome. For example, research on monoterpene biosynthesis in spearmint (*Mentha spicata*) peltate GTs has shown that the three enzymes responsible for the biosynthesis of the major monoterpene (-)-carvone, (a cyclase, a hydroxylase and a unique carveol dehydrogenase) are all located exclusively in GTs (Gershenzon *et al.* 1989). A study by the same group later detected a carveol dehydrogenase in the other peppermint tissues as well, but the authors showed the trichome enzyme was unique and different from the similar enzymes found in the rest of the leaf. The authors suggested that the enzyme found in the other leaf tissues utilizes carveol only as an adventitious substrate (Gershenzon *et al.* 1992). In another Lamiaceae species, *Schizonepeta tenuifolia*, three enzymes involved in the proposed biosynthetic pathway for the monoterpene *p*-menthane ((+)-limonene synthase, (+)-cis-isopiperitenol dehydrogenase and (+)-cis-isopiperitenone reductase) have been detected in extracts of peltate GTs (Liu *et al.* 2018). More extensive studies showed that the glandular head portion is the part of the trichome capable of producing PSMs. In addition, diterpene divatrienediol synthesis was observed in detached, intact glandular heads from trichomes of *Nicotiana tabacum* (Keene and Wagner 1985). The head portion of the trichome was identified as the primary or possibly the only site of synthesis. The study also shows that chloroplasts, which are abundant in glandular heads, are involved in the biosynthesis (Keene and Wagner 1985). Similarly, in cannabinoid biosynthesis, THCA synthase is expressed in the storage cavity of GTs (Sirikantaramas *et al.* 2005). It shows that the secretory cells not only secrete the synthesised metabolites but also the enzymes responsible, to the glandular cavity. Functional THCA synthase was present in the storage cavity suggesting that the trichome storage cavity is the site of synthesis of the cannabinoid (1)-tetrahydrocannabinol (Sirikantaramas *et al.* 2005). A study by Mahlberg and Kim (2004) also supports the synthesis of cannabinoids in the storage cavity by showing that cannabinoids are synthesized outside the specialized disc cells in GTs of *Cannabis* (Cannabaceae) and are accumulated in the adjacent cavity. Furthermore, a study on basil peltate glands showed them as the site of storage and most likely the site of synthesis of phenylpropenes (Gang *et al.* 2001). The peltate trichomes transcribe the genes encoding the enzymes of phenylpropene biosynthesis pathway at very higher rate indicating they

are the major site of synthesis (Gang *et al.* 2001). Another strong line of evidence is the presence of phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), which are key components in flavonoid biosynthesis, in all biosynthetically active trichome stages suggesting that flavonoid biosynthesis occurs within the trichomes (Göpfert *et al.* 2006).

Evidence also supports at least the partial biosynthesis of alkaloids in specialised secretory structures. Despite alkaloids being present in almost all types of secretory structures, evidence for the location of biosynthesis has been found only from research on idioblasts and laticifers. The two types of secretory structures have been identified as the sites where vindoline - the main precursor of the two alkaloids vincristine and vinblastine - biosynthesis occurs (Mekky *et al.* 2018). Similarly, the final synthesis of the benzyloquinoline alkaloids occur in laticifers while the early steps occur in sieve elements and companion cells (Beaudoin and Facchini 2014). For example in *Catharanthus roseus*, the enzymes involved in the early stage of vindoline (Indole alkaloid) biosynthesis are found in the epidermis of stems, immature leaves, and flowers, and in the apical meristem of the root tips, whereas the enzymes involved in the later stages are limited to the laticifer and idioblast cells (St-Pierre *et al.* 1999). The same multi-stage biosynthesis occurs in opium poppy, where earlier steps in the biosynthesis of the benzyloquinoline alkaloids take place in neighbouring sieve elements and companion cells, with the final steps localized in laticifers (Beaudoin and Facchini 2014). In these examples, resin ducts and laticifers seem to act as transporting channels to a degree, rather than solely acting as secretory and storage tissue.

As mentioned, the most extensive studies on PSM biosynthesis come from GT work while there is much less knowledge available on the synthesis of PSMs in oil glands, likely due to the difficulties in isolating these embedded structures. It is generally accepted that formation of oil in glands occurs in the secretory epithelial cells lining gland lumina. Evidence for this comes from studies on *Citrus* species where cDNA from the MEP pathway of monoterpene synthesis was isolated and expression detected in both developing and mature oil glands (Yamasaki and Akimitsu 2007). Such studies have focused on pathway genes relating to overall oil production rather than the biosynthesis of individual PSM components.

Oil production in glands has been described in families Leguminosae and Rutaceae in greater detail. Production of oil apparently begins before lumen formation (Machado *et al.* 2017). The epithelial cells of the gland wall are active for a limited

period, primarily during the early development of the gland, and later they become senescent or die just after the lumen fills with oil components. The walls of the epithelial cells facing the lumen undergo continuous peeling, allowing the newer cells to secrete the metabolites. The parenchyma sheath, likely a meristematic sheath that surrounds the gland, divides and acts to gradually replace the epithelial cells that undergo lysis in the process of exudate release (Machado *et al.* 2017; Rodrigues *et al.* 2011). Nevertheless, a contrasting description about oil gland formation in *Citrus* species says that the secretory cells are longer lived and remain active in oil production throughout development (Knight *et al.* 2001). The amount of oil within glands is physically limited by gland capacity and that is linked with leaf expansion rather than the biosynthetic capacity of glands (King *et al.* 2006). No studies are available on the biosynthesis of any non-volatile PSMs in oil glands.

Secretory structures should possess mechanisms to have maximum production including the supply of energy and precursors needed to carry out secondary metabolite biosynthesis efficiently (Balcke *et al.* 2017). The structural arrangement of these structures supports to have a maximum production. For example, the secretory wall of GTs undergoes several cell divisions, thickening of the cuticle, and an enlargement of the subcuticular space as the oil begins to accumulate (Huang *et al.* 2008). In some instances the volume of the trichome head swells into multiples to maximise the storage of metabolites for an efficient biogenesis (Balcke *et al.* 2017). Although there is only limited knowledge of the energy supplying mechanisms that support high productivity of PSMs, there is some evidence from certain secretory structures, particularly from GTs. For example, GTs of tomato are photosynthetic and the energy and reducing power from photosynthesis is used to support the PSM production (Balcke *et al.* 2017). Supporting this phenomenon, it has been identified that trichome heads of Solanaceae species have abundant chloroplasts (Akers *et al.* 1978). Nevertheless, they acquire their carbon requirement from leaf sucrose (Balcke *et al.* 2017). Trichomes also possess mechanisms to increase the supply of precursors for the metabolic pathways to synthesise PSMs. Particularly in tomato glandular trichomes, the citrate-malate shuttle supplies cytosolic acetyl-CoA which is precursor of the MEV pathway (Balcke *et al.* 2017). Plastidic glycolysis and malic enzymes support the formation of plastidic pyruvate (Balcke *et al.* 2017).

1.4. Role of compounds localised to secretory structures

One of the major roles of PSMs sequestered to plant secretory structures is chemical defence against herbivore and pathogen attacks. Many PSMs present in secretions have shown biological activities related to plant defence such as toxicity. Evidence includes the widely found monoterpene from oil gland and GTs, 1,8-cineole acting as a foliar feeding deterrent to large herbivores (Croteau *et al.* 2000). Similarly in *Nicotiana* species, the secretions of trichomes containing nicotine are resistant to aphids while the diterpenes in the secretion, α - and β -duvatrienediols, inhibit germination of fungal spores (Cruickshank *et al.* 1977; Thurston *et al.* 1966). Bioassays using MAGEs found in oil glands have shown inhibitory effects on bacteria and fungi and cytotoxic activity, therefore, they may perform such defensive functions *in planta* (Hou *et al.* 2000). Furthermore, *Pinus* synthesise a terpenoid resin as a defence mechanism against insects and microorganisms (Zulak and Bohlmann 2010).

The PSMs stored in secretory structures may also help provide a level of physical protection by forming barriers to prevent pest entrance, particularly in laticifers and resin ducts. When injured plants possessing these structures exude latex exudates that act as a deterrent to feeding against insect herbivores (Becerra 1990; Dussourd and Eisner 1987). Oleoresin secreted by resin ducts in conifers that acts against herbivores also contains volatile mono- and sesquiterpenes and non-volatile diterpene resin acids. Oleoresin physically pushes insect herbivores out of the site of entry with oleoresin flow. After the volatile components have evaporated the non-volatile diterpene resin acids harden to seal the wound (Keeling and Bohlmann 2006). On the other hand, secretory structures that are located towards the periphery of tissues and are therefore more exposed to the environment may serve a role protecting inner developing tissues. For example, trichomes located on the surface of plants not only accumulate phytotoxic oils, but position the compounds as a first line of defence that is encountered by herbivores often before plant tissue is damaged (De Barros *et al.* 2017; Wagner 1991).

In addition to defensive roles, the PSMs may also perform diverse roles in ecological interactions due to the volatility of particular compounds enabling them to act as signalling molecules to interact with the environment. In flowers and fruits, secretory structures serve to attract and/or reward pollinators and consumers. For example, 1,8-cineole emitted by flowers can serve as attractants for pollinators (Dudareva *et al.* 2006). Secretory structures in flowers involve in protection apart from functions related to reproduction. Phenolic idioblasts in Leguminosae indicate a protective function for the

inner developing floral organs. Idioblasts in vascular bundles of floral organs may act as a barrier against the entry of pathogens by releasing volatiles and could act to protect valuable reproductive organs from pathogens (De Barros *et al.* 2017; Dudareva *et al.* 2006). Similarly, fruits have a secretory endocarp that exists even in young stages (Augusta Dantas Tolke *et al.* 2017). Some other ecological roles involve chemical defence against damage by UV radiation by flavonoids and monoterpenes providing a competitive advantage as allelopathic agents that inhibit the seed germination of other species (Croteau *et al.* 2000). Some PSMs are also involved in controlling associated insect behaviour, for example, conifer terpenes affect the insect behaviour such as feeding and reproduction and act as attractants of parasitoids and predators of some herbivorous insects (Raffa 2014; Seybold *et al.* 2006).

Despite the many known roles performed once the compounds are secreted or released to the environment, their roles within the secretory structures themselves are unclear. These compounds may accumulate in specialised structures only as a mean of storage to avoid autotoxicity. However, internal roles of non-volatiles can be assumed as some of them are spatially segregated to the exterior of cavity lumina (Heskes *et al.* 2012b). For example, MAGEs in *Eucalyptus* glands could perform a protective function as a barrier layer between the toxic oils housed in the centre of cavity lumina and the functioning epithelial cells of gland walls (Heskes *et al.* 2012a). In support of this hypothesis, oil droplets have been observed within the non-volatile layer indicating that volatiles may diffuse through the protective layer and into the cavity centre (Heskes *et al.* 2012a). Therefore, MAGEs could play a role in protection, biosynthesis and mobilization of monoterpene oils from the site of synthesis presumably in the epithelial cells lining the gland lumen to the extracellular lumen (Hakki *et al.* 2010). However, it is not known if other compounds localised to the secretory structures perform similar roles.

Triketones are likely compartmentalised to glands to avoid auto-toxicity and to reduce the possibility of photolysis of the compounds. Triketones have absorption maxima between 270 and 350 nm, therefore they absorb solar light and can potentially undergo photolysis (Dumas *et al.* 2017). On the other hand, triketones cause photodynamic bleaching under high light intensity, therefore, they can be toxic to plant cells. This property has been harnessed in their use as commercial herbicides.

1.5. Conclusion

Plant glandular secretory structures accumulate different groups of PSMs possibly to avoid autotoxicity to secretory epithelial and other living cells. This sequestration provides plants with the ability to accumulate biologically active compounds in high concentrations, thereby protecting them from herbivory and facilitating other ecological roles. Despite some structures such as trichomes being relatively well researched, at least in some plant families, the contents of other secretory structures in most plant families is under-surveyed. In particular, more non-volatile PSMs are likely to be present in embedded oil glands, but the ability to isolate or otherwise analyse their specific contents and modes of biosynthesis remains a challenge. The aim of this thesis is to explore the glandular non-volatile content in *Eucalyptus*, a genus characterised by a high abundance of embedded oil glands and large quantities of the terpenic oils they contain.

1.6. Thesis approach

Plants from the family Myrtaceae have oil glands that contain abundant volatile and non-volatile PSMs. In particular, species in the genus *Eucalyptus* are rich in PSMs such as terpenoids and phenolics. Some of these compounds, such as volatile terpenes and some non-volatile terpenoids, have been identified from oil glands; however, the localisation of most of the other PSMs, like terpene phloroglucinols and phenolics, remains unclear. The focus of this thesis is to explore the distribution of the non-volatile PSMs localised to the foliar oil glands in the genus *Eucalyptus*, and to measure their relationships to volatile oil components.

Eucalyptus (L'Herit) is a tree genus distributed widely in the Australian landscape. The genus is rich in species diversity, consisting of over 700 species in seven subgenera. The two largest subgenera with a higher number of species are *Symphyomyrtus* and *Eucalyptus* while the other subgenera contain relatively few species in each taxon (Brooker 2000; Nicolle 2015). The majority of *Eucalyptus* species are native to Australia and some islands to the north of the continent (Ghisalberti 1996). Nevertheless, it is the world's most widely planted genus as it is cultivated widely as a timber species. Leaves are usually isobilateral, vertically oriented and concolorous, i.e.

having approximately the same morphology and colour on both leaf sides (Brooker and Kleinig 2006; King 1997). The trees of this genus have many adaptations to the varied climates in the region such as for drought tolerance and resistance to fire (Gill 1997). However, the species from the two major subgenera exhibit differences in relation to their habitat and physiology. *Symphyomyrtus* species exhibit a higher growth rate and survival in both native and exotic habitats than subgenus *Eucalyptus* species, which could be related to their difference in respiratory parameters (Anekonda *et al.* 1999; Noble 1989). Also, the species from the two subgenera show a differential distribution pattern throughout Australia, where subgenus *Eucalyptus* show a limited distribution compared to the *Symphyomyrtus* species (Ladiges *et al.* 2010). Despite these differences in the two subgenera, in general, the species from this genus synthesize defensive chemicals some of which are widely used by humans. Leaves, roots and fruits of eucalypts have been used in traditional folk medicine in Australia and many other regions in the world mainly for cold and influenza (Gomez-Estrada *et al.* 2011; Navarro *et al.* 1996; Smith 1991; Tabuti *et al.* 2010).

The presence of embedded foliar oil glands is a characteristic of the genus. The oil glands occur in most tissues such as leaves, phloem and the pith and flowers. In addition to glands, a system of oil containing canals named “oil ducts” have also been observed in the pith, midrib and petiole in some *Eucalyptus* species (Carr and Carr 1969; Carr *et al.* 1970). The oil gland frequency in leaves can vary to a great extent; for example, *E. megacarpa* has less than 50 glands cm⁻² while *E. protensa* has a frequency as high as 3400 glands cm⁻² (Brooker and Nicolle 2013). Oil glands are initiated in the eucalypt embryo at a very early stage and the gland development pattern is uniform throughout the genus. They are formed of multiple layers of overlapping outer cells and an inner layer of epithelial cells that surround the central cavity (Bohte and Drinnan 2011; Carr and Carr 1970).

Eucalyptus oil glands are known as repositories of essential oils and the genus is popular for its oil which contains mainly monoterpenes and sesquiterpenes (Gilles *et al.* 2010). Oil of *Eucalyptus* species has been investigated since very early studies. Brophy and Southwell (2002) summarised the oil constituents in eucalypts. The oil composition shows a great diversity between species. Monoterpenes dominate the oil profile of most of the species, with α -pinene and 1,8-cineole being the most dominant components. In contrast, some species have a sesquiterpene dominant oil composition; for example *E. coccifera* (Brophy and Southwell 2002). The oil components have many biological

activities against fungi, bacteria, insects, mites and weeds (Batish *et al.* 2008). These properties have given advantageous ecological effects to the plant such as protecting the leaves from herbivores/pathogens and providing allelopathic properties to the tree which result in retarding the growth of associated vegetation (Liu *et al.* 2008). It has long been believed that *Eucalyptus* oil glands contain exclusively essential oil components, particularly mono- and sesquiterpenes. Chemical analyses have been generally focused on elucidating the oil extracted from whole leaves rather than characterising the entire biochemistry of oil glands. King *et al.* (2006) analysed the chemistry of individual oil glands showing for the first time that *Eucalyptus* glands house both monoterpenes and sesquiterpenes. In eucalypts, the exact site of terpene biosynthesis is unknown; however, it is assumed to be in the oil glands based on the evidence from other species that accumulate terpenes in glands (Voo *et al.* 2012).

Until recent years, *Eucalyptus* glands were accepted to contain only the volatile oil components; however, non-volatile terpenoids and few phenolics were reported from glands in the last decade, changing the long believed concept. Goodger *et al.* (2009) showed that glands not only house volatile terpenoids, they also contain non-volatile terpene components, particularly MAGEs. Following this discovery, the flavanone pinocembrin was also found localised to the foliar glands of *Eucalyptus* (Heskes *et al.* 2012b). Marilia and Diego (2008) have also reported the presence of phenolic PSMs in the epithelium and the lumen of *E. cinerea* glands; however, it was confined without structural identification of the compound due to the nature of the test.

Many other non-volatile PSMs are known from *Eucalyptus* leaf extracts though it is uncertain about their localisation in oil glands. In particular, triterpenoids have been identified from some species, for example leaf wax of 17 species from *Symphyomyrtus* (Li *et al.* 1997; Pereira *et al.* 2005). Formylated phloroglucinol compounds (FPCs) is another special group of PSMs unique to the family Myrtaceae that had an uncertainty about their localisation in oil glands. Very recently, matrix-assisted laser desorption ionization-mass spectrometry imaging (MALDI-MSI) revealed that FPCs are also associated with embedded oil glands in two *Eucalyptus* species (Santos *et al.* 2019). FPCs are mono to tetra formylated phloroglucinol-based derivatives with an attached terpene moiety with various monoterpenes and sesquiterpenes linked to the formylated phloroglucinol ring (Eyles *et al.* 2003). To date, all FPCs, except one which is also from the family Myrtaceae, have been identified from *Eucalyptus* species. Reports indicate that they are present in leaves and buds of eucalypts and also in wound wood. For

example, macrocarpal A and B, and sideroxylonal A and B were detected in wound wood of *E. globulus* and *E. nitens* (Eyles *et al.* 2003). The two major groups of FPCs identified from eucalypts are macrocarpals, which incorporate a sesquiterpene, and euglobals, which incorporate either a monoterpene or a sesquiterpene (Kozuka *et al.* 1982). Apart from these two groups, some other FPCs that lack the terpene adduct such as grandinol, jensenone and sideroxylonal also occur in eucalypts (Eschler *et al.* 2000). It is possible that only the FPCs associated with a terpene moiety are present in oil glands but not the other FPCs. There is indirect evidence to predict that FPCs with a terpene adduct occur in oil glands. For example, in two different chemotypes of *E. globulus*, FPC concentrations have shown a strong positive correlation with concentrations of the monoterpene 1,8-cineole, which is an abundant eucalypt oil gland component (Lawler *et al.* 1999; Moore *et al.* 2004). In the recent study by Santos *et al.* (2019), ions corresponding to sideroxylonals were not detected in glands. On the other hand, macrocarpals and euglobals have been reported only from subgenus *Symphyomyrtus* species, suggesting a possible phylogenetic relationship (Eschler *et al.* 2000). FPCs show a great structural diversity with at least 47 different compounds found, and more compounds are likely to be found given the fact that various monoterpenes and sesquiterpenes can incorporate in different manners (Konoshima and Takasaki 2002; Singh *et al.* 2006). Among them, the chances are higher that more FPCs will be found in oil glands, at least the structures that incorporate a terpene adduct.

Although some MAGEs are now known from glands, there is more that are still known only from leaf or other organ extracts. Despite the MAGEs localised to oil glands, about 40 different MAGEs are known from plants to date mostly extracted from whole leaves. MAGEs are present in the plant families such as Adoxaceae, Fabaceae, Lamiaceae, Myrtaceae, Oleaceae and Plantaginaceae. However, the distribution of MAGEs within most of the families except Myrtaceae does not seem to be consistent. They have been reported only from a few species from each family; for example *Cunila spicata* from Lamiaceae, *Portulaca oleracea* from Portulacaceae and *Jasminum hemsleyi* from Oleaceae (Li *et al.* 2014; Manns and Hartmann 1994; Tanahashi *et al.* 1995; Taskova *et al.* 1998). In contrast, MAGEs have been identified from a range of species from family Myrtaceae and some of them were directly identified from oil glands (Heskes *et al.* 2012b). Therefore, there is a higher chance for more MAGEs to be localised to secretory structures and more compounds to be found from the same family.

In addition to FPCs and MAGEs, some other non-volatile phytochemicals are common in eucalypts. These include phenolics, particularly flavonoids with both unsubstituted and substituted B-ring (Saraf *et al.* 2017), hydrolysable and condensed tannins, long chain ketones (Fox and Macauley 1977; Moore *et al.* 2004) and cyanogenic glycosides (Gleadow *et al.* 2008). Despite these large number of compounds identified from whole tissue extracts only some terpenoids and a single flavonoid have been identified from oil glands.

The non-volatile compounds in eucalypt oil glands can comprise up to 50% of gland lumen volume (Goodger *et al.* 2010). The volume of the oil obtained from a leaf does not match quantitatively with the estimated gland lumen volume (King *et al.* 2006). This highlights the fact that there is still an undiscovered proportion of compounds localised within the eucalypt oil glands. Despite the importance of oil glands as repositories of many biologically and ecologically important volatile and non-volatile compounds, the physiology of these tissues have been poorly described. One of the major reasons for this is the difficulty of physically isolating these embedded structures. Although many studies have addressed the chemical nature of volatile oils, little work has focussed on the non-volatile contents in oil glands. Only a few detailed studies on the chemistry of non-volatiles in oil gland have been reported. Compared to the volatile terpenoids, the non-volatiles localised to the oil glands in *Eucalyptus* have gain less attention. Hence, this thesis attempts to understand the oil gland chemistry in more detail focusing on non-volatiles in relation to volatile oil components.

1.6.1. Thesis aims

The identification of PSMs localised in eucalypt oil glands is important for a variety of reasons including the mapping of biosynthetic pathways and the discovery of physiological roles. Moreover, PSMs synthesised in secretory structures have a high commercial importance in pharmaceutical, cosmetics and food industries due to their biological activities. The localisation of such compounds will have a positive impact on selection of high yielding species or genotypes for commercial cultivation purposes. Despite this biological and commercial importance, only a low number of species have been specifically studied for the presence of non-volatile PSMs in this large genus.

It is hypothesised that all *Eucalyptus* species synthesize non-volatile PSMs in their oil gland cavities in addition to volatile oil components. The finding of MAGEs, particularly cuniloside B and cypellocarpin C, localized to the glands of many species

suggest that some NVCs could be ubiquitous in the genus. Therefore, MAGEs are likely to occur in more *Eucalyptus* species as well as other NVCs like phenolics and FPCs. Recent investigation on techniques to isolate oil glands opened up new possibilities for exploration of the compounds in these embedded structures. This thesis attempts to address this gap in our knowledge on the *Eucalyptus* oil gland metabolome.

The goal of this thesis is to characterise the non-volatile components localised to the glands in a selected range of *Eucalyptus* species, in relation to volatile oils. In the first part of Chapter 2, MAGEs present in the foliar oil glands of *Eucalyptus* species representing the two major subgenera *Symphyomyrtus* and *Eucalyptus* are characterised. The identity, abundance and distribution of MAGEs in oil glands is then presented. The second part of the chapter is a study of *E. froggattii* to understand how MAGEs and oil vary within a natural population. The third part of the chapter describes the establishment of cell suspension cultures of *E. polybractea* as a potential system to study the biosynthesis of NVCs. Biotransformation of *p*-coumaric acid in *E. polybractea* cell cultures was used to identify the enzymatic processes in these cells that may be involved in the production of phenolic containing MAGEs. The results of Chapter 2 showed *E. subg. Eucalyptus* species are high in phenolic compounds in their glands, therefore, these species were analysed in Chapter 3 and the compounds were identified as the flavanones and other structurally related compounds. The flavanones were quantified, and the volatile components localised to glands were also studied to better understand the gland metabolome. Chapter 4 characterises the two different types of oil glands present in *E. brevistylis* for the storage/synthesis of β -triketones and sesquiterpenes. Overall, this thesis characterises the PSMs of *Eucalyptus* foliar oil glands, particularly MAGEs, flavanones and other structurally related NVCs in relation to volatile oils.

Chapter 2

Monoterpene acid glucose esters in *Eucalyptus* oil glands

2.1. Introduction

Monoterpene acid glucose esters (MAGEs) are prevalent in trees of the family Myrtaceae, particularly in the genus *Eucalyptus*. To date, a total of about 40 MAGEs have been identified from different plant families while 18 of them are known only from eucalypts. The first known MAGEs from the genus *Eucalyptus*, resinoside A and B, were identified from the leaves of *E. resinifera* (Hyodo *et al.* 1992). Subsequently, several structurally different varieties of MAGEs have been identified commonly from leaves and less often from fruit capsules of many *Eucalyptus* species (Boulekbache-Makhlouf *et al.* 2010; Goodger and Woodrow 2013; Tian *et al.* 2010; Yang and Guo 2007). Some MAGEs appear to be present in numerous *Eucalyptus* species. For example, cuniloside B was found to be present in glandular extracts of all 29 species examined by Hakki *et al.* (2010) and Heskes *et al.* (2012b). Cypellocarpin C, another frequently found MAGE, was identified from 23 of the species and froggattiside A was identified from 22 species of *Eucalyptus* examined. Moreover, cuniloside B has also been identified from other Myrtaceae species such as *Melaleuca armillaris* (Heskes *et al.* 2012b). Many other known and tentatively identified MAGEs have been identified from a range of *Eucalyptus* species belonging to different subgenera (Goodger and Woodrow 2011; Heskes *et al.* 2012b). This evidence suggests that MAGEs are widespread in *Eucalyptus* species and may be ubiquitous in the genus.

The different MAGEs identified from eucalypts vary in chemical structure. The two monoterpenoid acids found in MAGEs are oleuropeic acid ((S)-4-(1-hydroxy-1-methylethyl)-1-cyclohexene-1-carboxylic acid) and menthiafolic acid (linalool-1-oic acid; 6-hydroxy-2,6-dimethyl-2,7-octadienoic acid). Oleuropeic acid is the more common acid moiety found in MAGEs of eucalypts. The only two compounds that contain menthiafolic acid are froggattiside A and eucalmalduside A (Begum *et al.* 2011;

Goodger *et al.* 2009; Heskes *et al.* 2012b). The monoterpene acid is attached mostly at the C6 position of glucose as the primary aglycone. In addition to the monoterpenoid aglycone, most of the MAGEs also contain a second aglycone that is generally a non-monoterpenoid attached to the glucose moiety. This arrangement increases the structural diversity of this compound group. MAGEs possessing a single aglycone have also been identified at a lesser frequency. The only such example from eucalypts is eucalmaidin A isolated from the leaves of *E. maideni*, which contains only oleuropeic acid attached at the C6 primary hydroxyl position of glucose (Tian *et al.* 2009). Three of the MAGEs identified from eucalypts possess two monoterpene acids. The two stereoisomers, cuniloside A identified from *E. globulus* and cuniloside B identified from more than 20 *Eucalyptus* species, have oleuropeic acid attached to both primary hydroxyl and C1 anomeric positions (Hasegawa *et al.* 2008; Heskes *et al.* 2012b). Similarly, froggattiside A isolated from many *Eucalyptus* species contain oleuropeic acid and menthiafolic acid as the two aglycones moieties (Goodger *et al.* 2009).

In addition to the monoterpene acid, a non-monoterpenoid group is present in all the other MAGEs identified from eucalypts, and all of these are phenolic aglycones. Although terpenoid aglycones like iridoid and triterpenoids are known from a number of MAGEs from other plant families, none of them have been found from any eucalypt to date (Noté *et al.* 2009; Taskova *et al.* 1998). The phenolic aglycone glycosylates mostly at the anomeric position of the glucopyranose ring. Among them, gallic acid derivatives are more common. Cypellocarpin A, eucaglobulin, eucalmaidin B and C contain gallic acid glycosylated to the anomeric position of glucose. Moreover, a methylated form of gallic acid is found in eucaglobulin B, which has been isolated from three *Eucalyptus* species (Heskes *et al.* 2012b). In globulosin B, gallic acid is attached at the primary hydroxyl position, and it is the only example where a non-monoterpenoid aglycone is attached to the C6 position (Hasegawa *et al.* 2008). In addition, MAGEs incorporating flavonol moieties have also been identified from eucalypts. The flavonol glycosides resinoside A and B from *E. resinifera* contain kaempferol as the anomeric aglycone (Hyodo *et al.* 1992). The flavonols quercetin and isoquercetin are found in eucalmaidin D from *E. maideni* and in cypellogins A and B from *E. cypellocarpa*, respectively (Kasajima *et al.* 2005; Tian *et al.* 2009). Another flavonol glycoside structurally similar to cypellogin B that contains a di-hydro-derivative of oleuropeic acid as the monoterpene moiety, namely cypellogin C, has been found from *E. cypellocarpa* (Kasajima *et al.* 2005). Chromanone derivatives are present in cypellocarpin B and C isolated from many *Eucalyptus* species and eucalmalduisides A, B and C isolated from *E. camaldulensis*

leaves (Begum *et al.* 2011; Hakki *et al.* 2010; Ito *et al.* 2000). Therefore, MAGEs in eucalypts are highly structurally diverse and further chemical screening is likely to uncover additional MAGE structures.

The exact function of MAGEs in plants is unclear, even though they may be involved in oil production, possibly related to transport and oil diffusion (Goodger and Woodrow 2011). Esterification to glucose increases the water solubility of aglycones and binding with glucose prevent free diffusion of molecules. MAGEs are now known to be exclusively localised to the foliar glands in eucalypts and co-occur with volatile oils (Goodger and Woodrow 2011; Heskes *et al.* 2012b). Out of the total of cuniloside B and froggattiside A, the two most abundant MAGEs in *E. polybractea*, 99% was found to be localised in the glands (Goodger and Woodrow 2011). This suggests that MAGEs are biosynthesised within the glands rather transported to them after being biosynthesised in other tissues. Moreover, fluorescence lifetime images of isolated glands from three *Eucalyptus* species including *E. polybractea* showed that the non-volatiles are restricted to the periphery of the gland lumen. In contrast, the terpenoid essential oil components are accumulated in the centre of the lumen (Heskes *et al.* 2012b). This indicates a possible function of MAGEs to protect the gland wall cells, including the important secretory cells, from potential autotoxic effects of oil components.

MAGEs possess many biological activities that could be related to a role in plant defence. Protection against common mammalian and insect herbivores, and also microorganisms such as bacteria, virus and fungi, is important for plants (Crous *et al.* 2000; Edwards *et al.* 1993). Particular structural features of MAGEs support possible defence related activities. For example, both monoterpene acids found in MAGEs, oleuropeic and menthiafolic acids, share the same molecular formula ($C_{10}H_{16}O_3$) and all MAGEs consist of at least one α , β -unsaturated carbonyl group. This group acts as a Michael Reaction acceptor that gives the compound many biological activities, including features related to defence. Michael type acceptors can interact with electron rich macromolecules and result in adverse effects and lead to cell damage and cytotoxicity (Amslinger 2010). Many of the glucose esters having two aglycones contain two α , β -unsaturated carbonyl groups that possibly increase their activity (Dinkova-Kostova and Talalay 1999). Although the exact defence functions of MAGEs are unclear, there is positive evidence for such activity. Phlebotricoside, a menthiafolic acid glucose ester isolated from *Viburnum phlebotrichum* (Adoxaceae) leaves, has been reported as a compound with bitter taste, a characteristic that can be related to defence against

herbivores (Hase *et al.* 1982). Many oleuropeic acid glucose esters have been studied for their biological activities including the properties associated with plant defence. Eucaglobulin isolated from *E. globulus* leaves has shown inhibitory effects on microorganisms. The minimum inhibitory concentration reported for the bacterium *Escherichia coli* was 0.5 mM and for the yeast *Candida albicans* was 1 mM (Hou *et al.* 2000). In addition, several MAGEs have been tested for their cytotoxicity on mammalian cells. The activity of cuniloside B (reported as eucalmaidin E), cypellocarpins A, B and C, eucalmaidins A, B and C, as well as free oleuropeic acid isolated from fresh leaves of *E. maiden*, were tested on African Green Monkey kidney cells (Vero cells) using a cell cytotoxicity assay (Tian *et al.* 2010; Tian *et al.* 2009). All three cypellocarpins, cuniloside B, eucalmaidins B and C showed cytotoxicity to different degrees and the effect of all of them was higher than their precursor oleuropeic acid. Cypellocarpin A and B were more cytotoxic than any other compound tested with IC₅₀ values of 0.03 mM and 0.02 mM respectively. The evidence also supports the idea that the overall structure of the compound is important for the activity rather than the individual moieties. Eucalmaidin E (cuniloside B) reported a maximal non cytotoxic concentration of 0.20 mM against Vero cells while oleuropeic acid reported 1.09 mM (Tian *et al.* 2009). Similarly, resinoside A inhibits the blue mussel (*Mytilus edulis*) attachment more than its components oleuropeic acid and kaempferol (Hyodo *et al.* 1992). In addition, MAGEs have also been shown to be important for the protection against oxidative damage. In *Portulaca oleracea*, glucose esters were produced in response to stress elicited by copper and showed antioxidative activities stronger than precursor monoterpenes (Wu *et al.* 2012). All these properties support a possible role of MAGEs in plants as defensive metabolites.

