

Article

Chemical Constituents of Kino Extract from *Corymbia torelliana*

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Abstract: Seven flavanones were identified from kino exudate of *Corymbia torelliana* by spectroscopic and spectrometric methods including UV, 1D and 2D NMR and UPLC-HR-MS. The study identified seven molecules, namely 3,4',5,7-tetrahydroxyflavanone (**1**), 3',4',5,7-tetrahydroxyflavanone (**2**), 4',5,7-trihydroxyflavanone (**3**), 3,4',5-trihydroxy-7-methoxyflavanone (**4**), (+)-(2*S*)-4',5,7-trihydroxy-6-methylflavanone (**5**), 4',5,7-trihydroxy-6,8-dimethylflavanone (**6**) and 4',5-dihydroxy-7-methoxyflavanone (**7**) from this eucalypt species. This is the first report of these natural products from *C. torelliana* kino exudate.

Keywords: *Corymbia*; *Eucalyptus*; NMR spectroscopy; kino; natural products

1. Introduction

Corymbia torelliana belongs to the Myrtaceae, a family of plants with at least 133 genera and more than 3800 species [1]. This family includes the eucalypts, *Eucalyptus*, *Corymbia* and *Angophora*, which are the world's most widely planted hardwood trees. *C. torelliana* is native to rainforest fringes in tropical Australia and it has an unusual mutualism with stingless bees. Stingless bees are strongly attracted to the resin of *C. torelliana* fruits and the bees subsequently help to disperse the seeds [2–5], thereby making this tree species invasive where it has been introduced for amenity plantings outside its natural range.

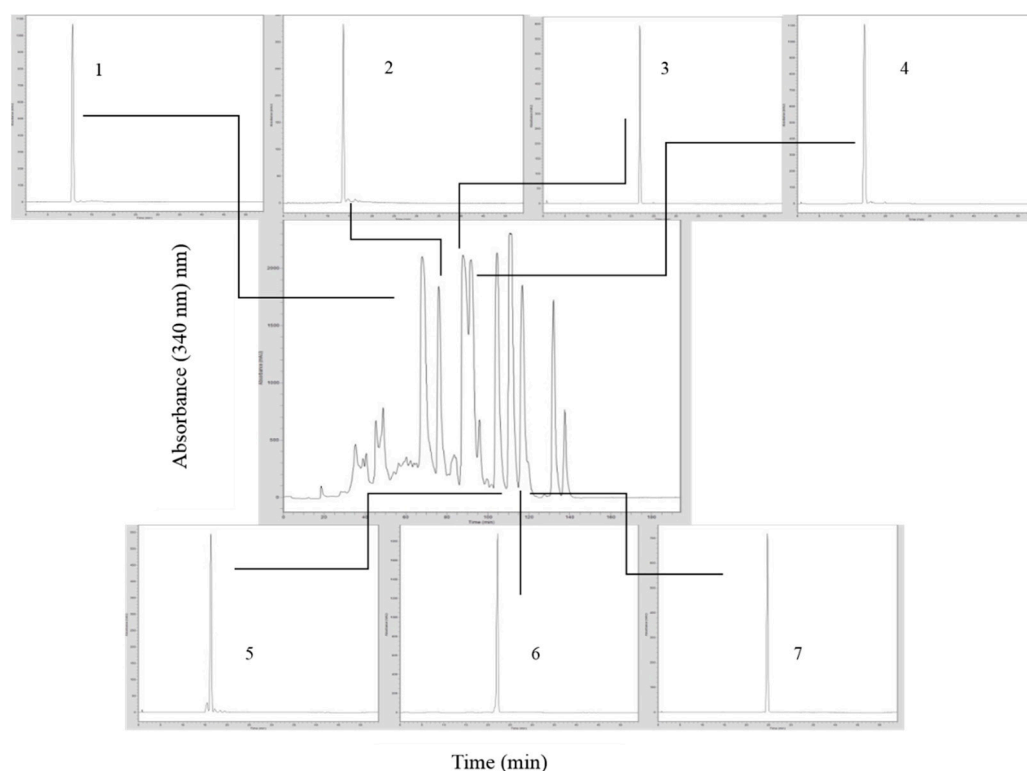
Kino is a class of wood exudate found in many Myrtaceae species including the eucalypts. Kinins are characterized by their deep rich colouration, high tannin content, polyphenols and astringency [6,7]. Kinins have been used by Aboriginal people in Australia for thousands of years for medicinal purposes [8]. The secondary metabolite chemistry of kinins was initially examined 50 years ago by paper partition chromatography and two-dimensional co-chromatography and, more recently, by NMR, although only a small number of eucalypt species have been studied [9–16]. Different classes of phenolic compounds have been identified from kino extracts, and these compounds include aromadendrin, kaempferol, ellagic acid, gallic acid, gallocatechin, epicatechin, catechin, naringenin, hemiphloin, isohemiphloin, leucoanthocyanin, gallic acid and engletin [9–16]. In a recent study using NMR, 3,5,4',5''-tetrahydroxy-7-methoxy-6-[1-(p-hydroxy-phenyl)ethyl] flavanone and 3,5,7,4',5''-pentahydroxy-6-[1-(p-hydroxy-phenyl)ethyl] flavanone have been identified from kino exudate of *C. citriodora*—the first record of phenylethyl-dihydro flavonols in plant exudates [9].

