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# Quantitative Analysis of Various B-ring Unsubstituted and Substituted Flavonoids in Ten Australian Species of *Eucalyptus*

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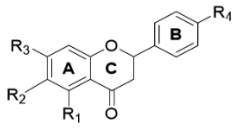
Flavonoids (in particular unsubstituted B ring flavanones) in *Eucalyptus* foliage play an important role in mediating animal plant interactions, and there is a need for methods to analyse the diverse profiles found in leaves. A simple, high-performance liquid chromatographic (HPLC) method with in-line connected photodiode-array (PDA) detection was developed and validated to identify and quantify nine B-ring unsubstituted and three B-ring substituted flavonoids in ten Australian species of *Eucalyptus*. Of these, eight compounds were detected and quantified in the crude methanolic extracts of leaves of various *Eucalyptus* species (*E. sieberi*, *E. rossii*, *E. fastigata*, *E. macrorhyncha*, *E. fraxinoides*, *E. agglomerata*, *E. consideniana*, *E. pauciflora*, *E. dives* and *E. obliqua*) based on comparison with the retention times and λmax values of pure compounds. This rapid and sensitive HPLC/PDA method was coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) for qualitative analysis to corroborate the identification of compounds by HPLC/PDA analysis.

**Keywords:** *Eucalyptus*, Ecology, Marsupials, Antifeedant, Flavanones, HPLC/PDA quantitation, HPLC-ESI-MS.

Chemical variation within and between closely related species of plants is common, and can involve either quantitative variation in the concentration of particular compounds, or qualitative variation in the structure of compounds from specific classes [1]. There is strong evidence that variation amongst animals in their tolerance of individual compounds can lead to major differences in their diet, and in the long term success of those plants [2-5].

Chemical variation within and between different *Eucalyptus* species has attracted much attention because of the strong ecological and economic consequences of that variation. For example, conserving koalas depends on understanding their diet choices, and the growth of forest plantations is severely constrained by insect grazing. The chemical basis of this variation has been largely approached through bioassay guided fractionation, including the identification of formylated phloroglucinol compounds as key mediators of the diets of koalas [6-7]. However, recently, untargeted metabolomics using 1H NMR spectroscopy [8] was used to identify flavanones with unsubstituted B rings as likely mammalian antifeedants. This hypothesis was confirmed following in vivo feeding studies with common brushtail possums with a range flavones and flavanones. Feeding rates of animals were significantly depressed by B ring unsubstituted flavanones but not by flavanones with substitutions in the B ring or unsubstituted flavones [9]. Critically, it appears that B ring unsubstituted flavanones only occur in the subgenus Monocalyptus (= *Eucalyptus*) of the *Eucalyptus* genus [8, 10].

Understanding the actions and effects of these flavanones depends on a robust method for their quantification, as it is likely that each species of *Eucalyptus* contains a diversity of related structures. Several C-methylated flavones (eucalyptin, 8-demethyl-eucalyptin, sideroxylin and 8-demethyl-sideroxylin) and three closely related flavanones (pinocembrin, alpinetin and O, O-dimethyl pinocembrin) have been reported earlier in the leaf of *E. sieberi* [11-12]. Recently, we reported the presence of pinocembrin in three species of *Eucalyptus*, namely *E. sieberi*, *E. fraxinoides* and *E. pauciflora* [13], and Goodger et al. reported several related flavonoids from other species of *Eucalyptus* [10]. In this study, we report on the identification and quantification of 12 flavonoids in the crude methanolic extracts of leaves of various *Eucalyptus* species from the subgenus Monocalyptus.




