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The potential of bioactive constituents of *Eucalyptus* foliage as non-wood products from plantations

A report for the RIRDC/Land & Water Australia/FWPRDC/MDBC Joint Venture Agroforestry Program

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November 2004

RIRDC Publication No 04/154
RIRDC Project No ANU-56A

ISBN 1 74151 062 7
ISSN 1440-6845

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Published in November 2004
Printed on environmentally friendly paper by Canprint

Foreword

A range of perennial plant species which occur, or are suited to growth in low rainfall environments of Australia are currently being screened for their potential to produce products other than wood. Uses such as fodder, new compounds and secondary products from extracts such as *Eucalyptus* oil, provide the opportunity to obtain multiple products from plantations and other on-farm tree-planting designs. This is especially important for low rainfall areas where the scale of planting of deep-rooted perennials which is required to reduce the effects of dryland salinity, dictates that we search for on-farm commercial solutions together with conservation and environmental services.

Formylated phloroglucinol compounds show a range of biological activities such as antifouling effects, antibacterial actions and antiviral properties. To test these compounds at commercial scale requires, however, identification of the best species, provenances and extraction methods to obtain the quantities required. This project surveyed a range of current and prospective low rainfall eucalypt and *Melaleuca* species for the relative content of formylated phloroglucinol compounds in the foliage. Preliminary analysis of different extraction methods was also undertaken.

This project was funded by the Joint Venture Agroforestry Program (JVAP), which is supported by three R&D Corporations — Rural Industries Research and Development Corporation (RIRDC), Land & Water Australia, and Forest and Wood Products Research and Development Corporation (FWPRDC), together with the Murray-Darling Basin Commission (MDBC). These agencies are funded principally by the Australian Government.

This report, a new addition to RIRDC's diverse range of over 1000 research publications, forms part of our Agroforestry and Farm Forestry R&D program, which aims to integrate sustainable and productive agroforestry within Australian farming systems.

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Simon Hearn
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Acknowledgements

This is a joint research project between The Australian National University and Phytochemical Services. We thank Dr Ian Wallis (ANU), Dr Joe Brophy (UNSW), and Dr Rick Willis (Australian Institute of Marine Science) for access to analytical instrumentation and for help in analysis of samples in different parts of this study. For their help in collecting plant material we are grateful to Dr Deane Nicolle (University of Adelaide), Mr John Bartle (CALM, WA), Mr D. Cooper (CALM WA), Mr Geoff Davis (G.R. Davis Pty Ltd) and Mr Ian Cowie (Northern Territory Herbarium). In understanding the range of material being planted in low-rainfall experiments we are grateful for the advice of Dr David Bush (ALRTIG Co-ordinator, CSIRO Forestry and Forest Products Canberra), Dr Rod Bird (Agriculture Victoria), Dr Christine Stone (State Forests of NSW), Dr Paul Ryan (DPI Queensland) and Dr Rod Keenan (Bureau of Rural Sciences). Dr Bart Eschler (ANU) carried out the cyclodextrin and large-scale extraction studies. In that regard, we are grateful to Professor Chris Easton for a sample of β-cyclodextrin and Dr Steve Colgate for discussion about cyclodextrins as agents for trapping organic toxins. The detailed studies of Eucalyptus loxophleba were conducted in collaboration with Mr John Bartle and Dr Peter Grayling (CALM WA).

Abbreviations

ALRTIG	Australian Low Rainfall Tree Improvement Group
ANU	Australian National University
CALM	Department of Conservation & Land Management, Western Australia
DM	Dry matter
FPC	Formylated Phloroglucinol Compound
GC-MS	Gas Chromatography-Mass spectrometry
HPLC	High Pressure Liquid Chromatography
MeOH	Methanol
TLC	Thin Layer Chromatography
UNSW	University of New South Wales

Glossary

angiotensin	A neuroactive hormone involved in the regulation of blood osmolarity and water balance, and in drinking behaviour
chromatography	The separation of chemical substances by making use of differences in the rates at which the substances travel through or along a stationary medium
cohobation	A method of steam distillation in which the water is separated from the distillate and returned to the vessel holding the sample
Da	Dalton (a unit of mass of a molecule)
eluting	The process of extracting one material from another by washing with a solvent to remove adsorbed material from an adsorbent
eV	Electron volt (a unit of energy)
extract	A solution obtained by soaking a substance
Fourier transformation	As used here, a mathematical technique for increasing the sensitivity of mass spectrometric analysis
hydrophilic	Having a strong affinity for water
lipophilic	Having a strong affinity for fats or oils
mass spectrometry	An analytical method that allows for the identification of components of a mixture by separating the molecules by their mass
moiety	A component part of a complex molecule
solvent	A liquid used to dissolve another substance
sonication	Using high frequency sound waves to assist in dissolving a substance

Contents

Executive summary

Plantations of deep-rooted native vegetation in low-rainfall areas of Australia yield many environmental benefits including a reduction in the height of the water table and consequently in ground salinity. These plantings also fix CO_2 and potentially increase biodiversity. Tree planting is encouraged if there is clear financial return in the form of saw logs, wood for the production of charcoal or biological products such as essential oils. This project, conducted jointly between the Australian National University (Dr W. J. Foley) and Phytochemical Services (Dr E.V. Lassak), sought to identify sources of new bioactive products that might provide another income stream from plantings of low-rainfall eucalypts.

We focused attention on a group of recently described compounds from *Eucalyptus* known as formylated phloroglucinol compounds (FPCs) that show a wide range of biological activities including potency as antifouling agents, tumour suppression and antibacterial and antiviral properties.

We surveyed 39 species of eucalypt and four species of *Melaleuca* that are either the main plantation species in low-rainfall areas or else have been investigated as having potential for planting in these environments. We used mass spectrometry and a variety of chromatographic techniques to survey extracts of these plants for FPCs and in some cases for essential oils.

We identified several rich sources of FPCs particularly amongst some Western Australian oil mallees. Most notable was *E. loxophleba*, with sideroxylonal concentrations as high as 9% of the dry leaf mass – the richest source of sideroxylonal recorded. Sideroxylonal was the most abundant compound recorded with high concentrations also observed in *E. cinerea*, *E. pulverulenta* and *E. mannifera*. Large quantities of macrocarpals were observed in *E. kartzoffiana* and *E. pulverulenta* as well as in some *E. viminalis*.

In those species that contain sideroxylonals there is a strong association between the concentration of 1,8-cineole and the concentration of FPCs, suggesting that selection for cineole (as is currently occurring in *E. loxophleba*) will result also in selection for FPCs.

We investigated several methods for extracting and purifying FPCs from a medium scale extraction (i.e. 1 kg of dry leaves). The success of these procedures depended on the target compound. Large amounts of jensenone could be extracted and purified readily from *E. jensenii* whereas moderate amounts of macrocarpals could be purified from *E. viminalis*. Sideroxylonals could only be obtained sparingly. Our attempts to purify FPCs using two different types of cyclodextrins were unsuccessful.

There is no doubt that there are rich sources of bioactive products available in existing low-rainfall plantings. However, better ways of preparing enriched and purified extracts must be found before enough of these products can be isolated for research into their uses with a view to eventually marketing them. One method that should be investigated further is the use of heated and pressurized water since this approach is environmentally friendly, fast and compatible with industrial-scale operations.

Introduction

Chemical constituents of *Eucalyptus*

Eucalyptus contains many chemical compounds that play several roles in the plant. These include defence against insect and vertebrate herbivores and protection against UV radiation and against cold stress. The best-known compounds are the terpenoids, which form most of the essential oil giving *Eucalyptus* foliage its characteristic smell. However, Eucalyptus is also a rich source of phenolic constituents such as tannins and simpler phenolics. Some of these have formed the basis of industries in the past. For example, tannins were extracted from *Eucalyptus astringens* and rutin from *Eucalyptus macrorhyncha* (Lassak and McCarthy 1992).

However, the most recent interest in phenolic compounds from *Eucalyptus* has focused on a newly identified group called the formylated phloroglucinol compounds (FPCs) (Lawler et al. 2000). This grouping includes the subtypes known informally as euglobals, macrocarpals and sideroxylonals. All FPCs have the same fully substituted, formylated, aromatic moiety, but vary in the structure of the side chain. In macrocarpals and euglobals the sidechain is a C_{10} or C_{15} unit derived from common foliar terpenes such as bicyclogermacrene, α-pinene or β-phellandrene but in the sideroxylonals and the simple FPCs (e.g. jensenone), the side chain is a C_5 unit (Ghisalberti 1996).

Formylated phloroglucinol compounds have a wide range of biological actions such as antifouling properties (Singh et al. 1996, Terada et al. 1999), antibacterial activity (Murata et al. 1990), inhibitory activity of HIV-Rtase (Nishizawa et al. (1992), angiotensin-converting enzyme, aldose reductase (Murata et al. 1992), tumour inhibition (Takasaki et al. 1995), and glucosyltransferase (Osawa et al. 1998). In addition, they play a major ecological role in Australian forests since they act as powerful antifeedants against insect and marsupial herbivores (Pass et al. 1998, Lawler et al. 2000).

Formylated phloroglucinol compounds probably do not occur in all eucalypts. Preliminary studies (Eschler et al. 2000) suggest that FPCs are concentrated in the informal subgenus *Symphyomyrtus*. They seem to be absent from the informal subgenus *Monocalyptus* and from the monospecific informal subgenus *Idiogenes* (*Eucalyptus cloeziana*). The compounds occur sparingly and at low concentrations in the *Corymbia* and *Blakella* grouping (bloodwoods and paper fruited bloodwoods). The sole member of the informal subgenus *Eudesmia* surveyed (*Eucalyptus phoenicia*) appeared rich in euglobals. Although it is clear that more species need to be surveyed, the preliminary results suggest that research should concentrate on the informal subgenus *Symphyomyrtus*.

The relationship between FPC concentrations and essential oils in *Eucalyptus*

Preliminary studies reported a strong correlation between foliar concentrations of the commercially valuable terpene 1,8-cineole and sideroxylonal in *E. polyanthemos* (Lawler et al. 2000). This correlation is important ecologically because marsupial folivores use the concentration of the cineole as a cue to the concentration of the FPC. In other words, if they detect high concentrations of cineole they will eat little, if any, of

the foliage. Likewise, low concentrations of cineole suggest to the folivore that the foliage is palatable (Lawler et al. 1999). If this relationship is confirmed in other species of *Eucalyptus* it suggests that any selection towards increasing the concentrations of essential oils might also yield similar increases in the concentrations of FPCs. In contrast, there are some indications that eucalypts that have been selected for rapid growth rate may contain lower concentrations of FPCs than do conspecifics with an inherently lower growth rate (Eschler et al. 2000).