We have found that crude extract from the kino of *C. torelliana* has antimicrobial activity against both Gram-negative and Gram-positive bacteria *in vitro*. This finding led us to isolate and characterise the major chemical components of *C. torelliana* kino extract with a view to future screening of their antimicrobial activities.

2. Results and Discussion

The crude extract of kino displayed a HPLC-UV trace of eight major compounds with flavonoid-like spectra (Figure 1). Seven compounds were recovered in sufficient purity and quantity by preparative HPLC for spectroscopic identification.

Figure 1. HPLC-UV/DAD chromatogram of crude extract from kino of *Corymbia torelliana*. UV trace of crude extract (with UV spectra of each major peak).

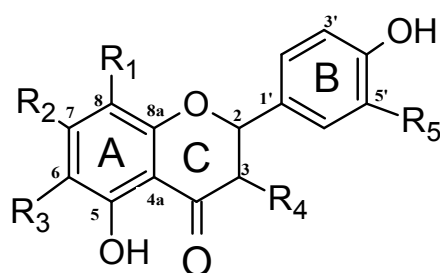


The isolated compounds were identified via spectroscopic analyses, including $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and high resolution mass spectroscopy. The NMR data and assignments for identified compounds are provided (Tables 1–3). The chemical formulas of the compounds were determined by liquid chromatography coupled to the high resolution mass spectrometry of $[\text{M-H}]^-$ ions (Table 4). The structures of these compounds are shown (Figure 2).

All seven flavanones isolated from kino extracts of *C. torelliana* have a 4',5-dihydroxyflavanone core structure that is further substituted with hydroxyl, methoxy and/or methyl groups. Compounds were identified respectively as 3,4',5,7-tetrahydroxyflavanone (**1**), 3',4',5,7-tetrahydroxyflavanone (**2**), 4',5,7-trihydroxyflavanone (**3**), 3,4',5-trihydroxy-7-methoxyflavanone (**4**), (+)-(2*S*)-4',5,7-trihydroxy-6-methylflavanone (**5**), 4',5,7-trihydroxy-6,8-dimethylflavanone (**6**) and 4',5-dihydroxy-7-methoxyflavanone (**7**). All compounds were cream- to pale yellow-coloured powders, and all compounds are reported from the kino extract of this species for the first time.

3,4',5,7-tetrahydroxyflavanone (**1**) has been identified previously from kino exudates of *C. calophylla* and *C. gummifera* [13,14]. 4',5,7-trihydroxyflavanone (**3**) has been reported as naringenin from kino exudate of *C. maculata* [17], while 3,4',5-trihydroxy-7-methoxyflavanone (**4**) has been reported from kino exudates of *C. citriodora* [18]. Interestingly, only these three compounds have been reported previously from kino samples of eucalypt species. We are reporting four other known compounds for the first time from kino exudates, although these have been reported previously from other plant extracts. 3',4',5,7-Tetrahydroxyflavanone (**2**), *i.e.*, eriodictyol, was isolated and identified for the first time from leaves of *Eriodictyon californicum* (Boraginaceae) [19]. (+)-(2*S*)-4',5,7-Trihydroxy-6-methylflavanone (**5**) has been found in *Pseudotsuga* spp. (Pinaceae) [20,21] and 4',5,7-trihydroxy-6,8-dimethylflavanone (**6**) has been reported from bulbs of *Pancratium maritimum* (Amaryllidaceae) [22] and stems of *Bauhinia glauca* (Leguminosae) [23]. 4',5-Dihydroxy-7-methoxyflavanone (**7**) has been obtained from resin of *Xanthorrhoea* spp. (Xanthorrhoeaceae) [24].

Figure 2. Structures of isolated flavanones (**1–7**) identified from kino of *Corymbia torelliana*.



Compound	R ₁	R ₂	R ₃	R ₄	R ₅
1	H	OH	H	OH	H
2	H	OH	H	H	OH
3	H	OH	H	H	H
4	H	OMe	H	OH	H
5	H	OH	Me	H	H
6	Me	OH	Me	H	H
7	H	OMe	H	H	H

Table 1. ^1H and ^{13}C -NMR spectral data for compounds **1** and **2** from *Corymbia torelliana*.