| Trivial name | [M-H]⁻ | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
| **Flavanone type** | | | | | |
| Flavanone **[1]** | 223 | H | H | H | H |
| 6-Hydroxy flavanone **[2]** | 239 | H | OH | H | H |
| 7-Hydroxy flavanone **[3]** | 239 | H | H | OH | H |
| 5-Methoxy flavanone **[4]** | 253 | $OCH_3$ | H | H | H |
| 6-Methoxy flavanone **[5]** | 253 | H | $OCH_3$ | H | H |
| 7-Methoxy flavanone **[6]** | 253 | H | H | $OCH_3$ | H |
| 5,7-Dihydroxy flavanone (Pinocembrin) **[7]** | 255 | OH | H | OH | H |
| 5-Hydroxy-7-methoxy flavanone (Pinostrobin) **[8]** | 269 | OH | H | $OCH_3$ | H |
| 5,7-Dimethoxy flavanone **[9]** | 283 | $OCH_3$ | H | $OCH_3$ | H |
| 4',5,7-Trihydroxy flavanone (Naringenin) **[10]** | 271 | OH | H | OH | OH |
| **C- Methylated flavone type** | | | | | |
| 5-Hydroxy-4',7-dimethoxy-6,8-dimethyl flavanone (Eucalyptin) **[11]** | 325 | OH | $OCH_3$ | $CH_3$ | $OCH_3$ |
| 5-Hydroxy-4',7-dimethoxy-6-methyl flavanone (8-Demethyl eucalyptin) **[12]** | 311 | OH | $OCH_3$ | H | $OCH_3$ |

**Figure 1:** Structures of the different flavonoids.

An RP-HPLC/DAD method was developed to detect and/or quantify flavonoids in *Eucalyptus* leaves. The proposed HPLC/DAD method enables determination of ten flavanones and two flavones [flavanone (**1**), 6-hydroxy flavanone (**2**), 7-hydroxy flavanone (**3**), 5-methoxy flavanone (**4**), 6-methoxy flavanone (**5**), 7-methoxy flavanone (**6**), 5,7-dihydroxy flavanone/pinocembrin (**7**), 5-hydroxy-7-methoxy flavanone/pinostrobin (**8**), 5,7-dimethoxy flavanone (**9**), 4',5,7 trimethoxy flavanone/naringenin (**10**), 5-hydroxy-4',7-dimethoxy-6,8-dimethyl flavanone/eucalyptin (**11**), 5-hydroxy-4',7-dimethoxy-6-methyl flavanone/8-demethyl-eucalyptin (**12**)] in 10 different *Eucalyptus* species. To corroborate the results

of HPLC/DAD analysis, the fragmentation pattern of selected mass ions were studied using HPLC-MS. The structures of all studied compounds are shown in Figure 1.

***HPLC/PDA analysis***

*Optimization of HPLC conditions:* After trying several solvent systems as detailed in experimental section, appropriate resolutions were obtained in 25 min using the following gradient: 0-2 min 60% B in A, 3-10 min 60-70% B, 10-20 min 70-100% B, 20-25 min 100% B. Post run time was 10 min (Figure 2A). This gradient was used for all further analyses of the twelve compounds in different species. This method was rapid as compared with previous reported methods [10].

*Method validation:* The proposed method was validated with respect to linearity, LOD, LOQ, intra- and inter-day precision and accuracy (Table 1). The calibration curves for compounds (**1-12**) were obtained with six concentrations. The correlation coefficient values ($r^2$ = 0.999) indicated the appropriate linearity between concentration and the corresponding peak areas.

LOD and LOQ for all analytes were determined by diluting the standard stock solutions of the corresponding compounds sequentially. The LOD (S/N = 3) and LOQ (S/N = 10) for compounds quantified were in the range of 6.3 - 175.7 ng and 21.1 - 585.7 ng, respectively (Table 1). Also, this method was found to be accurate with an overall recovery of 95 - 105% (RSD range 1.0–3.2%). The developed HPLC method showed good reproducibility for the quantitation of 12 compounds, with intra- and inter-day variations of these compounds less than 2.6% (RSD) and 3.5% (RSD), respectively (Table 1). The RSD values for robustness studies were below 5% for the parameters studied.

*Analysis and quantitation of Eucalyptus samples:* Of the twelve compounds used in method development, eight were detected and quantified in the crude methanolic extracts of leaves of the selected ten *Eucalyptus* species. Linear equations developed from calibration curves were used to determine the percentage of individual components in the extracts (Table 2). The HPLC chromatogram of the crude methanolic extract of *E. sieberi* at 330 nm is shown in Figures 2B. Pinocembrin (**7**) was the major unsubstituted B ring flavanone in leaves of *E. sieberi* and *E. fraxinoides*, and was present at high concentrations (8.8 and 6.5% of dried methanolic extract respectively). In contrast, the methanol extract of leaves of *E. consideniana* was a rich source of pinostrobin (**8**). All other eucalypt species contained lower concentrations of the quantified unsubstituted B ring flavanones. The flavone, eucalyptin (**11**) was