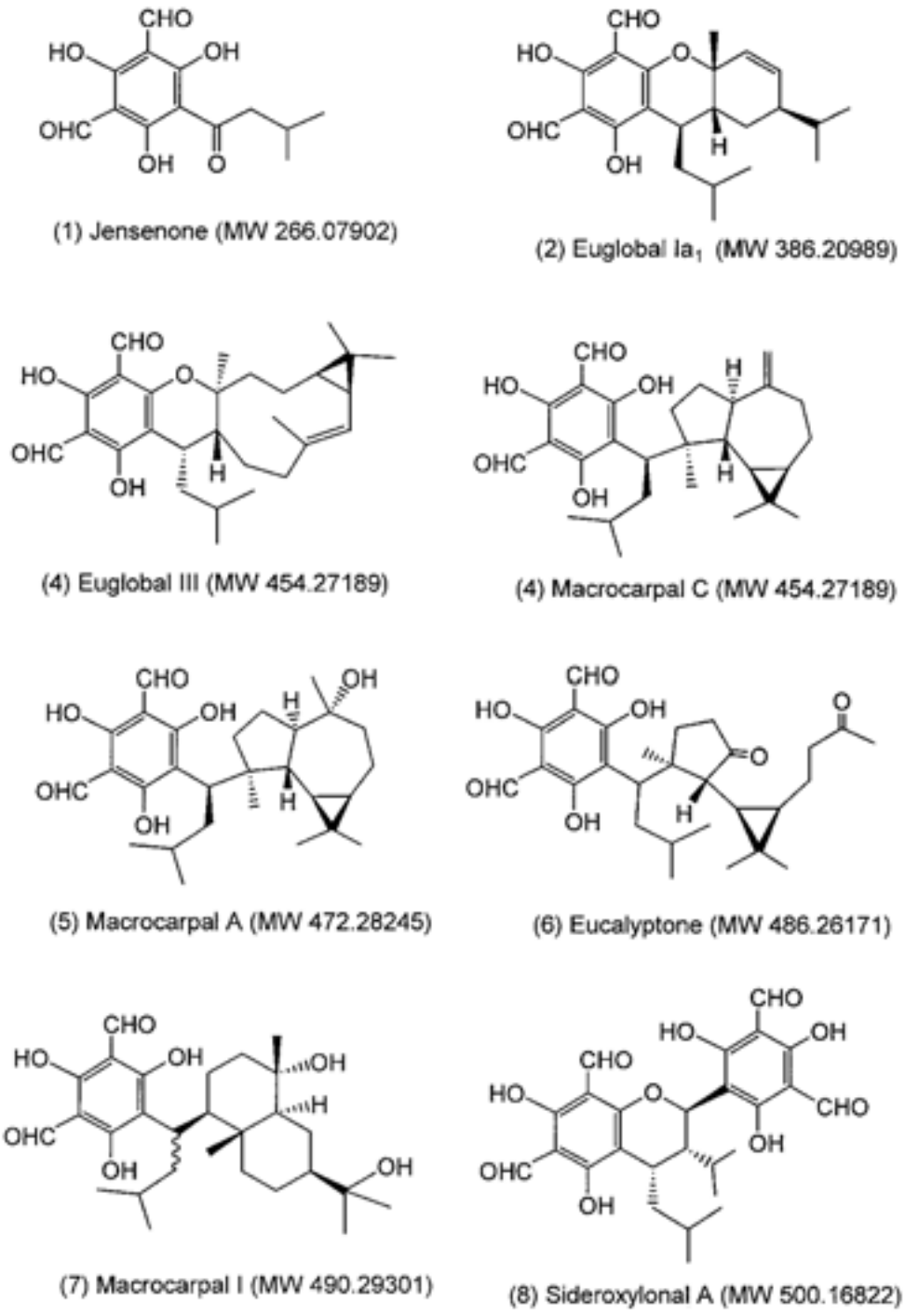

Figure 1. Structures of some known formylated phloroglucinol compounds isolated from *Eucalyptus* with their molecular weights

Rationale for the project

The *Eucalyptus* plantation industry is growing at more than 10% per annum and has been identified by all levels of government as the most ecologically and socially desirable way to produce forest products. Consequently, large sums of public and private money are being committed to the industry. Several investigative studies have indicated that *Eucalyptus* plantations can produce chemical compounds that have potential medical or other industrial applications (Wondu 2000). The compounds specifically considered are the macrocarpals, euglobals and sideroxylonals. These compounds are probably restricted to *Eucalyptus*, although there is one report of a single compound in the genus *Choriocarpa* – another Myrtaceous Australian plant (Brophy et al. 1994).

There has been intense interest in these compounds, mainly from Japanese sources, resulting in the issuing of many patents covering their extraction, synthesis and applications. The applications are diverse and include distinct potential markets. Sideroxylonals are the most potent natural anti-fouling agents known (Singh et al. 1996). Natural antifouling agents are sought to replace the use of toxic tin compounds in Australian waterways. Several different macrocarpals have been incorporated into mouthwashes, toothpastes and skin creams. Finally, the diverse biological actions of these compounds suggest other medical uses. Of all these uses it seems as though the markets in antifouling agents and personal care through skin creams and antibacterial mouthwashes are most attainable.

The feasibility of developing a new non-wood product from *Eucalyptus* leaves would enhance the attractiveness of growing *Eucalyptus* in low-rainfall areas. In particular, the eucalypts being grown for the production of essential oils are especially attractive. First, the foliage of mallee species such as *E. horistes* and *E. polybractea*, grows quickly. Secondly, the foliage is already harvested and extracting an additional product from the same foliage is an attractive proposition.

This project had three principal objectives:

1. We aimed to survey, using a variety of chemical techniques, the foliage from a range of *Eucalyptus* species that are currently being exploited or else have been considered for planting in low-rainfall areas of Australia.
2. We sought to establish whether plants containing commercially valuable essential oils also contained rich concentrations of other bioactive metabolites such as FPCs.
3. We aimed to test several methods of producing enriched extracts of FPCs for market development.

Methods

Rationale for selection of species

We chose the species to include in this survey on the basis that they were either currently planted in low-rainfall areas or else being investigated by other organisations as having potential for planting in low-rainfall areas. Still other species were collected because they exhibit special characteristics. For example, *Eucalyptus mannifera* grows widely on the southern tablelands of NSW but is unattractive to marsupial folivores, suggesting that its foliage might contain high concentrations of secondary chemicals. We recognized from the outset that markets for these compounds had not yet developed and so, at this stage, they were unlikely to be the primary determinant for new plantings. However, by identifying the compounds as potential second income streams from existing plantings, we hoped to enhance the viability of those planting. We restricted our studies to eucalypts in the *Symphyomyrtus* and *Corymbia* groupings and excluded those in the subgenus *Monocalyptus* (= subgenus *Eucalyptus*) because previous studies had not detected FPCs in species from this grouping (Eschler et al. 2000).

Accordingly we investigated species that were subject of genetic improvement experiments by the Australian Low-Rainfall Tree Improvement Group (ALRTIG) (http://www.ffp.csiro.au/alrtig/). The species being investigated have potential to provide genetically improved eucalypts for farm forestry in the 400-600 mm rainfall zone, particularly in areas of intensive cropping where significant clearing of native vegetation has occurred. The ALRTIG group has chosen five eucalypts as the focus of their studies of hardwoods. These are: *Corymbia maculata* (spotted gum), *Eucalyptus sideroxylon* and *E. tricarpa* (red ironbarks), *E. occidentalis* (swamp yate), *E. cladocalyx* (sugar gum) and *E. camaldulensis* (river red gum).

Secondly we focused on the oil mallees that are planted in south-western NSW (*E. polybractea* and *E. horistes*) and also in Western Australia. The latter were identified by the Oil Mallee Project, which aims to develop an industry based on planted eucalypts that can provide essential oils, charcoal and electricity in areas of dryland cropping in Western Australia. In south-western NSW, the emphasis is on the production of 1,8-cineole-rich oils for medicinal and other markets. We obtained 13 eucalypt taxa (12 species) that are currently being planted in Western Australia and two species from south-western NSW. We also obtained four *Melaleuca* species from NSW plantations. Although FPCs have not been recorded from *Melaleuca*, their extensive plantings, especially *M. alternifolia*, for the production of medicinal oils in Australia warrant their inclusion in this survey.

Finally, we investigated a group of species that have been either tested in trials for their potential in low-rainfall environments, mostly in south-eastern Australia, or else are thought to be potentially useful in these regions. We identified these species through literature reviews (e.g. RIRDC), discussions with researchers familiar with farm forestry experiments in Victoria (e.g. Dr Rod Bird), NSW (Dr Christine Stone) and Queensland (Dr Rod Keenan) and by searching for species lists in a range of published and unpublished sources. We obtained a further 21 species from these sources giving a total of 39 species of *Eucalyptus* and four species of *Melaleuca*.

Finally, our initial results identified very high concentrations of sideroxylonals in *E. loxophleba* (York Gum) from Western Australia. Therefore we investigated this

species in more detail in collaboration with Mr John Bartle and Dr Peter Grayling of the CALM (WA) Revegetation Systems Unit.

Collection and treatment of foliage samples

We obtained samples of foliage from all species from between four and eight individual trees of each species. Through earlier work we knew that the concentrations of FPCs vary widely between individuals within a species, so collecting foliage from multiple trees of each species was essential. However, analysing all of these samples would be prohibitively expensive so in compromise we analysed composite samples. This ensured that we would detect FPCs if they occurred in a species while minimising the high analytical costs. The treatment of samples depended on their point of collection. Those obtained locally were divided. One part was frozen and later subjected to steam distillation for the extraction of terpenes using whole leaves. The other part was freeze-dried and then ground to pass a 1mm sieve in a Cyclotec 1093 Mill (Tecator, Sweden) for later extraction of FPCs. Samples sent to us by various collaborators were air-dried immediately after collection and, on arrival, either ground as described above or left whole for steam-distillation.