Even though MAGEs have been characterised predominantly from eucalypts, relatively few species have been studied in this large genus. Out of the relatively low number of species examined, the majority are from the subgenus *Symphyomyrtus*, while the other subgenera have been poorly examined. Only four species (*E. gregsoniana*, *E. muelleriana*, *E. olsenii* and *E. pauciflora*) have been studied from the subgenus *Eucalyptus*, the second largest subgroup in the genus. A single species each have been studied from the two smaller subgenera, i.e. *E. erythrocorys* from subg. *Eudesmia* and *E. cloeziana* from subg. *Idiogenes* (Hakki *et al.* 2010; Heskes *et al.* 2012b). To date, only about 30 species have been explored in great detail specifically for MAGEs, suggesting that more species need to be studied (Goodger and Woodrow 2011). Studying the

ubiquitous nature of this group of compounds within the genus may help us better understanding the role and ecological importance of MAGEs.

The primary aim of research described in this chapter was to characterise MAGEs in *Eucalyptus* species to find whether they are ubiquitous within the genus. MAGEs were characterised from a range of *Eucalyptus* species belonging to the two major subgenera *Symphyomyrtus* and *Eucalyptus* using high-performance liquid chromatography (HPLC) coupled with UV and mass spectrometer detectors. MAGEs are already known to be exclusively localised to the glands in *Eucalyptus* (Goodger and Woodrow 2011); therefore, in this study MAGEs were extracted from collections of isolated foliar oil glands. The oil components of these species were also analysed using gas chromatography (GC) analyses of whole leaf extracts to examine the relationship between the co-housed oils and MAGEs. In the second part of this chapter, a population of *E. froggattii* was studied to identify how MAGEs vary at the population level in nature. The aim of the third part of the chapter was to establish cell suspensions of *E. polybractea* and *E. froggattii* and then use biotransformation studies to explore the possible reaction types that occur in cells, and thereby predict the possible biosynthetic pathway of MAGEs or other potential non-volatile compounds (NVCs). As a majority of the MAGEs identified from eucalypts to date contain a phenolic group attached to the glucopyranose ring at the C1 anomeric position, a simple phenolic phenylpropanoid (*p*-coumaric acid) was selected as the feeding agent.

2.2. Materials and methods

The experiments in this chapter are described in three sections as (2.2.1) MAGEs survey, (2.2.2) *E. froggattii* population study and (2.2.3) biotransformation study.

2.2.1. MAGEs survey

i. Plant material

A range of 30 *Eucalyptus* species representing the two major subgenera *Symphyomyrtus* and *Eucalyptus* were studied for the presence of non-volatile secondary metabolites in their foliar glands. The NVC extraction was purely from isolated oil glands and therefore, species with a high gland density were selected based on the

published descriptions of foliar glands (Brooker and Nicolle 2013). Species representing different sections from within the two largest subgenera, *Symphomyrtus* and *Eucalyptus*, were selected (Table 2.1). Fully expanded leaves were collected from mature trees growing in the Currency Creek Arboretum, South Australia (35°25'44.85" S, 138°45'45.75" E) in April 2014. Leaves were harvested using a pole pruner, immediately sealed in snap-lock bags and returned to the laboratory on dry ice. Samples were then stored at -80°C until analysed.

ii. Isolation of glands and extraction of non-volatiles

Foliar glands were isolated from the leaves of 30 *Eucalyptus* species following the method described in Goodger *et al.* (2010) using pectinase P-4716 (Sigma-Aldrich, St. Louis, USA) from *Aspergillus niger*. The enzyme was diluted with distilled water (1:2 v/v). Each leaf was dissected into approximately 4 × 20 mm strips and these strips were immersed in 1500 µL of diluted pectinase enzyme solution and were incubated in a thermo-mixer (Eppendorf, Hamburg, Germany) at 30°C with agitation at 600 rpm for 30 s at 1-min intervals. The incubation time was altered ranging from 12-24 h for different species depending on the time taken for complete digestion. At the time the epidermis was easily peeled with forceps from the other leaf tissues and the leaf strips were dissected under a dissecting microscope. The oil glands were then separated from mesophylls and vasculature and were collected using a sieve into an Eppendorf tube.

Finally, the isolated glands were immediately ground using a vial micropestle and were extracted for non-volatiles in 200 µL of 100% acetonitrile. All extracts were passed through 0.45 µm membrane filters (Phenex PTFE, Phenomenex) to remove any cellular debris prior to chromatographic analyses. The extracts were stored in -20°C until analysed.

iii. Chromatographic analyses and mass spectrometry of non-volatile components

Non-volatile extracts of glands were fractionated using a high-performance liquid chromatography system comprised of a Shimadzu LC-20AT pump, SIL-20AHT autosampler and SPD-M20A detector (Shimadzu Prominence, Kyoto, Japan) with photo diode array UV detection (190–500 nm). A Gemini C18 analytical column (5 µm, 150 X 4.6 mm; Phenomenex, Torrance, USA) was used and eluted at a flow rate of 0.8 mL min⁻¹, resulting in operating pressures below 120 bar with a 38 min run time. The mobile

phase comprised of 0.1% formic acid in deionised water and 0.1% formic acid in acetonitrile. The LC gradient was 30-50% acetonitrile over 7 min, followed by 50-95% over 7 min and then isocratic at 95% for a further 12 min, finally a gradient of 95–100% over 0.25 min. The column was then re-equilibrated with 30% acetonitrile for 5 min.

Electro-spray ionisation liquid chromatography mass spectrometry (ESI-LCMS/MS) was operated on an Agilent 6520 QTOFMS system (Santa Clara, CA, USA) in both positive and negative modes. The positive mode was operated using the following conditions: nebulizer pressure 37 psi, gas flow rate 12 L min⁻¹, gas temperature 350°C, capillary voltage 4000 V, fragmentor 150 V and skimmer 65 V. Mass spectra were collected in the range of 70–1700 *m/z*. In negative ion mode, a nebulizer pressure of 45 psi and a capillary voltage of 3500 V were used. The analytical method comprised two scan experiments: the first was a full scan with a dwell time of 493 msec spectrum⁻¹, followed by a second MS/MS experiment with a dwell time of 493 msec spectrum⁻¹ where a collision energy of 20 V was applied. Analytes were also monitored by a diode array detection (DAD) UV-Vis scan at specific wavelengths of 221 nm, 278 nm and 340 nm with the bandwidth set at 4 nm. Mass spectra were evaluated using Mass Hunter Workstation software version B.06.00 (Agilent Technologies, USA). The mass spectral data along with UV absorbance and retention time were used to identify MAGEs. The unique mass fragmentation pattern of MAGEs was used to identify any unknowns (Heskes *et al.* 2012b).

iv. Extraction of volatile essential oil components

The volatile components of selected *Eucalyptus* species were extracted following the method described in Heskes *et al.* (2012b). A leaf from each species was ground to a fine powder in liquid nitrogen in a mortar with pestle and the powder was then extracted in 3 mL of hexane containing 100 mg L⁻¹ tridecane as an internal standard in glass vials. Samples were then incubated at 50°C for 5 days on an orbital shaker (Ratek Instruments Pty Ltd, Boronia, Australia) at 100 rpm. Following the incubation, the hexane extracts were removed, dehydrated with anhydrous Na₂SO₄, vortexed and centrifuged for 1 min at 11,200 *g*. The supernatant was transferred into 2 mL glass vials for GC analysis.

v. Analysis of volatile components by gas chromatography and mass spectrometry

Extracted volatile components were analysed by Gas Chromatography with Flame Ionisation Detection (GC-FID) prior to the mass spectrometry. The GC-FID system was a Perkin Elmer AutosystemXC (Perkin Elmer, Melbourne, Australia) fitted with a Zebron ZB-5 low polarity column (30 m × 0.25 mm i.d., Phenomenex, USA). Helium was used as the carrier gas at a flow rate of 1 mL min⁻¹. The injector temperature was 250°C and detector temperature was 220°C. The column temperature was held at 120°C for 1 min following injection, then ramped at 7°C min⁻¹ to 180°C and held at that temperature for a further 10 min. Monoterpenes and sesquiterpenes in extracts were identified by comparing to standards (1,8-cineole, *p*-cymene, limonene, sabinene, α -terpineol, terpinen-4-ol, myrcene, β -pinene, α -pinene, aromadendrene, caryophyllene (Sigma Aldrich, St Louis, USA)) and were quantified by comparing the integrated response signal to calibration series of the standards.

The hexane extracts were also analysed using Gas Chromatography-Mass Spectrometry (GC-MS) to identify any unknown constituents. The GC-MS system comprised a Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole mass spectrometer (MS; Agilent, Santa Clara, USA). Samples (1 μ L) were injected in splitless mode into the system. The MS was adjusted according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (FC-43). The GC analyses were performed on a 30 m VF-5MS column with 0.2 μ m film thickness and a 10 m Integra guard column (J & W, Agilent). The injection temperature was set at 250°C, the MS transfer line at 280°C, the ion source adjusted to 230°C and the quadrupole at 150°C. Helium was used as the carrier gas at a flow rate of 1.0 mL min⁻¹. For the analysis, the following temperature program was used: start at injection 70°C, a hold for 6 min, followed by a 10°C min⁻¹ oven temperature ramp to 250°C and hold for 5 min. Both chromatograms and mass spectra were evaluated using Agilent MSD ChemStation E.02.02.1431 for GC-MS. Mass spectra of eluting compounds were identified using either the NIST 11 or the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany or Adams mass spectra library (Adams 2007).

2.2.2. *E. froggattii* population study

i. Plant material

Eucalyptus froggattii Blakely (Kamarooka mallee), a species with high abundance of MAGEs, was selected as an ideal species to study the variation of MAGEs in natural populations (Goodger *et al.* 2009). Leaf samples were collected from the trees growing in the Kamarooka State Park (36°38'40.2"S 144°16'36.6"E) and Wychitella Flora and Fauna Reserve (36°19'22.7"S 143°41'37.9"E), Victoria, in September 2013. The two populations are located approximately 60 km apart from each other. Fully expanded mature leaves were randomly collected from 31 trees from each of the two populations using pole pruners and returned to the laboratory on ice.

ii. Analysis of leaf extracts

A leaf from each tree was ground to a fine powder in liquid nitrogen in a mortar with pestle. Half of the ground leaf tissue was used to extract non-volatiles with 3 mL of 30% acetonitrile in water at 25°C for 5 days. The extracts were then fractioned using 30 mg Strata-X reverse phase cartridges (Phenomenex). Cartridges were washed with 1 mL 30% acetonitrile in water. Cuniloside B and froggattiside A was then eluted with 3 mL 60% acetonitrile in water. This fraction was further separated by high-performance liquid chromatography (HPLC) as described in section 2.2.1-iii. Cuniloside B was quantified by comparison with a standard series of synthetic cuniloside B at 221 nm.

The remaining half of the ground leaf tissue was used to extract the volatile components. The tissues were extracted in hexane and were analysed using gas chromatography as described in the sections 2.2.1-iv and 2.2.1-v to quantify monoterpenes and sesquiterpenes. The data of the population study was compared with a t-test using IBM SPSS Statistics software, version 21 (IBM Armonk, NY, USA).

2.2.3. Biotransformation study

i. Plant material

E. polybractea and *E. froggattii* were selected for biotransformation experiments due to the known high abundance of MAGEs and oil in their tissues (Heskes *et al.* 2012b). Micropropagated *in vitro* plantlets of the two species were supplied by the Plant Physiology Laboratory of the University of Melbourne (Goodger *et al.* 2008). The

plantlets had been initiated from two maternal genotypes growing in the Systems Garden of the University of Melbourne. Young expanded leaves (4-6 leaves of 5-7 mm length from each plantlet) were used as ex-plants for callus initiation. The collected leaflets were immediately used for callus initiation in sterile conditions.

ii. Culture medium

Two media differing in growth regulators, designated as M1 and M2, were tested for the callus production from leaves of two species. Both media consisted of Lloyd & McCown woody plant basal salt mixture (Australtec, Australia), Murashige & Skoog modified vitamins (x1000) and different combinations of growth regulators thidiazuron (TDZ), 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). The growth regulator combination in M1 medium was NAA (0.10 mg L⁻¹) and BAP (0.5 mg L⁻¹) while M2 had NAA (0.02 mg L⁻¹) and TDZ (0.66 mg L⁻¹; **Appendix A**). Both media were supplemented with 2.5% sucrose. The components were dissolved in 1000 mL of deionised water and the pH of both media was adjusted to 5.6–5.8 using 0.1% KOH, before being autoclaved. Gelrite (2.5 g L⁻¹) was used as the solidifying agent for callus producing medium. The media were then autoclaved at 121°C for 20 min. Autoclaved solid medium was dispensed into Petri plates (approximately 20 mL per plate) before using. All glassware and other equipment were also sterilised before being used.

iii. Callus induction and maintenance

After trimming the bases and tips, leaflets were dissected into halves perpendicular to the midrib and both halves were used for callus initiation. Six ex-plants were placed in each sterilised Petri plate (9 cm) containing 20 mL of solid medium either M1 or M2. The plates were sealed with sealing film (Australtec, Australia), incubated in the dark at 25 ± 1°C and observed routinely over the first six weeks. When the callus formed, to multiply callus, approximately 0.2 g of calli were transferred to freshly prepared solid media every 6 weeks for five months.

iv. Cell suspension culture

Cell suspensions were initiated from *E. polybractea* and *E. froggattii* calli. Four weeks old callus from the fourth subculturing cycle were used for suspension initiation. To initiate suspension cultures, 3 g of callus from each species were transferred to 25 mL of corresponding autoclaved liquid media in 125 mL Erlenmeyer flasks and sealed with aluminium foil. Suspensions were incubated in the dark at $25 \pm 1^\circ\text{C}$ on a rotary shaker at 100 rpm. *E. polybractea* in M2 medium showed signs of a healthy suspension in about three weeks. *E. polybractea* in M1 medium and *E. froggattii* in M2 medium did not grow into good cell suspensions and so were discontinued. *E. polybractea* suspension, which was successful, was subcultured in 4-weeks intervals. After six subculturing cycles, the suspensions were transferred to and maintained in 100 mL of M2 medium in 250 mL Erlenmeyer flasks. Suspension cultures were routinely subcultured every 28 days. In each subculture, 5 mL of packed cell volume (PCV) and 25 mL of conditioned media were transferred to a new sterilised flask and 75 mL of freshly prepared media was added (It was experimentally determined 3 g of cells is equal to 5 mL of PCV in a pipette).

v. Measurement of the cell growth

The growth of *E. polybractea* cell suspension was measured using the changes in PCV. The total volume in a flask containing media and cells was transferred aseptically to an autoclaved measuring cylinder and the cells were left to settle for 5 min. Then the volume reading of the sedimented cells was recorded. The cells and media were then transferred back into the same flask and were incubated under the same conditions until the next measurement. This procedure was repeated at 4 days intervals for 35 days. The experiment was replicated for two similar flasks containing cell suspensions of same maturity.

vi. Determination of cell viability

The viability of cells was tested using fluorescein diacetate (FDA) dye. A stock solution was prepared using 5 mg of FDA dissolved in 1 mL acetone and was stored at 4°C (Widholm 1972). A diluted FDA solution was prepared by adding 5 mL acetone to 0.1 mL of stock solution. To test the viability, 1 mL of the diluted FDA solution was mixed with 1 mL of cell suspension (28 days old) and the mixture was incubated for 5

min at room temperature. An aliquot of solution containing FDA stained cells was placed on microscope slides and covered with a cover slip. The cells were viewed under a stereomicroscope with fluorescence (Leica M205) under UV illumination with GFP2 filter. For each sample the number of stained cells (viable cells) and the total number of cells were counted to determine the percentage cell viability.

vii. *p*-Coumaric acid feeding and analysis of biotransformation products

To test the biotransformation reactions, *E. polybractea* cell suspensions were fed *p*-coumaric acid (Sigma Aldrich, USA). A suspension culture was prepared in a 250 mL flask by subculturing an already continuing two years old culture. After 23 days of incubation period under the conditions described above (iv), 10 mg of *p*-coumaric acid dissolved in 250 μ L of 55% ethanol solution was administered through a 0.45 μ m membrane filter to 100 mL of cell suspension in a flask. The suspensions were returned to the shaker and were incubated in the same conditions for further 4 days. Two control samples, one which had not been treated and another which had been administered with only 55% ethanol solution without coumaric acid, were also tested. The experiment was replicated four times.

After the incubation period of four days, the cells and medium were separated by filtration using filter paper. The cells were air dried to remove any media and the dried cells were ground with liquid nitrogen with a mortar and pestle and were extracted with 100% methanol for 2 days. The methanol fraction was analysed by liquid chromatography-mass spectrometry (LC-MS) in both positive and negative modes using the conditions described earlier (2.2.1-iii). A Gemini C18 analytical column (Phenomenex, 5 μ m, 150 X 4.6 mm) was used and eluted at a flow rate of 0.8 mL min⁻¹. The mobile phase comprised of 0.1% formic acid in deionised water and methanol. The gradient was 5% methanol over 2 min, followed by 5-100% over 16 min and then isocratic at 100% for a further 10 min. The structures of the products were determined by comparison with the mass spectral data with published literature.

2.3. Results

2.3.1. Species selected for the study

As the major focus of this study was to investigate the MAGEs and other non-volatiles localised to glands, species from the genus *Eucalyptus* that have been reported as having a considerable visible oil gland frequency were selected (Brooker and Nicolle 2013). Species ranging from an oil gland frequency of 260 cm⁻² (in *E. approximans*) to very high frequencies as high as 2100 cm⁻² (in *E. suberea*) were included. In the selection process, which was primarily based on the abundance of foliar oil glands, the two major subgenera of the genus *Eucalyptus*, i.e. subgenus *Symphyomyrtus* and subgenus *Eucalyptus*, were represented. The final list included 17 *Symphyomyrtus* and 13 *Eucalyptus* species from different sections and series within the two subgenera (Table 2.1).

2.3.2. *Eucalyptus* foliar glands

Glands were isolated from the leaves of *Eucalyptus* species following the enzymatic digestion of leaves (Figure 2.1). This approach made the extraction process more effective compared to the previous method of collecting non-volatiles manually from broken glands in dissected leaves (Heskes *et al.* 2012b). The glands were isolated from digested leaves within 24 h of incubation and were separated from the other leaf tissues (mesophyll and vasculature) using sieves. The sieve size was selected according to the size of the glands of each species with gland sizes ranging from 75 to 230 µm in diameter. All glands isolated from a single leaf of each species were directly used to extract the glandular contents.

Table 2.1: List of *Eucalyptus* species surveyed in this study and their taxonomic classification.

#	Species	Subgenus	Section	Series
1	<i>Eucalyptus ochrophloia</i>	<i>Symphyomyrtus</i>	<i>Adnataria</i>	<i>Subbuxaeales</i>
2	<i>E. thozetiana</i>	<i>Symphyomyrtus</i>	<i>Adnataria</i>	<i>Subbuxaeales</i>
3	<i>E. articulata</i>	<i>Symphyomyrtus</i>	<i>Bisectae</i>	<i>Loxophlebae</i>
4	<i>E. erythronema</i>	<i>Symphyomyrtus</i>	<i>Glandulosae</i>	<i>Elongatae</i>
5	<i>E. proxima</i>	<i>Symphyomyrtus</i>	<i>Glandulosae</i>	<i>Clinatae</i>
6	<i>E. protensa</i>	<i>Symphyomyrtus</i>	<i>Glandulosae</i>	<i>Erectae</i>
7	<i>E. tenera</i>	<i>Symphyomyrtus</i>	<i>Glandulosae</i>	<i>Erectae</i>
8	<i>E. cyanophylla</i>	<i>Symphyomyrtus</i>	<i>Dumaria</i>	<i>Rufispermae</i>
9	<i>E. dumosa</i>	<i>Symphyomyrtus</i>	<i>Dumaria</i>	<i>Rufispermae</i>
10	<i>E. exigua</i>	<i>Symphyomyrtus</i>	<i>Dumaria</i>	<i>Ovulares</i>
11	<i>E. incrassata</i>	<i>Symphyomyrtus</i>	<i>Dumaria</i>	<i>Tetrapterae</i>
12	<i>E. pimpiniana</i>	<i>Symphyomyrtus</i>	<i>Dumaria</i>	<i>Dissonae</i>
13	<i>E. pumila</i>	<i>Symphyomyrtus</i>	<i>Latoangulatae</i>	<i>Pumilae</i>
14	<i>E. gillenbergii</i>	<i>Symphyomyrtus</i>	<i>Exsertaria</i>	<i>Erythroxylon</i>
15	<i>E. badjensis</i>	<i>Symphyomyrtus</i>	<i>Maidenaria</i>	<i>Lanceolatae</i>
16	<i>E. aromaphloia</i>	<i>Symphyomyrtus</i>	<i>Maidenaria</i>	<i>Acaciiformes</i>
17	<i>E. parvula</i>	<i>Symphyomyrtus</i>	<i>Maidenaria</i>	<i>Acaciiformes</i>
18	<i>E. mitchelliana</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Longitudinales</i>
19	<i>E. stellulata</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Longitudinales</i>
20	<i>E. oreades</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Fraxinales</i>
21	<i>E. agglomerata</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Pachyphloiae</i>
22	<i>E. gregsoniana</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Pauciflora</i>
23	<i>E. falciformis</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Radiatae</i>
24	<i>E. nitida</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Radiatae</i>
25	<i>E. approximans</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Strictae</i>
26	<i>E. apiculata</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Strictae</i>
27	<i>E. dendromorpha</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Strictae</i>
28	<i>E. obstans</i>	<i>Eucalyptus</i>	<i>Eucalyptus</i>	<i>Strictae</i>
29	<i>E. suberea</i>	<i>Eucalyptus</i>	<i>Frutices</i>	<i>Subereae</i>
30	<i>E. brevistylis</i>	<i>Eucalyptus</i>	<i>Longistylus</i>	<i>Pedaria</i>

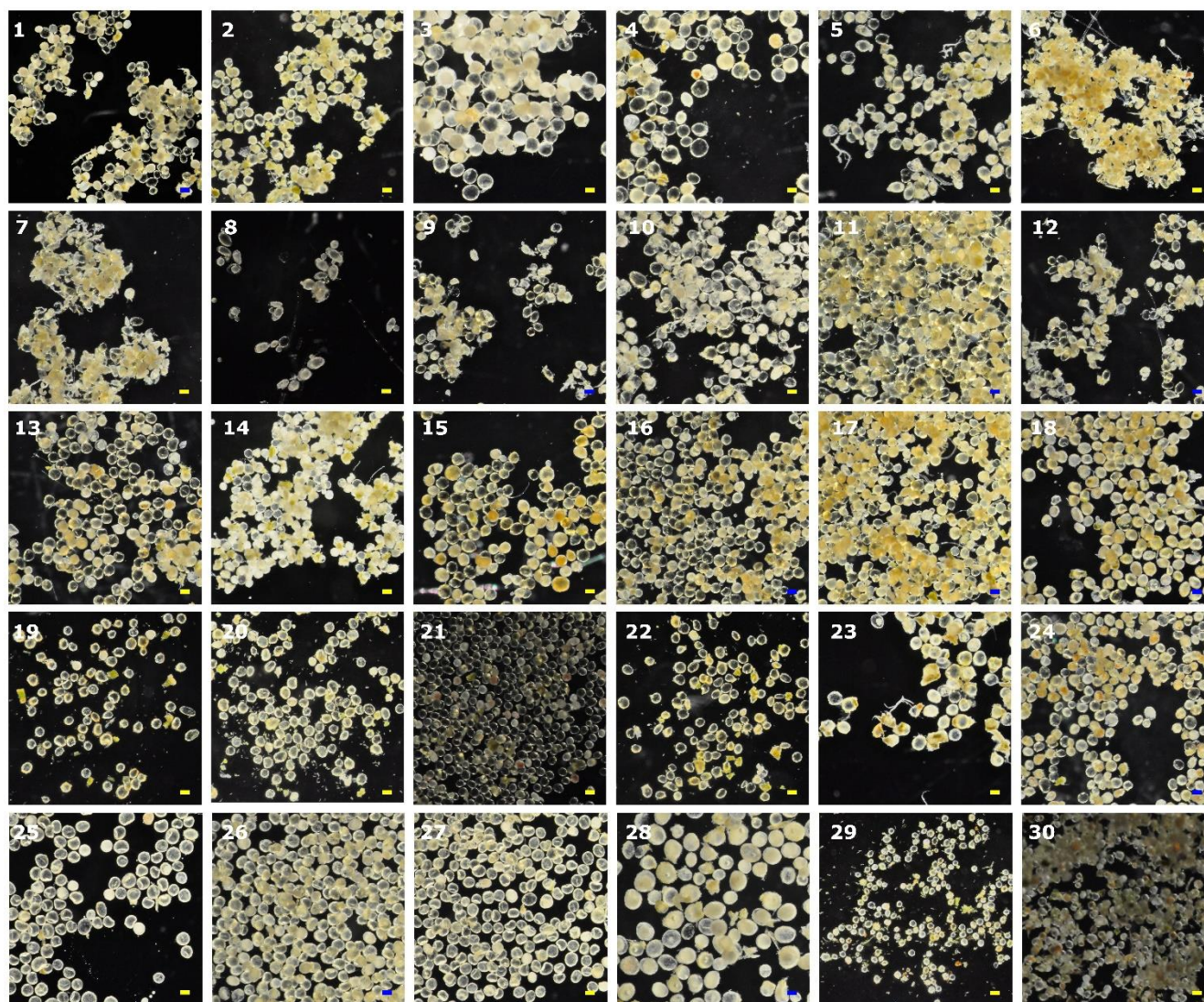


Figure 2.1: Representative foliar oil glands isolated from the *Eucalyptus* species belonging to the subgenera *Symphyomyrtus* and *Eucalyptus*. The glands were separated after enzymatic digestion of leaf tissues and only small amounts of contaminating mesophyll cells are visible. The number denotes the species as given in the list in **Table 2.1**. All scale bars represent 200 μm .

2.3.3. Non-volatile compounds in foliar glands

The preliminary studies indicated that all 30 species contain NVCs in their glands. ESI-LCMS/MS with UV absorbance detection was used to identify the constituents in glandular acetonitrile extracts. The glands of the species from the two subgenera contained an array of peaks corresponding to NVCs eluting at two major ranges of mobile phase concentrations (**Figure 2.2**). The species belonging to the subgenus *Symphyomyrtus* were dominated by compounds eluting in the range of 30–50% acetonitrile with UV absorbance maxima of 221 nm. The rest of the species, which belong to the subgenus *Eucalyptus*, were dominated by compounds eluting at later retention times with higher concentrations of acetonitrile and their UV absorbance maxima range was 278–340 nm. The UV absorbance maxima of the early eluting compounds (221 nm) matched with the previously reported records of MAGEs; therefore, they were further analysed to identify and elucidate their structures. The absorbance maxima of the latter group, which eluted at later retention times, was consistent with phenolics and were further studied in Chapter 3.

2.3.4. MAGEs localised to foliar glands

Identification of MAGEs in acetonitrile extracts was based on the characteristic mass fragmentation pattern of oleuropeyl glucose esters. In previous studies by Goodger *et al.* (2009) and Heskes *et al.* (2012b), two highly abundant C16 fragments of m/z 311.14 and 329.15 and a less abundant C16 fragment with m/z 347.16 have been observed in mass spectral data. The two more abundant fragments correspond to an oleuropeic acid attached to a glucopyranose ring via an ester bond with the loss of one or two water molecules, respectively. The less abundant C16 fragment corresponds to an oleuropeyl glucose ester with no loss of water (Goodger *et al.* 2009; Heskes *et al.* 2012b). This characteristic mass fragmentation pattern was observed in the acetonitrile extracts of oil glands in the present study; therefore, the mass spectral data were further analysed to identify the putative MAGEs located in glands.

Based on the mass spectral data, six previously reported MAGEs and 19 novel compounds matching the unique fragmentation pattern were identified from the glandular extracts. Majority of species had ion peaks at m/z 513.2666 $[M+H]^+$, m/z 530.2954 $[M+NH_4]^+$, and at m/z 535.2505 $[M+Na]^+$ corresponding to the molecular formula $C_{26}H_{40}O_{10}$ matching published data for cuniloside B and froggattiside A (Goodger *et al.* 2009). The fragmentation of parent ions resulted in the ion peaks of m/z

329.15, 311.14, 495.25, 477.24 and 347.16 indicating the presence of oleuropeic acid attached to glucopyranose (**Figure 2.4**). The differentiation between the two compounds was based on the retention time. Cuniloside B eluted at 4.6 min while froggattiside A had a retention time of 5.8 min. Similarly, the diagnostic fragmentation patterns of ions matching several other known MAGEs were also observed (**Figure 2.5, Table 2.2**). Eucaglobulin, cypellocarpin A and eucalmaidin B were present at 2.3 min. The ion peaks were detected at m/z 516.2062 $[M+NH_4]^+$, m/z 521.1641 $[M+Na]^+$ corresponding to the molecular formula $C_{23}H_{30}O_{12}$. The specific identification of these three compounds separately needed further analysis. Eucalmaidin C was identified by the $[M+NH_4]^+$ at m/z 544.2370 and $[M+Na]^+$ at m/z 549.1940 (Tian *et al.* 2009). In addition, the fragmentation patterns of eucaglobulin B and cypellocarpin C were also identified from the extracts. Eucaglobulin B was identified by the ion peaks detected at m/z 544.2378 $[M+NH_4]^+$ and m/z 549.1968 $[M+Na]^+$ corresponding to the molecular formula $C_{25}H_{34}O_{12}$ (Heskes *et al.* 2012b). An ion peak was present in most species at m/z 521.2009 $[M+H]^+$ corresponding to the molecular formula $C_{26}H_{32}O_{11}$ matching published data for cypellocarpin C (Ito *et al.* 2000).

MAGEs were present in the glandular extracts of 26 of the 30 species examined, with the exception of *E. nitida*, *E. dendromorpha*, *E. suberea* and *E. brevistylis*. Some MAGEs appeared to be more widespread in the genus. The extracts of 25 species contained cuniloside B and cypellocarpin C, and froggattiside A was detected in 22 species (**Table 2.2**). A total of 19 novel MAGEs, which did not match with the previously published mass spectral data, were detected from many species. Most of the novel MAGEs were from *Symphyomyrtus* species where the subgenus was rich in this compound group. The only subgenus *Eucalyptus* species that showed a novel MAGE was *E. gregsoniana* from which ‘unknown 14’ was putatively identified. All *Symphyomyrtus* species had MAGEs as the dominant non-volatile group, while species from some sections had a greater number of compounds than others. Particularly, *E. tenera* from the section Glandulosae (subgenus *Symphyomyrtus*) had at least 12 peaks corresponding to MAGEs where seven of them appeared to be novel MAGEs (**Figure 2.3**) and *E. proxima* from the same section had a total of eight such peaks.

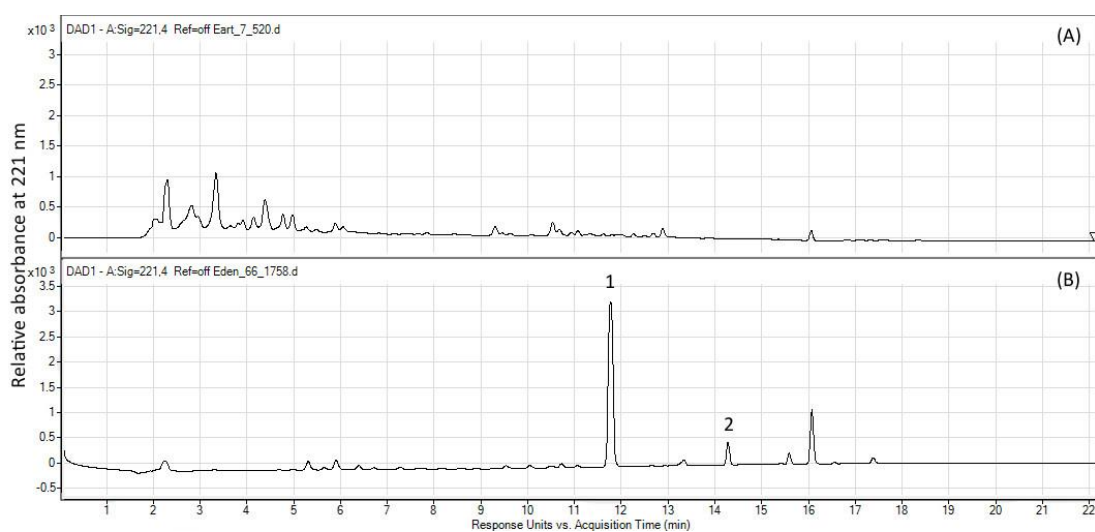


Figure 2.2: Representative LC-UV chromatograms of glandular extracts (in 100% acetonitrile) from *Eucalyptus* species measured at 221 nm. (A) The chromatogram of *E. articulata* from subg. *Symphyomyrtus* showing the peaks corresponding to MAGEs and (B) the chromatogram of *E. dendromorpha* from subg. *Eucalyptus* indicating the major unsubstituted B-ring flavanones dimethylpinocembrin (1) and pinostrobin (2).