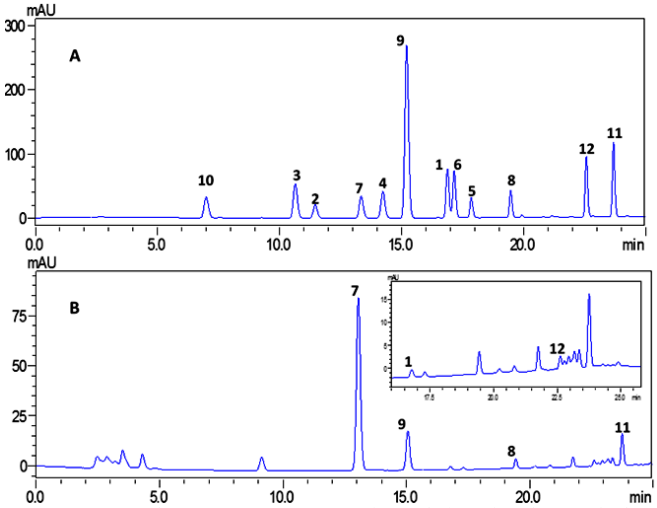



**Figure 2:** HPLC chromatogram at 330 nm of A) solution of twelve standards, **1**: flavanone; **2**: 6-hydroxy flavanone; **3**: 7-hydroxy flavanone; **4**: 5-methoxy flavanone; **5**: 6-methoxy flavanone; **6**: 7-methoxy flavanone; **7**: 5,7-dihydroxy flavanone/pinocembrin; **8**: 5-hydroxy-7-methoxy flavanone/pinostrobin; **9**: 5,7-dimethoxy flavanone; **10**: 4',5,7-trimethoxy flavanone/naringenin; **11**: 5-hydroxy-4',7-dimethoxy-6,8-dimethyl flavanone/eucalyptin; **12**: 5-hydroxy-4',7-dimethoxy-6-methyl flavanone/8-demethyl-eucalyptin; B) Methanol extract of *E. sieberi*, using 0.1% formic acid in water (A) and methanol (B): 0-2 min 60% B in A, 2-10 min 60-70% B, 10-20 min 70-100% B, 20-25 min 100% B.

**Table 1:** Chromatographic characteristics of the flavonoids and validation results of the analytical method.

| Sr. No. | Analytes | Test wave-length (nm) | Retention time (min.) | Test range (ng) | Regression equation (y = mx + c) | $(r^2)^A$ | LOD[B] (ng) | LOQ[B] (ng) | Repeatability[C] (1 day, n = 3) RSD (%) | Inter-mediate precision[C] (3 days, n = 9) RSD (%) | Accuracy[D] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | Recovery | RSD (%) |
| 1 | Flavanone (**1**) | 322 | 16.87 | 40-350 | y = 9820.9x + 85462 | 0.9999 | 11.9 | 39.7 | 2.3 | 3.5 | 94.9 | 2.2 |
| 2 | 6-Hydroxy flavanone (**2**) | 256 | 11.45 | 20-350 | y = 4042.7x + 45244 | 0.9997 | 6.3 | 21.1 | 0.6 | 1.9 | 103.4 | 1.4 |
| 3 | 7-Hydroxy flavanone (**3**) | 277 | 10.64 | 200-4000 | y = 4927.8x - 567464 | 0.9999 | 56.3 | 187.8 | 1.8 | 2.3 | 97.9 | 1.0 |
| 4 | 5-Methoxy flavanone (**4**) | 332 | 14.23 | 400-4000 | y = 8786.3x - 2156723 | 0.9998 | 96.5 | 321.9 | 2.6 | 3.3 | 105.3 | 1.2 |
| 5 | 6-Methoxy flavanone (**5**) | 352 | 17.85 | 40-350 | y = 1314.3x – 37090 | 0.9993 | 10.3 | 34.3 | 0.8 | 1.5 | 95.5 | 2.3 |
| 6 | 7-Methoxy flavanone (**6**) | 275 | 17.15 | 200-3500 | y = 1568.4x - 136693 | 0.9998 | 51.1 | 170.2 | 1.2 | 3.1 | 99.6 | 1.6 |
| 7 | Pinocembrin (**7**) | 289 | 13.34 | 600-4000 | y = 3219.6x - 843570 | 0.9995 | 175.7 | 585.7 | 0.6 | 1.0 | 96.2 | 3.2 |
| 8 | Pinostrobin (**8**) | 288 | 19.47 | 400-3500 | y = 3689.5x - 565968 | 0.9993 | 109.4 | 364.8 | 0.7 | 1.4 | 98.5 | 1.8 |
| 9 | 5,7-dimethoxy flavanone (**9**) | 284 | 15.34 | 200-4000 | y = 4139.1x - 977512 | 0.9998 | 149.4 | 498.1 | 1.3 | 3.0 | 99.8 | 1.5 |
| 10 | Naringenin (**10**) | 289 | 6.98 | 600-4000 | y = 5861.6x + 189755 | 0.9998 | 155.1 | 517.1 | 0.9 | 1.5 | 105.4 | 1.9 |
| 11 | Eucalyptin (**11**) | 324 | 23.68 | 100-4000 | y = 1515.3x + 59664 | 0.9999 | 32.4 | 108.1 | 0.7 | 1.4 | 97.7 | 2.1 |
| 12 | 8-Demethyl-eucalyptin (**12**) | 329 | 22.57 | 300-5000 | y = 3184.3x - 697691 | 0.9993 | 82.4 | 274.9 | 2.1 | 3.5 | 94.9 | 3.2 |