We extracted terpenes from 100 g samples of whole, air-dried leaf by steam distillation with cohobation. In this process, the volatile components are released from the plant, vaporize and are present in the steam. The steam and vapour condense back to a liquid state. Due to differences in density, the terpenes separate from the water. The separated water contains water-soluble essential oil components and is termed the hydrosol, or hydrolate. In the interests of yield efficiency, this distillation water is returned in many designs of distillation and this process is termed distillation with cohobation. This is the method used for the commercial distillation of essential oils. Although it is likely that this method results in the rearrangement of some components, we aimed to choose methods that were likely to reflect the oils obtainable commercially.

Chemical methods for identification of formylated phloroglucinol compounds and terpenes in *Eucalyptus*

Rationale for the methods chosen

Our approach in this work was to conduct a rapid survey of the occurrence of FPCs in a range of eucalypts and then to conduct more detailed studies of a subset of species that appeared promising from the survey results. There has been little study of the FPCs to date (the compounds were only discovered in the late 1980s and early 1990s) and consequently there is no rapid and simple method to determine whether they are present in a particular sample of *Eucalyptus* foliage. We used a method based on mass spectrometry to detect the compounds. In this approach, we use high resolution mass spectrometry to search crude extracts of *Eucalyptus* leaves for molecular masses exactly the same as those of isolated and identified FPCs. Although it remains possible that there are other unknown compounds that have the same mass as FPCs, measurement of an exact molecular mass corresponding to that of known FPCs is good evidence that they are present.

We then chose a subset of samples that appeared promising and extracted the volatile oil component in a way that was consistent with commercial operations. We then used chromatography and mass spectrometry to separate and identify the components of

these complex oils. Finally, we used another chromatographic method (HPLC) to separate and quantify the amount of FPC in the target species. Since this procedure produced data that were readily interpreted, we applied the method to all species collected as a check on the original survey results.

Detailed description of chemical methods used

Extraction of formylated phloroglucinol compounds

Formylated phloroglucinol compounds vary in their polarity but are essentially insoluble in polar solvents such as alcohol and water. We used a mixture of 20% acetone in light petroleum spirit to span the range of polarities expected in the structures of FPCs. To obtain an FPC-rich extract, 1.5500 ± 0.0500 g of dried, ground leaf was weighed into a cellulose extraction thimble (80 x 20 mm, Whatman) and refluxed with 100 mL of 4:1 light petroleum spirit:acetone in a Soxhlet extractor (40 mL siphoning volume) connected to a 250 mL round-bottom flask, heated on a water bath (85°C) for 4 h. After refluxing ceased, the solvent was removed by rotary evaporation at 50°C. The resulting crude extract was transferred quantitatively into a pre-weighed 20 mL glass vial with 4:1 dichloromethane:methanol, which was dried under a stream of air for 24 h and then left exposed to air in a fumehood for a further 48 h before being reweighed. The extract was then scraped from the walls and bottom of the vial and transferred to a second, clean vial which was capped and stored in the dark. Subsequent extraction of the leaf residue with methanol and examination of the extract by TLC showed no evidence of FPC compounds when treated with the ketone/aldehyde specific stain, 2,4-dinitrophenyl hydrazine/phosphoric acid.

Chromatographic separation of formylated phloroglucinol compounds (HPLC)

The chromatographic separations were carried out on an SGE International Pty Ltd (NSW, Australia) 250 x 4.0 mm GL Wakosil II 3C18RS 3 μm column connected to a Waters Alliance HPLC system consisting of a Waters 2690 separation module, an autosampler fitted with a 250 μL syringe and a 100 μL sample loop, and a Waters 996 diode array detector. Just prior to HPLC, 15 mg was weighed into a glass vial and dissolved by sonication in exactly 5 mL of 20% methanol in acetonitrile, containing the internal standard 2-ethyl phenol (1.0 g.L-1). The optimal separation was obtained with a gradient elution using acetonitrile (A) and water (B), both containing 0.1% trifluoroacetic acid. The flow rate was 0.75 mL/min with a column temperature of 40ºC and a run time of 15 min. The gradient was 0-5 min 60% A, 40% B; increasing linearly to 90% A, 10% B at 60 min where it remained until 70 min before declining to the starting conditions at 80 min. Typical injection volumes ranged from 10-25 μL.

Fourier Transform Ion Cyclon Resonance Mass Spectrometry

For FTMS analysis, about 5 mg of dried crude extract was dissolved in 10mL MeOH and 10 μL diluted to 1 ml in MeOH. This solution was continually infused at a flow rate of 1μL min-1 into the external electrospray source (Analytica of Bradford, Bradford, CT) of a Bruker BioApex 47e Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTMS) operating in negative ion mode with broadband (low resolution (6-10 k FWHM at m/z 500)) detection. Typically, the signal was averaged over 16 transients prior to Fourier transformation, requiring a data acquisition time of about 1 min, and the consumption of about 1 μg of the crude extract. Since the nature of the compounds we expected to detect was well known from previous investigations, no attempt was made for precise calibration or accurate mass measurement, measured mass

within 0.05 Da being considered an acceptable confirmation of the presence of the compounds of interest.

Chromatographic separation & mass spectrometric identification of terpenes (GC-MS)

We used gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS) to separate and identify components of these oils. Analytical gas chromatography was carried out on a Shimadzu GC17A gas chromatograph. A Megabore column of DB-Wax (60 m x 0.5 mm x 1μm) which was programmed from 50 to 220^{o}C at 3^{o}C/min was used with helium carrier gas. GC integrations were performed on a SMAD electronic integrator. GC-MS was performed on a VG Quattro mass spectrometer operating at 70 eV ionisation energy. The GC column used was a DB-Wax (60 m x 0.32 mm x 0.25 um) programmed from 35 to 220^{o}C at 3^{o}C/min with helium as carrier gas. Mono- and sesquiterpenes were identified by their identical GC retention time relative to known compounds and by comparison of their mass spectra with either known compounds or published spectra (Adams 1995; Stenhagen et al. 1974; Heller and Milne 1978, 1980, 1983; Swigar and Silverstein 1981; Joulain and König 1998).

Improved methods of extraction and purification of FPCs from *Eucalyptus*

If FPCs are to be of any commercially use, it is necessary to find methods to either purify the compounds or else prepare enriched extracts. The purification/enrichment experiments in this project focused on species that contained large concentrations of the compounds and which were readily available locally since each experiment required more than one kg of dry foliage. We investigated several methods of extracting the compounds using either steam distillation or organic solvents. We then used these extracts to test two separate methods of purifying the resultant extracts. These were either the standard method practised from the time of discovery of the compounds which involves extensive column chromatography on silica, or a cheaper and quicker method, using base/acid extraction. We recognized from the outset that this second procedure was unlikely to produce a highly purified extract but its simplicity was attractive.

We studied the extraction of the simple FPC jensenone from *E. jensenii*, macrocarpals from *E. viminalis*, and sideroxylonal from *E. melliodora*. In each case, the plant material (1 kg dry mass) was soaked in 8 L of solvent in a 20L flask and mixed periodically over the next 3 days. The solvent was then removed by filtration and concentrated by evaporation. An additional volume of solvent was added and these washings were removed and added to the first extract. Steam-distillation (only for *E. jensenii*) was carried out by immersing 1 kg of foliage in water in a 20L flask and boiling it for 24 hr. The distillate was collected from which jensenone was filtered.

In a second series of experiments, we studied whether FPCs could be purified with the aid of cyclodextrins. These are cyclic sugar-based compounds that form a truncated cone structure that is hydrophilic on the exterior but lipophilic on the interior surface. Provided they are smaller than the cyclodextrin molecule, lipophilic organic compounds such as macrocarpals and euglobals should be trapped in the interior of the cyclodextrin molecule and thus be removed selectively from the extract. We studied the ability of

various molar ratios of two types of cyclodextrins (β-cyclodextrin and β-methyl cyclodextrin) to selectively remove macrocarpals from an enriched extract of *E. viminalis* foliage.

Detailed study of *Eucalyptus loxophleba*

The high concentrations of sideroxylonal observed in *Eucalyptus loxophleba* ssp. *lissophloia* in our initial experiments prompted us to investigate this species in more detail. *E. loxophleba* is already the subject of genetic improvement to select material that yields a large amount of 1,8-cineole as part of the Western Australian Oil Mallee programme (Wildy et al. 2000). In collaboration with Mr John Bartle of CALM Revegetation Systems Unit we collected foliage from 60 trees that were planted as part of a replicated progeny experiment at Toollbin in Western Australia. These trees were open-pollinated progeny grown from parents that had yielded the highest amount of cineole in a broader survey of *E. loxophleba* throughout its range.

Foliage was sampled from a single tree of each family and a subsample retained for the analysis of 1,8-cineole by Dr Peter Grayling (CALM, WA) using the method described by Ammon et al. (1985). We oven-dried the remaining foliage at 35^{o}C for 4 d. This material was then ground in liquid nitrogen to pass a 1 mm screen in a cyclone grinder and the sideroxylonal concentration determined as described above.

Results

Survey of formylated phloroglucinol compounds in various taxa of *Eucalyptus* and *Melaleuca* (Tables 1 and 2)

The notable feature of the analysis was the general absence of FPCs and related compounds in many of the species analysed. We did not detect any FPCs in the four samples of *Melaleuca*, although there was a trace of an unknown substance eluting at 13.3 minutes that may be an FPC. Of the 37 eucalypts, 16 did not contain any FPCs or they contained only traces of compound, especially the unknown compound that elutes at 11.3 minutes. Compounds that eluted at 20.3 and 24.8 minutes were seen in several samples. The former is unknown, while the latter may be traces of jensenone although we often observe another peak co-eluting with jensenone. Five of the eucalypt samples contained measurable but very low concentrations (<5 mg per g DM) of FPCs. These were *E. viridis* and *E. occidentalis* (which contained traces of macrocarpals) and *E. cneorifolia*, *E. horistes* and *E. argophloia* (sideroxylonals).

Of the 16 samples that contained notable concentrations of FPCs (>5 mg per g DM), those rich in sideroxylonals prevailed (*E. cinerea, E. leptopoda, E. loxophleba, E. myriadena, E. pulverulenta, E. mannifera, E. brookeriana, E. cornuta*, and *E. tricarpa*). Among these, *E. cinerea, E. mannifera, E. pulverulenta* and *E. loxophleba* all had extremely high concentrations of sideroxylonals (>30 mg per g DM). The latter deserves further mention because both of the subspecies analysed (*E. l. gratiae* and *E. l. lissophloia*) both had concentrations of sideroxylonals totaling more than 4% of the dry leaf. Similarly, *E. pulverulenta* is notable because its leaves also contained 7.0 mg per g DM of macrocarpal G.