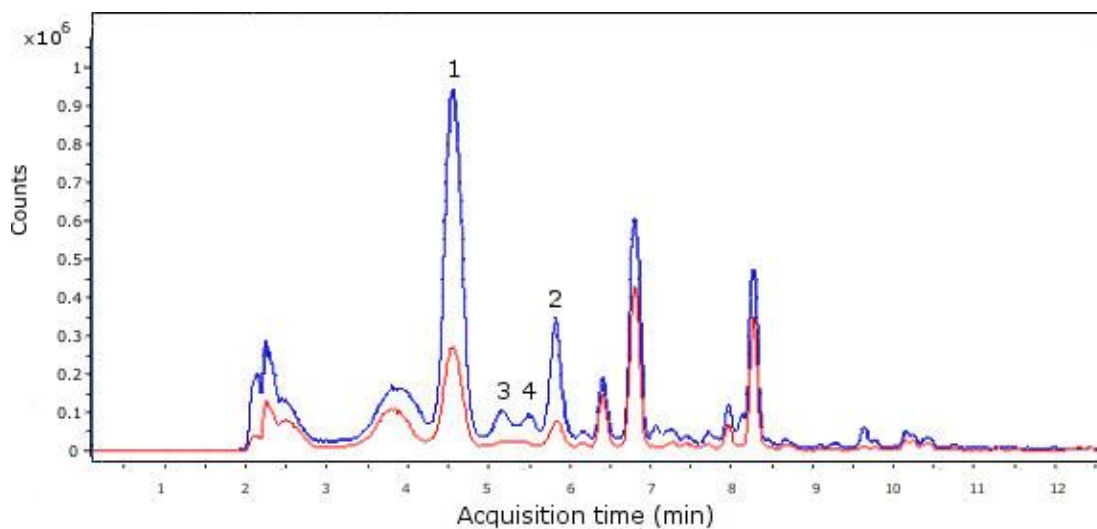


Figure 2.3: Extracted ion chromatogram of m/z 311.14 (blue line) and 329.15 (red line) ions from oil gland extracts of *Eucalyptus tenera*. Peak numbers indicate cuniloside B (1), froggattiside A (2), eucaglobulin B (3), and cypellocarpin C (4).

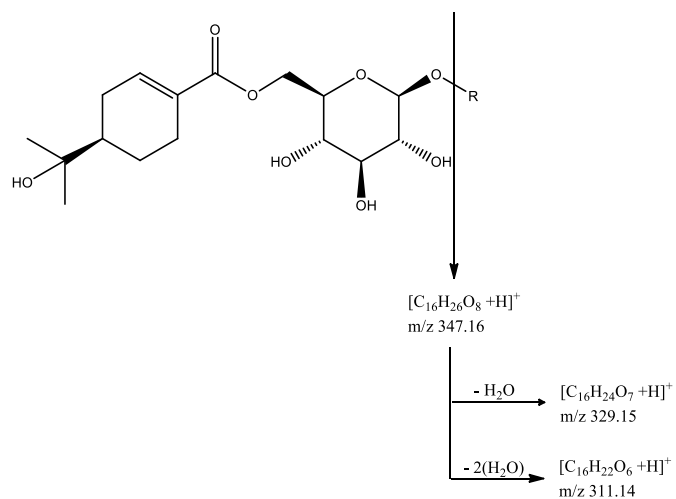


Figure 2.4: Schematic of the fragmentation of oleuropeic acid glucose esters resulting in the characteristic ion fragments of m/z 311.14 and 329.15.

Table 2.2: Electrospray ionisation mass spectral analysis of MAGEs identified from *Eucalyptus* foliar gland extracts.

RT (min)	Parent ions	Product ions	Formula	MAGE	Species #
2.30	516.2062 [M+NH ₄] ⁺	311.1487	C ₂₃ H ₃₀ O ₁₂	Eucaglobulin/	16
	521.1641 [M+Na] ⁺	329.1576		Cypellocarpin	
		293.1355		A/	
		463.1594		Eucalmaidin B	
		481.1773			
3.40	537.1967 [M+H] ⁺	401.1640	C ₂₆ H ₃₂ O ₁₂	Unknown 1	2, 3
	503.1932	463.1614			
		311.1490			
		149.0976			
3.76	530.2224 [M+NH ₄] ⁺	495.1879	C ₂₄ H ₃₂ O ₁₂	Unknown 2	10
	535.1799 [M+Na] ⁺	477.1773			
		311.1489			
3.97	544.2370 [M+NH ₄] ⁺	311.1488	C ₂₅ H ₃₄ O ₁₂	Eucalmaidin C	5, 7
	549.1940 [M+Na] ⁺	329.1593			
4.2	478.2633 [M+NH ₄] ⁺	311.1483	C ₂₂ H ₃₆ O ₁₀	Unknown 3	4
	483.2186 [M+Na] ⁺	329.1587			
	461.2388 [M+H] ⁺	293.1385			
		239.1273			
4.62	530.2954 [M+NH ₄] ⁺	329.1592	C ₂₆ H ₄₀ O ₁₀	Cuniloside B	1-17, 19-23,
	535.2505 [M+Na] ⁺	311.1488			25, 26, 28
	513.2666 [M+H] ⁺	495.2539			
5.13	561.2323 [M+Na] ⁺	557.2790	C ₂₇ H ₃₈ O ₁₁	Unknown 4	16
	556.2767 [M+NH ₄] ⁺	311.1498			
		329.1596			
		149.0983			
5.21	544.2378 [M+NH ₄] ⁺	311.1488	C ₂₅ H ₃₄ O ₁₂	Eucaglobulin B	5, 7
	549.1968 [M+Na] ⁺	509.2027			
		491.1926			
		329.1591			
		167.1063			

Continued next page

RT (min)	Parent ions	Product ions	Formula	MAGE	Species #
5.43	536.2702 [M+NH ₄] ⁺	311.1488	C ₂₄ H ₃₈ O ₁₂	Unknown 5	9
	541.2258 [M+Na] ⁺	329.1595			
		149.0959			
		183.0660			
		213.0727			
		167.1060			
5.63	523.2883[M+Na] ⁺	497.2359	C ₂₆ H ₄₄ O ₉	Unknown 6	8
	501.3022 [M+H] ⁺	311.1486			
		329.1588			
5.77	521.2009 [M+H] ⁺	503.1898	C ₂₆ H ₃₂ O ₁₁	Cypellocarpin C	1-23, 25, 26
		311.1520			
5.89	530.2952 [M+NH ₄] ⁺	311.1487	C ₂₆ H ₄₀ O ₁₀	Froggattiside A	2-17, 19, 20, 22,
	535.2522 [M+Na] ⁺	329.1588			23, 25, 26
	513.2697 [M+H] ⁺				
6.16	530.2224 [M+NH ₄] ⁺	285.1127	C ₂₄ H ₃₂ O ₁₂	Unknown 7	7, 10, 15
	535.1799 [M+Na] ⁺	235.1678			
		311.1485			
		329.1573			
6.27	518.2610 [M+NH ₄] ⁺	351.1434	C ₂₄ H ₃₆ O ₁₁	Unknown 8	17
	523.2187 [M+Na] ⁺	329.1606			
		311.1498			
		360.2179			
		149.0954			
		293.1392			
6.30	540.2805 [M+NH ₄] ⁺	311.1488	C ₂₇ H ₃₈ O ₁₀	Unknown 9	8
	545.2374 [M+Na] ⁺	329.1584			
6.36	476.2884 [M+NH ₄] ⁺	471.1743	C ₂₃ H ₃₈ O ₉	Unknown 10	7
	481.2415 [M+Na] ⁺	439.2343			
		329.1605			
		311.1499			
		149.0971			
		293.1480			
		207.1742			
6.42	518.2599 [M+NH ₄] ⁺	351.1429	C ₂₄ H ₃₆ O ₁₁	Unknown 11	17
	523.2183 [M+Na] ⁺	329.1606			
	501.2368 [M+H] ⁺	311.1498			

Continued next page

RT (min)	Parent ions	Product ions	Formula	MAGE	Species #
6.45	501.2106 [M+Na] ⁺ 496.2547 [M+NH ₄] ⁺	311.1490 329.1596	C ₂₅ H ₃₄ O ₉	Unknown 12	8
6.55	602.3945 [M+NH ₄] ⁺ 607.3498 [M+Na] ⁺ 585.2751 [M+H] ⁺	311.1537 329.1627 549.3464 531.3362	C ₃₁ H ₅₂ O ₁₀	Unknown 13	5-7
6.67	521.2075 [M+H] ⁺	503.1967 311.1488	C ₂₆ H ₃₂ O ₁₁	Unknown 14	10, 11, 13,17, 22, 24
7.08	530.2983 [M+NH ₄] ⁺ 535.2536 [M+Na] ⁺	530.2920 477.2468 311.1507 149.0966 329.15	C ₂₆ H ₄₀ O ₁₀	Unknown 15	7, 10, 12
7.99	549.2675 [M+Na] ⁺ 544.3151 [M+NH ₄] ⁺	207.1747 311.1471 329.1576 149.0974	C ₂₇ H ₄₂ O ₁₀	Unknown 16	7
8.13	602.3927 [M+NH ₄] ⁺ 607.3485 [M+Na] ⁺	531.3313 549.3355 311.1506 329.1613	C ₃₁ H ₅₂ O ₁₀	Unknown 17	5, 7
8.28	589.3367 [M+Na] ⁺ 584.3812 [M+NH ₄] ⁺	657.2583 621.2781 329.1613 311.1509 549.3438 531.3334	C ₃₁ H ₅₀ O ₉	Unknown 18	5, 7
9.55	521.2430	561.2381 311.1488 211.0982	C ₃₆ H ₃₄ O ₂	Unknown 19	2, 16, 17

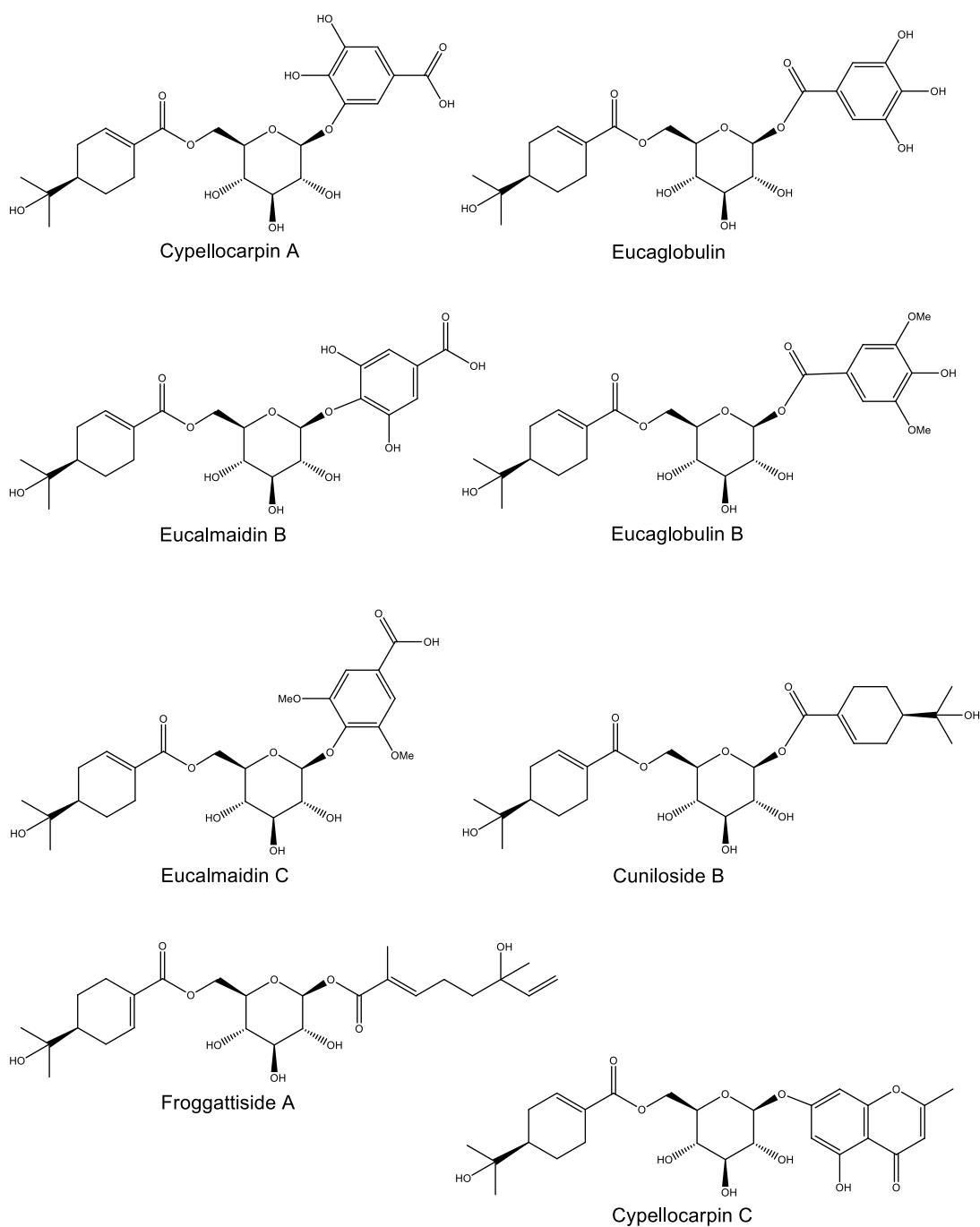


Figure 2.5: Structures of monoterpenoid acid glucose esters (MAGEs) identified from isolated *Eucalyptus* foliar glands.

2.3.5. Volatile components in *Eucalyptus* foliar glands

The volatile oil components in hexane extracts of all 30 species were analysed using gas chromatography. The most abundant oil components detected were monoterpenes and sesquiterpenes. The total oil ranged from a low of 9.6 mg g⁻¹ DW (in *E. stellulata*) to a high of 106.6 mg g⁻¹ DW (in *E. erythronema*). Some species had an oil profile rich in monoterpenes (e.g. *E. badgensis*) while some were rich in sesquiterpenes (e.g. *E. brevistylis*). In general, the most abundant oil components were 1,8-cineole, α -pinene, and *p*-cymene. Limonene, β -pinene, linalool and α -terpineol were present in most of the species in comparatively low levels (**Table 2.3**). The major sesquiterpenes identified were β -eudesmol, α -caryophyllene and spathulenol. The total oil in the two subgenera *Symphyomyrtus* and *Eucalyptus* did not differ significantly ($t = -1.143$, $p = 0.263$; **Figure 2.6**). The oil of subgenus *Eucalyptus* species was analysed further in Chapter 3 at the glandular level.

Table 2.3: Quantification of monoterpenes and sesquiterpenes in *Eucalyptus* leaf extracts. Total oil is in mg g⁻¹ leaf DW.

#	Species	Total oil	% of monoterpenes								% of sesquiterpenes		
			1,8-cineole	limonene	α -pinene	β -pinene	linalool	α -terpineol	p-cymene		β -eudesmol	α -caryophyllene	spathulenol
1	<i>E. ochrophloia</i>	10.2	40.7	0	0.2	0.2	0	1.5	3.6		0.1	0	0.2
2	<i>E. thozetiana</i>	15.7	14.6	0.9	6.5	14.4	0.3	4.4	0.8		3.1	1.0	0.3
3	<i>E. articulata</i>	59.4	33.8	0.4	7.0	5.4	0	4.7	4.6		0	0.1	0.1
4	<i>E. erythronema</i>	106.6	48.0	2.2	28.9	0.5	0.1	0.3	0.7		0.1	4.2	0.2
5	<i>E. proxima</i>	42.3	6.1	0.6	13.5	0.3	0.1	0.2	1.9		0	5.4	0.2
6	<i>E. protensa</i>	46.1	26.9	0.6	31.6	0.4	0	0.3	3.4		0.8	1.6	1.7
7	<i>E. tenera</i>	38.3	16.9	0.2	22.4	0.2	0	0.6	1.2		0.1	7.0	0.3
8	<i>E. cyanophylla</i>	48.9	16.4	0.6	15.3	0.3	0	0.2	0.6		0.3	23.2	0.1
9	<i>E. dumosa</i>	27.7	58.2	1.0	11.6	0.2	0	0.4	0.7		0.2	5.5	0.3
10	<i>E. exigua</i>	47.1	4.6	0.8	9.2	13.8	0	5.8	14.1		0	1.4	1.8
11	<i>E. incrassata</i>	72.6	34.3	1.5	37.7	0.7	0.1	0.5	0.7		0.4	1.7	0
12	<i>E. pimpiniana</i>	22.9	6.5	1.4	14.7	5.5	0	5.2	8.8		0.1	0.1	8.8
13	<i>E. pumila</i>	55.2	75.5	1.5	5.0	0.1	0	0.7	1.1		0.1	1.0	0
14	<i>E. gillenii</i>	21.9	58.1	1.4	0.9	0.3	0	1.1	4.5		0.1	0	9.2
15	<i>E. aromaphloia</i>	68.0	62.9	3.4	12.7	0.2	0.1	1.9	1.5		0.1	2.0	0
16	<i>E. badjensis</i>	46.12	33.9	3.5	14.4	0.2	0.1	2.2	8.2		0.3	0.2	0.1
17	<i>E. parvula</i>	37.9	69.2	2.7	3.2	0.1	0.4	0.4	0.9		0.2	3.9	0.2
18	<i>E. mitchelliana</i>	31.0	7.3	0.4	1.8	0.1	0	0.5	5.7		0.5	0.7	2.1
19	<i>E. stellulata</i>	9.6	5.1	0.7	0.6	0	3.0	2.3	29.6		0.3	1.8	6.1
20	<i>E. oreades</i>	28.8	3.7	0.5	0.8	0.1	0.4	4.5	26.7		0.2	0.1	1.1
21	<i>E. agglomerata</i>	10.8	7.8	1.1	56.4	1.2	0	0	0.6		0	0	0
22	<i>E. gregsoniana</i>	57.6	7.0	0.6	15.3	0.1	0.2	0.6	0.1		1.0	0.5	0.6
23	<i>E. falciformis</i>	74.0	68.9	6.0	3.5	1.0	0.1	1.4	0.3		0.1	0	0.1
24	<i>E. nitida</i>	51.7	12.1	0.3	0.5	0	0.6	1.6	24.5		0.2	0.2	2.7
25	<i>E. approximans</i>	20.8	0.4	0.1	0	0	0	0.8	14.7		0	0	0
26	<i>E. apiculata</i>	19.5	27.6	0.5	6.1	10.3	0	8.4	7.8		0.3	0	2.1
27	<i>E. dendromorpha</i>	30.5	0.3	0.8	0.2	0	0.1	1.8	20.1		0	0.1	0.9
28	<i>E. obstans</i>	26.9	44.0	0.7	0.7	0.1	0	0.3	9.3		0.2	0	0.1
29	<i>E. suberea</i>	27.4	0.2	0	0	0	0	0	0		0	0.1	0
30	<i>E. brevistylis</i>	51.1	0.2	0.1	0.1	0	0	0.1	2.0		0.5	0.1	0.3

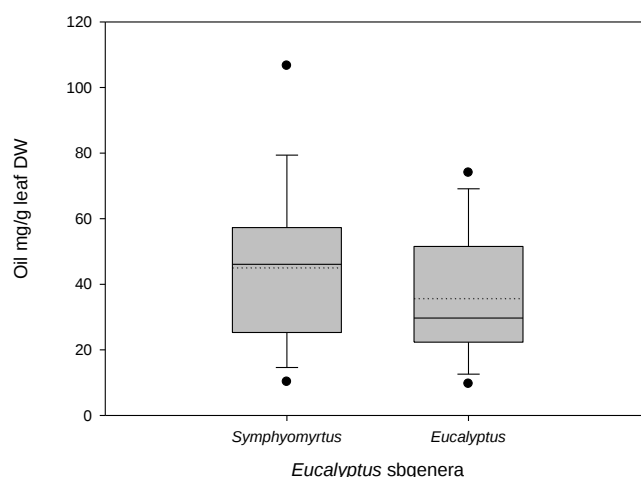


Figure 2.6: Variation of oil in *Eucalyptus* subgenera. Box plots represent oil for two *Eucalyptus* subgenera *Symphyomyrtus* and *Eucalyptus*. Mean values are represented by dotted lines and median values by solid lines.

2.3.6. Variation of MAGEs and oil in natural populations

Inter and intra-population variations of foliar cuniloside B and terpene oils were studied in two populations of *E. froggattii* (**Figure 2.7**). Total oil content in population 1 ranged from 8.6 to 33.5 mg g⁻¹ DW with a mean ($\pm$ SE) of 18.1 ± 1.3 mg g⁻¹ DW. In population 2, the oil content ranged from 6.0 to 28.7 mg g⁻¹ DW with mean of 13.6 ± 1.0 mg g⁻¹ DW. Cuniloside B ranged between 0.003 to 17.5 mg g⁻¹ DW in population 1 and 1.0 to 24.9 mg g⁻¹ DW in population 2. The mean cuniloside B concentrations of population 1 and 2 were 5.3 ± 0.7 and 5.6 ± 1.0 mg g⁻¹ DW (mean $\pm$ SE) respectively. The mean cuniloside B concentration in population 2 was significantly higher than that of population 1 ($t = -2.35$, $p = 0.02$). The total oil was compared with the cuniloside B (mg g⁻¹ DW) in two populations. The relationship between total oil weight and MAGEs was not significant (Pearson correlation: $p = 0.31$ in population 1 and $p = 0.29$ in population 2).

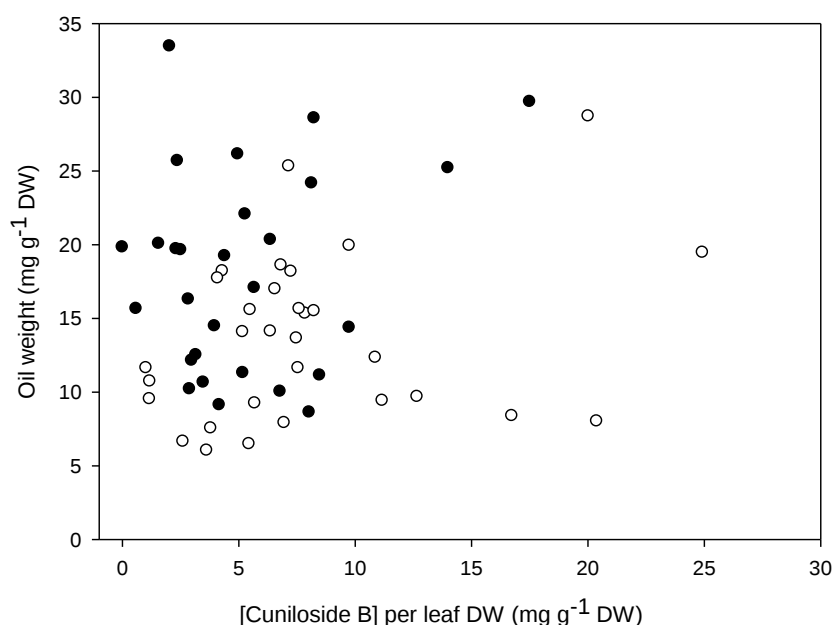


Figure 2.7: Comparison between total oil (mg g⁻¹ DW) and cuniloside B (mg g⁻¹ DW) in leaves from two populations of *Eucalyptus froggattii*. Filled circles represent the population 1 and hollow circles represent the population 2.

2.3.7. Biotransformation study

i. *E. polybractea* and *E. froggattii* callus cultures

Preliminary investigations in this section were aimed to test the potential of obtaining cell suspensions of *E. polybractea* and *E. froggattii* through leaf-derived callus. Firstly, experiments were conducted to select media suitable to produce friable callus. Explants of the two species were tested on two different media compositions, M1 and M2 supplemented with different growth regulator combinations. Both species started to develop light yellow calli in about four weeks. By the sixth week vigorously growing light yellow calli of *E. polybractea* were obtained. It was observed that M2 medium elicited a better response than M1 for *E. polybractea*. *E. froggattii* calli in both media started to turn brown over the time (**Figure 2.8**). The primary callus of both species was subcultured at six-weeks intervals on the same parental medium composition on which the callus was initiated. *E. froggattii* callus grown on M1 medium showed signs of shoot regeneration at the end of the second subculturing cycle. The observations on callus induction are summarised in **Table 2.4**.

ii. *E. polybractea* and *E. froggattii* cell suspension cultures

After two passages of subcultures, calli of *E. polybractea* grown on M1 and M2 media, and *E. froggattii* grown on M2 medium, were used to establish suspension cultures. *E. froggattii* callus grown on M1 was not used for cell suspensions due to the signs of shoot regeneration. To initiate the suspensions approximately 3 g of callus were transferred into the liquid medium of the same composition as that used for callus cultures. *E. polybractea* suspension was established successfully in the medium supplemented with NAA and TDZ (M2 medium) in about two weeks. The calli progressively disintegrated to small cell aggregates in the liquid medium. These were subcultured at four-weekly intervals. *E. polybractea* on M1 had big clumps of callus while only some parts of them disintegrated into suspension. The observations on cell suspensions are summarised in **Table 2.4**.

The callus of *E. froggattii* transferred into M2 liquid media did not show any growth or signs of a suspension after four weeks. Large cell clumps were found in the liquid medium without establishing a proper suspension. The flasks were maintained further and in about six weeks the calli showed signs of shoot regeneration. Therefore, the experiments on *E. froggattii* cell suspension were discontinued.

iii. The established *E. polybractea* cell suspension

E. polybractea cell suspension in M2 liquid medium showed signs of a proper suspension. After six subculturing cycles, the suspensions were transferred to and maintained in 250 mL Erlenmeyer flasks for further experiments (**Figure 2.9**).

The growth curve for the suspension in 250 mL flasks was established by measuring the packed cell volume to determine the right time for subculture and precursor feeding. The curve showed a typical sigmoidal shape (**Figure 2.10**). The latency period (lag phase) was 6 days and then the suspension moved onto the exponential growth phase and a linear phase prior to a plateau phase. The biomass increased till about the 28th day. Therefore, the *E. polybractea* suspensions in 250 mL flasks were routinely sub-cultured every 28 days as the cells achieved the maximum growth in the cell cycle.

Suspensions had mostly round, and sometimes elongated cells and the percentage viability of cells was recorded as 92.6%. The cells aged 28 days in suspension were observed under the microscope for the greenish fluorescence of viable cells (**Figure**

2.11). The fluorescent green colour of fluorescein was liberated as a reaction of the non-fluorescent stain with cellular esterase accumulated within viable cells. Only viable cells can retain fluorescein as it is unable to pass through the living cell membrane (Khafagi 2007; Singh and Chaturvedi 2012).

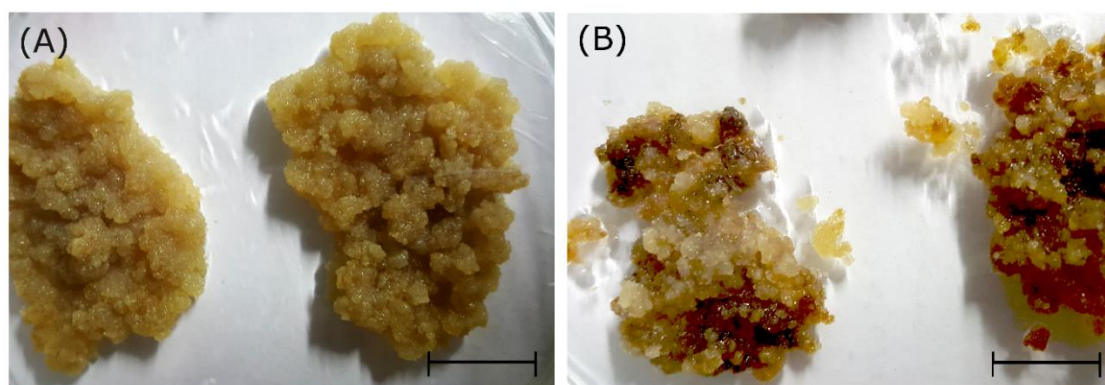


Figure 2.8: Six weeks old calli of *E. polybractea* and *E. froggattii* on M2 medium. (A) Friable light-yellow callus of *E. polybractea* and (B) light yellow callus of *E. froggattii* with browning. Scale bars represent 1 cm.

Table 2.4: Observations on callus formation and cell suspensions of *E. polybractea* and *E. froggattii* on M1 and M2 solid and liquid media.

Species	Medium	Callus morphology (on solid medium)	Cell suspension observations (in liquid medium)
<i>E. polybractea</i>	M1	Hard callus	Big clumps of callus
	M2	Light yellow and friable callus	Pale yellow suspension
<i>E. froggattii</i>	M1	Delayed callus initiation, hard callus turned brown as ages. Showed signs of shoot regeneration	-
	M2	Light yellow hard callus turned brown as ages	Clumps of callus, no further growth observed. Signs of shoot regeneration

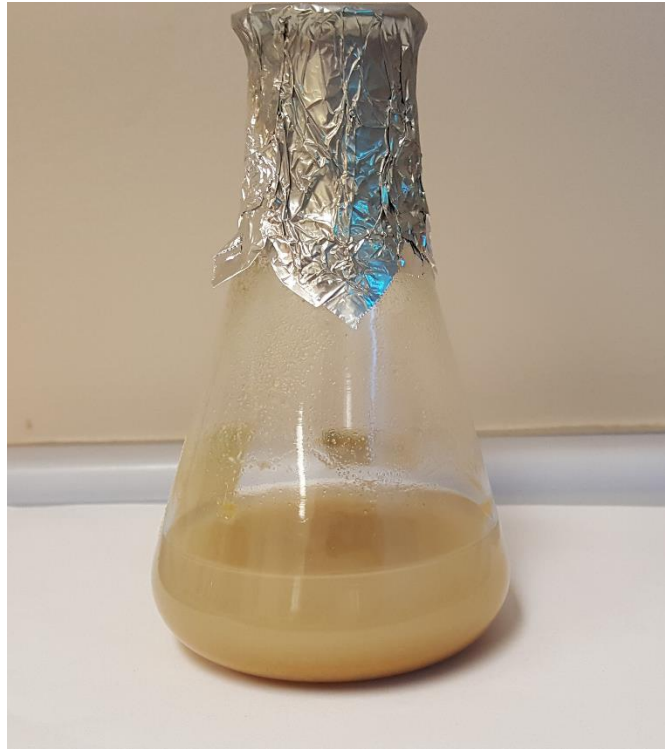


Figure 2.9: A 28 day old dense cell suspension of *E. polybractea* grown in M2 medium in flask.

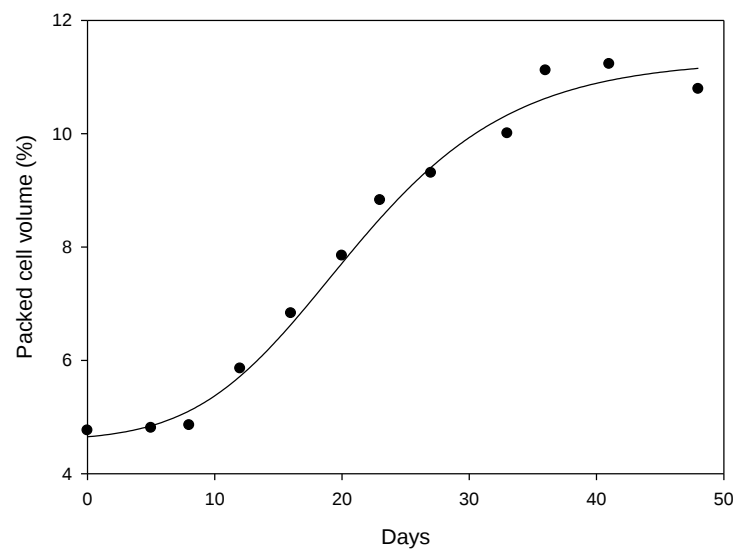


Figure 2.10: The growth curve of *E. polybractea* cell suspension culture showing packed cell volume (PCV) in relation to time as it pertains to each of the growth phases (lag phase, exponential or log phase, linear phase and plateau or stationary phase).