[A] $r^2$ is correlation coefficient of each calibration curve
[B] LOD and LOQ were estimated by successively diluting the standard solutions, considering a signal-to-noise ratio of 3 and 10, respectively
[C] 100 ng/μL of each analyte was used for the determination of repeatability and precision
[D] Spiked amount (ng) for each analyte is the lowest amount used in the test range (column 4).

**Table 2:** Quantitation of 12 flavonoids in methanol extracts of ten *Eucalyptus* species

| Sr. No. | Analytes[B] | % of dried methanol extract [A] (% DM) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *E. sieberi* | *E. rossii* | *E. fastigata* | *E. macro rhyncha* | *E. fraxinoides* | *E. agglo merata* | *E. consi deniana* | *E. pauciflora* | *E. dives* | *E. obliqua* |
| 1 | Flavanone (**1**) | 0.03±0.01 (0.01) | | 0.02±0.01 (0.01) | | | | 0.02±0.01 | | | |
| 3 | 7-OH flavanone (**3**) | | | 0.14±0.02 (0.06) | | | | | | | |
| 6 | 7-$CH_3O$ flavanone (**6**) | | | | | 0.09±0.01 (0.03) | | | | | |
| 7 | Pinocembrin (**7**) | 8.80±0.33 (3.52) | 0.08±0.02 (0.04) | | 0.07±0.01 (0.03) | 6.50±0.29 (2.47) | 1.12±0.23 (0.53) | | 0.82±0.03 (0.29) | 0.04±0.01 (0.01) | 0.03±0.01 (0.01) |
| 8 | Pinostrobin (**8**) | 0.48±0.06 (0.19) | 0.19±0.02 (0,08) | | | 0.21±0.03 (0.08) | 0.13±0.02 (0.06) | 2.80±0.19 (0.81) | 0.21±0.02 (0.07) | 0.19±0.02 (0.06) | 0.19±0.02 (0.07) |
| 9 | 5,7-dimethoxy flavanone (**9**) | 1.54±0.01 (0.62) | 0.23±0.01 (0.10) | | | 0.42±0.03 (0.15) | | | 0.12±0.02 (0.04) | 0.20±0.03 (0.06) | 0.90±0.03 (0.31) |
| 11 | Eucalyptin (**11**) | 0.29±0.03 (0.12) | 0.15±0.02 (0.07) | 0.57±0.03 (0.26) | 0.27±0.02 (0.11) | 0.11±0.02 (0.04) | 0.31±0.02 (0.15) | 0.15±0.02 (0.04) | 0.38±0.02 (0.13) | 0.18±0.01 (0.06) | 0.30±0.02 (0.10) |
| 12 | 8-demethyl eucalyptin (**12**) | 0.16±0.02 (0.06) | | 0.18±0.02 (0.08) | 0.19±0.02 (0.07) | 0.10±0.01 (0.04) | 0.21±0.02 (0.09) | 0.10±0.01 (0.03) | 0.24±0.02 (0.08) | 0.16±0.01 (0.05) | 0.27±0.02 (0.09) |