Several trees had a mixture of macrocarpals and sideroxylonals with a total concentration of between 10 and 20 mg per g DM (*E. vegrandis, E. dorrigoensis, E. kartzoffiana* and *E. porosa*). In no case did the concentration of macrocarpals exceed 18 mg per g DM – that of *E. kartzoffiana* where the macrocarpal G (12 mg per g DM) dominated the profile.

Table 1: The occurrence of formylated phloroglucinol compounds in a range of low-rainfall eucalypts and melaleucas as inferred by observation of exact molecular masses in petrol-acetone extracts of each species**.** '+' indicates definite presence. '-' indicates absence. '+/-' indicates probable presence.

Species	code	Grandinol	Jensenone	Monoterpene Euglobals & Macrocarpals	Sesquiterpene Euglobals & Macrocarpals	Macrocarpals A,B,D,E,F,H	Eucalyptone (Macrocarpal am-1)	Macrocarpal I, J	Sideroxylonal
		251.0915	265.0708	385.2007	453.2631	471.2736	485.2529	489.2841	499.1596
ALRTIG Hardwoods									
Corymbia maculata	CC1	-	-	-	-	-	-	-	-
E. cladocalyx	CC6	+/-	-	+	+/-	-	+/-	+/-	+
E. camaldulensis	BM1	+	-	+	+	+	+	-	+
E. occidentalis	CC17	-	-	+/-	+/-	-	_		+
E. tricarpa	CC20	-	-	-	-	-	-	-	+
WA Oil Mallees									
E. angustissima	EL1A	-	-	+/-	+/-	-	-	-	-
E. cinerea	EL12	+	-	+	+		-	-	+
E. cneorifolia	EL13	+/-		+/-	+	-	-	-	+
E. horistes	EL15	+	-	+/-	+/-	-	-	-	+
E. kochii	EL16	+/-	-	-	+/-	_	-	-	+
E. leptopoda	EL17	+		+	+	+/-	+/-	+	+
E. loxophleba ssp. *gratiae*	EL18	+	-	-	-	-	-	-	+
E. loxophleba ssp. *lissophloia*	EL19	+	-	-	-	-	-	-	+
E. myriadena	EL20	+	-	-	+/-	-	-	-	+
E. plenissima	EL21	-	-	+	+	-	-	-	-
E. polybractea	EL22	-	-	+/-	-	-	-	-	-

Table 1 (cont)

Species	code	Grandinol 251.0915	Jensenone 265.0708	Monoterpene Euglobals & Macrocarpals 385.2007	Sesquiterpene Euglobals & Macrocarpals 453.2631	Macrocarpals A,B,D,E,F,H 471.2736	Eucalyptone (Macrocarpal am-1) 485.2529	Macrocapal I,J 489.2841	Sideroxylonal 499.1596
E. pulverulenta	EL23	+	-	+	+	+	-	-	+
E. vegrandis	EL24	+		+	+	+/-	+	+/-	+
NSW Oil Mallees									
E. polybractea (Prov 1)	EL1	+	-	+	+	+	+	-	+/-
E. polybractea (Prov 2)	EL2	+	-	+	+	-	-	-	-
E. polybractea (Prov 3)	EL3								
E. viridis (Prov 1)	EL6	+/-	-	+/-	+	+	+	+	+
E. viridis (Prov 2)	EL7	-	-	-	+	+	+	-	-
Cultivated Oil Bearing Non-Eucalypts									
Melaleuca alternifolia	EL8	-	-	-	-	-	-	-	-
M. linariifolia	EL9	-	-	-	-	-	-	-	-
M. dissitiflora	EL10		-	-	-	-	-	-	-
M. uncinata	EL11	-	-	-	-	-	-	-	-
Other Potential Low-rainfall Eucalypts									
Corymbia eximia	EL14	-	-	-	-	-	-	-	-
E. mannifera	EL25	-	-	-	+/-	-	-	-	+
E. macarthurii	BM4	-	-	-	-	-	-	-	+

Table 1 (cont).

Species	Code	Grandinol 251.0915	Jensenone 265.0708	Monoterpene Euglobals & Macrocarpals 385.2007	Sesquiterpene Euglobals & Macrocarpals 453.2631	Macrocarpals A,B,D,E,F,H 471.2736	Eucalyptone (Macrocarpal am-1) 485.2529	Macrocarpal I,J 489.2841	Sideroxylonal 499.1596
E. albens	CC2	-	-	-	+/-	-	-	-	-
E. argophloia	CC3	-	-	-	+/-	-	-	-	+/-
E. botryoides	CC4	+	_	+	+/-	-	+/-	-	+
E. brookeriana	CC5	+	+/-	+	+	+	-	-	+
E. coolabah ssp. *arida*	CC7	+	-	+	+	-	+/-	-	+/-
E. cornuta	CC8	+	+/-	+	-	-	-	-	+
E. dawsonii	CC9	+/-	-	+	-	-	-	-	+
E. dorrigoensis	CC10	+	+/-	+	+	+	+	+	+
E. dundasii	CC11	+/-	+/-	+	+	-	+/-	-	+/-
E. famelica	CC12	+/-	-	+/-	+/-	-	+/-	-	+/-
E. kartzoffiana	CC13	+	+	+	+	+	+	+	-
E. largiflorens	CC14	-	-	-	-	-	-	-	-
E. microcarpa	CC15	-	-	+/-	-	-	-	-	-
E. moluccana ssp. *queenslandica*	CC16	-	+/-	-	-	-	-	-	-
E. petiolaris	CC18	-	-	+	+	+	+	-	+
E. porosa	CC19	-	-	+	+	+	+	-	+
E. viminalis	BM3	-	-	+	+	+	+	-	+
E. jensenii	BM2	+/-	+	+	+/-	-	-	-	+/-

Table 2: The concentration of formylated phloroglucinol compounds in low-rainfall eucalypts and melaleucas measured by HPLC. 't' = trace; blank = not detected, M = major unknown compound.

Species	Peaks - retention time (minutes), identification and concentration (mg per g dry matter)											
	9.7	11.3	13.3	16.2	20.3	24.8	28.0	32.0	37.0	44.5	45.5	71.2
				Grandinol		Jensenone	Eucalyptone	Macrocarpal-A	Macrocarpal-B	Sideroxylonal A	Sideroxylonal C	Macrocarpal-G
ALRTIG Hardwoods												
Corymbia maculata		t			t	t						
E. cladocalyx		t				t						
E. camaldulensis												
E. occidentalis	t							0.5	0.3			2.3
E. tricarpa										4.5	1	
WA Oil Mallees												
E. angustissima		t										
E. cinerea	t			0.6						25	8	1.9
E. cneorifolia		t				t				1.7		
E. horistes		t								1.5		
E. kochii		t								0.5		
E. leptopoda	t	t								4.0	1.5	0.8
E. loxophleba ssp. *gratiae*	t	t								36	9	
E. loxophleba ssp. *lissophloia*										44	16	
E. myriadena		t								16.5	6	
E. plenissima		t	t									
E. polybractea		t										

Table 2 (cont)

Species	Peaks - retention time (minutes), identification and concentration (mg per g dry matter)											
	9.7	11.3	13.3	16.2	20.3	24.8	28.0	32.0	37.0	44.5	45.5	71.2
				Grandinol		Jensenone	Eucalyptone	Macrocarpal-A	Macrocarpal-B	Sideroxylonal A	Sideroxylonal C	Macrocarpal-G
E. pulverulenta		t								24.5	8.5	7.0
E. vegrandis			t				1.5	2.0	1.2	2.3	0.7	4.5
NSW Oil Mallees												
E. polybractea		t										
E. polybractea												
E. polybractea		t										
E. viridis		t	t		M			0.8	0.5	0.6		2.0
E. viridis		t	t		M			0.8				2.2
Cultivated Oil Bearing Non-Eucalypts												
Melaleuca alternifolia			t									
M. linariifolia												
M. dissitiflora												
M. uncinata												
Other Potential Low-rainfall Eucalypts												
Corymbia eximia		t				t						
E. mannifera										8-35	2-12	
E. macarthurii												

Table 2 (cont)

Species	Peaks - retention time (minutes), identification and concentration (mg per g dry matter)											
	9.7	11.3	13.3	16.2	20.3	24.8	28.0	32.0	37.0	44.5	45.5	71.2
				Grandinol		Jensenone	Eucalyptone	Macrocarpal-A	Macrocarpal-B	Sideroxylonal A	Sideroxylonal C	Macrocarpal-G
E. albens		t										
E. argophloia	t	t		0.25						2.0	0.8	
E. botryoides		t			t	t						
E. brookeriana										16	5	
E. coolabah ssp. *arida*		t		t								
E. cornuta				0.8						9	3	
E. dawsonii		t	M									
E. dorrigoensis		t		0.3	t	t		2	1	4.5	1.0	5
E. dundasii		t										
E. famelica		t										
E. kartzoffiana		t			t	t	1.7	2.5	1.7			12
E. largiflorens		t										
E. microcarpa		t										
E. moluccana ssp. *queenslandica*						t						
E. petiolaris							1.4	1.8	1			5.3
E. porosa							1.2	1.3	0.9	9	3	2.5
E. viminalis												
E. jensenii		t				38						

Detailed study of *Eucalyptus loxophleba* (Figure 2)

The extensive survey confirmed that *E. loxophleba* contains greater concentrations of sideroxylonal than any other material that we have examined. However, there was significant variation amongst the 60 samples (see Figure 2). One tree contained 92 mg/g of sideroxylonal A + C whereas 6 samples contained almost no sideroxylonal.

Relationship between terpenes and FPCs and the characters of distilled oils (Tables 3 and 4 and Figure 3)

The yield, refractive index, optical rotation and distillation time of essential oils of all species investigated are described in Tables 3 and 4. There were insufficient species containing sideroxylonal to test whether there was a general relationship between the concentration of 1,8-cineole and the concentration of sideroxylonal across a range of species. However, within the species *E. viminalis* and *E. melliodora*, and in the more extensive set of *E. loxophleba* there was a strong correlation between the concentration of 1,8-cineole and the concentration of sideroxylonal (Figure 3).