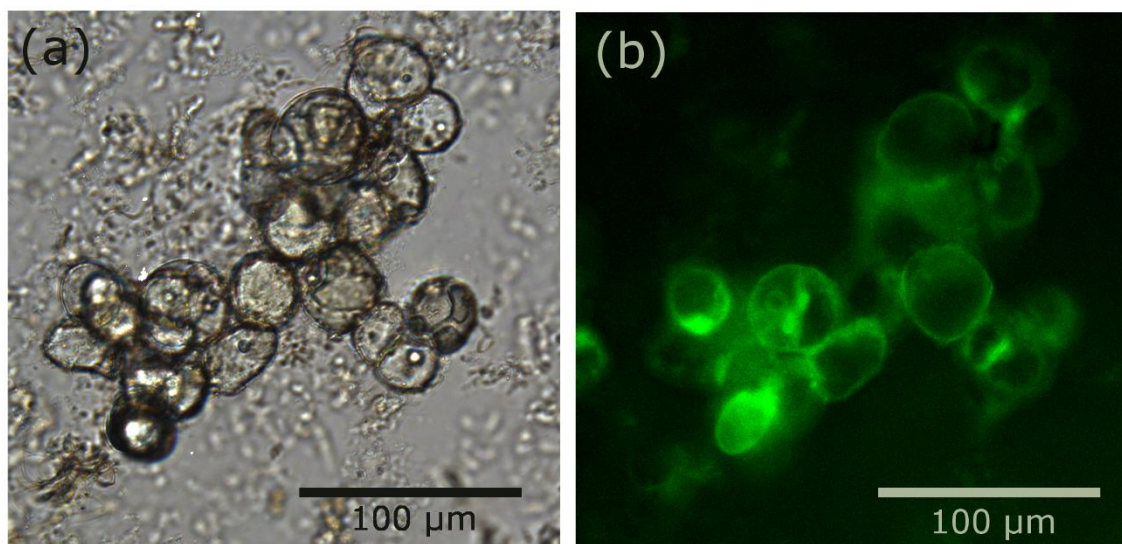


Figure 2.11: Four weeks old cells of *E. polybractea* suspension. (a) Cells without the fluorescence filter, and (b) fluorescence of viable cells stained with 1% fluorescein diacetate solution.

iv. Biotransformation of *p*-coumaric acid by *E. polybractea* cells

To study the biosynthesis pathways of MAGEs/NVCs in cells, *p*-coumaric acid was fed to cell suspensions. *p*-Coumaric acid is a simple phenolic phenylpropanoid that commonly occurs in plants and is a precursor of many PSMs (Ferreira *et al.* 2019). A majority of the MAGEs identified from eucalypts to date contain a phenolic group attached to the glucopyranose ring. Therefore, *p*-coumaric acid was selected as a suitable precursor to feed in this study. *E. polybractea* cells in suspension were fed with *p*-coumaric acid on the 23rd day of the cell cycle. After four days of incubation, the biotransformation products were isolated from the cells. Structural elucidation was done by LC-MS and three compounds were obtained (**Figure 2.12**). Coumaric acid glucoside was tentatively identified at RT 13.8 min. Mass fragment ions of m/z 325.0936 at 9.96 min and 325.0928 at 10.44 min also showed the presence of two possible monoglucosides: *p*-coumaric acid β -D-glucopyranosyl ester and 4-O- β -D-glucopyranosylcoumaric acid at RT 9.96 and 10.44 min, respectively. The control using the ethanol gave the same product ion peaks similar to the non-treated cells confirming that there is no effect of the solvent used to dissolve coumaric acid on cells.

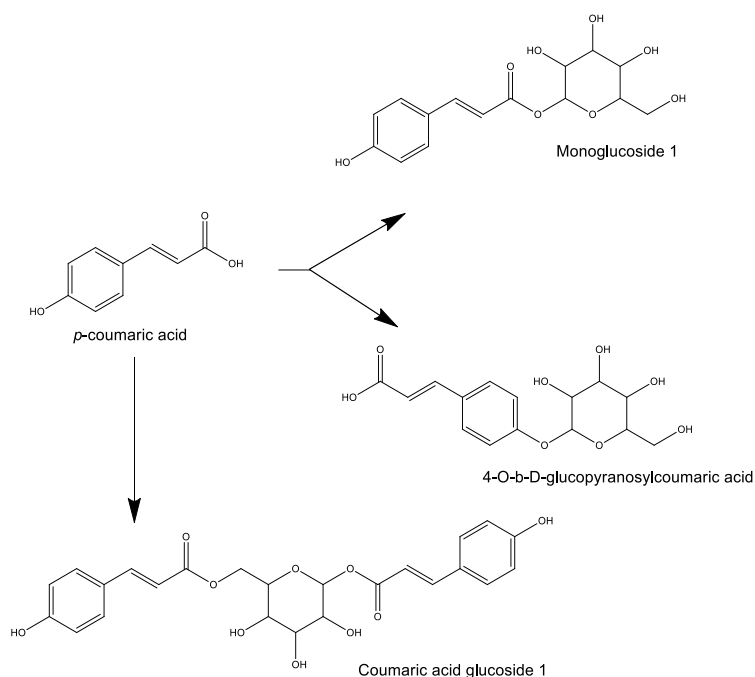


Figure 2.12: Putative biotransformation products of *p*-coumaric acid feeding to *E. polybractea* cell suspensions.

2.4. Discussion

The main aim of this study was to discover the MAGEs and other novel non-volatiles localised to foliar glands of *Eucalyptus* species and find whether MAGEs are ubiquitous in the genus. The results show that the oil glands of the majority of species examined contain MAGEs.

2.4.1. MAGEs localised to glands and their widespread occurrence

This study shows that the foliar glands of eucalypts contain various MAGEs in different abundances. Several known and novel MAGEs from foliar glands were identified based on the unique MS fragmentation producing m/z 311.14 and 329.15 $[M+H]^+$ ions. The results show that some MAGEs are widespread in the genus *Eucalyptus* with cypellocarpin C, cuniloside B and froggattiside A found in more species than any of the other compounds. These three MAGEs have been identified from a range of species in previous studies as well (Hakki *et al.* 2010; Heskes *et al.* 2012b). Cuniloside B seems to be the most widespread in the genus and has also been identified in the sister genus *Corymbia* (Hakki *et al.* 2010).

A total of 19 novel MAGEs were tentatively identified based on the characteristic mass fragmentation pattern. Two of them, “Unknown 13” and “Unknown 14”, have been reported previously in other *Eucalyptus* species by Heskes *et al.* (2012b). In that publication, “Unknown 13” (detected at RT 6.55 min) was reported from five species in subgenus *Symphyomyrtus* and “unknown 14” was identified from nine *Eucalyptus* species including three subgenus *Eucalyptus* species. “Unknown 14” is the only compound that was identified in the present study from a subg. *Eucalyptus* species in addition to the three more frequently occurring compounds: cuniloside B, froggattiside A and cypellocarpin C. Structural elucidation of the unknown compounds by NMR was not possible in this study due to the difficulty of purifying sufficient quantities of the compounds.

The data obtained from some species raises a question about the ubiquitous nature of MAGEs in the genus. For example, no MAGEs were detected in glandular extracts of four species from subgenus *Eucalyptus*: *E. nitida*, *E. dendromorpha*, *E. brevistylis* and *E. suberea*. In previous studies, predominantly *Symphyomyrtus* species have been studied while only a comparatively small number of species from subg. *Eucalyptus* were screened. None of the species from the subg. *Eucalyptus* sections to which *E. brevistylis* and *E. suberea* belong (*Longistylus* and *Frutices*) had been studied previously.

2.4.2. Relationship between MAGEs and oil components

There could be a relationship between MAGEs and oil components in eucalypts given their shared site of storage and/or synthesis in the glands. The highest amount of α -terpineol (2.8 mg g⁻¹) was observed in *E. articulata*, the species which also had the highest number of peaks likely corresponding to MAGEs (19 different retention times) according to the mass fragmentation pattern. This finding is compatible with the fact that α -terpineol is a possible precursor of oleuropeic acid, the monoterpene acid esterified to glucopyranose in many oleuropeic acid glucose esters. Guo and Yang (2006) proposed that oleuropeic acid is possibly synthesised from α -terpineol. They suggested that the C-7 position of α -terpineol is preferentially oxidized to produce 7-hydroxy- α -terpineol and then oleuropeic acid (8-hydroxy-p-menth-1-en-7-oic acid; **Figure 2.13**). Similarly, a study by Heskes *et al.* (2012b) showed that the amount of volatile essential oils and MAGEs are strongly correlated in *E. polybractea*. In particular, cuniloside B and froggattiside A from two natural populations of *E. polybractea* in Bendigo region in

Victoria were shown to be highly correlated with the concentrations of total terpene oils in both populations (Heskes *et al.* 2012b). In contrast, the population study on *E. froggattii* presented here did not show a strong correlation between oil constituents and MAGEs. However, it is noteworthy that though MAGEs have been discovered from *E. froggattii*, the total oil yield is relatively low and its oil profile is dominated by sesquiterpenes whereas *E. polybractea* oil is largely comprised of monoterpenes (Goodger *et al.* 2009).

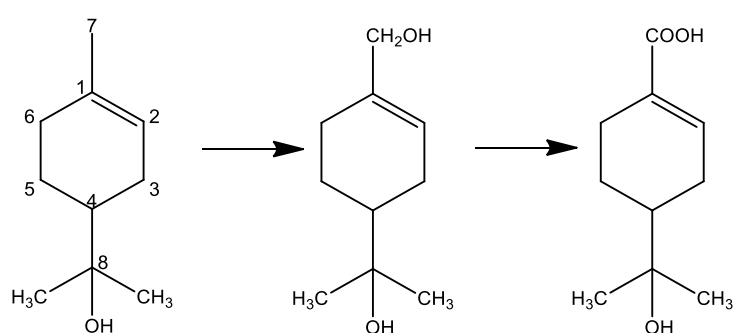


Figure 2.13: Proposed biosynthetic pathway for (+)-oleuropeic acid (Guo and Yang 2006). The C-7 position of α -terpineol is oxidized to produce 7-hydroxy- α -terpineol and then oleuropeic acid.

2.4.3. Chemotaxonomy and ecological aspects

Several strong phylogenetic trends in chemical profiles of *Eucalyptus* foliar glands were observed. Species from the two largest subgenera *Symphyomyrtus* and *Eucalyptus*, which contain about 300 and 120 species, respectively, were selected for this study (Brooker 2000). It is clear from the results that the species within the subgenus *Symphyomyrtus* are rich in MAGEs. Although a quantification of total MAGEs was not possible in this study due to a lack of standards for the majority of compounds, based on the peak area on LC-UV absorbance chromatograms, the MAGEs in the subgenus *Eucalyptus* species were found in comparatively lower abundance. In addition, *Symphyomyrtus* species generally contained a greater variety of MAGEs. Subgenus *Eucalyptus* species had limited MAGE structural diversity with almost all of them having only cuniloside B, froggattiside A and cypellocarpin C. The novel MAGEs were largely lacking in the subgenus with only a single novel compound detected in *E.*

gregsoniana. In contrast, subg. *Eucalyptus* species were dominated by compounds containing fragmentation ions corresponding to phenolics. Such a difference has been observed in previous studies supporting the present results. For example, MAGEs have been identified as the dominant non-volatile group in selected species from the two subgenera *Symphyomyrtus* and *Eudesmia* (Heskes *et al.* 2012b). In that study, cuniloside B was identified from all species examined including four subgenus *Eucalyptus* species. Furthermore, sections *Adnataria*, *Bisectae* and *Dumaria* from subgenus *Symphyomyrtus* had a higher diversity of MAGEs than species from other sections (Heskes *et al.* 2012b).

The MAGEs may play a role in ecological interactions of eucalypts. Though the structure and biological activity evidence supports a possible defence function, studies that directly explore the association between the MAGEs and herbivore interactions of plants are completely absent. Though it is not yet clear, some studies indicate the compounds in *Eucalyptus* foliage may play a role in leaf selection pattern by herbivores. For example, two “mosaic trees” of *E. melliodora* and *E. sideroxylon* have been identified which have intra-individual variation in foliar chemistry and susceptibility to insect herbivory (Padovan *et al.* 2012). Analysis of the chemical profile of leaves showed that the resistant leaves had lower concentrations of sesquiterpenes and higher concentrations of formylated phloroglucinol compounds than susceptible leaves on the same tree in both species. Furthermore, the two types of leaves have similar concentrations of nitrogen in both species highlighting that the only difference between the two mosaic trees is the levels of monoterpenes. The resistant leaves from the mosaic *E. melliodora* have a higher monoterpene concentration compared to the susceptible leaves. In contrast, the leaves from *E. sideroxylon* have shown a reversed pattern on this scenario, in which the susceptible leaves contained a higher level of monoterpenes than resistant leaves. These variations in the chemical profiles suggest that single or a group of compounds seem to play the actual role in deterring herbivory in these species. Though it has not been particularly analysed, being *Symphyomyrtus* species from the section *Adnataria*, both *E. melliodora* and *E. sideroxylon* potentially have MAGEs in high abundance. In addition to the herbivory-related differences, *Symphyomyrtus* species have other characteristics; for example, they show a greater capacity to deal with abiotic stresses such as drought (Anekonda *et al.* 1999; Merchant *et al.* 2006). However, the relationship between the species choice by herbivores and the foliage chemistry has not yet been clearly understood, therefore, further research is required in this area.

2.4.4. Glycosylation of coumaric acid by cultured cells of *E. polybractea*

E. polybractea was used as a model system to study the reaction types that occur in *Eucalyptus* oil gland secretory cells and/or the gland lumina. Cells in suspension successfully transformed coumaric acid to its glycosides. It has been previously reported that suspension cells of *E. perriniana* can convert phenylpropanoids including coumaric acid into 4-O- β -D-glucopyranosylcoumaric acid, *p*-coumaric acid β -D-glucopyranosyl ester, 4-O- β -D-glucopyranosylcoumaric acid β -D-glucopyranosylester, caffeic acid, and 3-O- β -D-glucopyranosylcaffeic acid (Katsuragi *et al.* 2010). Mono-glucosides of precursors have been previously obtained from biotransformation in *Eucalyptus* cells. Cultured *E. perriniana* cells converted exogenously added thymol, carvacrol, and eugenol into their mono-glucosides (Shimoda *et al.* 2006). Biotransformation pathways of phenylpropanoids seems to be different in plants and cultured plant cells compared to other organisms. In contrast to the plant systems, no formation of phenylpropanoid glycosides have been observed in algae. For example, a green unicellular alga, *Hematococcus pluvialis*, converted the phenyl propanoids ferulic acid and *p*-coumaric acid into vanillin, vanillic acid, vanillyl alcohol, and protocatechuic acid rather than glycosides (Shimoda *et al.* 2007).

E. polybractea cell suspensions were established successfully in this study for the first time. Cell suspensions provide a convenient system to study enzyme regulation and explore the biosynthesis pathways of secondary metabolites. The undifferentiated cells, relatively uniform state of development of the cells, the absence of interfering microorganisms and the compressed vegetative cycle are some of the advantages of using cell cultures over whole-plant studies (Croteau *et al.* 2000). Cell cultures have commonly been used to discover the enzymes involved in biosynthesis of natural compounds, for example in alkaloid biosynthesis. More than 80 enzymes that catalyse the steps in biosynthesis of indole, isoquinoline, tropane, pyrrolizidine, acridone, and purine classes of alkaloids have been discovered with the help of cell suspensions (Croteau *et al.* 2000). The future work on the *E. polybractea* cell suspension system developed in the present study may help elucidate the biosynthetic pathway of MAGEs, possibly by feeding predicted precursors such as α -terpineol.

2.5. Conclusion

This study shows that *Eucalyptus* foliar glands contain numerous known and novel MAGEs. In general, MAGEs were widespread in the genus and appeared to be ubiquitous in the subgenus *Symphyomyrtus*. They were the dominant group of NVCs found in the glands of subgenus *Symphyomyrtus* species. MAGEs were not the dominant NVCs in the glands of subgenus *Eucalyptus* species, but cuniloside B, froggattiside A and cypellocarpin C were still detected, albeit at lower levels. In contrast, the NVCs of subgenus *Eucalyptus* species were dominated by a later eluting group of phenolic non-volatiles, which will be studied in detail in Chapter 3. This evidence suggests a clear difference in chemotaxonomy between the two major subgenera of *Eucalyptus*, which may lead to a difference in herbivory and other ecological aspects. This study also provides evidence for a possible relationship between oil components and MAGEs in eucalypts.

The established cell suspensions of *E. polybractea* is a potential system to investigate more detail on biosynthesis of non-volatiles. The results obtained with cultured cells showed that the cells can glycosylate coumaric acid to give the corresponding glycosides. The enzymes related to glycosylation reactions can be further studied using *E. polybractea* suspension cultures.

Chapter 3

Flavonoids in *Eucalyptus* foliar glands

3.1. Introduction

Phenolic compounds including flavonoids are found widely in species from the genus *Eucalyptus*. Different types of flavonoids have been identified in extracts of eucalypt tissues, most commonly from leaf extracts. The first identified flavonoid from eucalypts was eucalyptin (5-hydroxy-7,4'-dimethoxy-6,8-dimethylflavone), a C-methylated flavone, identified from leaf wax of *Eucalyptus* species (Lamberton 1964). Following that discovery, the unsubstituted B-ring flavanones alpinetin, pinocembrin and O,O-dimethylpinocembrin were identified in extracts of *E. sieberi* leaves (Bick *et al.* 1972). More recently, pinocembrin has been detected in leaf extracts of five *E.* subg. *Eucalyptus* species (Saraf *et al.* 2015; Tucker *et al.* 2010) and foliar glandular extracts of a further four species from that subgenus (Heskes *et al.* 2012b). Flavonols have also been identified from several *Eucalyptus* species. For example, kaempferol was identified from *E. sideroxylon* leaves (Hillis and Isoi 1965) and the flavonol aglycone quercetin and glycosides of quercetin (3-glucosides, 3-rhamnosides, 3-rutinosides and 3-glucuronides) were identified from leaves of *E. sideroxylon*, *E. camaldulensis* and *E. rudis* (Conde *et al.* 1997; Hillis 1966; Hillis and Isoi 1965). Flavonol glycosides and acylated flavonol glycosides have been reported from *E. rostrata* leaf extracts (Okamura *et al.* 1993). In addition, flavonoid glycosides (apigenin-7-glucuronide, luteolin-7-glucoside and 7-glucuronide) have been isolated from *E. camaldulensis* (Abd-Alla *et al.* 1980). Flavonoids have also been identified from kino (gum) and honey of *Eucalyptus* species (Lee *et al.* 2017; Yao *et al.* 2004).

Though flavonoids appear to be common in eucalypts, especially in leaf extracts, the exact foliar tissue type in which they are located is unclear. Some early studies provide evidence for the presence of flavonoids in *Eucalyptus* leaf wax. For example, the flavones eucalyptin and 5-hydroxy-7,4'-dimethoxy-6-methylflavone were isolated from the leaf wax of *E. torelliana* (Lamberton 1964). Following that discovery, eucalyptin and

three other C-methylated flavones 8-desmethyl-eucalyptin, sideroxylin, and 8-desmethyl-sideroxylin were identified from the leaf cuticular wax of eight *Eucalyptus* species (Wollenweber and Kohorst 1981). Similarly, eucalyptin and 8-desmethyl eucalyptin were identified from leaf wax of several species of Myrtaceae including *Eucalyptus* (Courtney *et al.* 1983). However, these flavonoids are not as abundant as the other compounds found in the cuticle (Kolattukudy 1970). Therefore, it can be hypothesised that the flavonoids in cuticle may be exudates that are released from an inner tissue like oil glands. As a specialised secretory and storage tissue type, oil glands have a higher chance for containing flavonoids within. The lack of evidence for the presence of flavonoids in oil glands could be due to the impracticalities of isolating compounds from these embedded structures, rather than their absence from within glands. *Eucalyptus* oil glands are now known to contain other groups of non-volatile compounds (e.g. monoterpene acid glucose esters (MAGEs; Goodger *et al.* 2009)) while recent evidence from four species of *E.* subg. *Eucalyptus* species confirms that the flavanone pinocembrin is also present in foliar oil glands (Heskes *et al.* 2012b). This evidence suggests that there is a possibility of more flavonoids or related non-volatile compounds localised in the oil glands of *Eucalyptus* species.

There are many known roles for flavonoids in plants. In particular, plant flavonoids generally act as pigments that give colour and taste. For example, anthocyanins give colour to flowers and fruits. The rich taste of some plant structures due to flavonoids may act as an attractant or repellent to pollinators or pests (Ghasemzadeh and Ali 2011). Nevertheless, the exact role of the flavonoids localised to secretory structures in *Eucalyptus* or any plant species is unclear. Like the volatile components localised to secretory structures, flavonoids may also play a role in plant defence. Pinocembrin, which has been identified previously from eucalypt oil glands, has biological activities related to plant defence such as antibacterial, antifungal and antifeedant activities (Hanawa *et al.* 2001; Napal *et al.* 2009; Shain and Miller 1982). Similar to the suggested roles for MAGEs, flavonoids may also form a protective layer for secretory cells, shielding them from toxic oil components stored in gland lumina; however, their localisation and arrangement within the glands is not yet known. To further understand these concepts, it is a worthwhile exercise to identify whether more flavonoid compounds other than pinocembrin are localised to the oil glands in *Eucalyptus* species.

In addition to their *in planta* roles, plant flavonoids are also important as commercial compounds such as pharmaceuticals and natural antioxidants. The biological activities of flavonoids, including the compounds already known from secretory structures, have been well researched and many of them possess activities that make them beneficial for human health (Ghasemzadeh and Ali 2011). For example, pinocembrin has shown positive results as a pharmaceutical drug for cardiovascular diseases and cancer (Lan *et al.* 2016; Rasul *et al.* 2013). The methylated form pinostrobin has many proven pharmacological activities such as anti-microbial, anti-malarial and anti-cancer properties (Patel *et al.* 2016). Moreover, methylated flavanone compounds are more important as pharmaceuticals than non-methylated forms (Wen and Walle 2006). Flavonoids are also important as natural antioxidants. Although antioxidants can be manufactured synthetically there are side-effects when consumed. Also, natural antioxidants have shown to be more effective than synthetics (Chen *et al.* 1992). For example, the antioxidant activity of acylated flavonol glycosides isolated from *E. rostrata* is higher than that of synthetic antioxidants (Okamura *et al.* 1993). Therefore, natural flavonoids have a higher importance in the food industry as well. Due to this pharmaceutical importance, a better understanding of natural flavonoids in plants would be advantageous commercially. Given the presence of pinocembrin in four *Eucalyptus* species within the same subgenus, there is a higher chance of having the same or structurally similar compounds with methyl substitutions in more taxonomically related species. The qualitative and quantitative information, such as the amounts of different flavonoids present in various species, will facilitate the selection process of species that contain the desired compound profiles for commercial cultivation.

Research described in this chapter aims to identify and quantify flavanones and other major non-volatile and volatile components in foliar glands of *E. subg. Eucalyptus* species. In both physiological and commercial aspects, it is important to identify the purity of each flavonoid component and to know whether flavanones are exclusively or predominantly localised to the oil glands. In this study, the foliar gland extracts of 11 species were characterised using high-performance liquid chromatography (HPLC) coupled with UV and mass spectrometry (LC-MS) to identify and quantify the flavonoid compounds localised within them. Compounds were extracted from isolated foliar oil glands, and their localisation to other leaf tissues was also examined. The oil components in glandular extracts of these species were also analysed using gas chromatography-mass spectrometry (GC-MS) analyses to better understand the glandular contents to help elucidate their physiological role.

3.2. Materials and Methods

3.2.1. Plant material

Based on the preliminary data obtained in the previous chapter, 11 species from *E. subg. Eucalyptus* were selected to characterise the contents in their foliar oil glands. The acetonitrile extracts of the glands of these species predominantly had compounds eluting at later retention times with UV absorbance matching to flavanones (278 nm) in a significantly high abundance. Leaves harvested from the trees growing in the Currency Creek arboretum described in Chapter 2 (2.2.1-i) were used for this study.

3.2.2. Identification and quantification of flavanones in glandular extracts

The species rich in later eluting phenolic non-volatiles were further studied to identify and quantify the compounds present in their oil glands. The glands were isolated from the leaves of selected subgenus *Eucalyptus* species following the enzyme digestion protocol described in Chapter 2 (2.2.1-ii). From an isolated pool of glands from a single leaf of each species, two sets of 200 glands were randomly collected into separate Eppendorf tubes. One 200 gland set was used for the experiment described in 3.2.3. The other set of glands was immediately ground using a micropestle and non-volatile compounds extracted in 200 μ L of 100% acetonitrile as described in the extraction protocol above (2.2.1-ii). The glands in acetonitrile were incubated for 3 days at 25°C prior to the transfer of extract to glass vials. Flavanones in glandular extracts were fractionated using an HPLC system and electrospray ionisation liquid chromatography-mass spectrometry (ESI-LCMS/MS) as described in the previous chapter (2.2.1-iii). Comparisons with commercial standards and mass fragmentation patterns were used to help identify flavanones and other structurally related compounds.

The flavonoid components were quantified compared to a series of commercial standards (pinocembrin and pinostrobin - purchased from Sigma-Aldrich (St. Louis, MO, USA), dimethylpinocembrin and dimethylchrysin - purchased from Indofine Chemical Company (Hillsborough, NJ, USA)). The compounds were dissolved in 100% acetonitrile and standard series were produced and analysed using Liquid Chromatography (LC) with detection at the maximum UV absorbance for the respective compounds. The UV absorbance wavelengths used were 289 nm for pinocembrin, 288 nm for pinostrobin, 283 nm for dimethylpinostrobin and 263 nm for dimethylchrysin.

Alpinetin was quantified at 288 nm using the standard series of pinostrobin, due to the lack of a commercially available standard.

3.2.3. Identification and quantification of volatile oil components

Volatile components in glands of each species were also identified and quantified. From each species, 200 glands from the same pool isolated in 3.2.2 were ground and extracted in 200 μ L hexane containing 100 mg L⁻¹ tridecane as an internal standard and incubated for 3 days at 50°C to extract the volatile components. Hexane extracts were then dehydrated with anhydrous Na₂SO₄ and analysed using gas chromatography with flame ionisation detection (GC-FID) and GC-MS as described in (2.2.1-v). Volatile components were quantified using mass spectral data and standard series of key monoterpenes and sesquiterpenes as described in Goodger *et al.* (2007). Mass spectra were evaluated using Agilent MSD ChemStation E.02.02.1431 for GC-MS. Identification of β -triketones was done using either the NIST 11 or Adams 2012 mass spectra libraries and quantified based on a standard series of the sesquiterpene caryophyllene oxide (Sigma-Aldrich) due to the unavailability of commercial triketone standards.

A separate set of leaves was used for leaf area determinations and calculations of compound quantities per leaf dry weight. A leaf from each species was scanned using a flatbed scanner and weighed after drying in an oven at 60°C for 5 days. Leaf mass per unit area was calculated using these values.

3.2.4. Structural elucidation of C-methyl flavanones

HPLC analysis of glandular extracts of *E. agglomerata* showed two large unknown peaks and these two compounds were purified for structural elucidation. Oil glands were isolated from mature leaves, ground using a vial micro-pestle and then extracted in 100% acetonitrile. Peaks were collected following fractionation using the HPLC eluent system described in section 2.2.1-iii with FRC-10A fraction collector. The sample was separated on a Gemini C18 semi-preparative column (5 μ m, 150 $\times$ 10 mm; Phenomenex) run at a flow rate of 5 mL min⁻¹. The mobile phase consisted of water and acetonitrile and the LC gradient method was as follows: acetonitrile was ramped from 30 to 95% over 20 min and then the column was re-equilibrated at 30% acetonitrile for 5

min. Each compound was collected in several fractions and pooled together and finally dried under nitrogen gas flow.

3.2.5. Nuclear magnetic resonance spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy was used to identify the two unknowns collected in 3.2.4 and to confirm the identity of pinocembrin in extracts. LC-MS analysis gave an ion peak at 15.58 min RT which matched with mass fragmentation pattern of pinocembrin. This peak was also purified for confirmation using NMR. Approximately 1 mg of each partially purified extracts from *E. agglomerata* were dissolved in 0.75 mL deuterated chloroform (CDCl₃; Cambridge Isotope Laboratories., USA), filtered, and transferred to a 5 mm NMR tube. ¹H NMR spectra were acquired at 25°C using a Bruker Avance III 600 MHz spectrometer running the standard Bruker “zg30” pulse sequence. CDCl₃ was used as an internal reference (7.26 ppm) for all spectra.

3.2.6. Exclusive localisation of flavanones in foliar oil glands

Different leaf tissues were partitioned and analysed separately to test whether flavanones are present in tissues other than oil glands. Six randomly selected fully expanded mature leaves from *E. nitida* were used in this study. Each leaf was dissected into halves along the midrib and the area of each half was measured prior to extraction. One half of each leaf was digested using pectinase enzyme following the gland isolation protocol described in Chapter 2 (2.2.1-ii). After the digestion, the different tissue types in the leaf (epidermis with cuticle, veins and oil glands) were separated and purified. Each tissue type was frozen in liquid nitrogen and ground with a micro-pestle. Each ground component was extracted in acetonitrile as described previously (2.2.1-ii). The other half of the leaf was also ground to a fine powder in liquid nitrogen and extracted in acetonitrile in the same manner. The left and right halves were used alternatively for grinding and enzymatic digestion of leaf components to avoid any errors due to differences in gland localisation pattern in two sides of the leaves. Each extract was analysed using HPLC following the method given in Chapter 2 (2.2.1- iii) and the amount of the two key flavanones in *E. nitida*, pinocembrin and pinostrobin, per unit leaf area in the leaf and gland extracts were determined. The data were compared with t-test using IBM SPSS Statistics software, version 21 (IBM Armonk, NY, USA).

3.3. Results

3.3.1. Species selection

A group of non-volatile compounds eluting at later retention times with absorbance spectrum consistent with flavonoids (absorbance maxima of 278 nm) were observed in the glandular acetonitrile extracts from leaves of subgenus *Eucalyptus* species studied in Chapter 2. Based on the peaks observed in LC-UV chromatograms, 11 species rich in compounds matching the UV maxima of flavonoids were selected to study in the present chapter to identify their glandular contents. Nine species sampled were from section *Eucalyptus* representing numerous series while the other two species were from sections *Frutices* and *Longistylus* (**Table 2.1**).

3.3.2. Identification of two C-methyl flavanones

Several novel phenolic compounds were present in some species in varying abundances while two such compounds were particularly abundant in the glandular extracts from *E. agglomerata*. The acetonitrile extract was dominated by these two later eluting compounds at RT 17.16 and 18.78 min. These two compounds were purified from *E. agglomerata* oil glands and identified using LC-MS and ^1H and ^{13}C NMR analyses as C-methyl flavanones. The ^1H and ^{13}C NMR spectrum appeared almost identical to that of cryptostrobin (5,7-dihydroxy-8-methylflavanone) and demethoxymatteucinol (6,8-dimethylpinocembrin) reported in literature (Cannon and Martin 1977). The position of methyl group was confirmed by key gHMBC correlations of between C5 and OH5 (Figure 3.1; for full NMR results see Appendix; Shrestha *et al.* 2007). This is the first identification of these two compounds from a *Eucalyptus* species.

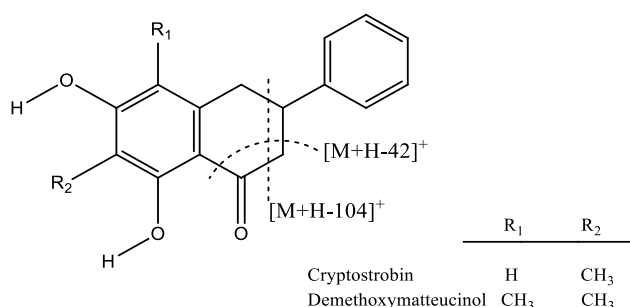


Figure 3.1: Chemical structures of two C-methyl flavanones (cryptostrobin and demethoxymatteucinol) identified from *Eucalyptus agglomerata* glandular extracts.

3.3.3. Unsubstituted B-ring flavanones from subgenus *Eucalyptus* species

LC-MS analysis of almost all species gave an ion peak $[M-H]^-$ at m/z 255.06 with the molecular formula structurally related to pinocembrin. This compound peak was collected and 1H NMR analysis identified this compound as pinocembrin (5,7-dihydroxyflavanone; for full NMR results see Appendix B). Mass spectral data and comparisons with commercial standards were used for the structural elucidation of the rest of the structurally related compounds. Unsubstituted B-ring flavanones characteristically produce fragments with neutral losses of 104 and 42 Da (Ma *et al.* 1997). The major compounds eluted at later retention times were consistent with flavanone standards and identified as structurally related unsubstituted B-ring flavanones. The ion peak at 11.79 min with a m/z 285.11 $(M+H)^+$ was identified as dimethoxylated flavanone dimethylpinocembrin (5,7-dimethoxyflavanone, pinocembrin dimethylether). Similarly, the mono-methoxylated flavanone pinostrobin (5-hydroxy-7-methoxyflavanone) was identified at 14.33 min with a m/z 271.09 $(M+H)^+$. Alpinetin (5-methoxy-7-hydroxyflavanone) was detected at 8.76 min with a m/z 271.09 $(M+H)^+$ in a comparatively low abundance. Structurally related dimethoxylated flavone dimethylchrysin (5,7-dimethoxyflavone) was present (**Figure 3.2**). These flavonoids, except alpinetin, were present in the glandular extracts of the section *Eucalyptus* species in very high abundance (based on LC-UV chromatogram peaks). However, they were not present in the two species from sections other than *Eucalyptus*: *E. suberea* and *E. brevistylis* (taxonomic classification of species is given in **Table 2.1**).