[A]Concentration = mean ± s.d. (n = 3); [B]2, 4, 5 and 10 were not detected in any species

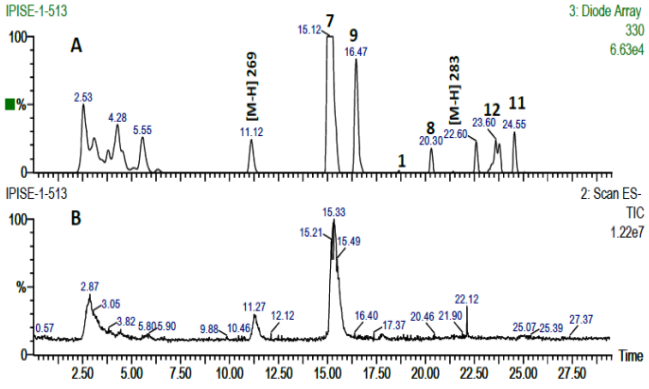



**Figure 3:** HPLC–DAD–ESI–MS analysis of the methanol extract of the leaves of *E sieberi* (A) HPLC–UV monitored at 330 nm and (B) LC-negative ESI–MS total ion current (TIC) profile, using using 0.1% formic acid in water (A) and methanol (B): 0-2 min 60% B in A, 2-10 min 60-70% B, 10-20 min 70-100% B, 20-25 min 100% B.

present in all ten species of *Eucalyptus*, and 8-demethyl-eucalyptin (**12**) was present in nine species out of ten. This is not surprising, as these flavones occur commonly in eucalypts [8]. Most of the plants contained between four and six of the 12 quantified flavonoids, except *E. macrorhyncha* where only two were observed.

### *LC-MS analysis*

HPLC coupled to electrospray ionization mass spectrometry was used to clarify the mass spectrometric behaviour of the naturally occurring flavonoids in *Eucalyptus*. The methanol extracts of various species of *Eucalyptus* were first investigated by mass spectrometry to obtain the ions of the molecular species. Negative mass spectra were then obtained from these $[M-H]^-$ ions and were analyzed to study the fragmentation.

Figure 3 shows the HPLC–DAD (330 nm) and total ion current (TIC) profiles of the crude methanolic extract of *E. sieberi* leaves, while Figure 4 shows the LC-MS spectra obtained from the $[M-H]^-$ ions for the six flavonoids present, namely flavanone, pinocembrin, pinostrobin, 5,7-dimethoxy flavanone, eucalyptin and 8-demethyl-eucalyptin. Along with these flavonoids, an other flavonoid was identified as alpinetin on the basis of mass fragmentation and absoption maximum [10]. An unsubstituted B-ring flavanone was also observed at 22.60 min. LC-MS spectra of other *Eucalyptus* species are shown in supplementary information (Figures S1-S9).

The characteristic fragments observed for compounds present in the ten species of *Eucalyptus* are shown in Figure 4. Unsubstituted B-ring flavanones produce characteristic fragments with the loss of