Improved methods of isolation of formylated phloroglucinol compounds (Table 5)

Large scale extractions

The results from the large-scale extractions are shown in Table 5. For the simple FPC jensenone, the macrocarpals and the sideroxylonals, at least one of the large-scale procedure recovered reasonable amounts (30-43%) of the theoretical yield, determined using an analytical procedure with small amounts (ca 1.5 g) of dry ground leaf. There was no difference between the hot and cold extractions combined with base extraction for recovering jensenone (30% versus 28%), although steam distillation alone returned poor yields (13%). Similarly, hot solvent extraction did not apparently affect the yield of macrocarpals. However, the recovery of macrocarpals differed widely between the later work-up procedures with the base and standard extractions yielding 43% and 12% of the theoretical yield, respectively. In contrast to the extractions of both the jensenone and macrocarpal-rich material, the cold extraction failed to remove most of the sideroxylonals from the starting material. Any sideroxylonals removed by either the hot or the cold extractions were promptly hydrolysed by base extraction. The procedure that appeared to give reasonable yields (31%) was hot solvent extraction followed by the standard work-up procedure – column chromatography on silica.

Table 3. The percentage composition of the steam volatile oils of a range of low-rainfall eucalypts. 't' = trace; blank = not detected

	angustissima	***cinerea***	***cneorifolia***	***horistes***	***kochii***	***leptopoda***	***loxophleba* ssp. *lissophloia***	***loxophleba* ssp. *gratiae***	***myriadena***	***plenissima***	***polybractea***	***pulverulenta***	***vegrandis***	***viridis***	***eximia***
3-methylbutanal		0.7		t			0.8	0.2	0.3	-	t	0.7	t	t	-
α-pinene	0.4	4.3	0.3	4.0	1.9	6.2	13.9	15.2	23.6	0.9	4.7	5.5	15.0	7.4	12.2
4-methylpentyl-2-acetate							6.5	3.4							
β-pinene	0.1	t	t	2.0	1.9	0.2	t	t	0.3	1.2	0.5	0.1	0.4	0.3	1.1
sabinene	0.4		t	0.4	2.2		t	0.4		0.7	t				3.9
myrcene	t	t	t	0.4	0.6	0.2	0.5	0.7	0.2	0.1	0.2	0.3	0.2	0.2	2.2
α-phellandrene		0.5			t		t	1.6	0.1			t	2.3		0.2
α-terpinene		t	t	t	0.3		t	t	t	0.1	t	t	0.1	t	0.4
limonene	1.3	5.0	0.5	2.1	2.3	2.1	3.5	2.2	2.2	1.3	1.6	5.7	1.7	1.6	8.6
β-phellandrene	t	t	t	0.4	0.8		0.2	6.2	0.4	t	0.2		0.7	0.1	1.3
1,8-cineole	91.9	62.1	52.1	82.3	78.1	83.4	65.4	24.2	54.8	84.7	85.4	65.8	40.0	22.8	0.2
Z-β-ocimene		t													0.5
γ-terpinene	t	t	t	0.2	0.6	0.4	0.4	0.4	0.2	0.3	0.1	0.5	0.1	0.1	0.7
E-β-ocimene													t		0.1
p-cymene	1.0	1.3	23.0	1.8	1.4	0.8	0.8	4.4	1.9	2.2	2.5	0.6	5.0	0.3	0.1
terpinolene	t	t	t	t	0.2	t	t	0.1	t	0.1	t	0.1	0.2	t	0.5
*iso*amyl *iso*valerate		t	t	t		t	0.3	t	0.1		t	t	t	t	
α-cubebene												t		t	0.1
trans-p-menth-2-en-1-ol			0.4												
bicycloelemene															0.3
α-copaene															t
α-gurjunene		t										t		t	0.4
linalool															t
linalyl acetate															t
β-elemene					t			0.3	t	t			t	0.4	0.8
pinocarvone	t	t		0.1	0.1	0.4	0.1	t	0.8	0.2	0.1	t	0.7		

Table 3 (cont)

	angustissima	*cinerea*	*cneorifolia*	*horistes*	*kochii*	*leptopoda*	*loxophleba* ssp. *lissophloia*	*loxophleba* ssp. *gratiae*	*myriadena*	*plenissima*	*polybractea*	*pulverulenta*	*vegrandis*	*viridis*	*eximia*
β-caryophyllene		1.0	0.2				t	1.9	0.3		t	0.2	1.4	0.2	0.6
terpinen-4-ol		0.4	1.1	t			0.2	1.7	0.3	t	0.3	1.1	0.9	1.0	0.8
aromadendrene	1.1	0.5		1.0	1.5	1.3	0.7	1.2	0.5	1.3	0.5	0.6	0.4	0.4	1.4
cis-p-menth-2-en-1-ol			0.4												
α-bulnesene															0.1
alloaromadendrene	0.1	0.3		0.1	0.2	t	t	0.9	0.1	0.2	0.1	0.5	0.3	0.8	1.4
citronellyl acetate															0.1
trans-pinocarveol			0.6	0.2	0.1	0.8	0.6				0.6	0.1			0.2
humulene															0.1
cryptone	0.2		9.1	0.2	t	0.3				t	t				
viridiflorene				t	1.4		t			1.1	0.4	0.1			0.2
phellandral			0.3												
α-terpineol	1.1	5.1	t	1.1	2.0	2.8	1.8	2.0	1.1	1.1	0.5	2.9	0.9	1.9	
bicyclogermacrene		0.7		0.1	0.1	t	0.1	5.1	0.7	0.2	0.5		2.1	0.2	15.9
piperitone			0.4												
carvone			0.5												
δ-cadinene							t				0.2		0.1		0.1
α-farnesene															t
trans-piperitol			t												
cuminal			4.3	0.2	0.6	t	t			0.2			t	0.5	
trans-p-mentha-1(7),8-dien-2-ol	0.2	0.3		0.1	0.4	t		0.2	0.2	0.5	0.2	t	t	t	
trans-p-mentha-1,8- dien-6-ol	t	t	0.2	0.2	0.2	0.2	0.1	0.1	0.4	0.3	0.2	0.2	0.4	t	
p-cymen-8-ol	0.3	t	1.1	0.2	0.1	0.1	0.2	t	0.2	0.2	t	t	0.1	t	
cis-p-mentha-1,8 dien-6-ol	t	t		0.1	0.1	t		t	0.1	0.1	0.1	t	t		
β-phenylethyl butyrate			0.3												

Table 3 (cont)

	angustissima	*cinerea*	*cneorifolia*	*horistes*	*kochii*	*leptopoda*	*loxophleba* ssp. *lissophloia*	*loxophleba* ssp *gratiae*	*myriadena*	*plenissima*	*polybractea*	*pulverulenta*	*vegrandis*	*viridis*	*eximia*
cis-p-mentha-1(7),8-dien-2-ol	t	0.3	t	t	t	0.1	t	t	0.2	t	t	0.2	0.2	t	
palustrol		0.2	t				t	0.3	t			t	0.3		0.3
caryophyllene oxide	t			t	t			0.4	0.4	0.1			0.6		
*epi*globulol	t	0.5		t		t	t	0.4	t			0.5	0.6	0.8	0.2
ledol	t	0.2	t	t	t	t	t	0.3	t	t		0.1	0.3	0.5	0.3
cubeban-11-ol		0.9	t	t	0.1	t	t	0.5	0.2	0.1		0.3	0.7	0.9	0.7
globulol		2.1	0.2	0.4	0.1	0.5	0.8	3.0	0.8	0.1	0.1	2.7	5.0	5.8	11.1
elemol											t				3.7
viridiflorol	0.2	1.4	t	0.1	t	t	0.1	1.2	0.3	t		0.4	1.7	1.5	2.1
carvacrol			1.2												
spathulenol		1.8	0.5	0.4	0.6		0.5	8.3	2.3	0.5	0.1	0.1	6.3	8.1	2.6
γ-eudesmol				t			0.3				0.1				5.7
australol			1.1												
α-eudesmol							0.6				0.2				5.0
β-eudesmol							0.7				0.5				4.7
E,E-farnesol															t

Table 4: Yield, optical rotation, refractive index and distillation time of eucalypt oils examined in this study. 'nd' indicates not determined

Species	Oil yield (% dry mass)	Optical rotation (^{0}) at 20^{o}C	Refractive index at 20^{o}C	Distillation time (h)
E. angustissima	6.00	+2.8	1.4614	5
E. cinerea	4.76	+3.9	1.467	7
E. cneorifolia	1.41	-5.4	1.4749	2.5
E. horistes	6.55	+4.2	1.4619	4.5
E. kochii	10.00	+3.6	1.4633	4.5
E. leptopoda	5.70	+4.6	1.4618	5
E. loxophleba ssp. *gratiae*	7.11	+8.4	1.4814	11
E. loxophleba ssp. *lissophloia*	7.82	+7.6	1.458	3
E. myriadena	4.70	+8.4	1.469	4
E. plenissima	5.71	+3.2	1.4629	4
E. polybractea (whole)	2.85	+1.7	1.459	3.5
E .polybractea (distilled)	0.22	nd	nd	6
E. pulverulenta	8.98	+3.6	1.4642	9
E .vegrandis	3.33	+4.4	1.469	5.5
E. viridis	3.37	+60.4	1.5007	15
Corymbia eximia	1.94	+18.1	1.4994	30