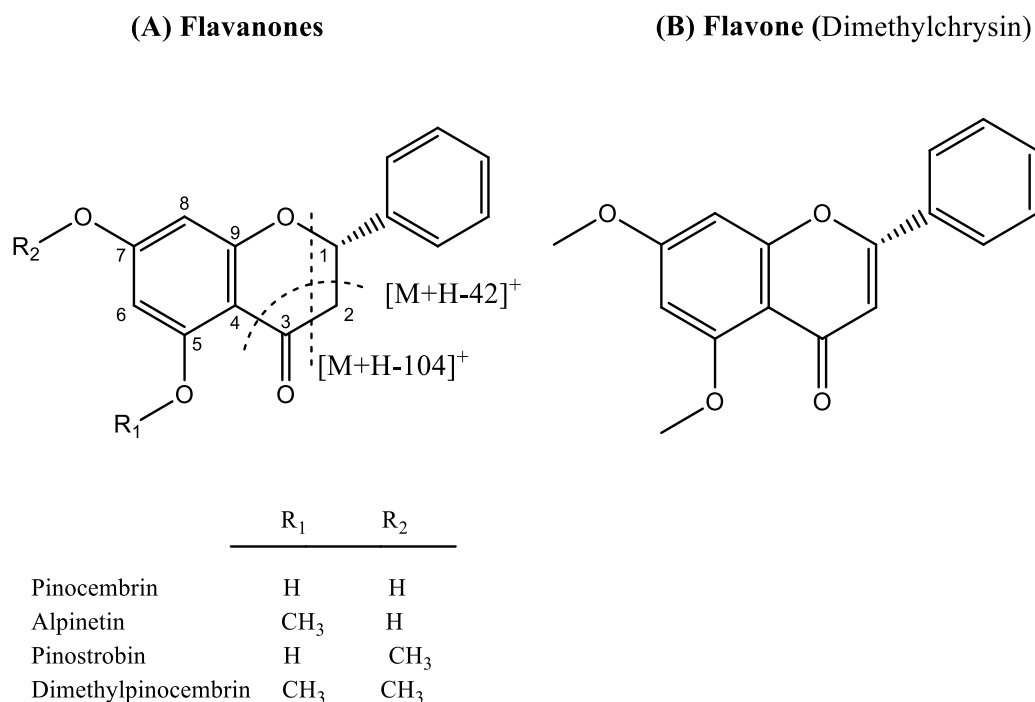


Figure 3.2: Structures of (A) flavanones and (B) the flavone dimethylchrysin localised to foliar oil glands of *E. subg. Eucalyptus* species.

3.3.4. Quantification of flavanones in oil glands

Unsubstituted B-ring flavanones dominated the non-volatile fraction of glandular extracts from subgenus *Eucalyptus* species and were quantified on a per gland and per leaf dry weight basis (**Table 3.1, Figure 3.3**). Eleven species from the subgenus were further studied to quantify the above flavonone group and the related flavones in their oil glands. On a per gland basis, the highest concentration of total flavonoids was found in the glandular extracts of *E. oreades* (0.7 µg gland⁻¹). The relative proportion of each flavonoid in glands varied among species and certain compounds were remarkably high in some of them. For example, *E. nitida* was dominated by pinostrobin with a proportion as high as 91% (0.35 µg gland⁻¹) of the total flavonones (0.38 µg gland⁻¹). The concentration of dimethylpinocembrin in *E. dendromorpha* and *E. oreades* glands was 84% (0.23 µg gland⁻¹) and 75% (0.53 µg gland⁻¹), respectively. Pinocembrin was a constituent in extracts of all species from *E. subg. Eucalyptus* section *Eucalyptus*. The maximum amount of pinocembrin was detected in *E. stellulata* glands (0.07 µg gland⁻¹), where it was 23% of the total flavanones in glands. It is noteworthy that in a majority of

species dimethylpinocembrin was the most abundant flavanone. Six species (*E. apiculata*, *E. approximans*, *E. dendromorpha*, *E. falciformis*, *E. oreades* and *E. stellulata*) had dimethylpinocembrin as the highest abundant flavanone ranging from 60-84% of the total flavonones. In contrast to the above species with higher concentrations of flavonoids, a few species had a very low amount of flavonones. The total quantified flavonones in *E. agglomerata* and *E. gregsoniana* were 0.05 and 0.003 $\mu\text{g gland}^{-1}$, respectively. Pinocembrin was present as the highest abundant flavonoid component in both species. In *E. agglomerata*, 75% of the total flavonoids was pinocembrin. *E. gregsoniana* had 90% pinocembrin out of the total flavonoids. Compared to the other three flavanones, alpinetin was in lower abundance or not detected at all in some species. While the proportion of alpinetin in glands was low as 0.1-2.5% in other species, in *E. agglomerata* 23% of the quantifiable glandular flavonones was alpinetin (0.01 $\mu\text{g gland}^{-1}$). However, it should be noted that other groups of flavanones were abundant in *E. agglomerata*, including C-methyl flavanones and other unidentified compound peaks.

Concentration of flavanoids per unit leaf mass in each species was calculated using the amounts per gland, average gland number per leaf (gland density) and average leaf dry mass (the data used for these calculations are attached in Appendix C). *E. oreades* and *E. nitida* had the highest amounts of total flavanones, 12.87 and 12.72 mg g^{-1} DW respectively; however, the combinations of individual compounds were different. The major constituent of *E. oreades* was dimethylpinocembrin (9.68 mg g^{-1} DW) while *E. nitida* had pinostrobin at a higher percentage (11.53 mg g^{-1} DW). *E. gregsoniana* reported the lowest quantity of total flavonoids with a value of 0.18 mg g^{-1} DW (**Figure 3.3**).

Table 3.1: Amounts of flavonones in *E. subg. Eucalyptus* glandular extracts. The values are in **(a)** per gland basis ($\mu\text{g gland}^{-1}$) and **(b)** per leaf dry weight (mg g^{-1} leaf dry weight).

*Species #	Species	Dimethylpinocembrin		Pinocembrin		Pinostrobin		Alpinetin		Dimethylchrysin (flavone)		Total flavonones	
		(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
20	<i>Eucalyptus oreades</i>	0.53	9.68	0.03	0.63	0.13	2.44	<0.01	0.04	<0.01	0.09	0.71	12.87
24	<i>E. nitida</i>	-	-	0.04	1.18	0.35	11.53	trace	0.01	-	-	0.38	12.72
26	<i>E. apiculata</i>	0.23	4.12	0.06	1.08	0.05	0.87	0.01	0.16	0.01	0.21	0.36	6.44
19	<i>E. stellulata</i>	0.19	5.20	0.07	1.96	0.05	1.33	0.01	0.21	-	-	0.32	8.69
27	<i>E. dendromorpha</i>	0.23	5.06	<0.01	0.08	0.03	0.75	<0.01	0.04	0.01	0.12	0.28	6.05
25	<i>E. approximans</i>	0.16	1.99	0.02	0.24	0.05	0.68	<0.01	0.02	<0.01	0.04	0.24	2.96
23	<i>E. falciformis</i>	0.11	3.76	0.02	0.57	0.03	1.13	trace	0.01	-	-	0.16	5.47
21	<i>E. agglomerata</i>	<0.01	0.03	0.04	1.11	trace	<0.01	0.01	0.35	-	-	0.05	1.49
22	<i>E. gregsoniana</i>	trace	0.02	<0.01	0.16	-	-	trace	<0.01	-	-	<0.01	0.18
29	<i>E. suberea</i>	-	-	-	-	-	-	-	-	-	-	-	-
30	<i>E. brevistylis</i>	-	-	-	-	-	-	-	-	-	-	-	-

*The species number assigned in this table is the same number allocated for each species in **Table 2.1** in Chapter 2.

Species are listed in the order of total flavonones per gland, from highest concentration to the lowest.

The amounts less than 0.001 have been reported as a trace value.

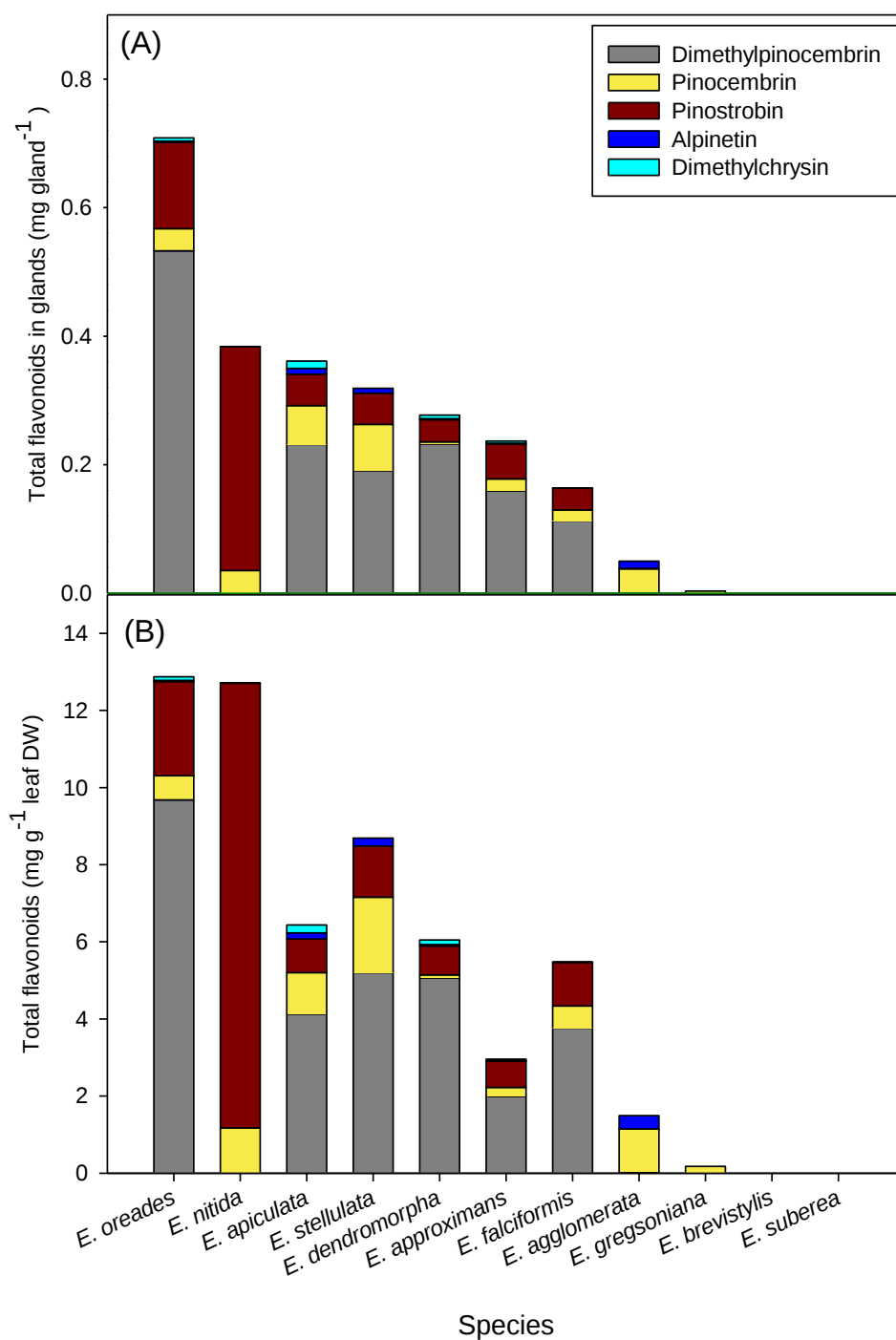


Figure 3.3: Total flavonoids in foliar oil glands of *Eucalyptus* subg. *Eucalyptus* species on (A) per gland basis and (B) per leaf dry weight basis.

3.3.5. Other non-volatile components localised to oil glands

Several other unknown PDA peaks were detected in UV chromatograms of some of the *E. subg. Eucalyptus* species suggesting their glands house more complex compounds containing flavanone moieties in addition to flavonones. The structures of these compounds were elucidated by comparing their ESI-LCMS/MS data to the fragmentation patterns of known compound classes, and the following compound groups were identified.

Two flavanone-glucosides were present in the glandular extracts of *E. stellulata* (**Table 3.2**). Pinocembrin-7-*O*-glucoside was identified by the ion peaks at m/z 419.13 $[M+H]^+$ and at m/z 417.13 $[M-H]^-$ corresponding to the molecular formula $C_{21}H_{22}O_9$ at 6.41 min retention time. Ion peaks at m/z 257.08 $[M+H-162]^+$ and m/z 255.07 $[M-H-162]^-$, due to the loss of glucose, indicate the presence of pinocembrin in this structure. Fragmentation with neutral losses of 104 Da were observed in both positive and negative ionisation modes. Characteristic neutral loss of glucose (162 Da) and characteristic fragmentation of unsubstituted B ring flavanones were used to identify these compounds (Domon and Costello 1988). Similarly, 5-methoxy-pinocembrin-7-*O*-glucoside was identified by the $[M+H]^+$ at m/z 433.10 corresponding to $C_{22}H_{24}O_9$. A fragment ion peak at m/z 271.09 $[M+H-162]^+$ corresponding to pinostrobin was identified in this compound after the loss of glucose.

β -triketone heterodimers linked by an *iso*-pentyl moiety were detected in non-volatile extracts from the glands of *E. gregsoniana* (**Table 3.2**). They are the compounds corresponding to molecular formulas $C_{24}H_{32}O_7$ at 16.8 min ion peak at m/z 431.20 $[M-H]^-$, $C_{26}H_{36}O_7$ at 18.5 min with a m/z of 459.24 $[M-H]^-$, $C_{25}H_{34}O_7$ at 17.6 min with a m/z of 445.22 $[M-H]^-$ and $C_{27}H_{38}O_7$ at 21.3 min with a m/z 473.25 $[M-H]^-$. In these compounds, fragment pairs were observed as a result of the characteristic fragmentation (Appendino *et al.* 2002). For example, the β -triketone heterodimer with m/z of 445.22 showed characteristic fragmentation on either side of the *iso*-pentyl bridge creating fragment pairs with m/z 277 and 167 and m/z 235 and 209.

Based on the characteristic fragmentation pattern, C-glucosides were identified from the glandular extracts (**Table 3.2**). Two 6-C chromone glucoside isomers were detected in *E. gregsoniana* at 2.6 and 4.3 min with m/z of 397.15 $[M+H]^+$ corresponding to molecular formula $C_{19}H_{24}O_9$ (Tian *et al.* 2012).

Flavanone-triketone conjugates linked by an *iso*-butyl moiety were found in *E. agglomerata* (**Table 3.2**). An ion peak with m/z of 491.21 $[M-H]^-$ was identified at 16.9

min retention time corresponding to $C_{29}H_{32}O_7$. The fragmentation resulted in m/z 255 corresponding to pinocembrin. Another flavanone-triketone conjugate was identified at 17.99 min with m/z of 505.2248 $[M-H]^-$ corresponding to $C_{30}H_{34}O_7$. The characteristic fragmentation creates a mass difference of 55 Da between the fragment ions m/z 181 and the loss of 236 Da (m/z 255 fragment) from the first compound, and the fragment ion m/z 181 and the loss of 236 Da (m/z 269 fragment) from the second compound. This suggests the fragmentation occurs in either side of an *iso*-butyl bridge (Tsui and Brown 1996).

3.3.6. Exclusive localisation of flavanones to oil glands

The oil glands and other leaf tissues of *E. nitida* were examined to test whether the flavanones are exclusively localised to glands or present in other leaf tissues. The concentrations of the compounds identified from the glands were compared with that of the intact leaves. No statistical difference was observed between the concentrations of pinostrobin ($t = -1.09$, $P = 0.33$) or pinocembrin ($t = -0.73$, $P = 0.498$) extracted from isolated glands or from intact leaves. This evidence suggests that unsubstituted B-ring flavanones are exclusively localised in glands. The other leaf tissues, i.e. epidermis with cuticle and vasculature, which were isolated along with the glands, were also tested separately for the same two compounds. Both pinocembrin and pinostrobin found in epidermis was less than 0.6% (pinocembrin - 0.6%, pinostrobin - 0.41%) of the total while the compounds in vasculature was less than 0.06% (pinocembrin – 0.06%, pinostrobin – 0.03%).

Table 3.2: Electrospray ionisation mass spectral analysis of flavanones and other non-volatile compounds identified from *E. Subg. Eucalyptus* foliar gland extracts.

RT (min)	Parent ions	Product ions	Formula	Compound	Species # *
2.60	397.15 [M+H] ⁺		C ₁₉ H ₂₄ O ₉	6-C Chromone glucoside	22
4.31	397.15 [M+H] ⁺		C ₁₉ H ₂₄ O ₉	6-C Chromone glucoside (isomer of the peak at 2.6 min)	22
6.41	419.13 [M+H] ⁺ 417.13 [M-H] ⁻	257.08 255.07	C ₂₁ H ₂₂ O ₉	Pinocembrin-7-O-glucoside	19
8.61	433.1007 [M+H] ⁺	271.0941	C ₂₂ H ₂₄ O ₉	5-methoxy-pinocembrin-7-O-glucoside	19
8.76	271.0937 [M+H] ⁺ 269.0828 [M-H] ⁻		C ₁₆ H ₁₄ O ₄	Alpinetin	19-27
10.78	283.0994 [M+H] ⁺		C ₁₇ H ₁₄ O ₄	Dimethylchrysin	20, 25-27
11.79	285.1156 [M+H] ⁺	181.0504	C ₁₇ H ₁₆ O ₄	Dimethylpinocembrin	19-23, 25-27
11.9	255.0598 [M-H] ⁻	213.0477 187.0761	C ₁₅ H ₁₂ O ₄	Pinocembrin	19-27
14.33	271.0986 [M+H] ⁺ 269.0532 [M-H] ⁻	167.0343	C ₁₆ H ₁₄ O ₄	Pinostrobin	19–21, 23-27
16.84	431.2042 [M-H] ⁻		C ₂₄ H ₃₂ O ₇	β-Triketone heterodimer	22
16.91	491.2118 [M-H] ⁻	309.1142 181.0868 255.0665	C ₂₉ H ₃₂ O ₇	Flavanone triketone conjugate	21
17.64	445.22 [M-H] ⁻	277.1392 167.0649 235.1280 209.0757	C ₂₅ H ₃₄ O ₇	β-Triketone heterodimer	22
17.99	505.2248 [M-H] ⁻	323.1292 181.0864 269.0819	C ₃₀ H ₃₄ O ₇	Flavanone triketone conjugate	21
18.50	459.24 [M-H] ⁻	291.1526 167.0634 235.1256 223.0900	C ₂₆ H ₃₆ O ₇	β-Triketone heterodimer	22
21.32	473.25 [M-H] ⁻		C ₂₇ H ₃₈ O ₇	β-Triketone heterodimer	22

* The species numbers are according to the numbers allocated in **Table 2.1** in Chapter 2.

3.3.7. Volatile components in oil glands of subgenus *Eucalyptus* species

The volatile oil components in the glandular extracts of *E. subg. Eucalyptus* were quantified on a per gland basis (**Figure 3.3**). Monoterpenes and sesquiterpenes were present as the major volatile compound groups in oil. In general, the monoterpene content was higher than that of sesquiterpenes and flavanones (**Table 3.1, Table 3.3**). The highest concentration of monoterpenes was found in *E. falciformis* ($1.27 \mu\text{g gland}^{-1}$) of which the majority is 1,8-cineole (80%). The most abundant monoterpene components in glands of the most species were *p*-cymene, α -pinene and 1,8-cineole. *E. stellulata* had the highest percentage of *p*-cymene (61%) in monoterpene class. The highest percentage of α -pinene was detected in *E. agglomerata* (79%) while the highest percentage of 1,8-cineole was found in *E. falciformis* (83%). *E. suberea* and *E. brevistylis* had the lowest concentrations of monoterpenes (0.006 and $0.01 \mu\text{g gland}^{-1}$, respectively). In both species sesquiterpene concentration was higher than monoterpenes. *E. gregsoniana* also had more sesquiterpenes than monoterpenes, while it was the species that had the highest amount of sesquiterpenes among all species ($0.14 \mu\text{g gland}^{-1}$). The most abundant sesquiterpene in five of the species was β -eudesmol. In *E. gregsoniana*, which had the most sesquiterpenes, 50% of this class of compounds was β -eudesmol (**Table 3.3**).

The glandular hexane extracts of *E. brevistylis* and *E. suberea* had β -triketones, which come from a rare group of compounds found mainly in myrtaceous plants. *E. brevistylis* had the β -triketone conglomerone (100% of β -triketone) in high abundance ($0.02 \mu\text{g gland}^{-1}$). In *E. suberea*, the β -triketones conglomerone (60%), aglomerone (30%) and isobaeckol ME (5%) were present (**Table 3.3**). These triketone compounds were not detected in any of the other subg. *Eucalyptus* species studied.

Table 3.3: Monoterpene and sesquiterpene oil components identified from the glandular extracts of *Eucalyptus* subg. *Eucalyptus* species. All concentrations are given in µg per gland.

Species	Total oil per gland	Abundant monoterpenes		Abundant sesquiterpenes	
		compound	Total	compound	Total
<i>E. oreades</i>	1.09	<i>p</i> -cymene (41%), trans- <i>p</i> -menth-2-en-1-ol (22%) trans-piperitol (16%)	1.01	β-eudesmol (40%), C ₁₅ H ₂₆ O (20%) spathulenol (6%)	0.07
<i>E. nitida</i>	1.05	<i>p</i> -cymene (44%), 1,8-cineole (20%), piperitone (17%)	0.99	β-eudesmol (19%) spathulenol (18%), C ₁₅ H ₂₆ O (18%)	0.07
<i>E. apiculata</i>	0.85	1,8-cineole (45%), cis-piperitol (14%), trans- <i>p</i> -menth-2-en-1-ol (10%)	0.83	caryophyllene oxide (30%) aromadendrene (17%), ledene (10%)	0.02
<i>E. stellulata</i>	0.22	<i>p</i> -cymene (61%) 1,8-cineole (21%) d-terpinene (5%)	0.19	spathulenol (23%) viridiflorol (13%) α-caryophyllene (10%)	0.03
<i>E. dendromorpha</i>	0.74	<i>p</i> -cymene (36%) piperitone (35%), trans- <i>p</i> -menth-2-en-1-ol (11%)	0.72	trans-caryophyllene (25%), C ₁₅ H ₂₄ (16%), calarene (13%)	0.03
<i>E. approximans</i>	1.07	piperitone (70%) limonene (18%) trans- <i>p</i> -menth-2-en-1-ol, (4%)	1.05	α-gurjunene (23%), aromadendrene (22%), C ₁₅ H ₂₆ O (17%)	0.02
<i>E. falciformis</i>	1.30	1,8-cineole (83%) limonene (6%) α-pinene (4%)	1.27	β-eudesmol (24%) calarene (15%) d-cadinene (7%)	0.03
<i>E. agglomerata</i>	0.28	α-pinene (79%) 1,8-cineole (11%) trans- <i>p</i> -menth-2-en-1-ol (3%)	0.24	α-caryophyllene (15%) caryophyllene oxide (11%) spathulenol (10%)	0.04
<i>E. gregsoniana</i>	0.24	α-pinene (59%) 1,8-cineole (35%) limonene (3%)	0.10	β-eudesmol C ₁₅ H ₂₆ O eudesmol	0.14
<i>E. brevistylis</i> *	0.11 ^a	<i>p</i> -cymene (41%), trans- <i>p</i> -menth-2-en-1-ol (22%), trans-piperitol (16%)	0.01	β-eudesmol (40%), C ₁₅ H ₂₆ O (20%) spathulenol (6%)	0.08
<i>E. suberea</i> *	0.07 ^b	trans-piperitone (trace), cryptone (trace)	<0.01	bicyclogermacrene (48%), C ₁₅ H ₂₄ (28%)	0.01

* *E. brevistylis* and *E. suberea* had triketones as an additional group. a. including 0.02 µg gland⁻¹ triketones. b. including 0.06 µg gland⁻¹ triketones. All other species had monoterpenes and sesquiterpenes as the only volatile compound classes found in glands.

3.3.8. Flavanoid and terpene relationships

The concentrations of compounds in the section *Eucalyptus* species were statistically tested (Pearson's correlation) for any correlations between the flavonoid and terpene components (**Figure 3.4**; The taxonomic classification of species is given in **Table 2.1**). From the monoterpene class, *p*-cymene, trans-para menth-2-en-1-ol and trans-piperitol showed strong positive correlations with total flavanones. *P*-cymene, which was the most abundant monoterpene found in five of the species studied, was positively correlated with the total flavanone concentrations ($r = 0.75$, $P = 0.02$; **Figure 3.4**, **Table 3.3**). Also, the total flavanones had a strong positive correlation with the terpene alcohols trans-para menth-2-en-1-ol ($r = 0.878$, $P = 0.002$) and trans-piperitol ($r = 0.861$, $P = 0.003$). However, they did not show a relationship with any of the individual flavanones. From among the flavanones, pinostrobin was strongly positively correlated with *p*-cymene ($r = 0.8$, $P = 0.009$). The monoterpenes α -phellandrene ($r = 0.969$, $P < 0.001$) and linalool ($r = 0.984$, $P < 0.001$) also showed strong correlations with pinostrobin. None of the two monoterpenes showed a relationship with total flavanones. The other three flavanones, pinocembrin, dimethylpinocembrin and alpinetin, individually did not show a significant correlation with either *p*-cymene, α -phellandrene or linalool. Also, no significant relationship was observed between the flavanones and the two other most abundant monoterpenes, 1,8-cineole and α -pinene. From the sesquiterpene class, spathulenol was positively correlated with pinostrobin ($r = 0.876$, $P = 0.002$) and caryophyllene oxide was positively correlated with alpinetin ($r = 0.757$, $P = 0.018$; **Figure 3.4**). None of the other identified mono- or sesquiterpenes showed a relationship with the flavanoid components. Many of the compounds that showed a positive correlation with flavanoids were among the most abundant terpene components identified from the glands (**Table 3.3**).

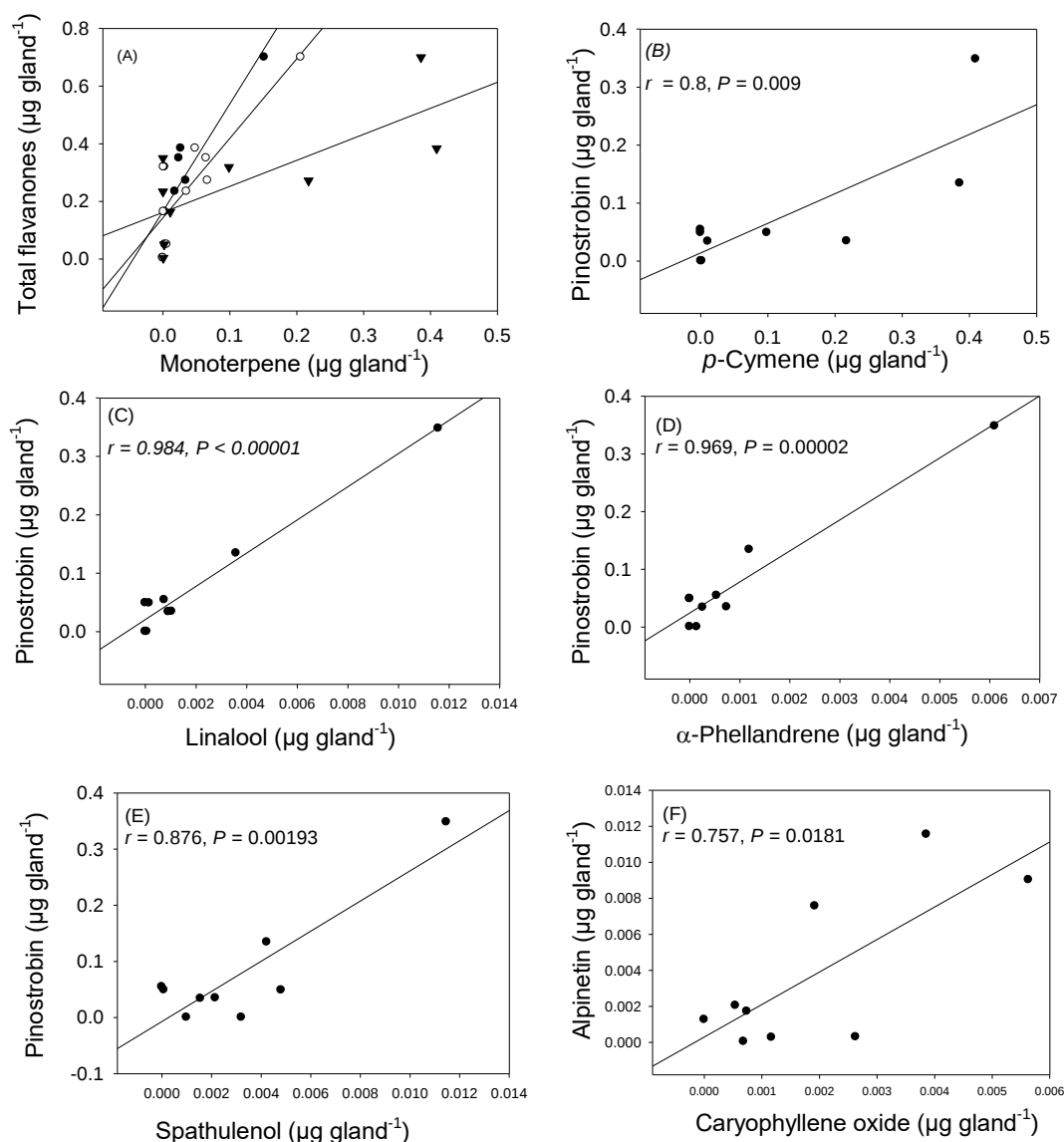


Figure 3.4: Relationships between terpene and flavanone concentrations in foliar oil glands of the section *Eucalyptus* species. (A) Total flavanones plotted against the monoterpenes p -cymene, trans-piperitol and trans-para menth-2-en-1-ol. The black circle symbols represent trans-piperitol ($r = 0.861$, $P = 0.003$), the white circle symbols represent trans-para menth-2-en-1-ol ($r = 0.861$, $P = 0.00285$) and black triangle symbols represent p -cymene ($r = 0.75$, $P = 0.02$). (B), (C), (D) Pinostrobin concentration variation with the monoterpenes p -cymene, linalool and α -phellandrene. (E) Variation in pinostrobin compared with the sesquiterpene spathulenol. (F) the variation of alpinetin with the sesquiterpene caryophyllene oxide. The Pearson's correlation coefficient (r) and the associated significance level (P) are listed on each figure. All concentrations are given in μg per gland. Each point represents an individual species.

3.4. Discussion

The primary aim of work described in this chapter was to characterise the flavanones and related compounds localised to foliar glands of *E. subg. Eucalyptus* species. The volatile components were also studied to understand any relationships with non-volatiles. Structurally related flavonoids and volatile terpenes and β -triketones from some species were identified as the major compound classes present. All compounds were extracted from isolated glands, therefore, the compounds identified are either constituents of gland lumina and/or the gland wall cells including the secretory epithelium.

3.4.1. Flavonones localised to oil glands

This study shows that the unsubstituted B-ring flavanones pinocembrin, dimethoxylated flavanone dimethylpinocembrin, the structurally similar monomethoxylated flavanones pinostrobin and alpinetin, and the related dimethoxylated flavone dimethylchrysin are likely localised to the oil glands in *Eucalyptus*. This is the first evidence for the localization of the latter three compounds to the foliar oil glands in any *Eucalyptus* species. Moreover, the above flavonones dominated the glandular extracts of all species from *E. section Eucalyptus*. Pinocembrin has earlier been detected in glandular extracts of *E. gregsoniana* and three other *Eucalyptus* species: *E. muelleriana*, *E. pauciflora* and *E. olsenii*, all from subgenus *Eucalyptus* (Heskes *et al.* 2012b). There is no evidence for flavone dimethylchrysin or other related structures from glands of *Eucalyptus* or glands of any other species. However, different flavones have been identified from the glandular trichomes (GTs) of species from other plant families including alfalfa (Fabaceae; Aziz *et al.* 2005), *Lychnophora* (Asteraceae; GobboNeto *et al.* 2008), oregano (Lamiaceae; Bosabalidis *et al.* 1998) and mint (Lamiaceae; Voirin *et al.* 1993).