Position	Compound 1 (d ₆ DMSO)			Compound 2 (CD ₃ OD)		
	^{13}C	^1H	HMBC	^{13}C	^1H	HMBC
2	82.7 (CH)	5.04 (d,11.4)	3,4,8a,1',2'	80.4 (CH)	5.27 (dd, 12.6, 3.0)	1',2',6'
3	71.3 (CH)	4.56 (d,11.4)	2,4,1'	44.1(CH ₂)	A 3.05 (dd,17.0, 12.6) B 2.69 (dd, 17.0, 3.0)	2,4,1' 4
4	197.2 (C)			197.2 (C)		
4a	100.1 (C)			103.3 (C)		
5	163.1 (C)			165.1 (C)		
5-OH		11.9 br s				
6	96.0 (CH)	5.89 (d, 1.8 Hz)	4a,5,7,8	97.6 (CH)	5.88 (d, 1.7)	4a,7
6-Me						
7	167.1 (C)			169.0 (C)		
7-Me						
8	95.0 (CH)	5.83 (d,1.8 Hz)	4a,6,7,8a	96.5 (CH)	5.86 (d, 1.7)	4a,7,8a
8a	162.4 (C)			164.6 (C)		
1'	127.5 (C)			132.0 (C)		
2'				114.7 (CH)	6.91 br s	2,1',3',4',6'
3'				116.3 (CH)	6.78 s	1'
4'	157.6 (C)			146.6 (C)		
5'				143.2 (C)		
6'				119.3 (CH)	6.78 s	2,2',4'
2'/6'	129.3 (CH)	7.31 (d, 8.25)	2,1',3'/5',4'			
3'/5'	114.8 (CH)	6.78 (d, 8.25)	1',2'/6',4'			

Table 2. ^1H and ^{13}C -NMR spectral data for compounds **3** and **4** from *Corymbia torelliana*.

Position	Compound 3 (d ₆ DMSO)			Compound 4 (CD ₃ OD)		
	^{13}C	^1H	HMBC	^{13}C	^1H	HMBC
2	78.4 (CH)	5.43 (dd, 12.9, 2.9)	3,4,1',2'	83.7 (CH)	5.01 (d, 11.4)	3,4,1',2'
3	41.9 (CH ₂)	A 3.25 (dd, 17.2, 12.9) B 2.68 (dd, 17.2, 2.9)	2,4,1' 2,1'	72.3 (CH)	4.57 (d, 11.4)	2,4,1'
4	196.8 (C)			197.6 (C)		
4a	101.7 (C)			101.2 (C)		
5	162.9 (C)			163.7 (C)		
5-OH		12.13 (br s)	4a,5,6			
6	95.8 (CH)	5.85 (br s)	5,7,8,8a	94.6 (CH)	6.08 (d, 2.4)	5,7,8,4a
6-Me						
7	166.7 (C)			168.4 (C)		
7-Me				54.9 (CH ₃)	3.8	7
8	94.9 (CH)	5.85 (br s)	5,7,8,8a	93.6 (CH)	6.04 (d, 2.4)	4a,6,7,8a
8a	163.4 (C)			163.0 (C)		
1'	128.8 (C)			127.7 (C)		
2'						
3'						
4'	157.6 (C)			157.8 (C)		
5'					6.8	
6'					7.35	
2'/6'	128.3 (CH)	7.31 (d, 8.5)	2,1',3'/5',4'	128.4 (CH)	7.35 (d, 8.4)	2,3',4'
3'/5'	115.1 (CH)	6.79 (d,8.5)	1',2'/6',4'	114.7 (CH)	6.80 (d, 8.4)	1',2',4'

Table 3. ^1H and ^{13}C -NMR spectral data for compounds 5–7 from *Corymbia torelliana*.