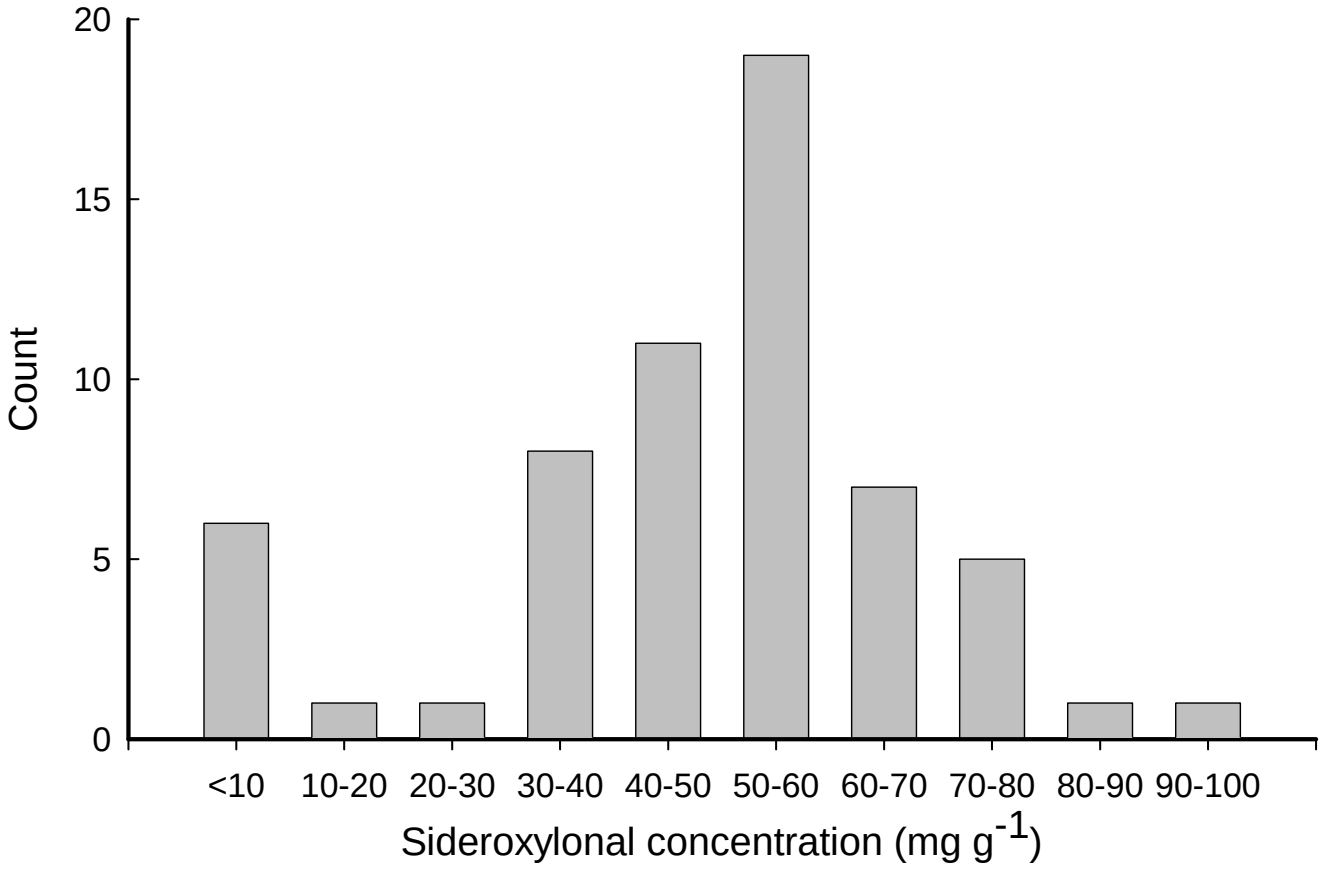

Figure 2: Frequency distributions of foliar sideroxylonal amongst 60 open pollinated progeny grown from high-cineole yielding *Eucalyptus loxophleba*

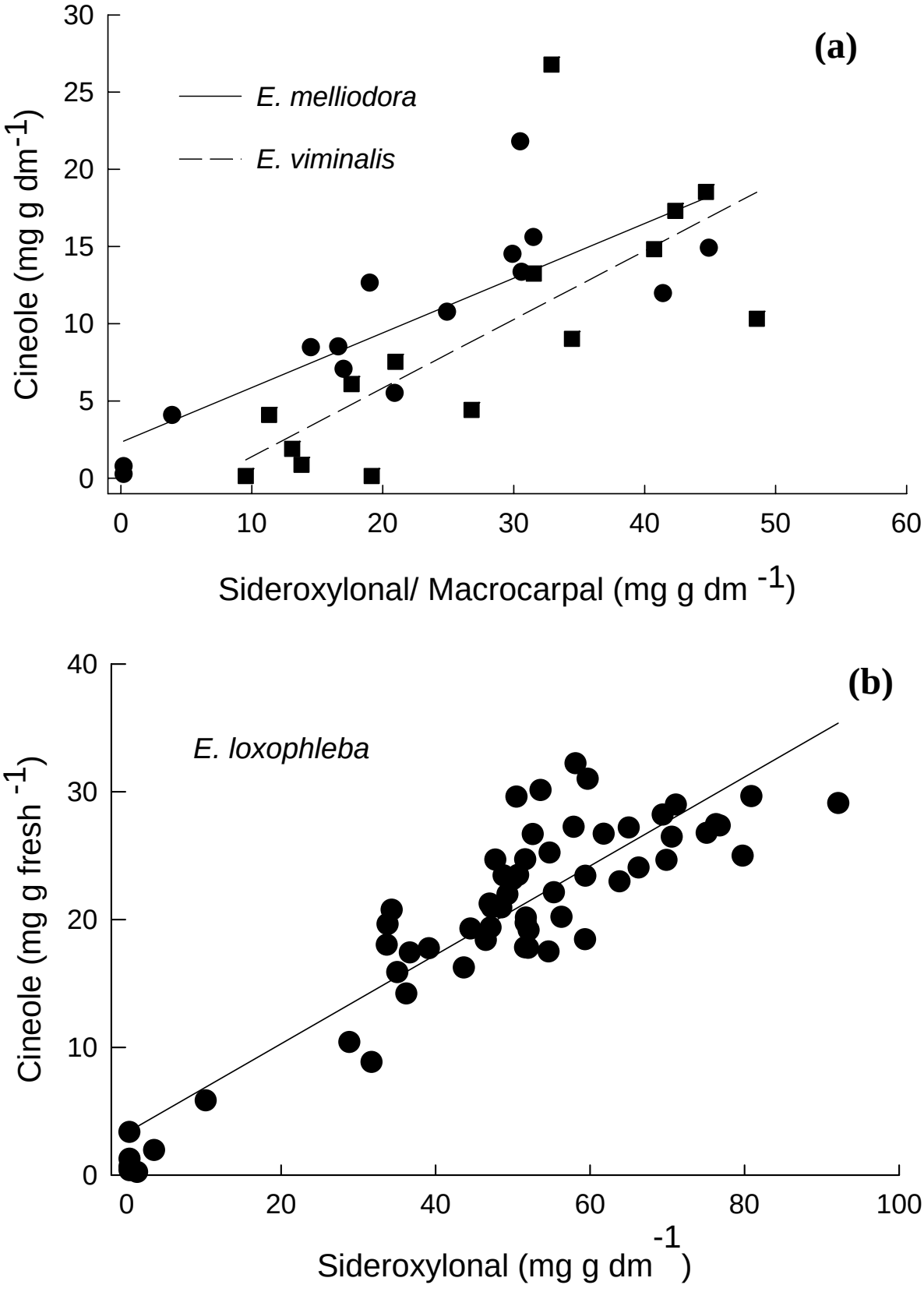

Figure 3. The relationship between foliar concentrations of sideroxylonals (*E. melliodora*, *E. loxophleba*) or macrocarpals (*E. viminalis*) and the concentration of cineole in three species of *Eucalyptus*

Table 5: The yield of formylated phloroglucinol compounds resulting from different treatments of one kilogram of foliage from *Eucalyptus* species rich in jensenone, macrocarpals or sideroxylonals Yields expressed as % dry matter (DM)

Species	Constituents[1]	Extraction procedure	Work-up procedure[2]	Large Scale yield[3]	Analytical Yield[4]
E. jensenii	Simple FPC (jensenone)	Steam distillation	Nil	0.4-0.6%	3.8%
		Hot Solvent extraction	Base extract	1.2%	4.0%
		Cold Solvent extraction	Base extract	1.0%	3.6%
E. viminalis	Macrocarpals	Hot Solvent extraction	Standard	0.3%	2.1%
			Base extract	0.9%	2.1%
		Cold solvent extraction	Standard	~0.2%	2.1%
			Base extract	0.9%	2.1%
E. melliodora	Sideroxylonals	Hot solvent extraction	Standard	1.2%	3.9%
			Base extract	Nil - Compound hydrolysed	3.9%
		Cold solvent extraction	Standard	~ 0.2%	3.8%
			Base Extract	Nil – Compound hydrolysed	3.8%

1 The dominant formyl phloroglucinol constituents present in the foliage.

2 The work up procedure describes the technique used to separate the extracted FPC from other undesired constituents (mainly fatty acids and triterpenoids). 'Standard' work-up refers to the techniques described by Eschler and Foley (1999).

3 Large-scale yield describes the yield of purified compound obtained from the extraction and work-up of 1 kg of foliage.

4 The analytical yield refers to the amount of compound calculated to be in the foliage on the basis of extraction and HPLC analysis of a 1 g sample following the standard protocol described for sideroxylonals by Wallis et al. (2003) or for macrocarpals in the Methods section of this report

Cyclodextrins as adjuncts to aid purification of macrocarpals

The combinations of β-cyclodextrin and β-methyl cyclodextrin investigated failed to capture the various FPCs (macrocarpals A, B, G, eucalyptone and sideroxylonals A and C) in an FPC-rich extract of *E. viminalis*. One possible explanation is that the FPC molecules are too big to fit inside the conical structure of the cyclodextrin.

Discussion

Occurrence of FPCs in low-rainfall eucalypts – overview and market prospects

This study showed that FPCs occur in a wide variety of low-rainfall eucalypts. The focus of this study was on species that were already being planted (or considered for planting) in low-rainfall situations either as a direct counter to increasing dryland salinity or else to provide various wood-products in farm forestry operations. The discovery of rich sources of FPCs amongst this material as well as the finding that there is a positive relationship between 1,8-cineole concentration and FPC (in those species where the compounds co-occur) are key steps to developing products based on novel bioactive eucalypt metabolites. The concentrations of sideroxylonals in a number of species being investigated in the WA Oil Mallee project were exceptionally high. In particular, *E. loxophleba* contained the highest concentrations of sideroxylonals that have ever been recorded reaching a maximum of 9% of dry matter. The WA Oil Mallee project seeks to develop dryland plantings that can yield multiple products including cineole-rich oils and wood for charcoal production. Our finding that there was a strong correlation between the concentration of 1,8-cineole and the concentration of sideroxylonal in this species suggests multiple benefits from genetic improvement activities. Strategies for the selection of cineole-rich provenances from within the target species have already been undertaken and this will have the side benefit of also selecting for high concentrations of FPCs. Therefore we conclude that there are already plant-based resources for developing FPCs as an extra by-product of dryland plantings.