Two C-methyl flavanones, cryptostrobin and demethoxymatteucinol, were identified from *Eucalyptus* species. A very recent study reports cryptostrobin together with strobopinin and desmethoxymatteucinol from leaves of white mahogany and stringybark eucalypt species, which are from the sections *Amentum* and *Capillulus* of the monocalypt subgenus (Marsh *et al.* 2019). Cryptostrobin and demethoxymatteucinol have previously been found together in other plant species and to be present also with putative flavanones. Cryptostrobin have been found existing with its isomer strobopinin

in *Pinus* species (Lindstedt and Misiorni 1951). Although the two isomers are generally known from trees from family Pinaceae (Lamberton 1964) the compounds seem to be present in Myrtaceae as well. Strobopinin and desmethoxymatteucinol ((2S)-5,7-dihydroxy-6,8-dimethylflavanone) have been isolated from *Heteropyxis canescens* (Myrtaceae) leaves (Mohammed *et al.* 2009) while strobopinin was isolated from fruit resins of *Corymbia torelliana* (Myrtaceae; Massaro *et al.* 2014). Cryptostrobin and demethoxymatteucinol, together with unsubstituted B-ring flavanones pinostrobin and pinocembrin and other structurally related compounds including strobopinin and dimethylstrobopinin (5,7-dimethoxy-6-methylflavanone), have been identified from leaf extracts of *Campomanesia adamantium* (Myrtaceae; Coutinho *et al.* 2008). Strobopinin is known to occur together with dimethylpinocembrin in leaves and stems of *Leptospermum scoparium* (Myrtaceae; Mayer 1990), while Killeen *et al.* (2015) reported some unidentified C-methyl flavanones from the embedded foliar glands of *L. scoparium* and *L. morrisonii*.

Many other structurally related flavonoids were also identified from eucalypt glandular extracts. The two flavanone glucosides identified from *E. stellulata* have been detected together previously from *Penthorum chinense* (Penthoraceae; Guo *et al.* 2015). The 6-C chromone glucoside isobiflorin, which was detected from the glands of many species, has been identified earlier from leaf extracts of *E. globulus* (Al-Sayed *et al.* 2014), *E. cypellocarpa* (Ito *et al.* 2000), and *Baeckea frutescens* L. (Myrtaceae; Kamiya and Satake 2010).

The flavanones identified in this thesis differ from each other by the degree of methylation of A-ring hydroxyl and carbons. Methylated flavonoids are important as pharmaceuticals as they can be more effective than non-methylated forms due to properties such as improved intestinal absorption and metabolic stability (Wen and Walle 2006). Structurally related unsubstituted B-ring flavanones with different degrees of methoxylation are known to co-occur in some plant species. For example, the non-methylated form pinocembrin has been isolated with mono-methoxylated alpinetin from *Alpinia katsumadai* (Li *et al.* 2012). Similarly, pinocembrin, alpinetin and dimethylpinocembrin have been isolated together from *E. sieberi* leaves (Bick *et al.* 1972). Furthermore, C-methyl flavanones were also identified together with other flavanones as described above. Dimethylpinocembrin and the flavone dimethylchrysin have also been detected in leaf extracts of *Leptospermum scoparium* (Häberlein and

Tschiersch 1998), the liverwort *Tylimanthus renifolius* (Feld *et al.* 2003) and rhizome extracts of *Boesenbergia pandurata* (Zingiberaceae; Jaipetch *et al.* 1983).

The results presented here suggest that species-specific quantitative variations occur in flavonoids in *Eucalyptus* oil glands. The total flavonoids in glands varied from $<0.01 \mu\text{g gland}^{-1}$ in *E. gregsoniana* to $0.7 \mu\text{g gland}^{-1}$ in *E. oreades*. The values in the two species per leaf dry weight are 0.18 and 12.87 mg g^{-1} respectively. The concentrations of individual compounds reported in this thesis are comparable to the sources found in literature. For example, pinocembrin concentration ranged from 0.08 to 2.0 mg g^{-1} in species. Pinocembrin contents reported in *E. pauciflora*, *E. fraxinoides* and *E. sieberi* were 0.3, 2.4 and 3.3 mg g^{-1} , respectively (Saraf *et al.* 2015). The concentrations based on leaf dry weight are important for selecting better species for commercial cultivation purposes as pinocembrin and other compounds reported here have a high economical value. For example, pinostrobin has high importance as a medicinal drug due to many pharmacological activities including the anti-microbial, anti-malarial, anti-alzheimer and anti-cancer activities (Patel *et al.* 2016). The present study shows that *E. nitida* has a significantly high pinostrobin concentration (11.53 mg g^{-1}) compared to the other species. However, it is likely that flavanones also show intra-specific variations as seen for monoterpenes and related compounds (Goodger and Woodrow 2012; Heskes *et al.* 2012b). In this thesis, leaves from a single randomly selected tree of each species were studied. Therefore, there is a chance that the amounts of the compounds can vary in other trees of the same species with higher or lower concentrations. Such chemotypic variations between individuals within species have been observed in *Eucalyptus* species with regard to unsubstituted B-ring flavanones (Marsh *et al.* 2019). This suggests that for commercial applications the quantitative studies should be extended to selecting high yielding chemotypes of species.

3.4.2. Sequestration and exclusive localisation of flavanones to oil glands

The most likely reason for the sequestration of flavanones into the oil glands would be to avoid cell damage from autotoxicity. Since the compounds have shown biological activities related to plant defence, the role of these flavonoids in eucalypts is also likely, at least in part, to be related to defence against herbivores and pathogens. For example, pinocembrin has proven antibacterial, antifungal and antifeedant activities (Napal *et al.* 2009; Shain and Miller 1982). Another study showed that the antibacterial activity of Australian stingless bee propolis extracts is due to the C-methylated

flavanones including cryptostrobin and unsubstituted B ring flavanones (Massaro *et al.* 2014).

The present study also shows for the first time that flavanones, particularly with the unsubstituted B-rings, are exclusively localised to the glands in the leaves of at least one *Eucalyptus* species – and there is no reason to assume that this is not indicative of the genus more widely. More than 99% of pinocembrin and pinostrobin, the two major flavanones found in *E. nitida*, were localised in glands. Cuticle/epidermis and vasculature together had only less than 0.7% of total flavanones and no evidence of flavanones were found from mesophyll cells. Similar findings have been observed for MAGEs in *Eucalyptus* oil glands and phenylpropenes in basil (*Ocimum basilicum*) trichomes. MAGEs were shown to be exclusively localised to the oil glands in *E. polybractea* leaves (Goodger and Woodrow 2011). Similarly in basil, the composition of oil in peltate glands and isolated peltate trichomes was similar to the composition of oil extracted from intact leaves (Gang *et al.* 2001). Flavanones have earlier been detected in leaf wax of *Eucalyptus* leaves (Lamberton 1964). In the present study only 0.6% of pinocembrin and 0.41% of pinostrobin were detected in leaf cuticle/epidermis. Although the maximum effort was taken to clean each tissue type, the minute concentrations found in veins could be due to possible contaminations in the dissection process.

The exclusive localisation of most if not all of the foliar flavanone complement to an enclosed extracellular compartment – the oil gland lumen – may be to protect the cells from autotoxicity. In contrast, in some other plant families epidermal and mesophyll cells have been identified as the primary sites of flavonoid accumulation – e.g. *O*-methylated flavonol glucosides in *C. americanum* (Saxifragaceae; Charest *et al.* 1986). However, this may depend on the structural nature and biological activities of the compounds.

3.4.3. Non-terpene volatile components in oil glands

In addition to mono- and sesquiterpene components, β -triketones were found in the volatile fraction of foliar oil glands in *E. suberea* and *E. brevistylis*. *E. suberea* had the β -triketones conglomerone (60%), agglomerone (30%) and isobaeeckol methylether (5%) in glandular volatile extracts. In *E. brevistylis*, the β -triketone fraction was purely conglomerone. *E. suberea* and *E. brevistylis* were the only two species from which free β -triketones were identified out of the all 30 species studied in this thesis. It is known that the monoterpene and sesquiterpene components of oil are localised to oil glands in

Eucalyptus (King *et al.* 2006). The present study is the first evidence for the localisation of β -triketones to the oil glands of any *Eucalyptus* species. Though triketones have previously been identified from some *Eucalyptus* leaf extracts their tissue level localisation was not known. The only evidence for triketones in oil glands involves grandiflorone from two myrtaceous species, *Leptospermum scoparium* and *L. morrisonii* (Killeen *et al.* 2015). However, there is no evidence for β -triketones in oil glands of *Eucalyptus* or any species other than *Leptospermum*.

The two species that contained triketones, *E. suberea* and *E. brevistylis*, did not have any flavonones and had the lowest identified concentrations of monoterpenes in their oil glands (<0.01 and $0.01 \mu\text{g gland}^{-1}$, respectively). In contrast, sesquiterpenes were more abundant compared to monoterpenes in these two species (0.01 and $0.08 \mu\text{g gland}^{-1}$, respectively). A similar profile has been observed in *Leptospermum morrisonii* (Myrtaceae) in which the triketone grandiflorone accounted for the majority (25-64%) of oil while monoterpenes were scarce with only camphene (0.1–2%) and (E)- β -ocimene ($<0.2\%$) being detected; however, as in the eucalypts, sesquiterpenes were more abundant than the monoterpenes (Brophy *et al.* 2000). This suggests that there could be a relationship between the terpene and triketone components present in oil glands both in quantitative and qualitative aspects. The lack of knowledge on how β -triketones are localised in oil glands with the other oil components forms the basis for Chapter 4.

3.4.4. Flavanoid-terpene relationship

Several mono- and sesquiterpenes had medium to strong positive correlations with the concentrations of either a single flavanone component or total flavanones. This correlation could be simply due to the gland size where larger glands may contain more of all PSMs. Nonetheless, the absence of such correlations between the other components raises the question why a relationship exists between only some of the terpenes and flavanones. Particular terpenoids seemed to have a stronger relationship with pinostrobin than the other flavanoids. For example, the monoterpenes *p*-cymene, linalool and α -phellandrene, and the sesquiterpene spathulenol had positive correlations with the flavanone pinostrobin (**Figure 3.4**). Some evidence from the literature supports the finding of a correlation between pinostrobin and monoterpenes. For example, the monoterpene *p*-cymene has been identified as a major monoterpene constituent in pinostrobin-containing species in Amaranthaceae family (Álvarez-Ospina *et al.* 2013). When the structural arrangements of these terpenes are considered, it can be speculated

that aromatic terpenes *p*-cymene and α -phellandrene could possibly be degradation products of the flavanone pinostrobin (**Figure 3.5**). Cleavage of the B-ring is common in 4-hydroxylated flavanones, however, no such evidence is found from flavanones with an unsubstituted B-ring like pinostrobin (Nikolic and van Breemen 2004).

The mevalonic acid (MVA) pathway and methylerythritol phosphate (MEP) pathway that synthesise terpenoids and the shikimate pathway that synthesise phenolics in plants are well characterised. The possible link that may exist between the biosynthesis of these two compound groups seems to be at gene expression level. Studies on basil GTs suggest that the MEP pathway and shikimate pathway are tightly co-regulated at transcriptional level (Xie *et al.* 2008). A good correlation exists among mRNA, protein, enzyme activity and terpenoid and phenolic metabolite levels in different chemotypic lines with terpene and phenolic profiles. The two pathways compete for carbon and carbon flow into these pathways is well regulated. The control of gene expression plays a crucial role in the control of the direction of carbon flow between the two metabolic pathways (Xie *et al.* 2008). In addition, post-translational modifications of proteins are also involved in determining the metabolite profile. For example, the ubiquitination leading to rapid degradation of enzyme and phosphorylation can lower enzyme activity and result in reduced synthesis of corresponding metabolites (Xie *et al.* 2008).

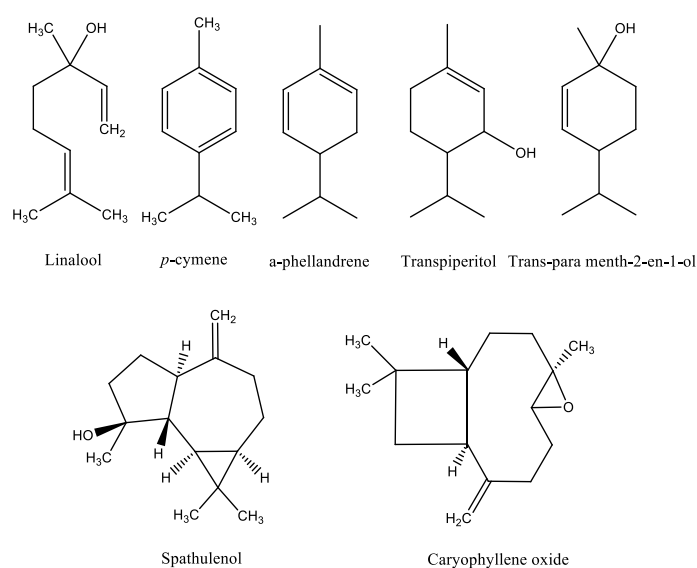


Figure 3.5: Structures of the monoterpenes and sesquiterpenes that show a positive correlation with flavanoids.

3.4.5. Phytochemical ecology and chemotaxonomy

In the present study, characteristic chemotaxonomical patterns were observed in *E. subg. Eucalyptus* species. While unsubstituted B-ring flavanones were present in all *E. section Eucalyptus* species examined, none of those flavonoids were found in the two species from *E. sections* other than *Eucalyptus* (sections *Frutices* and *Longistylus*). Unsubstituted B-ring flavanones have earlier shown activities related to the interactions between plants with their environment. For example, pinocembrin has shown antifeedant activity against insect pest larvae in *Flourensia oolepis* (Asteraceae; Napal *et al.* 2009). Recently, Marsh *et al.* (2015) showed that pinocembrin and simple flavanone were effective in suppressing feeding by common brushtail possums on palatable artificial diets, but flavones with unsubstituted B rings and flavanones with substitutions in the B ring were not. These findings suggest that glandular flavanones, particularly unsubstituted B-ring flavanones, may play an ecological role in plants. In the present study, both *E. brevistylis* and *E. suberea* did not contain any flavonoids in their glands. Both species are rare and have a severely restricted distribution, and grazing has been identified as one of the main potential threats to seedling recruitment (DOE 2014; DOPW 2017). In addition, a unique geographical distribution pattern has also been observed in *E. subgenus Eucalyptus* species. Though eucalypts are found throughout Australia as a dominant genus, subgenus *Eucalyptus* species are distributed only in western and eastern Australia and southern coastal and upland regions. They are not found in northern Australia and the arid zone (Ladiges *et al.* 2010). However, it is unclear whether these distributional variations have any relationship with the chemistry of eucalypts. Therefore, these aspects of chemotaxonomical traits need to be studied further to better understand the ecological role of flavonoids.

3.5. Conclusion

This study shows that the foliar glands of *Eucalyptus* subgenus *Eucalyptus* contain numerous flavanones and other non-volatile and volatile compounds. Flavanones were the dominant group of NVCs found in the glands. This is the first report of flavanone-*O*-glucosides and flavanone- β -triketone conjugates from glands of some *Eucalyptus* species. β -triketone heterodimers and chromone *C*-glucosides were also found in glandular extracts. Further, flavanones were exclusively localised to glands

rather than being distributed in the other leaf tissues. Monoterpenes and sesquiterpenes were found as common volatile constituents in glands. In addition, β -triketones were found in glandular extracts of two species belonging to the subgenus *Eucalyptus* that did not have any of the flavanones or related compounds. In conclusion, foliar oil glands of *E. subg. Eucalyptus* are rich in flavonones and other structurally related compounds and indicate different metabolic pathways of compound biosynthesis than other *Eucalyptus* subgenera.

Chapter 4

Different types of foliar oil glands synthesise β -triketones and sesquiterpenes in *Eucalyptus brevistylis*

4.1. Introduction

The genus *Eucalyptus* is well known for the volatile essential oils housed in their foliar oil glands. The oil profile varies among species within the genus, both in constituents and their abundances. The most abundant oil components identified from *Eucalyptus* species are the volatile terpenoids, particularly monoterpenes and sesquiterpenes, which have been proven to be located in glands (King *et al.* 2006). In addition to the commonly found terpenoids, β -triketones have also been identified from the volatile oils of some *Eucalyptus* species (Brophy and Southwell 2002; Hellyer 1968).

Triketones are a relatively rare group of compounds in nature and are found mainly in the family Myrtaceae (Hellyer 1968; van Klink *et al.* 1999). In particular, the triketones leptospermone, flavesone and agglomerone have been widely identified from the genus *Eucalyptus* (Hellyer 1968). In some eucalypts, β -triketones are found in very high proportions in their oil; for instance, steam-distilled oil from *E. agglomerata* and *E. mckieana* contains between 50% and 80% agglomerone (Hellyer 1964). In addition to *Eucalyptus*, triketones have also been identified from a range of other myrtaceous genera such as *Backhousia*, *Baeckea*, *Calytrix*, *Corymbia*, *Darwinia*, *Leptospermum*, *Melaleuca*, and *Xanthostemon*.

Despite triketones being found with the oil products of steam distillation, it has not been definitively shown that both are localised to the glands of intact plants. All β -triketones identified to date from eucalypts have been extracted with solvents or steam distillation of bulk samples of leaves or reproductive tissues. The findings of Chapter 2 of this thesis, using extracts of isolated glands, reveal that some *E. subg. Eucalyptus* species do contain β -triketones in their foliar glands. Similarly, in a previous study on

two *Leptospermum* species (Myrtaceae), β -triketones were localised to the foliar glands using Raman microscopy (Killeen *et al.* 2015). These results do not preclude the occurrence of β -triketones in leaf tissues other than glands such as epidermal cells, vasculature or, mesophyll layers. If β -triketones are consistently found in glands, then they may be simply mixed in with the terpenoid oil or they may be produced and stored in a separate type of specialised triketone gland. Such a differentiation of metabolic pathways has been observed for other compound groups in structurally different capitate and peltate glandular trichomes. For example, in *Lavandula pinnata* (Lamiaceae) capitate glandular trichomes contain both lipophilic and polysaccharidic substances, while peltate glandular trichomes only secrete lipophilic substances (Huang *et al.* 2008). Similarly in tomato, one trichome type produces acyl sugars and another type produces terpenoids (Balcke *et al.* 2017).

The aim of research described in this chapter is to explore how β -triketones are localised in glands in *Eucalyptus* species. In Chapter 2, both *E. brevistylis* and *E. suberea* were found to have β -triketones in their foliar glands. However, the glands of *E. suberea* were smaller in size and largely composed of β -triketones with only trace amounts of sesquiterpenes. In contrast, *E. brevistylis* had considerable amounts of both β -triketones and sesquiterpenes in glandular extracts and a larger gland size making the species more amenable to using the gland isolation technique to explore the components of individual glands. Therefore, *E. brevistylis* was selected as the species that best suits this study.

4.2. Materials and Methods

4.2.1. Plant material

Eucalyptus brevistylis Brooker (Rate's tingle; Family Myrtaceae) is classified as a Rare and Near Threatened tree species (DOPW 2015; DOPW 2017) and is endemic to the state of Western Australia (WA). Fully expanded leaves of *E. brevistylis* were harvested from 10 adult trees growing at two locations: seven trees growing along Boronia Road in Frankland State Forest, WA (34°48'48"S, 116°53'14"E) in October 2015, and three trees from the Currency Creek Arboretum in Adelaide, South Australia (SA) (35°25'45"S, 138°45'46"E) in March 2016. Leaves from the Frankland State Forest were collected under the permit numbers CE004912 and SW017061, procured

from the Department of Parks and Wildlife, WA. Pole pruners were used to harvest from trees at a height of 4-6 m from the state forest trees and 2-3 m from the arboretum trees. The harvested leaves were placed immediately in snap-lock bags and returned to the laboratory on dry ice. The leaf samples were stored at -80°C until analysed.

Seeds from two individual *E. brevistylis* trees were purchased from East Manjimup Nursery and Seed Centre, Forest Products Commission, Manjimup, WA in October 2015. The seeds had been collected from two trees in the population growing along Boronia Road, Frankland State Forest (34°48'46"S, 116°53'28"E). The collected seeds were sown as described in section 4.2.8 for seedling ontogenetic studies.

4.2.2. Isolation of glands and extraction of volatile components

Foliar oil glands were isolated from fully expanded mature leaves of *E. brevistylis* following the gland isolation protocol described in Chapter 2 (2.2.1-ii). Two types of glands of contrasting colours were visually distinguishable in the same leaf when viewed under the light microscope, appearing either bright golden-brown or translucent white. After the digestion of leaf tissues, the two types of glands were separated based on their colour difference and 200 glands of each type were collected using a pipette under a stereomicroscope (BH-2, Olympus, Centre Valley, PA, USA).

Isolated glands of each colour type were immediately ground separately using a vial micro-pestle and extracted in 1 mL of hexane containing 100 mg L⁻¹ tridecane as an internal standard. The glands in hexane were incubated for 3 days at 50°C on an orbital shaker (Ratek Instruments Pty Ltd, Boronia, Australia) at 100 rpm. After incubation, the hexane extracts were dehydrated with an aliquot of anhydrous Na₂SO₄, vortexed and centrifuged for 1 min at 6,300 *g*. The supernatant was then transferred into 2 mL glass vials for chromatographic analyses.

4.2.3. Analysis of volatile extracts by gas chromatography and mass spectrometry

Hexane extracts of glands were analysed by gas chromatography-flame ionisation detection (GC-FID) followed by mass spectrometry (GC-MS) as described in Chapter 2 (2.2.1-v). Mass spectra were evaluated using Agilent MSD ChemStation E.02.02.1431 for GC-MS. The compounds were identified using the NIST 11 and Adams mass spectra libraries (Adams 2012). Sesquiterpenes in hexane extracts were quantified based on standard series of major sesquiterpenes as described in Goodger *et*

al. (2007). The quantification of conglomerone, the major β -triketone found in *E. brevistylis* glands, was based on a standard series of the sesquiterpene caryophyllene oxide (Sigma-Aldrich), due to the unavailability of a commercial β -triketone standard.

4.2.4. Imaging of two types of glands

The isolated glands were imaged using fluorescence light microscopy and scanning electron microscopy (SEM) to further explore the morphology of the two colour types. Glands isolated from a mature leaf were first separated according to their colour under a stereo-light microscope. For fluorescence microscopy, representatives of each type of gland were imaged under UV illumination using a green fluorescent protein (GFP) filter. For SEM, glands were fixed in 2.5% glutaraldehyde overnight at room temperature. The glands were rinsed three times in distilled water, before being dehydrated in increasing concentrations of ethanol consisting of 10, 30, 50, 70 and 90% ethanol in distilled water for 30 min steps. The glands were then incubated in three changes of 100% ethanol for 1 h each step. The glands were critically point dried in a Balzers CPD030, carefully picked up and mounted onto SEM mounts with double-sided carbon tabs. The stubs were gold coated with a Dynavac Xenosput sputter coater and imaged in a Philips XL30 Field-Emission scanning electron microscope at 2.0 kV and a spot size of 2.

4.2.5. Spatial arrangement of glands in leaves

The arrangement of the two types of glands in the leaf was studied using images of six fully expanded leaves randomly selected from trees grown in both the Frankland State Forest (four leaves) and the arboretum (two leaves). Three leaf strips, each from the apex, middle and basal areas, perpendicular to the mid vein were dissected from each leaf using a scalpel. Images of each strip were taken using a camera attached to the dissecting microscope. Glands within 1 mm distance from the mid vein were considered as in the “vein region” while those within 1 mm of the margin were considered as in the “margin region”. All the other glands in between were considered as glands in the “mid region”. Images were analysed using Image-J software (version 1.50i, National Institutes of Health, USA) to count the number of glands in each region. Gland density (glands per cm^{-2}) was calculated for each selected leaf area. Glands on both the left and right sides of the leaf midrib were counted and the average was used as the final gland density.

4.2.6. *E. brevistylis* population survey

A series of *E. brevistylis* trees were surveyed to examine the variability of β -triketones and sesquiterpenes in a natural population and thereby to understand the distribution of two gland types in trees of this species. A total of 10 adult *E. brevistylis* trees, seven trees from Frankland State Forest, WA, and three trees from the Currency Creek Arboretum, SA, were surveyed for the volatile oil contents in their leaves. The state forest trees were estimated to be at least 50 years of age and arboretum trees were younger; they had been planted in 1995 and were thus aged about 20 years at the time of harvesting. A fully expanded leaf from each tree was ground to a fine powder with a pestle in a mortar containing liquid nitrogen and extracted following the leaf hexane extraction protocol (2.2.1-iv). The extracts were then analysed and quantified by GC-FID as described previously (2.2.1-v).

4.2.7. Gland types at different leaf ontogenetic stages

Young immature to fully expanded mature leaves from branches of four adult trees were studied to determine if the relative abundance of the two different gland types differed with leaf ontogeny. Two trees from the State Forest and two trees from the arboretum were randomly selected for this study. Five leaves along a single branch from each tree were harvested and extracted in hexane following the protocol described previously in Chapter 2 (2.2.1-iv). Prior to the extraction, each leaf was scanned using a flatbed scanner for area determination using Image-J. The hexane extracts were analysed by GC-FID as detailed in Chapter 2 (2.2.1-v) to quantify the β -triketones and sesquiterpenes in each leaf.

4.2.8. Gland types at different seedling ontogenetic stages

The abundance of the two types of glands across different stages of seedling development was studied using two open-pollinated, half-sibling families of *E. brevistylis*. Seedlings were grown from seed collected from two mother trees in the Frankland State forest. Seeds from each tree were sown in March 2016 in a tray containing a sterilized mixture of soil, vermiculite and sand (1:1:1, v/v/v). Individual seedlings were transferred to 300 mL forestry tubes one month after germination. Seedlings were then transferred into 1.65 L pots after 5 months and grown in a glass house at the University of Melbourne with mean day/night temperature of $23.2 \pm$

0.1°C/19.8 ± 0.1°C and a relative humidity of 55.5 ± 0.2%/63.4 ± 0.1%. Seedlings were fertilized every 3 months with Osmocote slow release fertilizer (Scotts Australia, Bella Vista, New South Wales, Australia) and watered twice daily.

The cotyledons and first formed fully expanded true leaves were harvested from eight seedlings from each family at intervals to test the glandular contents at different seedling stages in early ontogeny. Seedlings showed some individual growth variations and the time of the harvesting was dependent on the growth. From the first two seedlings of each family, two cotyledons per seedling were collected 38 and 44 days after sowing (DAS). From the third to eighth seedlings of each family, first formed true leaves were harvested 122, 149, 178, 254, 350 and 400 DAS (**Figure 4.1**). All samples were placed on ice and then stored at -80°C until analysed. Leaves were scanned using a flat-bed scanner just prior to grinding for extraction. Harvested cotyledons and leaves were ground to a fine powder in a mortar containing liquid nitrogen with a pestle and extracted in hexane for GC-FID as described in Chapter 2 (2.2.1-iv). The hexane volume used was 500 µL for cotyledons and smaller leaves that were harvested from the first four seedlings. For larger leaves, harvested from the rest of the seedlings, 3 mL of hexane was used. The extracts were incubated for five days at 50°C prior to transferring to 2 mL glass vials. The samples were then analysed and quantified using GC-FID as described previously in Chapter 2 (2.2.1-v). Leaf area was calculated from the scanned leaf images using Image-J software.

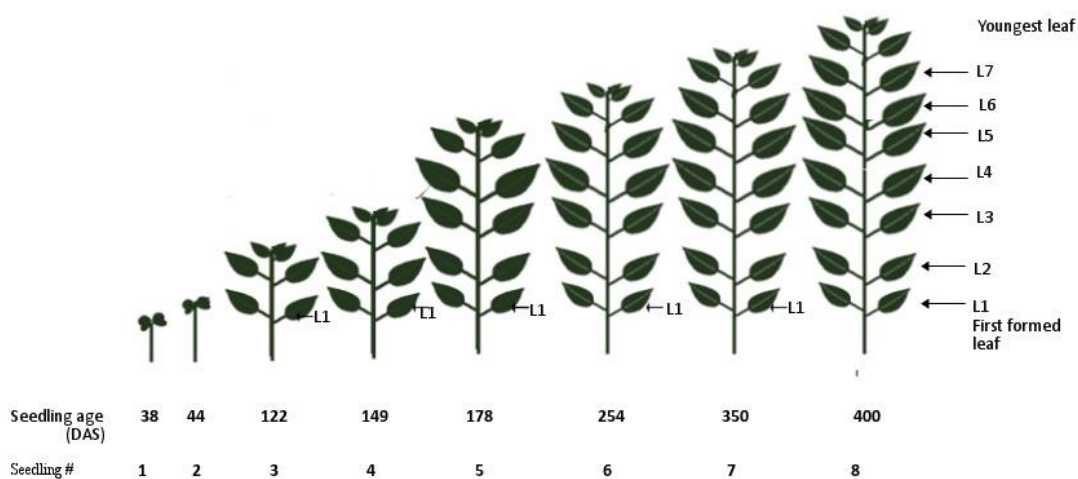


Figure 4.1. Sampling strategy of *E. brevistylis* seedlings. Leaf numbering was ordered from the first formed leaf (L1; basal leaf on seedling) to the last formed (apical position) in each seedling. Cotyledons were harvested from the first two seedlings and the fully expanded leaves were harvested from seedlings 3-8 of each family.

4.2.9. Gland lumen volume estimation

The average lumen volume of the ‘triketone glands’ and ‘sesquiterpene glands’ was estimated as described in Goodger *et al.* (2010). Stereomicroscopic images of isolated glands were used for volume calculations. The glands were assumed to have an ellipsoid shape and the equatorial (*a*) and polar (*b*) radii of 12 glands of each type were measured using the Image-J software. The lumen volume was calculated using the volumetric equation for an ellipsoid of $\frac{4}{3}\pi ab^2$. The data of the glands was compared with one-way ANOVA using Sigmaplot Version 13 (Systat Software, San Jose, CA, USA).

4.2.10. Exclusive localisation of β -triketones to foliar glands

Extracts of separated leaf tissues were analysed to test whether β -triketones were present in tissues other than glands. Six fully expanded mature leaves of *E. brevistylis* were randomly selected for this aspect of the study. All leaves were scanned prior to dissection using a flatbed scanner for leaf area determination. Each leaf was dissected into halves along the midrib and one half was digested using pectinase enzyme following the gland isolation protocol described in Chapter 2 (2.2.1-ii). After digestion, the different tissue types in the leaf (epidermis with attached cuticle, pooled vasculature tissues and glands) were separated and purified. Each tissue type was frozen in liquid nitrogen and ground with a micro-pestle in a tapered vial. Each ground component was extracted in hexane as described previously in Chapter 2 (2.2.1-iv). The other half of the leaf remained intact and was also ground to a fine powder in liquid nitrogen and extracted in hexane in the same manner. The left and right halves of leaves were used alternatively for intact and enzymatic separation of leaf components to avoid any possible errors due to gland localisation pattern in leaves. Each extract was analysed using GC-FID following the method given in Chapter 2 (2.2.1-v).

4.2.11. Determining if there are more than two types of glands in *E. brevistylis* leaves

Two gland types were analysed separately in batches to test whether there are more types of glands that cannot be distinguished using colour or other morphological traits. ‘Sesquiterpene glands’ and ‘triketone glands’ were isolated randomly in 10 batches of 10 glands of each type from a single leaf and tested for their volatile contents.

Firstly, ten sets of sesquiterpene-dominant glands were collected from an isolated pool of glands of *E. brevistylis* from a single leaf. Each set that consisted of ten glands were extracted in hexane following the above-mentioned protocol (4.2.2). The hexane volume used was 35 μ L. The extracts were analysed using GC-FID as described in Chapter 2 (2.2.1-v). A hexane extract from a single gland is the ideal way to test differences between glands, but the extract of a single gland did not produce a sufficient GC-FID signal. Therefore 10 glands were included in each extract to provide a reliable and reproducible signal. Given the triketone dominant glands also contained their own suite of sesquiterpenes, the same protocol was applied to them to determine if there were possibly two different types of glands within the golden-brown colour type.

4.2.12. Primary metabolites in the two types of glands

Glands were isolated from a single leaf from an adult tree and 50 glands from each type were randomly selected and derivatised to identify their constituent primary metabolites. Samples were analysed by Metabolomics Australia at the University of Melbourne. For derivatisation, 100 μ L of 1:3:1, CHCl_3 :MeOH:Milli-Q Water (mono-phasic) containing 1% internal standard from a stock of 0.5 mg mL⁻¹ were added to the isolated glands. The glands were homogenised using a cryo mill (Bertin Technologies) at -10°C. The mixture was shaken 15 min at 37°C, 850 rpm and centrifuged for 15 min at 25,200 *g*. The supernatant was collected and sonicated for 5 min, before being centrifuged for 15 min at 25,200 *g* at room temperature. The supernatant was transferred into a fresh 1.5 mL Eppendorf tube and 120 μ L of 1:3:3 CHCl_3 :MeOH:Milli-Q Water (bi-phasic) was added. The mixture was then vortexed for 30 sec and centrifuged for 15 min at 11,200 *g*. The top aqueous phase was collected and dried in a speed vacuum.