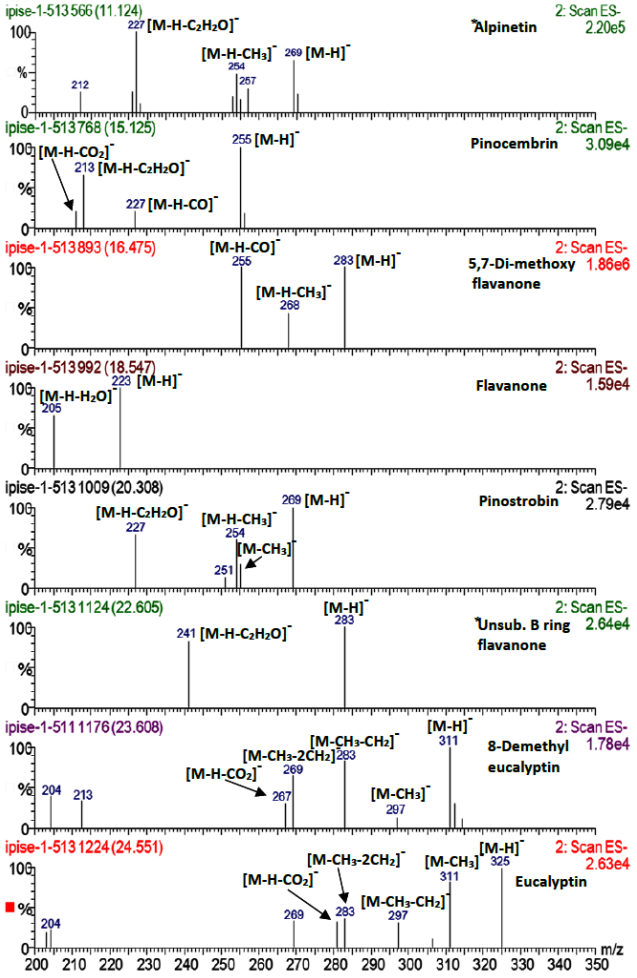



**Figure 4:** ESI-MS of flavonoids (1) [M-H] 269 possibly: Alpinetin, (2) [M-H] 255: Pinocembrin, (3) [M-H] 283: 5,7-Di-methoxy flavanone, (4) [M-H] 223: Flavanone, (5) [M-H] 269: Pinostrobin, (6) [M-H] 283, (7) [M-H] 311: 8-Demethyl eucalyptin and (8) [M-H] 325: Eucalyptin in methanol extract of *E sieberi*.
*Compound without standard, identified on the basis of mass fragmentation and absorption maximum.

$C_2H_2O$ (42 Da). Methoxylated flavanones produce $[M-H-CH_3]^-$ fragment with the loss of 15 Da from $[M-H]^-$ ions. Flavones and some flavanones like pinocembrin produce a $[M-H-CO_2]^-$ fragment with the loss of 44 Da. C-Methylated flavones produce $[M-CH_3]^-$, $[M-CH_3-CH_2]^-$ and $[M-CH_3-2CH_2]^-$ fragments, with losses of 15, 29, and 43 Da respectively. Proposed fragmentation from the anion of B ring unsubstituted flavanone type pinocembrin and details of

fragment pattern as well as observed fragments given in supplementary information (Figure S10-11 and Table S1).

Further analysis of extracts of *Eucalyptus* species resulted in tentative identification of three more flavonoids in addition to the twelve already identified. 5-Methoxy-7-hydroxy flavanone (alpinetin), C-methylated flavone sideroxylin and dimethyl sideroxylin were identified based on the characteristic fragmentation pattern of unsubstituted B-ring flavanone and C-methylated flavones. Alpinetin eluted at 11.1 min with *m/z* 269 $[M-H]^-$ , 254 $[M-H-CH_3]^-$ and 227 $[M-H-C_2H_2O]^-$ showed absorption wavelength 287 nm. C-Methylated flavone sideroxylin $[M-H]^-$ 311 and demethyl sideroxylin $[M-H]^-$ 297 were eluted at 21.4 and 19.3 respectively, showing fragments $[M-CH_3]^-$, $[M-CH_3-CH_2]^-$ and $[M-CH_3-2CH_2]^-$. Some other uncharacterized B ring unsubstituted flavanones were also observed at 15.02 $[M-H]^-$ 255, 18.05 $[M-H]^-$ 269, 19.45 283, 22.6 $[M-H]^-$ 283.

***Implications of the work:*** The discovery of the subgenus specific distribution of B ring unsubstituted flavanones in eucalypts [8, 10] is a major step in understanding the chemical ecology of eucalypts. Tucker et al. [8] predicted that B ring unsubstituted flavanones would be inactive against those marsupials that focused their diets on the Monocalyptus subgenus of Eucalypts, yet active against those that fed on Eucalypts from the other major subgenus, Symphyomyrtus. Recent *in vivo* studies have confirmed these predictions [9] (Beale, Foley, Marsh unpubl data). Both pinocembrin (**7**) and flavanone (**1**) were equally effective in suppressing feeding by common brushtail possums on palatable artificial diets, whereas flavones with unsubstituted B rings (e.g. chrysin) and flavanones with substitutions in the B ring (e.g. naringenin (**10**) were not. It is likely that all B-ring unsubstituted flavanones are effective antifeedants against brushtail possums. However, when applying this method to understand the distribution and quantitative variation of these compounds, it remains possible that we will encounter other novel structures that will need to be characterized and perhaps evaluated for their activity.