Although there are many potential uses of FPCs, these will not be realised until such time that we find better methods to first extract and purify suitable quantities for research (tens of grams). Extraction and purification is always the mostly costly part of any plant-based natural products industry with the cost of purification rising steeply with the removal of each minor impurity. The purity of the products required depends entirely on their end use. At present, there is no market for FPCs from Eucalyptus because in spite of their interesting properties in small-scale tests, the compounds have been too difficult to obtain to spur wider testing. For example, in Japanese studies typical yields from a kilogram of plant material have been only a few milligrams. These very low yields result from a lack of selection on the starting material and a desire to produce compounds, which were judged to be pure by sensitive chromatographic procedures. While researchers need compounds of such high purity, there is no reason that such purity is needed for most applications. For example, Amakura et al. (2002) recently reported that macrocarpals were part of the active fraction of a food additive derived from *Eucalyptus* and used in Japan. In this case, the food additive contained only about 0.2% macrocarpal. Elsewhere Sakei et al. (1999) reported that an aqueous ethanolic extract of *E. macrocarpa* and *E. globulus* containing macrocarpals was an effective angiotensin converting enzyme (ACE) inhibitor at 200 ppm (0.02%). Sakei et al. (1999) reported manufacturing a chewing gum containing this extract since ACE

inhibitors are effective hypotensive agents. They attributed the biological activity to macrocarpals in the extract but the final concentration of macrocarpals in the product must have been very low indeed. Finally, although there are no direct applications reported in the scientific or patent literature, one could envisage extracts of sideroxylonal-rich plants containing sufficient sideroxylonal to act as effective antifouling agents because of the potent antifouling properties of the compound.

These examples argue the case that production of chemically pure extracts may not necessarily be the aim of a commercial exploitation of FPCs from *Eucalyptus* for either industrial, food or drug uses. Of course, this is the situation with many commercial products that are marketed in various grades: industrial, food and the highly purified and expensive analytical grades. Nonetheless, testing that has been done to date has relied on very small samples of pure compounds and we argue that the lack of availability of compounds limits the development of markets for these natural products.

Occurrence of FPCs in *Eucalyptus* – taxonomic considerations (Table 6)

The influence of taxonomic affinity on the occurrences of FPCs in eucalypts is strong at the level of subgenera. Most striking is the absence of FPCs from eucalypts in the informal subgenus *Eucalyptus* (= *Monocalyptus*) which contains the stringybarks, peppermints and ashes. Limited sampling to date means that it is too early to evaluate whether there are other clear taxonomic signals in the distribution of FPCs in *Eucalyptus*. That none of the four species of oil-rich *Melaleuca* contained FPCs suggests however that FPCs are essentially a feature of eucalypts.

Relationship between 1,8-cineole and FPC concentrations

We anticipated that there would be a very strong relationship between the concentration of 1,8-cineole and the concentration of FPCs in eucalypts. However, initial inspection of the results of the oil and FPC analyses showed that many species that contained high concentrations of cineole did not contain any FPCs and so we could not seek a relationship across all species. Therefore we examined 15 trees of each of two species (*E. viminalis* and *E. melliodora*) in detail and observed a very strong relationship between FPC concentration (macrocarpals in the case of *E. viminalis*) and sideroxylonals in the case of *E. melliodora*) and cineole concentration. We then examined in more detail, a series of 60 trees of *E. loxophleba* and observed a very strong relationship between sideroxylonal concentrations and cineole concentrations. Together these results suggest that when both compounds occur together, their concentrations are linked but that in some species the enzymatic machinery to make FPCs is lacking or operating at a very low rate. However, we believe that the results show that in those species where the two compounds do in fact co-occur, selection of trees that are cineole rich will also result in selection of trees that are rich in FPCs. Therefore, provided that the FPC profile has been determined, improving the yield of cineole will also result in improvements in the yield of FPCs. This relationship should be checked for any species that is to be the subject of genetic improvement but in the case of *E. loxophleba* that we studied in detail, the relationship is very strong.

Table 6: A taxonomic listing of all known occurrences of euglobals, macrocarpals and sideroxylonals amongst *Eucalyptus* species. '+' indicates a positive report; '-' indicates absence and 'nd' indicates no data available. Additional sources: Eschler et al. (2000); Konoshima and Takasaki (2002); W.J. Foley and I.R. Wallis (unpublished data).

Subgenus	Section	Series	Species	Euglobals	Macrocarpals	Sideroxylonal
Blakella			*E.. tesselaris*	-	-	-
Coymbia	Ochraria	Eximiae	*C. eximia*	-	-	-
			C. peltata	-	-	-
			C. torreliana	+	-	-
	Politaria/Ochraria	Maculatae	*C. citriodora*	+	-	-
			C. maculata	-	-	-
			C. maculata	+	-	-
	Rufaria	Polycarpae	*C. clarksoniana*	-	-	-
		Gummiferae	*C. ficifolia*	-	-	-
Alveolata			*E. microcorys*	-	+	+
Monocalyptus (Eucalyptus)	Amentum		*E. acmenoides*	-	-	-
	Aromatica	Insulanae	*E. nitida*	-	-	-
		Radiatae	*E. dives*	-	-	-
			E. elata	-	-	-
			E. radiata	-	-	-
	Capillulus	Pachyphloius	*E. eugenioides*	-	-	-
			E. macrorrhyncha	-	-	-
	Cineraceae	Fraxinales	*E. delegatensis*	-	-	-
		Psathroxylon	*E. haemastoma*	-	-	-
			E. racemosa	-	-	-
		Pauciflorae	*E. pauciflora*	-	-	-
	Eucalyptus	Eucalyptus	*E. obliqua*	-	-	-
		Regnantes	*E. fastigata*	-	-	-
			E. regnans	-	-	-
	Longitudinales		*E. stellulata*	-	-	-
	Pseudophloius		*E. pilularis*	-	-	-
Eudesmia	Reticulatae	Miniatae	*E. phoenicea*	+	+	+
Idiogenes			*E. cloeziana*	-	-	-

Subgenus	Section	Series	Species	Euglobals	Macrocarpals	Sideroxylonal
Symphyomyrtus	Adnataria	Aquilonares	*E. coolabah*	+	+	-
		Buxeales	*E. largiflorens*	-	-	-
			E. porosa	+	+	+
			E. albens	-	+	-
			E. moluccana	-	-	-
			E. polybractea	-	-	-
			E. viridis	+	+	+
		Dawsonianae	*E. dawsonii*	+	-	+
		Siderophloiae	*E. crebra*	+	-	-
			E. drepanophylla	+	+	+
			E. jensenii	+	-	-
		Submelliodorae	*E. argophloia*	-	+	+
		Heterophloiae	*E. polyanthemos*	+	+	+
		Melliodorae	*E. leucoxylon*	+	-	-
			E. melliodora	+	+	+
			E. petiolaris	+	+	+
			E. sideroxylon	-	-	+
			E. tricarpa	-	-	+
	Bisectae	Angustissimae	*E. cneorifolia*	-	-	+
			E. angustissima	-	-	-
		Curviptera	*E. macrocarpa*	+	+	-
			E. leptopoda	+	+	+
		Subulatae	*E. socialis*	-	-	-
			E. horistes	-	-	+
			E. kochii	-	-	+
			E. kochii	-	-	-
		Clinatae	*E. vegrandis*	+	+	+
		Dundasianae	*E. dundasii*	-	-	-
		Erectae	*E. occidentalis*	+	+	-
		Loxophlebae	*E. loxophleba*	-	-	+
		Hadrotes	*E. cornuta*	+	+	+
	Dumaria	Rigentes	*E. famelica*	+	+	+
		Incrassatae	*E. incrassata*	+	nd	nd
		Ovulares	*E. myriadena*	-	+	+
	Equatoria		*E. deglupta*	-	-	+
	Exsertaria	Erythroxylon	*E. amplifolia*	+	+	
			E. blakelyi	+	nd	nd
			E. tereticornis	+	+	+

Subgenus	Section	Series	Species	Euglobals	Macrocarpals	Sideroxylonal
		Phaeoxylon	*E. exserta*	+	+	+
		Rostratae	*E. camaldulensis*	+	+	+
		Singulares	*E. rudis*	+	nd	nd
	Incognitae		*E. cosmophylla*	+	-	+
	Latoangulatae	Annulares	*E. botryoides*	+	+	+
			E. resinifera	-	-	+
			E. robusta	+	-	-
		Lepidotae-Fimbriatae	*E. punctata*	-	-	+
		Transversae	*E. grandis*	+	+	+
			E. pellita	+	-	+
			E. saligna	+	-	-
	Maidenaria	Argyrophyllae	*E. cinerea*	+	+	+
		Bridgesianae	*E. bridgesiana*	+	-	-
			E. dunnii	+	+	+
		Confines	*E. kartzoffiana*	+	+	+
		Globulares	*E. globulus*	+	+	+
			E. cypellocarpa	+	nd	nd
			E. nitens	+	+	+
		Orbiculares	*E. cordata*	+	nd	nd
			E. gunnii	+	nd	nd
			E. perriniana	+	nd	nd
			E. pulverulenta	-	+	+
		Viminales	*E. dalrympleana*	-	-	+
			E. rubida	+	-	+
			E. viminalis	+	+	
			E. parvula	+	nd	nd
		Acaciiformis	*E. acaciiformis*	+	-	+
		Foveolatae	*E. aggregata*	-	+	+
			E. brookeriana	+	+	+
			E. macarthurii	+	+	+
			E. ovata	+	+	+
		Microcarpae	*E. dorrigoensis*	+	+	+
			E. mannifera	-	-	+
			E. microcarpa	+	-	-
	Sejunctae		*E. cladocalyx*	+	+	+

It remains interesting to speculate on why there is a relationship between 1,8-cineole and FPC concentrations in those eucalypts in which the two groups of compounds co-occur since terpenes and FPCs are formed from very different biosynthetic pathways. The formation of terpenes such as 1,8-cineole is catalyzed by a group of enzymes called terpene synthases. These enzymes catalyze the folding of geranial pyrophosphate or farness pyrophosphate into a range of mono and sesquiterpenes. In contrast the FPCs are believed to be formed by a range of unrelated polyketides synthases. For example, valerophenone synthase (a chalcone synthase family enzyme) combines 3 malonyl CoA units with one isovaleryl CoA has recently been described as the key step in the formation of the phloroglucinol units in the bitter hop acid humulone (Zuurbier et al. 1995; Paniego et al. 1999). This, or a similar enzyme, must be responsible for the initial formation of simple FPCs such as jensenone.