Dried samples were prepared for derivatisation by adding 10 μ L of methoxy amine hydrochloride (30 mg/ml in pyridine) followed by shaking at 37°C for 2 h. Each sample was then derivatised with 20 μ L of *N,O*-bis (trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA with 1% TMCS, Thermo Scientific) for 30 min at 37°C. The sample was then left for 1 h before 1 μ L was injected onto the GC-MS using a hot needle technique.

GC-MS was performed on a 30 m VF-5MS column with 0.25 μ m film thickness and 0.25 mm internal diameter with a 10 m Integra guard column (Agilent). The analysis of derivatised samples was performed under the following temperature program: start at injection 70°C, a hold for 1 minute, followed by a 7°C min⁻¹ oven temperature ramp to

325°C and a final 6-minute hold at 325°C. Mass spectra were recorded at 2.66 scans s⁻¹ with an 50-600 *m/z* scanning range. Mass spectra were evaluated using the Agilent MassHunter Quantitative Analysis Version B.07.00 software and TMS derivatised compounds were identified using the commercial mass spectra library NIST (<http://www.nist.gov>), the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (<http://gmd.mpimp-golm.mpg.de/>) and an in-house Metabolomics Australia mass spectral library.

4.3. Results

4.3.1. Two different types of glands in *Eucalyptus brevistylis*

The key finding of this aspect of my research project is the occurrence of two distinct types of glands in *E. brevistylis* – a novel and exciting discovery in the genus. Initial observations of leaves of *E. brevistylis* under a light microscope showed two types of glands that were visually distinguishable due to their contrasting colours of translucent-white or brownish-gold. This visual difference was even more pronounced when glands were exposed by peeling away the epidermis after enzymatic digestion (**Figure 4.2**). The two types of glands were examined further to characterise their essential oil components. Hexane extracts of pools of each type of gland were analysed separately using GC-FID and the major compound groups were identified as sesquiterpenes and β -triketones. Interestingly, translucent glands contained exclusively sesquiterpenes including cubebols; cubebol and 10-*epi*-cubebol in high abundance, and cubenols; cubenol and *epi*-cubenol in lower abundance, with trace amounts of α -cubebene. These glands were termed ‘sesquiterpene glands’. In contrast, the brownish-gold glands contained predominantly the β -triketone conglomerone together with the sesquiterpenes α - and β -caryophyllene in much lower abundance (**Figure 4.3**, **Figure 4.4**), and so were termed ‘triketone glands’ (**Figure 4.6**).

It is possible that *E. brevistylis* leaves contain additional types of glands within the cohorts of ‘sesquiterpene’ and ‘triketone’ glands that account for the different constituents, but cannot be readily distinguished visually. To test this, hexane extracts from groups of 10 glands were analysed using GC-FID. The use of single glands would be ideal to test the difference among gland types, but the GC-FID signals from individual glands were insufficient to provide consistent and reproducible results.

Therefore, 10 gland pools were selected as they produced good GC data. The ratio between α - and β -caryophyllenes and conglomerone concentrations in ‘triketone glands’ was 1:8 and consistent in all batches. Similarly, the ratio of cubenol to cubebol concentration in ‘sesquiterpene glands’ was 1:5 and this was consistent throughout the 10-gland sets. The ratio between cubebol and 10-*epi*-cubebol was consistently 1:2. There was no difference between the compound concentration ratios of each gland set. Therefore, it is highly likely that there are only two types of glands present in leaves of *E. brevistylis*.

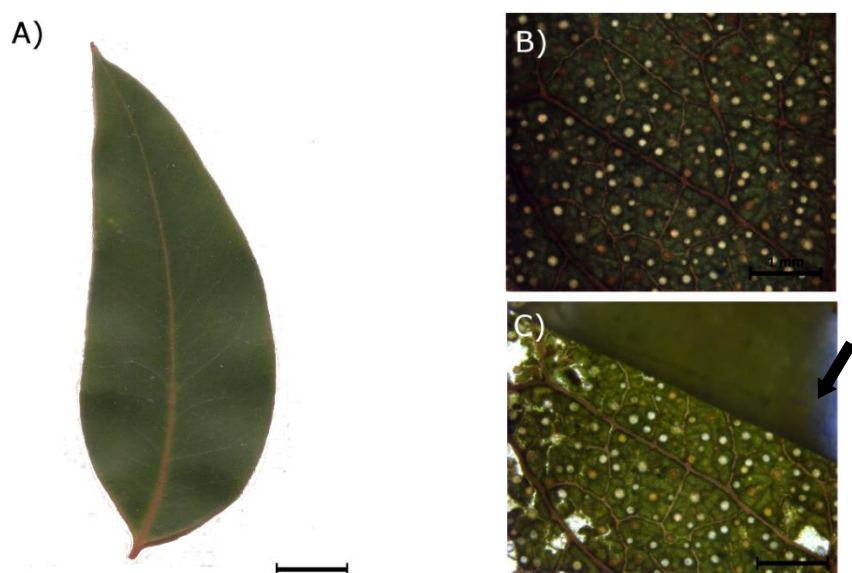


Figure 4.2: Different gland types in leaves of *E. brevistylis*. A) A fully expanded mature leaf from an adult tree. Scale Bar represents 1 cm. B) Two types of glands visually distinguished under light microscope as translucent or brownish-gold. C) The distinction between gland types is clearer after the leaf epidermis is removed. The arrow shows the epidermis and attached cuticle being peeled away from the inner leaf tissues. Scale bars represent 1 mm.

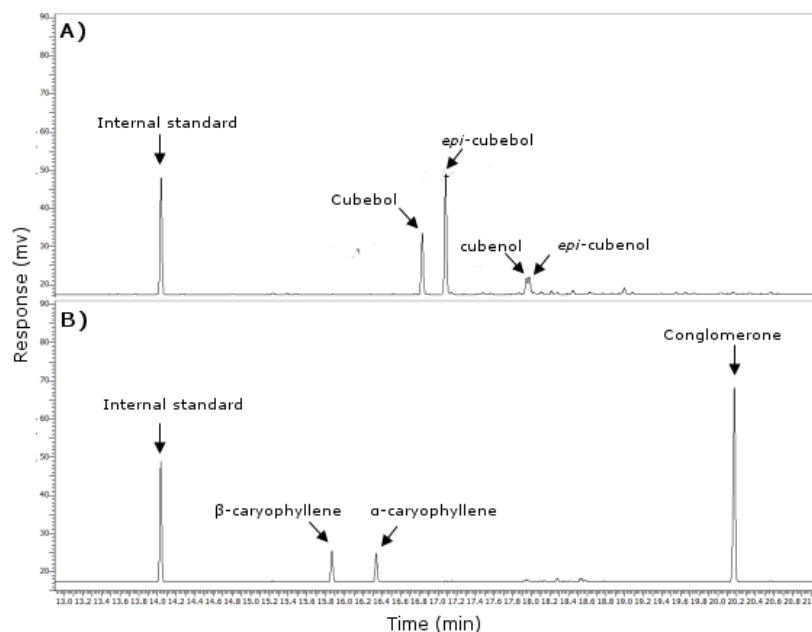


Figure 4.3: Representative GC-FID chromatograms of hexane extracts from two gland types in *Eucalyptus brevistylis* leaves. A) 'sesquiterpene glands' dominated by sesquiterpene cubebols and cubenols. B) 'triketone glands' dominated by the triketone conglomerone with lower amounts of sesquiterpene caryophyllenes.

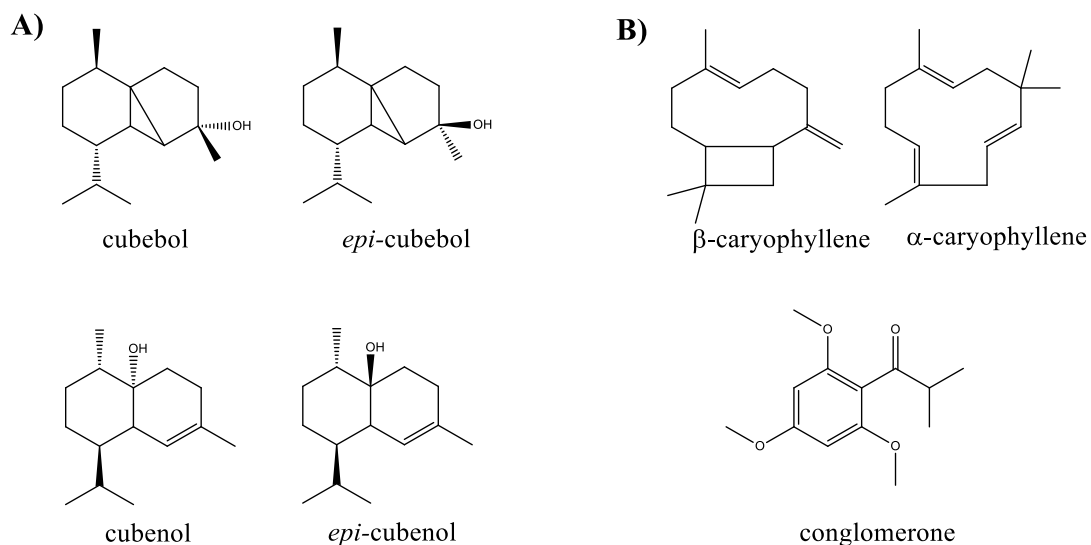


Figure 4.4: Structures of compounds identified from the hexane extracts of the two types of glands isolated from *Eucalyptus brevistylis* leaves. (A) Sesquiterpenes cubebols and cubenols identified from the 'sesquiterpene glands' (B) Sesquiterpene caryophyllenes and the β-triketone conglomerone identified from 'triketone glands'.

4.3.2. Distribution of two gland types in the leaf lamina

The mean density of all glands in fully expanded *E. brevistylis* leaves was 1056 cm⁻², and both gland types were present in all regions of the leaf lamina. Nonetheless, ‘triketone glands’ were more prevalent towards the leaf margin and basal region. In all three examined leaf regions (apex, middle and basal region), triketone glands were higher in the margin of that region compared to the area closest to the leaf mid vein and middle lamina areas. Overall, the percentage of ‘triketone glands’ was highest in the margin of the leaf base (35%; **Table 4.1, Figure 4.5**).

‘Triketone glands’ appeared slightly more elongated in shape and this was notionally supported by calculations of mean ($\pm$ S.E) circularity index for a randomly selected subset of isolated glands giving values of 0.99 ± 0.01 for ‘sesquiterpene glands’ and 0.97 ± 0.01 for ‘triketone glands’ (ANOVA $F = 5.2$, $P < 0.05$, $DF = 23$). Circularity index was calculated using Image-J where a value of 1.00 denotes a perfectly circular shape in a two-dimensional image. This data indicates sesquiterpene glands are closer to perfect sphericity while triketone glands are slightly more elongated in shape. Nonetheless, the two gland types did not differ significantly in volume (ANOVA $F = 0.7$, $P > 0.05$, $DF = 23$). The mean ($\pm$ S.E) gland volume was 1.2 ± 0.1 nL for ‘sesquiterpene glands’ and 1.3 ± 0.1 nL for ‘triketone glands’.

Table 4.1: Average gland count in each region of *Eucalyptus brevistylis* leaves. Data are the average number of glands in regions from six fully expanded leaves. The percentage of each gland type of total glands is given in within brackets.

Region in the leaf	Glands per cm ²		Total gland count
	Sesquiterpene glands	Triketone glands	
Leaf apex			
Margin	876 (75%)	287 (25%)	1163
Lamina	1145 (96%)	49 (4%)	1194
Mid Vein	1165 (98%)	26 (2%)	1190
Middle region			
Margin	754 (69%)	332 (31%)	1086
Lamina	786 (89%)	93 (11%)	879
Mid vein	970 (96%)	38 (4%)	1008
Basal region			
Margin	591 (65%)	319 (35%)	910
Lamina	896 (89%)	108 (11%)	1004
Mid vein	961 (89%)	113 (11%)	1074

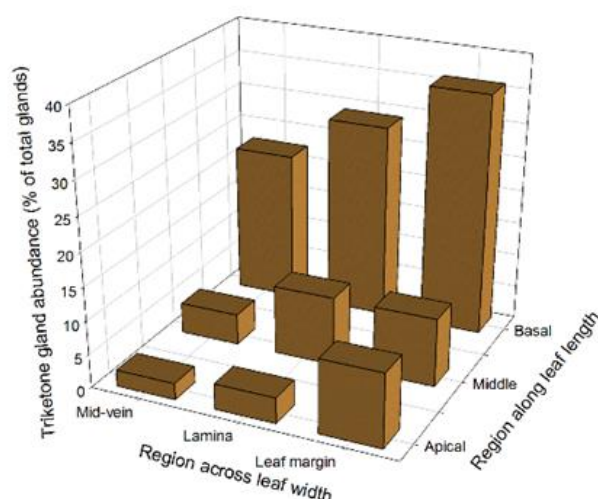


Figure 4.5: Spatial arrangement of two types of glands in *Eucalyptus brevistylis* leaf. Bars represent mean proportion of ‘triketone glands’ in each leaf region.

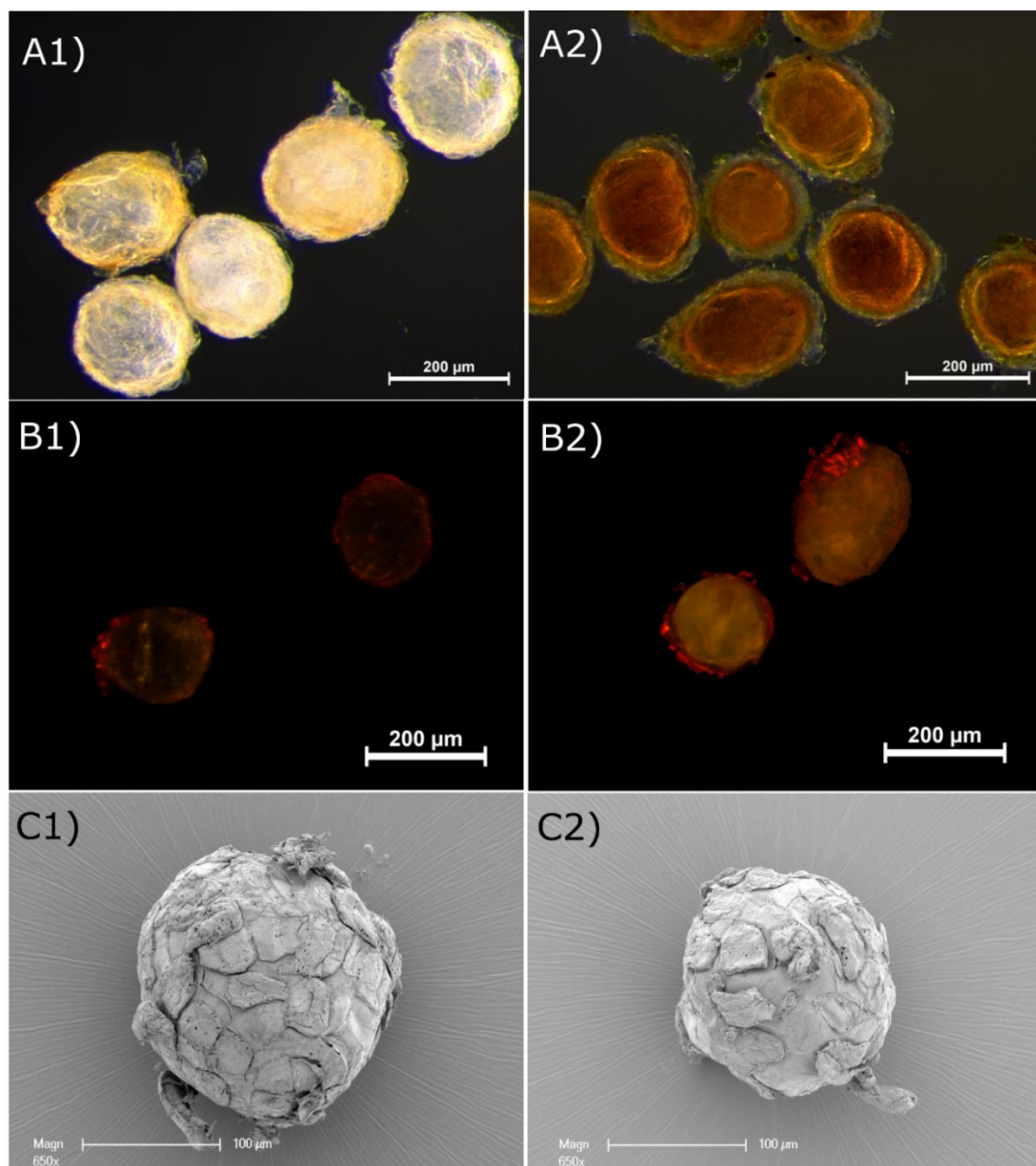


Figure 4.6: Microscopic images of the two gland types isolated from the leaves of *Eucalyptus brevistylis*. A) Stereomicroscopic images of (1) translucent-white 'sesquiterpene glands' and (2) 'triketone glands' in brownish-gold colour. B) Glands imaged under UV illumination with GFP filter (1) 'sesquiterpene glands' with no autofluorescence and (2) 'triketone glands' showing auto-fluorescence of the glandular contents – likely due to the triketone conglomerate. C) Scanning electron micrographs of (1) 'sesquiterpene glands' and (2) triketone glands. Both gland types show an overlapping arrangement of gland wall cells.

4.3.3. Exclusive localisation of β -triketones in glands

Conglomerone was quantified in different foliar tissue types to identify whether β -triketones are present in other tissues in addition to glands. Out of the total conglomerone identified from the leaf, $99.96 \pm 0.02\%$ was present in the glands with only trace amounts detected in the other tissue types. The leaf vasculature and epidermis/cuticle had only $0.01 \pm 0.01\%$ and $0.03 \pm 0.02\%$ of mean ($\pm$ SE) percentage conglomerone, respectively. It is possible that the small amounts of conglomerone in vasculature and epidermis was due to contamination from ruptured glands during the dissection and isolation process. The conglomerone in mesophyll tissue was calculated as the difference between the total conglomerone found in one half of the leaf and the amount found in all other tissues (glands, epidermis/cuticle and vasculature) in the other half of the leaf. The mesophyll did not show any evidence for the presence of conglomerone. These results strongly suggest that the β -triketones are exclusively localised in the glands in *E. brevistylis* (Table 4.2).

Table 4.2: The mean percentage conglomerone in different tissue types in *Eucalyptus brevistylis* leaves. The data are average values of six randomly selected leaves.

Leaf tissue	Mean percentage triketones (%)
Vasculature	0.014 ± 0.014
Epidermis and cuticle	0.030 ± 0.022
Oil glands	99.956 ± 0.023
Mesophylls	0 ± 0.000

4.3.4. Intra-specific variation of β -triketones and sesquiterpenes

To determine the level of intraspecific variation of triketones and sesquiterpenes in *E. brevistylis*, leaf extracts from seven trees from a natural forest and three trees grown in the arboretum were analysed. Leaf extracts from all plants contained triketones and sesquiterpenes indicating the presence of both gland types in all trees in the population (Figure 4.7). Triketone content ranged between 6.3 and 23.1 mg g⁻¹ DW with an average ($\pm$ SE) of 15.2 ± 5.1 mg g⁻¹ DW. The terpene profile was dominated by

sesquiterpenes. The only identifiable monoterpene was *p*-cymene, which ranged from 0.4% to a maximum of 2.0% of the total oil. Monoterpenes trans-p-menth-2-en-1-ol and trans-piperitol were also detected, but only in trace amounts. Total sesquiterpene content ranged between 3.2 and 36.6 mg g⁻¹ DW with an average ($\pm$ SE) of 22.2 $\pm$ 10.4 mg g⁻¹ DW. The sesquiterpene profile of trees from the adult population was clearly dominated by 10-*epi*-cubebol, which made up on average ($\pm$ SE), 59.3% $\pm$ 5.7 of total sesquiterpenes. The mean ($\pm$ SE) total glandular constituents in trees from the natural population, which were older than the arboretum trees, was 48.1 $\pm$ 2.6 mg g⁻¹ DW, while arboretum trees had 23.9 $\pm$ 5.6 mg g⁻¹ DW on average. Based on the total of constituents, the ratios of the two gland types were calculated. The triketone glands in arboretum trees varied from 60 – 96 % of total glands, while the older forest trees had 34 – 57% of triketone glands.

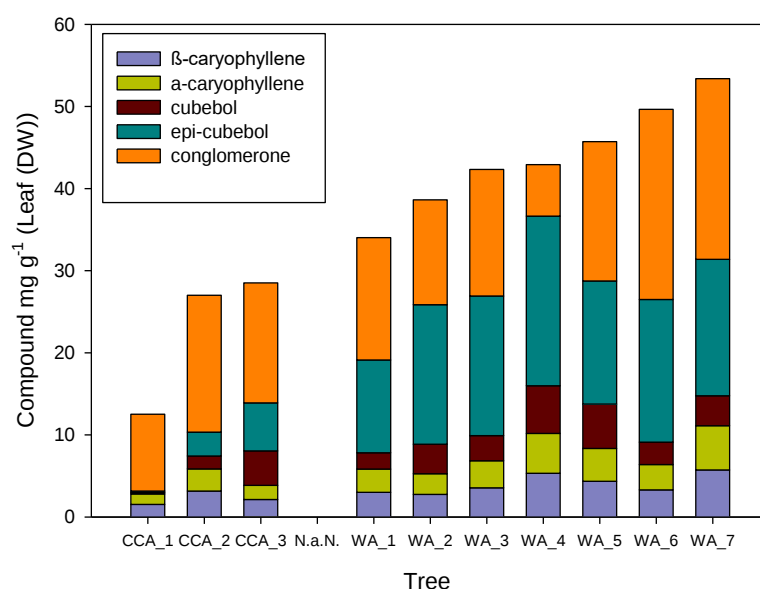


Figure 4.7: Concentrations of the β -triketone conglomerone and sesquiterpenes in a *Eucalyptus brevistylis* population from Western Australia (WA_1-WA_7) and the Currency Creek Arboretum in South Australia (CCA_1-CCA_3).

4.3.5. Variation of gland types with leaf ontogeny

The variation of triketones and sesquiterpenes was observed in leaves of different ontogenetic stages attached to the same branch of mature trees to determine if the two gland types develop in leaves concurrently or if there is ontogenetic variability in their relative formation. Given the constituents in each gland type have been shown to be consistent by exhaustive gland isolation and separation, simpler whole leaf solvent extractions were used for this study. **Figure 4.8** shows the total conglomerone and sesquiterpenes per unit leaf area in a representative tree from arboretum and WA. The concentrations of all compounds decreased with the leaf expansion while triketones showed the largest decrease. In younger leaves the triketone content in a given unit leaf area was considerably higher than any sesquiterpene. The youngest leaf (leaf no. 1) from the trees from WA had 9.4 mg cm^{-2} conglomerone while the total sesquiterpenes found in the 'triketone glands' was 1.8 mg cm^{-2} and sesquiterpene alcohols found in the 'sesquiterpene glands' was 2.4 mg cm^{-2} . Conglomerone in the arboretum tree, which was younger in age (about 20 yrs. old), was 31.8 mg cm^{-2} while the sesquiterpenes found in the 'triketone glands' was 13.5 mg cm^{-2} and sesquiterpenes in the 'sesquiterpene glands' was 19.4 mg cm^{-2} .

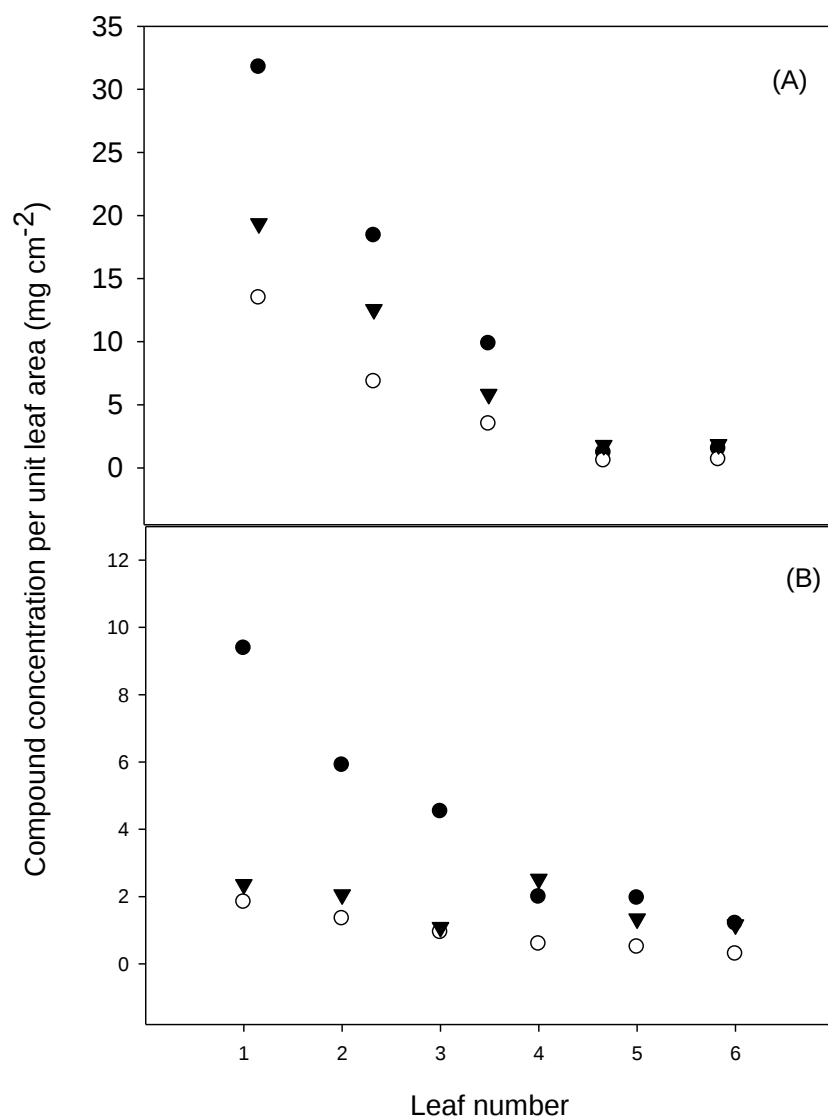


Figure 4.8: Variation of triketone conglomerone and sesquiterpenes per unit leaf area in representative trees from (A) the arboretum and (B) the natural population. The black circle symbols represent conglomerone and white circles the total sesquiterpenes in ‘triketone glands’. Triangle symbols represent total sesquiterpenes in ‘sesquiterpene glands’. Leaf #1 is the smallest, newly formed leaf, whereas leaf #6 is the most mature leaf.

4.3.6. Variation of gland types with seedling ontogeny

Seedlings from two half-sibling families (family A and family B) were tested to study the ontogenetic pattern of the two gland types during early plant development. The average conglomerone concentration in leaves of each seedling was calculated as a proxy for ‘triketone’ glands and the average of the two major sesquiterpenes cubebol and 10-*epi*-cubebol was used to represent ‘sesquiterpene glands’. In both families, average conglomerone concentration in a leaf was higher than the average of the total sesquiterpenes (Panels (A) and (B) in **Figure 4.9**). The cotyledons of the first seedling from families A and B had 5.1 and 4.2 mg g⁻¹ DW conglomerone, respectively. In both families, conglomerone showed a gradual increase with time. In family A, the maximum conglomerone concentration was reported 350 DAS as 24.9 mg g⁻¹ DW. Family B also reported the maximum conglomerone 350 DAS with a value of 19.1 mg g⁻¹ DW. These results suggest that ‘triketone glands’ are initiated very early in seedling ontogeny and the gland size and/or frequency of glands increase rapidly from the beginning during the first year of the plant development. In contrast, sesquiterpene concentrations were comparatively at lower levels (**Figure 4.9**). The average of sesquiterpenes in ‘sesquiterpene glands’ in family A and in family B was 0.38 and 0.43 mg g⁻¹ DW, respectively. Cubebol and 10-*epi*-cubebol were present in cotyledon stages as well, suggesting that ‘sesquiterpene glands’ are also initiated at very early stages in seedling development. However, these sesquiterpene levels remained at very low levels throughout the study period without a significant increase in seedlings.

Using a leaf formed at the same time from each seedling, conglomerone and sesquiterpene concentrations per leaf area were also calculated (Leaf position L1 from each seedling; the leaf harvesting pattern is shown in **Figure 4.1**). Conglomerone concentration per unit leaf area was very high in the early stages and decreased later to a more constant value in leaves of both families (Panels (C) and (D) in **Figure 4.9**). This suggests that as the leaf lamina expands ‘triketone’ glands are effectively diluted relative by an increased rate of ‘sesquiterpene’ gland formation.

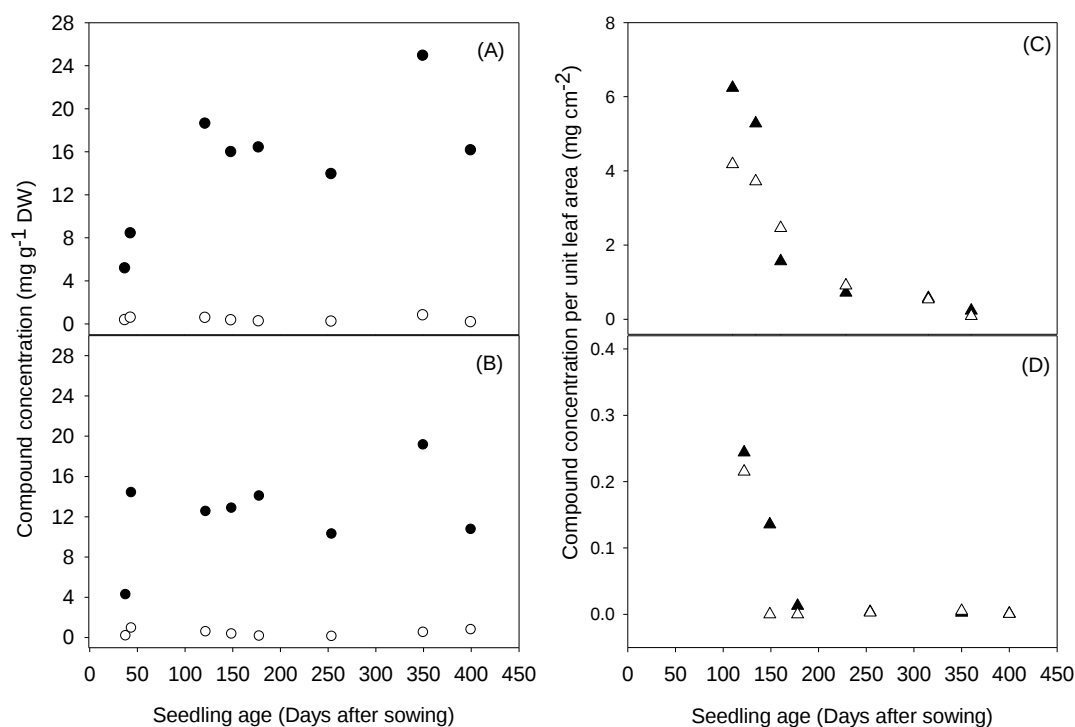


Figure 4.9: Conglomerone and sesquiterpene concentrations in leaves from seedlings of different ages of the two half-sibling families of *E. brevistylis*. Panels (A) and (B) show the variation of the concentrations of conglomerone and total sesquiterpenes in ‘sesquiterpene glands’ in the two families. (A) Family A and (B) family B. The black circle symbols represent the concentration of conglomerone, and hollow circle symbols represent the total of sesquiterpenes cubebol and 10-*epi*-cubebol in ‘sesquiterpene glands’. Panels (C) and (D) show the variation of concentrations of the major compounds per unit leaf area in leaves formed at the same time in each seedling (L1 leaf from each seedling) from the two families A and B. (C) Concentration of conglomerone and (D) concentration of sesquiterpenes cubebol and 10-*epi*-cubebol from ‘sesquiterpene glands’. The dark triangle symbols represent family A and hollow triangle symbols represent family B.

4.3.7. Primary metabolites in glands

To examine if the two gland types in *E. brevistylis* differed in contents other than their essential oils, primary metabolites were extracted, derivatised and analysed using GC-MS. The most abundant compound in triketone glands was sucrose with a normalised response value of 7.9 (normalized peak intensities relative to the Internal Standard). In contrast, the sesquiterpene glands had only a 0.7 normalised response value for sucrose. Both gland types contained hexadecanoic acid, octadecanoic acid, glycerol and glucose in high abundance. Many unknown compounds were detected in both gland types. **Table 4.3** details the primary metabolites identified from the two gland types.

Table 4.3: Normalised response values of primary metabolites detected in sesquiterpene and triketone glands isolated from *E. brevistylis* (normalized peak intensities relative to the Internal Standard).