## Experimental

***Chemicals and standards:*** All of the solvents used for extraction and purification were of laboratory grade. All chromatographic purifications were performed with silica gel #60-120, #230-400 and silica gel G (CDH India Ltd.), whereas TLC analyses were performed on silica gel coated Kieselgel 60 F254, 0.2 mm thick plates (Merck). HPLC grade methanol (JT Baker), acetonitrile (Sigma Aldrich), ultra-pure water (Elga®), acetic acid, formic acid and phosphoric acid were used for HPLC method development. Samples were prepared in methanol. Apart from chemical standards isolated from ether extract of leaves of *E. sieberi* (**7**, **11** and **12**), the remaining nine standards were purchased from Indofine Chemical Company Inc.- 121 Stryker Lane, Hillsborough, NJ 08844, USA. All standards were ≥98% pure.

***Plant materials:*** The leaves of ten species of *Eucalyptus* (*E. sieberi*, *E. rossii*, *E. fastigata*, *E. macrorhyncha*, *E. fraxinoides*, *E. agglomerata*, *E. consideniana*, *E. pauciflora*, *E. dives* and *E. obliqua*) were collected from the Palerang and Shoalhaven Shire regions of south-eastern New South Wales, Australia. All species are members of the subgenus Monocalyptus and are prominent in the diets of folivorous marsupials from south-eastern Australia. A voucher specimen of each was deposited in the herbarium of the Natural Products Field Laboratory, NIPER, S.A.S. Nagar, Punjab, India (NIP-NPM-CD-192 to NIP-NPM-CD-201). The leaves were freeze-dried, crushed coarsely and stored at -20 ºC until extraction.

***Preparation of extracts:*** Recently, we reported that Soxhlet extraction (SE) and accelerated solvent extraction (ASE) were more efficient than ultrasonic extraction (USE) for the extraction of pinocembrin from leaves of *E. sieberi*, and that ASE at 80 °C was more effective than ASE at either 60 °C or 100 °C [13]. Hence, we chose this method (ASE at 80 °C) to prepare extracts from the leaves of selected species of *Eucalyptus*.

Dried coarse leaves (10 g) of four species of *Eucalyptus* (*E. sieberi*, *E. rossii*, *E. fastigata*, *E. agglomerata*) were extracted by accelerated solvent extraction (ASE) with methanol at 80 °C for 20 min (two cycles). A Dionex ASE 100 accelerated solvent extractor was used for extractions. The extracts were filtered to remove particulate matter and were concentrated on a rotary evaporator (Buchi R-114, Switzerland) to yield 4.0 g (*E. sieberi*), 4.4 g (*E. rossii*), 4.5 g (*E. fastigata*) 4.6 g (*E. agglomerata*). Remaining extracts of six species of *Eucalyptus* were prepared previously by ASE to yield 3.9 g (*E. macrorhyncha*), 3.8 g (*E. fraxinoides*), 2.9 g (*E. consideniana*), 3.5 g (*E. pauciflora*), 3.1 g (*E. dives*) and 3.5 g (*E. obliqua*) of dried extracts [13].

***HPLC/PDA analyses:*** The HPLC analysis was carried out on a reversed-phased C18 column (InertSustain®, 4.6 X 250 mm; 5µm) connected to a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of a model LC-20 AD fitted with a SIL-20 AC HT autosampler and SPD-M20A photodiode-array detector. LC Solution software (Shimadzu) was used both for data collection and integration.

*Sample preparation:* Stock solutions (1 $mg.mL^{-1}$) of 12 standards (**1**-**12**) were prepared in methanol. Different working solutions were prepared by diluting the stock solutions with the same solvent. 100 µL from each of the 12 stock solutions were mixed to prepare the synthetic mixture. The sample solutions of methanolic extracts of various *Eucalyptus* species, (5 mg each of dried extract) were prepared by re-dissolving in methanol (1 mL) and filtering through a 0.20 µm PTFE syringe filter.