For macrocarpals and euglobals it is easy to envisage a role for terpenes in biosynthesis of the compounds. These compounds are presumed to result from a Diels-Alder condensation of an o-quinone-methide and a common leaf terpenoid. However, the C_5 unit in jensenone and sideroxylonals is most likely derived from isovalerate via enzymic condensation (and not from isoprene as we suggested earlier (Pass et al. 1998; Lawler et al. 2000)). Therefore, it is difficult to envisage any direct role of terpenes in the formation of jensenone and sideroxylonal. Nonetheless, presumed somatic mutations in some *Eucalyptus* trees (e.g. *E. melliodora* Edwards et al. 1990) have led to the occurrence of isolated branches expressing high concentrations of some terpenes together with sideroxylonals (W.J. Foley unpublished data) amongst a mass of branches showing only low concentrations of both groups of compounds. This suggests that although the pathways of formation of terpenes and sideroxylonals are likely to be quite separate, mutations can affect a key regulatory point of both biosynthetic pathways. Just what that regulatory point is remains unknown.

Leaf essential oils

The only species of *Eucalyptus* dealt with in this report which is at the present time a major commercial source of medicinal 1,8-cineole-rich *Eucalyptus* oil is *Eucalyptus polybractea*. Among the other species the following have cineole-rich oils which would comply with the requirements of the European (which now also includes the British Pharmacopoeia) (Monograph 0390 European Pharmacopoeia 4th Ed 2002) and of Australian Standards AS 2113.1 and AS 2113.2 for 70-75% cineole and 80-85% cineole "Oils of Australian *Eucalyptus"* respectively (Standards Australia 1998): *Eucalyptus angustissima, E. cinerea, E. horistes* (synonym *E. oleosa* var. *borealis*), *E. kochii, E. leptopoda, E. plenissima* and *E. pulverulenta*.

E. cinerea oil has been produced on a commercial scale at the beginning of the last century (Baker and Smith 1920). However, the 1,8-cineole content of the unrectified oil is relatively low ranging from about 54% to 78 % (Boland et al. 1991) and it is unlikely that the oil would regain its former commercial importance The botanically fairly closely re1ated *Eucalyptus pulverulenta* contains an oil which is qualitatively very similar to that of *E. cinerea* except that the 1,8-cineole content is significantly higher, up to about 82% (Brophy et al. 1985). Furthermore, we found much higher leaf oil yields, up 9% on a dry matter basis which is almost double the figure reported in Brophy et al. (1985). The five remaining species among the high-cineole oil group are all mallees which can be an advantage from an industrial point of view, as mallees are

amenable to mechanical harvesting, thus reducing production costs. Furthermore, their steam-distilled leaf oils do not contain undesirable constituents such as isovaleric aldehyde and α-phellandrene which makes them particularly suitable for medicinal use. Three amongst them, *E. kochii, E. plenissima* and *E. horistes,* (once considered to be varieties of *Eucalyptus oleosa*), are being used at the present time for the commercial extraction of essential oil. The chemical composition of their steam-distilled oils has been previously investigated, though only cursorily (Watson and Gardner (1947/48); Gardner and Watson (1947/48)*;* Brooker et al. (1988)). All three, but particularly *E. kochii,* are notable for their high essential oil yields (Table 4). The leaf oil of *E. leptopoda* has been briefly investigated by Marshall and Watson (1936/37) who reported an oil yield of 1.3% (fresh weight) and a 1,8-cineole content of about 68%. The oil yield as well as the cineole content of the oils reported here are significantly higher. The steam-distilled essential oil of *E. angustissima* was found to have the highest 1,8-cineole content (91.9%) of all species investigated here. It has not been, to our knowledge, previously investigated (but see Note at the end of this section).

None of the seven remaining species yielded oils which complied with the requirements of the European Pharmacopoeia for medicinal *Eucalyptus* oil. Details are as follows: *E. cneorifolia* oil contained very low amounts of 1,8-cineole but high levels of p-cymene, cuminal and cryptone; cryptone being the main reason the oil's negative optical rotation (Baker and Smith 1920; Berry 1947). Also the oil yield (1.4% on dry weight) was far too low for commercial profitability. *E. viridis* contains too little 1,8-cineole. It is possible that in both cases the foliage used in this project was obtained from aberrant populations as both species have been used in the past, although infrequently and in small amounts only for the commercial production of *Eucalyptus* oil (Lassak 1988). *E. myriadena* and *E. vegrandis* were found to contain relatively low levels of 1,8-cineole and very high levels of α-pinene. *Corymbia eximia* (synonym *Eucalyptus eximia*) contained only traces of 1,8-cineole and a lot of sesquiterpenoids. The two subspecies of *Eucalyptus loxophleba* contained significant quantities of 4-methylpentyl-2-acetetae a compound not normally found in *Eucalyptus* oil but considered to be a taxonomic marker for the *Eucalyptus loxophleba* group (Grayling and Knox 1991). It is though possible that these *E. loxophleba* oils could by virtue of their chemical composition as well as their high yield find use as solvents for fats and grease Their α-pinene and 4-methylpentyl-2-acetate content coupled with 1,8-cineole should confer to them superior solvent power. The use of cineole and cineole-rich *Eucalyptus* oil as a degreasing agent has been advocated by Barton (1989) and Barton and Knight (1997).

Note: Reports on the composition of the volatile oil of a number of the species investigated by us, obtained by vacuum distillation of dried foliage rather than by steam-distillation, have been published. Whilst vacuum distillation eliminates to a large extent, the chance of acid-induced rearrangements and degradations, such vacuum distillates are not representative of commercially traded essential oils. Also, the reported yields of volatiles obtained by vacuum distillation were, in most cases, much lower than oils obtained by steam distillation. Furthermore, the vacuum distillation method did not appear to fully extract some of the very high boiling sesquiterpenoid alcohols which, in turn, results in unrepresentative quantitative percentage compositions (Bignell et al. 1995b, 1996). Chemical compositions of vacuum distilled leaf oils of the following species investigated here by us have also been published: *E. angustissima* (Bignell et al. 1997a); *E. cneorifolia* (Bignell et al. 1997a); *E. horistes* (Bignell et al. 1995a); *E kochii* (Bignell et al. 1995a); *E. leptopoda* (Bignell et al. 1994); *E. loxophleba* ssp. *gratiae* (Bignell et al. 1997c); *E. loxophleba* ssp. *lissophloia* (Bignell et al. (1997c); *E. myriadena* (Bignell et al. 1997b); *E. vegrandis* (Bignell et al. 1998); *E. viridis* (Bignell et al. 1995b).

Extraction of FPCs from *Eucalyptus*

Our attempts to devise improved methods of extracting and purifying FPCs from eucalypts met with mixed success. We were able to develop simple ways of extracting and purifying jensenone from *Eucalyptus jensenii*. This compound is partially steam volatile but significantly more can be extracted using simple solvent washes. Of particular value is the ease with which pure jensenone can be produced from the extracts. The feasibility of carrying out this procedure of course depends on the final use to which the material is to be put. Jensenone is not a very potent antifouling agent (Professor Hideo Etoh, Shizuoka University, Japan, personal communication) although it is a very strong mammalian antifeedant with relatively long lasting effects since it induces a severe nausea and thus a conditioned aversion (Lawler et al. 1998, 1999) Providing a source of the compound should stimulate testing and lead to other uses.

In contrast, we were unable to improve the existing laborious small-scale methods for obtaining sideroxylonal. This was disappointing given the very high concentrations of sideroxylonal which are present in species such as *E. loxophleba* which are planted and processed extensively in Western Australia. The chemical nature of sideroxylonal means that it is not amenable to base-acid extraction which was the key to isolation of jensenone (and to a lesser extent the macrocarpals). Under basic conditions, even when the bases used are quite mild, sideroxylonal hydrolyses giving two fragments each of molecular weight (MW) 250 (MW of sideroxylonal is 500). Sideroxylonal is significantly more polar than the other FPCs and during purification, co-extracts with many fatty acids and other undesired material. We will continue to work on ways of extracting and purifying sideroxylonals because of their ecological importance and industrial potential.

We had some success in extracting macrocarpals using a base acid procedure but our hopes of using cyclodextrins to selectively remove macrocarpals from extracts were not met. We tried a wide range of conditions involving two cyclodextrins but little if any macrocarpal was taken up into the cyclodextrin core, probably because the macrocarpal molecule was too large to enter the cavity. The cost of cyclodextrin and the uncertainty over whether FPCs will prove to have commercial value suggests that for now this line of investigation requires more study in the laboratory.

Future directions

Difficulties of large-scale extraction remain the biggest hurdle to realizing the potential of FPCs as a secondary product from dryland plantings. Our studies indicate that *Eucalyptus loxophleba* is the best target for extracting FPCs because of the high yields of sideroxylonal available from this species together with the potentially large volume of material that could be processed. One possibility is that sideroxylonal could be extracted in conjunction with the Integrated Wood Processing (IWP) Plant operated by Western Power at Narrogin in Western Australia. This project is designed to produce cineole–rich oils as well as activated charcoal and electricity from extensive areas of mallees planted to reduce dryland salinity.

However, moving from laboratory scale extractions to a semi-industrial scale requires us to radically re-think our approach to extraction and purification. Rather than aiming to exhaustively extract all sideroxylonal from the plant material as we have done in the

laboratory, we have to consider extracting just a portion of the yield from the large volume of plant material being processed. Extraction methods that are suitable in the laboratory may not be feasible on the industrial scale of the IWP Plant. For example, the reliance on organic solvents such as petroleum spirit may not be feasible on an industrial scale. Two possible processing options that could be feasible are the use of supercritical carbon-dioxide extraction or the use of superheated, pressurized water.

Supercritical CO_2 is a mature technology that can selectively extract natural products of varying polarities from plant material (Lang and Wai 2001). Supercritical CO_2 extraction already operates on a pilot scale in Australia and elsewhere around the world. In contrast the notion of using superheated and pressurized water to extract natural plant products that are normally insoluble in water is attracting much attention because it avoids the problems of organic solvents, is potentially cheaper than using supercritical CO_2 and is quicker than using steam distillation for volatile constituents (Luque de Castro et al. 1999; Clifford 2002; Hawthorne and Kubatova 2002). In short, the polarity of water can be changed by modifying its temperature while also modifying the pressure in order to maintain it as a liquid. In this way, water can be used to extract moderately and highly non-polar compounds from plant material. This concept has already been successfully applied to the extraction of highly non-polar *Eucalyptus* leaf oils (Jimenez-Carmona and Luque de Castro 1999). If this could be done with sideroxylonals, it might prove to be a process that was suitable for incorporation as a secondary processing loop into an industrial scale plant such as Western Power's IWP Plant at Narrogin. We recommend that this possibility be investigated in further studies.

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