Compound	Sesquiterpene glands	Triketone glands
Sucrose	0.7031	7.9298
Hexadecanoic acid	3.5607	3.4931
Octadecanoic acid	3.1518	2.9542
Glycerol	1.8532	1.8569
Glucose	1.7365	1.5915
Fructose	0.2157	0.3040
Urea	0.2059	0.1821
Myo-Inositol	0.0739	0.0864
Tetradecanoic acid	0.1300	0.0845
4-hydroxy Cinnamic acid	0.0165	0.0791
Scyllo-Inositol	0.0661	0.0727
Triethanolamine	0.0705	0.0599
Octanoic acid	0.0808	0.0598
Pyroglutamic acid	0.0802	0.0493
Glycine	0.0376	0.0439
Dodecanoic acid	0.0587	0.0392
Docosane	0.0339	0.0334
Xylose	0.0636	0.0318
Cytosine	0.0427	0.0257
Alanine	0.0205	0.0230
Galactose	0.0410	0.0198
Threonic acid	0.0135	0.0187
4-hydroxy_Butyric acid	0.0239	0.0158
Glycolic acid	0.0172	0.0138
Manose	0.1214	0.0095
Isoleucine	0.0072	0.0070
Threonine	0.0046	0.0032
Threitol	0.0028	0.0021
2-methyl fumaric/maleic acid	0.0054	0.0003

4.4. Discussion

4.4.1. Metabolic differentiation of oil glands

The results of this chapter show for the first time the presence of two different types of foliar oil glands not only in *E. brevistylis* but also in any eucalypt. The first type appears translucent and are termed ‘sesquiterpene glands’ due to their abundant constituents being sesquiterpene cubenols and cubebols. The second type are golden-brown coloured and are termed ‘triketone glands’ due to them synthesising predominantly the β -triketone conglomerone with sesquiterpene caryophyllenes in lower abundance. As noted, the two types of glands can be visually distinguished based on their colour, but they are otherwise structurally and morphologically identical. The colour difference between the two gland types is likely due to the different compounds localised to them. For instance, both α - and β -caryophyllene in the golden-brown ‘triketone glands’ are known to be pale yellowish in colour (Database), whereas cubenol and cubebol in the translucent ‘sesquiterpene glands’ are known to be colourless oils (Jiangseubchatveera *et al.* 2015). As noted, this is the first report of the presence of two types of metabolically different glands in the same leaf of a myrtaceous species, or in any tree species. *Eucalyptus* oil has been subjected to a large number of studies in recent years, and the oil glands themselves have also been studied, albeit to a lesser extent, but in all cases glands have been reported or shown in images as translucent and colourless in appearance (Brooker and Nicolle 2013; Goodger *et al.* 2010). Similarly, in Chapters 2 and 3 presented here, the glands of 29 *Eucalyptus* species were isolated and none showed such a striking colour difference amongst glands.

A metabolic differentiation of glands is possible in other eucalypts, particularly in triketone bearing species and further use of the gland isolation protocol may facilitate their future discovery. Preliminary identification of glands in this study was based on leaf surface observations. However, identification of two colours of glands in leaves from more mature trees (i.e. leaves collected from older trees in Frankland State Forest) was more difficult using this method. Even in such leaves from older trees, the two types of glands were distinguishable when isolated and separated from the other leaf tissues using enzymatic digestion. However, most eucalypts contain terpene classes that co-occur in individual oil glands; therefore, it is unlikely that different gland types exist for mono and sesquiterpenes found in most *Eucalyptus* species (King *et al.* 2006).

Moreover, it was confirmed that only two gland types exist to produce different types of sesquiterpenes (cubebols and cubenols) and triketones and caryophyllenes in *E.*

brevistylis. Cubebols and cubenols have been found to co-occur in essential oil extracts from myrtaceous species from the genera *Corymbia*, *Eucalyptus*, *Kunzea*, *Leptospermum* and *Melaleuca* (Cornwell *et al.* 2000). Cubenols were considered by-products of acid-solvolysis of the sesquiterpene alcohols cubebol and *epi*-cubebol (Cornwell *et al.* 2000) supporting the finding here that they both are present in the same gland type.

Differentiation of secretory structures located on plant surfaces is known from trichomes in both structural and physiological aspects. Capitate and peltate types of glandular trichomes (GTs), which are different in morphology and ultrastructure, are common in trichome-containing plants, while differentiation of the composition of metabolites have also been observed. For example, in *Lavandula pinnata* capitate GTs contain mainly polysaccharides and to a lesser degree lipophilic substances. In contrast, peltate GTs only secrete lipophilic substances (Huang *et al.* 2008). Similarly in basil (*Ocimum basilicum*, Lamiaceae), peltate GTs store the phenylpropenes methylchavicol and eugenol while capitate GTs do not store these compounds or show any evidence for possessing the enzymatic activities needed for their biosynthesis (Gang *et al.* 2001). Another study reported phenolic compounds in peltate trichomes of *Rosmarinus officinalis* (Lamiaceae), but not in glandular trichomes (Marin *et al.* 2006). Two types of GTs named long trichomes and short trichomes have also been reported from the leaves of *Nicotiana tabacum*, *N. sylvestris* and *N. rustica* (Meyberg *et al.* 1991). The long and short trichomes produce a diterpene resinous material in chloroplasts and in small vacuoles of their head cells while short trichomes produce substances such as nicotine in a different type of plastid, in large vacuoles and in the apoplast of their head (Meyberg *et al.* 1991).

Metabolic differentiation of secretory structures embedded within plant tissues is far less common and has been reported only from a single plant family, the Hyperaceae. For example, *Hypericum* species contain two gland types termed ‘dark’ and ‘translucent’ (Soelberg *et al.* 2007). Further, a recent study on 17 *Hypericum* species suggests that there is a level of metabolic differentiation in gland types. Even though the results were not consistent in all species, in *H. maculatum* and *H. erectum* only the dark glands had the flavonoid rutin (Kucharíková *et al.* 2016).

4.4.2. Sesquiterpene biosynthesis

Sesquiterpene biosynthesis pathways including their associated enzymes have been documented to a great extent in literature. The present study found that the sesquiterpene hydrocarbons α - and β -caryophyllene co-occur with the triketone conglomerone in 'triketone glands'. The 'sesquiterpene glands' contained sesquiterpene alcohols while the sesquiterpene hydrocarbons were not detected in them. This suggests that sesquiterpene alcohols and sesquiterpene hydrocarbons synthesis is catalysed by different sesquiterpene synthases and these two enzyme groups/types are present/active in different gland types in *E. brevistylis*. The co-occurrence of α - and β -caryophyllene have been reported earlier and they have been shown to be biosynthesized together by a single, multi-product sesquiterpene synthase in *Arabidopsis* flower (Chen *et al.* 2003; Tholl *et al.* 2005). However, the reason for the cooccurrence of these two sesquiterpene hydrocarbons with the triketone conglomerone is not clear.

4.4.3. Gland ontogeny in leaves

The spatial arrangement pattern and leaf ontogeny results together suggest that triketone glands are formed earlier in leaf ontogeny and accumulate in the leaf margin as the leaf expands. Sesquiterpene glands appear to be formed later in leaf ontogeny. In eucalypts, the concentrations of volatile terpenes increase with leaf expansion (Goodger and Woodrow 2012). Large number of glands are initiated at early stages of development and then enlarge as the leaf expands. This results in a density drop in gland count but the total oil increases as the total gland volume increases (Goodger and Woodrow 2012). The results presented here show both gland types are distributed throughout the leaf, with triketone glands in greater abundance towards the margin and the base of leaf lamina. The elongation of triketone glands could be related to the position of glands in the leaf lamina as they are more present towards the leaf margin where they can be squashed against the leaf edges thereby modifying their spherical shape somewhat. Previous observations on glands in other *Eucalyptus* leaves indicate that a small proportion of the glands that are located along the leaf margin are more elongated in structure since they are adapting to a packed microenvironment. The glands distributed in the mid areas are more spherical in shape given that they have more space to enlarge equally in all sides.

4.4.4. Gland ontogeny in seedlings

The results suggest that triketone gland density is higher at early stages and later decreases. The variation pattern could also be due to gland size differences at different stages or thicker/heavier leaves effectively diluting glandular concentrations. This is likely to be associated with ecological role of phytochemicals. Young expanding leaves may need more resistance against herbivores. Glands observed in cotyledons of seedlings with a comparatively high concentration of triketones supports the idea that young plants have higher levels of defence compounds. *Eucalyptus* oil glands are known to be formed at a very early embryonic stages of development, shortly after the "heart stage". Oil-gland initials have been distinctly identified from the surrounding embryonic cells by their smaller size, darkly staining cytoplasm and obvious nuclei (Bohte and Drinnan 2011; Carr and Carr 1970). Other types of secretory organs have also been previously identified from cotyledons of seedlings in other plant families, for example excretory idioblasts in *Pharbitis* (Tretyn *et al.* 1996). A similar evidence has been observed in *Populus* species, where young expanding leaves were more resistant to damage from fungal pathogens and insects than the older leaves (Curtis and Lersten 1974; Shain and Miller 1982; Sharma *et al.* 1980).

4.4.5. Variation of triketones and sesquiterpenes in population

Conglomerone was the only triketone detected in *E. brevistylis* glands and was present in very high abundance. Triketones have previously been reported from *Eucalyptus* species and are a class of compounds relatively prevalent in the genus and more broadly in myrtaceous plants. Generally, triketones are rare in plants and if present they exist only as minor components in essential oils (Hellyer 1968). Nevertheless, oil profiles with up to 90% of triketones have been reported; for example, leptospermone from the myrtaceous plant *Xanthostemon chrysanthus* (Bick *et al.* 1965). In most of the species that contain triketones in oils, more than one β -triketone exists. For example, the triketones grandiflorone, leptospermone and flavesone were found together in oils of *Leptospermum flavesces* (Hellyer and Pinhey 1966). In most species where there is a large proportion of a certain β -triketone in the oil, no other β -triketones could be detected, such as tasmanone in *E. camfieldii*, agglomerone in *E. agglomerata*, and calythrone in *Calytrix tetragona* (Hellyer 1968).

Conglomerone and sesquiterpene concentration ratios showed variation between individuals in the population. The domination of conglomerone in the triketone profile

and 10-*epi*-cubebol in the terpene profile was consistent throughout the population (**Figure 4.7**). Conglomerone concentration ranged from 6 to 23 mg g⁻¹ DW with an average concentration of 15 mg g⁻¹ DW. The intraspecific variation of conglomerone has not been explored previously in any species. Other triketone compounds have shown a very high degree of intraspecific variation with some geographical patterns in other myrtaceous species (Douglas *et al.* 2004). However, *E. brevistylis* has a restricted geographical distribution where it is limited to an area near Walpole in the Warren region of WA and is known only from nine locations (Hearn *et al.* 2006). Therefore, minimal genetic variation with respect to triketone synthesis can be expected due to the small population sizes.

4.5. Conclusion

The results presented in this study show for the first time that the Myrtaceous species *E. brevistylis* has two different types of glands co-localised in their leaves. Translucent glands are specialised to produce and store sesquiterpenes while the golden-brown type is specialised for mainly β -triketone synthesis. Two sesquiterpenes α - and β -caryophyllene were found co-localised with β -triketones in the golden-brown type. The ‘sesquiterpene glands’ had cubebol and 10-*epi*-cubebol. The results also show that β -triketones are localised exclusively in glands and they are not present in other leaf tissues. Triketone glands were found to be dominant in younger stages in plant ontogeny. This study is the first record of such metabolic specialisation of oil glands for the synthesis of different groups of secondary metabolites in *Eucalyptus* or any tree species.

Chapter 5

General discussion and conclusions

5.1. Introduction

Trees in the genus *Eucalyptus* are characterised by their rich profile of plant secondary metabolites (PSMs), including an array of volatile essential oils and other non-volatile compounds (NVCs). Until recent years, it was thought that the embedded oil glands of eucalypts contained the volatile essential oil components exclusively. The oil profiles of species in this highly diversified genus have been widely explored and are known to contain mono- and sesquiterpenes as the main constituents. Recently, the use of a protocol to isolate embedded oil glands (Goodger *et al.* 2009) showed that *Eucalyptus* oil glands also house NVCs in addition to the well-known volatiles. In particular, monoterpene acid glucose esters (MAGEs) were found co-localised with oil components and were identified from the glands of an array of *Eucalyptus* species. Despite MAGEs being prevalent in eucalypts, the exact role of MAGEs and other NVCs within the glands is not clear. The tested and predicted bioactivity of many of the NVCs is suggestive of a possible defensive function. In addition, it has been proposed that MAGEs may play a physiological/protective role whereby they protect biosynthetic cells from auto-toxic effects of oil components. The flavanone pinocembrin has also been identified in the embedded glands of four *Eucalyptus* species. These findings suggest that *Eucalyptus* oil glands may house other unidentified MAGEs, flavonones and/or other groups of NVCs. Only a relatively low number of species have been assessed for the non-volatiles present in their glands. Therefore, it is important to explore more species to discover whether MAGEs are ubiquitous in this large genus and to help understand their physiological role within glands. The aim of this thesis was to address these gaps of knowledge and characterise the metabolome of *Eucalyptus* foliar glands focusing on NVCs in relation to volatile oils.

5.2. Summary of the key findings

5.2.1. Sequestration of NVCs in *Eucalyptus* foliar oil glands

This thesis shows that NVCs are common in *Eucalyptus* foliar oil glands. In Chapter 2, glandular NVCs from a diverse array of *Eucalyptus* species were studied to determine the presence and/or ubiquity of MAGEs and to identify whether they contain other compound classes as well. MAGEs were found to be widespread in *Eucalyptus*; however, they were not present in some taxonomically related species from *E.* subg. *Eucalyptus*. This survey included 30 species belonging to the two major subgenera *Symphyomyrtus* and *Eucalyptus*. MAGEs were identified from a total of 26 species studied. Several known MAGEs were identified based on their characteristic mass fragmentation patterns, retention times and UV absorbance using ESI-LCMS. Some of the species contained a higher abundance of certain MAGEs while some had a greater variety of MAGEs in their profile. For example, *E. tenera* had at least 12 MAGEs in glandular extracts. Some of the species that contained complex mixtures of MAGEs, such as *E. articulata*, are potential target species for future investigations on this compound group. Several novel MAGEs were also present in some species and these require structural elucidation using NMR spectroscopy. None of the known or novel MAGEs were detected in four of the species from *E.* subg. *Eucalyptus*: *E. nitida*, *E. dendromorpha*, *E. brevistylis* and *E. suberea*. In general, the abundance of MAGEs in *Symphyomyrtus* species was significantly higher than in species from subgenus *Eucalyptus*. Cuniloside B, cypellocarpin C and froggattiside A were the most widespread MAGEs in *Symphyomyrtus* and with the exception of the unknown reported from *E. gregsoniana*, were the only MAGEs identified from *E.* subg. *Eucalyptus* species. Previous studies have included mainly *Symphyomyrtus* species and fewer species from the subgenus *Eucalyptus*, leading to the idea that MAGEs are present in all species from the genus. However, the findings of this thesis suggest that MAGEs are predominantly found in the subgenus *Symphyomyrtus* with their occurrence, in subgenus *Eucalyptus* less abundant and more variable.

Despite the glands of *E.* subg. *Eucalyptus* species having MAGEs in lower abundance, they were still rich in NVCs, with flavonoids as the dominant group. Foliar glands of these species were further studied in Chapter 3 to characterise their chemical profiles. The unsubstituted B-ring flavanones pinocembrin, pinostrobin, alpinetin and dimethylpinocembrin, and the structurally related flavone dimethylchrysin, were identified as the major non-volatiles present in glands. This is the first report of

localisation of pinostrobin, dimethylpinocembrin and alpinetin, and the flavone specifically to glands of any plant species. This study shows for the first time that the unsubstituted B-ring flavanones are exclusively localised to glands in *Eucalyptus* species, rather than distributed in other leaf tissues. Further, the flavanones in subgenus *Eucalyptus* species were quantified and *E. oreades* and *E. nitida* were identified as species containing remarkably high amounts of total flavonoids (12.87 and 12.72 mg g⁻¹ DW). The species which had the lowest flavanones was *E. gregsoniana*, and it is noteworthy that it was the only species from which a novel MAGE was identified from subg *Eucalyptus*, in addition to the three commonly found MAGEs. Moreover, two C-methyl flavanones, cryptostrobin and demethoxymattucenol, were identified from *E. agglomerata* glands. This constitutes the first report of these two compounds from oil glands of any species, although cryptostrobin and desmethoxymatteucinol have been reported very recently from whole leaf extracts of two *Eucalyptus* species (Marsh *et al.* 2019).

5.2.2. β -Triketones in oil glands

Apart from the well-known volatile monoterpenes and sesquiterpenes, triketones were also found in some species. The results of chapters 3 and 4 show that triketones are sequestered to glands in *E. brevistylis* and *E. suberea*. Triketones are, amongst other things, toxic to the enzymes involved in carotenoid biosynthesis; therefore, the sequestration of them to oil glands seems to favour their storage away from cellular chloroplasts and chromoplasts (Dayan *et al.* 2009; Misawa *et al.* 1990). This is the first report of localisation of triketones in oil glands in any *Eucalyptus* species. This finding together with the known biological activity of these compounds suggests that triketones are likely exclusively localised to glands in all eucalypts where they occur and they are thus not found in other leaf tissues.

5.2.3. Metabolic differentiation of oil glands in *E. brevistylis*

The occurrence of two different types of glands in leaves of *E. brevistylis*, producing either β -triketones or sesquiterpenes, was a major discovery. In Chapter 4, two different gland types responsible for the synthesis of β -triketones and sesquiterpenes were identified from *E. brevistylis* leaves. Sesquiterpene glands, which are translucent-white in appearance, produce sesquiterpenes while triketone glands, which are golden-brown in appearance, produce mainly β -triketones and different sesquiterpenes in lower

abundance. This is the first evidence of metabolic differentiation in embedded oil glands in *Eucalyptus* or in any tree species. Leaves at three different stages of plant development were examined - leaves in seedlings including cotyledons, and leaves from two different ages of mature trees. The two gland types were observed together in leaves at all three stages. The ontogenetic variation suggests that triketone glands appear at a very early stage of plant development and are “forced” towards the leaf margins as leaves expand. Sesquiterpene glands develop relatively later in seedlings within the first six months of development. Although the glands differ in their contents, no structural difference was found between the two types.

5.3. *Eucalyptus* oil gland metabolome

Overall, the work presented in this thesis has contributed to our understanding of the glandular NVCs in *Eucalyptus* species and how they relate to their co-housed volatile oil components. The following aspects of the *Eucalyptus* gland metabolome are discussed in the light of this new knowledge.

5.3.1. Phytochemical ecology and chemotaxonomy

The findings of chapters 2 and 3 reveal that the distribution of NVCs in *Eucalyptus* species has a clear phylogenetic relationship, with a major difference observed between the two large subgenera *Symphyomyrtus* and *Eucalyptus*. MAGEs dominated the non-volatile fraction in *Symphyomyrtus* whereas flavanones dominated in subgenus *Eucalyptus* species. Flavanones were not detected at an identifiable level in any of the subgenus *Symphyomyrtus* species. Such differences in chemical profiles were observed even at lower taxonomic levels. For example, flavanones were identified only from the *E.* Section *Eucalyptus* while β -triketones were present only in two species that were selected from sections other than *Eucalyptus* (*E. brevistylis* from section *Longistylus* and *E. suberea* from section *Frutices*). Therefore, the presence of flavanones and absence of triketones may be a characteristic of *E.* Section *Eucalyptus* species. It is noteworthy that these two species also had the lowest concentrations of monoterpenes. A similar trend has been observed in earlier studies in eucalypts with flavanones and other compound groups. In a survey of 19 *Eucalyptus* species, pinocembrin was identified from all four subg. *Eucalyptus* species studied while it was not detected at all in any of

the *Symphyomyrtus* or *Eudesmia* species (Heskes *et al.* 2012). Similarly, Tucker *et al.* (2010) reported free flavanones in whole leaf extracts of subg. *Eucalyptus* species but not in *Symphyomyrtus* species. In addition, *Symphyomyrtus* species contain formylated phloroglucinol compounds (FPCs), particularly macrocarpals and euglobals, and cyanogenic glycosides; however, the subg. *Eucalyptus* species were lacking in these compound groups (Eschler *et al.* 2000; Gleadow *et al.* 2008). With regard to some compound groups in eucalypts, they can exist in both major subgenera with a significant difference in concentrations. For example, condensed tannins in *Symphyomyrtus* species were four times the concentration of those found in subg. *Eucalyptus* species (Fox and Macauley 1977; Noble 1989). This is similar to what was observed in the present study regarding the abundance of MAGEs in the two subgenera. Though the three MAGEs cuniloside B, froggattiside A and cypellocarpin C were detected in some subg. *Eucalyptus* species, based on the UV chromatogram peak data they were in lesser abundance compared to the *Symphyomyrtus* species. These results support a clear phylogenetic trend in *Eucalyptus* foliar oil gland chemistry.

The different chemical profiles in the trees of the two major subgenera may have a relationship with their ecology. For example, subgenus *Eucalyptus* species have been observed to have lower early growth rates and suffer less leaf damage from insects and fungal pathogens than *Symphyomyrtus* species (Noble 1989; Stone *et al.* 1999). In those studies, it was also evident that when insects and pests are highly controlled, *Symphyomyrtus* species have a higher growth rate. However, under natural conditions with high defoliation pressure from insects and pests, the tallest species were from the subgenus *Eucalyptus* (Stone *et al.* 1999). The resistance of subgenus *Eucalyptus* species may be related to the presence of high concentrations of unsubstituted B-ring flavanones and other structurally related flavanones as shown in Chapter 3 of this thesis. Nonetheless, both MAGEs and flavanones have proven biological activities related to plant defence against microorganisms to different degrees (Hou *et al.* 2000; Shain and Miller 1982). Chemical characteristics of foliage such as the presence of phenolics as well as terpenoids make them less palatable to herbivores (Gleadow *et al.* 2008; Moore *et al.* 2004). However, it is unclear to what extent the different PSM groups can deter herbivores. There is a lack of knowledge in this area and more ecological studies are essential to determine the association between the foliar chemistry and ecological behaviour of eucalypts.

5.3.2. Biosynthesis of MAGEs

Evidence from this study supports the idea that α -terpineol is a possible biosynthetic precursor of oleuropeic acid in MAGEs. *E. articulata*, the species which contained the highest number of likely MAGEs, also had the highest abundance of α -terpineol. The enzymes responsible for esterification and glycosylation seem to be present or active mainly in *Symphomyrtus* species and only in some *E.* section *Eucalyptus* species. Cytochrome P450 enzymes, glycosyltransferases and acyltransferases are the likely enzyme groups responsible for biosynthesis of MAGEs from monoterpene acid precursors. However, there is no knowledge on the specific enzymes responsible for the esterification or glycosylation in MAGE biosynthesis.

In the last section of Chapter 2, *E. polybractea* cell suspensions were established to investigate biosynthesis of MAGEs and potentially other NVCs through biotransformation studies. Coumaric acid glucosides were obtained after feeding *p*-coumaric acid to the cells in suspension. This biotransformation provides information relevant to possible biosynthetic pathways involved in *E. polybractea* and other related species. MAGE biosynthesis needs precursors derived from two different pathways. The majority of MAGEs contain both a monoterpene acid and a phenolic group attached to the glucopyranose ring. The few exceptions are cuniloside A and B, froggattiside A and eucalmaidin A, which contain either a single monoterpene acid aglycone or both aglycones are monoterpene acids. Feeding of coumaric acid, which is synthesised in the shikimic acid pathway, helps understand how phenolic groups incorporate into MAGEs. However, to study how the monoterpene acid incorporates by esterification, an ideal precursor is α -terpineol. Nevertheless, a reliable method needs to be developed to mobilise α -terpineol into cells in the suspension and also to identify α -terpineol and biotransformation products using HPLC. *E. polybractea* is a species that contains high concentrations of MAGEs (Heskes *et al.* 2012). Therefore, it is hoped that this cell line from *E. polybractea* will be used for future studies aiming to understand the reactions and enzymes responsible for MAGE biosynthesis.

5.3.3. Triketones in glands

Triketones were found only in *E. brevistylis* and *E. suberea*: species in which no flavonoids were detected. A similar observation pattern of flavonoids and triketones distribution in species was reported from the myrtaceous genus *Leptospermum* (Killeen *et al.* 2015). In that study, the triketone grandiflorone and C-methylated flavonoids were

identified from glands where the two compound groups showed an inverse relationship. In some *Leptospermum* species, the triketone to flavanone ratio was over 100:1. The authors suggest a common biosynthetic precursor for the two compound groups and have speculated that these two classes of compounds are regulated by same biosynthetic machinery with simple switches in metabolic flow based on the availability of cinnamoyl coenzyme A (CoA) or dihydrocinnamoyl CoA (Killeen *et al.* 2015). In this thesis, C-methylated flavonoids were identified from two *E. section Eucalyptus* species, but not from other species in which triketones were present. Since flavanones and triketones were found exclusively in the glands, it appears they are biosynthesised within glandular secretory cells rather than being transported from elsewhere in the leaf post biosynthesis.

5.3.4. Role of non-volatiles in glands

Although MAGEs were not directly quantified in this thesis, the finding of the highest oil concentration from the species that had the highest number of MAGEs (*E. articulata*) suggests that there is a relationship between the concentrations of MAGEs and oils, as expected by their co-localisation to glands. In addition, some other interesting findings can be highlighted. In *E. stellulata* (from subgenus *Eucalyptus*), MAGEs were in low abundance while having the lowest oil concentration per leaf dry weight (9.62 mg g⁻¹ LDW). The lowest per gland oil concentration (0.22 µg gland⁻¹) was also reported from *E. stellulata* (excluding the data from the two species (*E. brevistylis* and *E. suberea*) that did not contain any MAGEs or flavanones in their glands). This evidence suggests that the total oil concentrations may have a correlation with the concentration of MAGEs in glands. On the other hand, the species from subgenus *Eucalyptus* that were rich in flavanones, showed positive correlations with some mono- and sesquiterpenes suggesting a possible link between the two compound groups. The MEP pathway that synthesises terpenoids and shikimate pathway that synthesises flavonoids are largely well characterised (**Figure 1.2**). The correlations that exist between the terpenoid and flavonoid compositions seem to be tightly regulated at gene transcriptional level in secretory cells (Xie *et al.* 2008). Post-translational level regulation also controls the synthesis of specific metabolites. For example in glandular trichomes (GTs), the enzymes related to the production of phenylpropanoids are downregulated while the enzymes related to the terpenoid production are upregulated at mRNA and protein levels leading to a metabolic profile rich in terpenoids (Xie *et al.* 2008).

The two species that did not have any MAGEs or flavanones, *E. brevistylis* and *E. suberea*, had a total oil concentration of 51.13 and 27.38 mg g⁻¹ LDW, respectively. Nonetheless, their oil profile was different from the profiles of the other species. In *E. suberea*, the most abundant terpenoid component was the sesquiterpene bicyclogermacrene, which was not a constituent found in other species in considerable abundance, at least in the more related *E.* section *Eucalyptus* species. Therefore, it is arguable why the oil profiles are different in species that do not contain the non-volatiles MAGEs and flavanones. Biosynthetic mechanisms/pathways that are different to those found in the terpenoid rich glands of other species are possible in *E. brevistylis* and *E. suberea*, more broadly in the species belonging to *E.* sections other than *Eucalyptus*. A different unrecognised non-volatile group that are involved in mobilisation or protective functions may be present in these species.

5.4. Future research

The other triketone containing species in the genus *Eucalyptus* may, like *E. brevistylis*, also have different gland types specialised for triketone biosynthesis. *E. suberea* was the only other species that had triketones in this study and is a potential candidate for further studies of triketone bearing glands. It is possible that *E. suberea* also synthesises triketones in a separate type of glands. Its glands do not contain conglomerate and this triketone might be responsible for the appearance of *E. brevistylis* triketone glands. Alternatively, the golden-brown colour of triketone glands in *E. brevistylis* could be due to another compound related to triketone biosynthesis. The metabolome of *E. brevistylis* glands has not been fully elucidated and potentially unidentified compounds could be responsible for their unique colour. If an average glandular constituent density of 1 µg per nL were assumed, *E. brevistylis* glands should contain more compounds than identified here. The volume of sesquiterpene glands was 1.2 ± 0.1 nL and the volume of triketone glands was 1.3 ± 0.1 nL, but the total contents identified from an average gland was 0.11 µg. Also, it is noteworthy that these two gland types did not have any of the NVCs discovered from other species in this study. The question then arises how the secretory cells in the wall of glands in these species are protected from the auto-toxicity of the sesquiterpenes and triketones they contain. No MAGEs or flavonoids were detected in the two species *E. brevistylis* or *E. suberea*.

Therefore, future research should focus on fully characterising the NVCs localised to the glands.

Investigations on the spatial arrangement of flavonoids inside the gland will be important to gain further insights about their physiological role. MAGEs and flavanones may play the same role in the two subgenera as a protective barrier in oil glands in addition to a possible function in plant defence. Both compound groups have amphiphilic properties allowing them to interact with lipophilic oil components and hydrophilic cell wall substances. However, it is unknown whether flavanones are also restricted to the cavity periphery similar to what has been observed for MAGEs (Heskes *et al.* 2012). A recent study suggests that some FPCs are also localised to the periphery of glands based on the presence of mass fragment ions in the outer perimeter of the glands (Santos *et al.* 2019). Therefore, it is crucial to investigate the spatial arrangement of flavonoids in *Eucalyptus* oil glands.

5.5. Conclusion

Overall, the research presented in this thesis shows that *Eucalyptus* foliar oil glands are rich in NVCs including MAGEs and flavanones. In addition to monoterpenes and sesquiterpenes, oil glands of some species contain volatile β -triketones, which belong to a rare group of PSMs in nature. Metabolic specialisation of glands to biosynthesise and store β -triketones in separate glands to the majority of sesquiterpenes is a major novel finding of this work. The use of different types of glands may help with characterising the biosynthetic pathways of the different compound classes in future research. Similarly, the discovery of new suites of NVCs in glands may help biosynthetic studies and possibly open avenues for the development of novel natural products. Overall, the results presented in this thesis contribute substantially to our limited understanding of *Eucalyptus* oil gland chemistry and biology.

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Appendix A

Composition of media used for callus and cell suspensions of *Eucalyptus polybractea* and *E. froggattii*.

Components	Amounts in culture media	
	M1	M2
Lloyd & McCown woody plant basal salt mixture (g L ⁻¹)	2.3	2.3
Murashige & Skoog modified vitamins (x1000; mL L ⁻¹)	1.0	1.0
Sucrose (g L ⁻¹)	25	25
NAA (mg L ⁻¹)	0.10	0.02
TDZ (mg L ⁻¹)	-	0.66
BAP (mg L ⁻¹)	0.50	-
Gelrite (g L ⁻¹)*	2.5	2.5

* Gelrite was used for solid media only.

Appendix B

NMR (600 MHz) results for pinocembrin and C-methylated flavanones extracted from *E. subg. Eucalyptus* as compared to literature values. Deuterated chloroform was used as solvent.

Compound	δ (ppm) Multiplicity coupling constants (Hz)								
	H-2	H-3a	H-3b	H-5	H-6	H-7	H-8	Me-1	Me-2
Pinocembrin extracted from <i>Eucalyptus</i>	5.36	2.76	3.02	-	5.92	-	5.94	-	-
	dd	dd	dd		d		d		
	3.0	3.1	13.1		2.2		2.2		
	13.0	17.1	17.1						
Pinocembrin literature values	5.42	2.76	3.09	-	5.92	-	5.94	-	-
	dd	dd	dd		d		d		
	3.0	3.0	12.6		2.1		2.1		
	13.0	17.1	17.1						
6-C-Methylpinocembrin extracted from <i>Eucalyptus</i>	5.44	2.85	3.04	-	-	-	6.01	2.05	-
	dd	dd	dd				s	s	
	3.0	3.1	12.9						
	12.9	17.1	17.1						
6-C-Methylpinocembrin literature values	5.42	2.8-3.4	2.8-	-	-	-	6.02	2.05	-
	dd	m	3.4						
	5.0		m						
	12.0								
6,8-C-Dimethylpinocembrin extracted from <i>Eucalyptus</i>	5.41	2.85	3.04	-	-	-	-	2.079	2.084
	dd	dd	dd					s	s
	3.0	3.1	12.9						
	12.9	17.0	17.0						
6,8-C-Dimethylpinocembrin literature values	5.40	2.85	3.04	-	-	-	-	2.07	2.08
	dd	dd	dd					s	s
	3.3	3.1	12.1						
	12.1	17.1	17.1						

Appendix C

Leaf gland parameters of the species selected for quantification of flavanones in Chapter 3.

Species # *	Species	Leaf surface area (cm ²)	Gland density (glands cm ⁻¹)
19	<i>Eucalyptus stellulata</i>	6	560
20	<i>E. oreades</i>	23	580
21	<i>E. agglomerata</i>	14	816
22	<i>E. gregsoniana</i>	10	1400
23	<i>E. falciformis</i>	16	800
24	<i>E. nitida</i>	11	830
25	<i>E. approximans</i>	6	260
26	<i>E. apiculata</i>	4	530
27	<i>E. dendromorpha</i>	14	576
29	<i>E. suberea</i>	7	2100
30	<i>E. brevistylis</i>	14	600

*The species numbers allocated in this table are the same numbers allocated for each species in Chapter 2.