*Optimization of HPLC conditions:* A number of HPLC methods were tested in order to optimize separation of all 12 flavonoid standards. These included: a) a ternary mixture of water with 0.1% formic acid, acetonitrile (ACN) and methanol from 0 to 60 min; b) a binary gradient of water with 0.1% formic acid (solvent A) and CAN (solvent B) from 0 to 60 min; c) ACN: water with 0.05% formic acid (40:60) in an isocratic mode for 80 min; d) increasing the proportion of ACN from 25 min until it reached 100% at 50 min, then holding for 10 min; e) a gradient program consisting of water with 0.1% formic acid (solvent A) and methanol for 65 min; and finally, f) water with 0.1% formic acid (solvent A) and methanol in 25 mins.

*Final chromatographic conditions:* Appropriate separation of 12 flavonoids was achieved using a linear gradient of solvent A (0.1% formic acid in water) and solvent B (methanol). The optimized gradient was employed at a flow rate of 1.0 mL.min-1. That was: 0-2 min 60% B in A, 2-10 min 60-70% B, 10-20 min 70-100% B, 20-25 min 100% B. Post run time was 10 min. The chromatogram was monitored at 330 nm and the UV spectra of individual peaks were recorded in the range of 190-600 nm. The column temperature was 40 °C.

*Validation Procedure:* Stock solutions (1 $mg.mL^{-1}$) of standards (**1**–**12**) were diluted to appropriate concentrations for the establishment of calibration curves. Six concentrations of the 12 standards were injected in triplicate, and the calibration curves were constructed by

plotting the peak areas against the concentration of each analyte. The calibration curves were prepared to cover the expected concentration range of analyte in the samples. The limit of detection (LOD) and limit of quantification (LOQ) under the above stated chromatographic conditions were determined on the basis of response at a signal-to-noise ratio (S/N) of 3 and 10, respectively, by injecting serially diluted solutions.

Intra- and inter-day variations were used to determine the precision of the HPLC method. Intra-day precision was performed by triplicate analysis on a single day. The inter-day precision was carried out on three different days. Variations were expressed as the relative standard deviations (RSD). The accuracy of the quantitation method was evaluated by the recovery test. For this study, accurate amounts of the 12 analytes were added to known amounts of methanol extracts (ten selected *Eucalyptus* species) that were subsequently re-dissolved in methanol and analysed. The following formula was used to calculate average recoveries:

Recovery (%) = 100 × (amount found–original amount)/amount spiked,
and RSD (%) = (SD/mean) × 100%.

The robustness of the method developed was determined by making deliberate changes to the chromatographic conditions such as change in flow rate (1.0 ± 0.05 mL/min) and column temperature (40 ± 5°C). Each parameter was analysed in triplicate and the variation in the retention time were expressed as % RSD of the three determinations with respect to normal retention time.

*Quantitative Analysis:* Five µL of each extract (5 $mg.mL^{-1}$) was injected onto the HPLC column using an autosampler (in triplicate). Identification of the different compounds was made by comparing the HPLC retention times, UV absorption spectra with those of the standards as well as spiking of the present analytes. Quantitation was performed on the basis of linear calibration plots.

***HPLC-ESI-MS analyses:*** The LC–MS system consisted of a Waters 2767 Sample Manager and 2525 Binary Gradient Pump, coupled to a single quadrupole ZQ mass spectrometer (Micromass 4000), operating in the ESI mode. The MS experiment setup and data acquisition were conducted using the MassLynx software V 4.0.

*Preparation of sample solutions:* 10 mg dried methanol extract from each of the ten species of *Eucalyptus* was redissolved in methanol (1 mL) and filtered through a 0.20 µ PTFE syringe filter.

*LC–MS conditions:* The optimum values of MS analyses were as follows: ESI negative ion mode; capillary voltage, 3.82 kV; cone voltage, 44 V; dissolvation temperature, 350 °C; source temperature, 150 °C; extractor, 4 V; RF lens, 0.2 V; dissolvation gas, 150 L.h-1; cone gas 80 L.h-1. High-purity nitrogen was used as nebulizer and cone gas. MS analyses were performed in the mass range of 100–1500 *m/z*. Analytical method A was used for analysis using the same C-18 HPLC column. The flow-rate was 1 $mL.min^{-1}$. The mass detector received a flow of 150 $\mu L.min^{-1}$ from the splitter.

*LC–MS analysis:* Ions in the range of *m/z* 100-1500 were measured in the mass fragmentation of respective peaks. HPLC peaks were identified by the measured reference spectra of the 12 flavonoid standards.

**Supplementary data:** Supplementary material associated with this article is available.

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