Position	Compound 5 (d ₆ DMSO)			Compound 6 (CD ₃ OD)			Compound 7 (CDCl ₃)		
	^{13}C	^1H	HMBC	^{13}C	^1H	HMBC	^{13}C	^1H	HMBC
2	77.9 (CH)	5.32 (dd,12.4,2.8)	3,4,1',2'	78.6 (CH)	5.30 (dd, 12.9, 3.0)	1',2'	79.0 (CH)	5.34 (dd, 13.0, 3.0)	3,4,1',2'
3	42.0 (CH ₂)	A 3.12(dd,17.0,12.4) B 2.60 (dd,17.0,2.8)	2,4,1' 2,1'	42.7(CH ₂)	A 3.05 (dd,16.8, 12.9) B 2.71 (dd, 16.8, 3.0)	2,4,1' 4	43.2 (CH ₂)	A 3.08 (dd,17.3, 13.0) B 2.78 (dd, 17.3, 3.0)	2,4,1' 2,1'
4	194.3 (C)			196.7 (C)			196.2 (C)		
4a	100.6 (C)			102.8 (C)			103.9 (C)		
5	160.4 (C)			157.9 (C)			164.5 (C)		
5-OH		12.47 (br s)	4a,5,6						
6	103.3 (C)			103.5 (C)			95.1 (CH)	6.06 (d, 2.4)	4a,5,7,8
6-Me	7.1 (CH ₃)	1.83 (s)	5,6,7	6.8 (CH ₃)	1.99 (s)	5,6,7			
7	168.8 (C)			163.6			168.1 (C)		
7-Me							55.7 (CH ₃)	3.79 (s)	7
8	94.9 (CH)	5.82 (s)	6,7,8a	101.6 (C)			94.2 (CH)	6.03 (d, 2.4)	6,7,8a
8-Me				6.0 (CH ₃)	1.98 (s)	7,8,8a			
8a	160.2 (C)			158.8 (C)			163.2 (C)		
1'	129.2 (C)			130.2 (C)			127.7(C)		
2'/6'	128.0 (CH)	7.29 (d, 8.3)	2,1',3'/5',4'	127.4 (CH)	7.32 (d, 8.4)	2,3',4'	127.9 (CH)	7.31 (d, 8.6)	2,1',3'/5', 4'
3'/5'	115.0 (CH)	6.77 (d, 8.3)	1',2'/6',4'	114.9 (CH)	6.81 (d, 8.4)	1',2',4'	115.7 (CH)	6.88 (d, 8.6)	1',2'/6',4'
4'	157.5 (C)			157.4 (C)			156.5 (C)		

Table 4. The kino extract of *Corymbia torelliana* contained flavanones 1–7; analyses by UPLC-HR-MS.

Compound	Identification	Formula [M–H] [−]	Calculated Mass [M–H] [−] (<i>m/z</i>)	Found Mass
1	3,4',5,7-tetrahydroxyflavanone	C ₁₅ H ₁₁ O ₆	287.05553	287.0565
2	3',4',5,7-tetrahydroxyflavanone	C ₁₅ H ₁₁ O ₆	287.05553	287.0566
3	4',5,7-trihydroxyflavanone	C ₁₅ H ₁₁ O ₅	271.06063	271.0616
4	3,4',5 -trihydroxy-7-methoxyflavanone	C ₁₆ H ₁₃ O ₆	301.07119	301.0721
5	(+)-(2 <i>S</i>)-4',5,7-trihydroxy-6-methylflavanone	C ₁₆ H ₁₃ O ₅	285.07629	285.0773
6	4',5,7-trihydroxy-6,8-dimethylflavanone	C ₁₇ H ₁₅ O ₅	299.09695	299.0930
7	4',5-dihydroxy-7-methoxyflavanone	C ₁₆ H ₁₃ O ₅	285.07629	285.0771

3. Experimental Section

All solvents used for extraction and chromatographic analysis were analytical grade. Ethyl acetate (EtOAc), acetonitrile (ACN) and deuterated solvent (DMSO-*d*₆) were purchased from Merck Pty Ltd (Kilsyth, Australia). Water was obtained from an in-house Milli-Q Ultrapure water system.

3.1. Plant Material

Fresh kino samples were collected from *C. torelliana* trees on the Sunshine Coast (26°42'S, 153°02'E), Queensland, Australia. The botanical identity of the trees was verified by Dr. Tom Lewis, Queensland Department of Agriculture, Fisheries and Forestry, and voucher specimens were deposited in the University of the Sunshine Coast herbarium (USC14055, USC14056 and USC14057). Samples were collected into clean vials from naturally occurring kino exudates of the trees from late November to March (late spring through early autumn), transported to the laboratory on ice, and stored in the dark at −20 °C until tested. Collection of samples from crystallized kino was avoided because of the effect of sunlight exposure on the chemical composition of kino [25].

3.2. Extraction

The kino samples were extracted in EtOAc/water (4:3). The EtOAc extracts were stored at −20 °C until fractionation by preparative HPLC. The EtOAc dry extract (100 mg) was dissolved in ACN/water (1:1) for fractionation by preparative chromatography. Eight compounds were recovered, but one compound was found in very low quantities and its identification has not been completed.

3.3. Chromatography

3.3.1. Analytical HPLC-UV/DAD

Analytical chromatographic analyses were performed on a Perkin Elmer series 200 HPLC using Chromera software. The column was a Phenomenex Synergi Fusion 4 μm, 4.6 × 75 mm polar embedded RP column. Mobile Phase A was 95:5 water:ACN and Mobile Phase B was 10:90 water:ACN. The flow rate was 1.2 mL/min, gradient elution from mobile phase A to mobile phase B was 90/10 → 0/100, total run-time was 55 min, and detection was at 205, 260, 290 and 340 nm wavelength.

3.3.2. Preparative HPLC

The EtOAc extracts were evaporated and re-dissolved (10% w/v) in ACN/water (1:1) and fractionated by preparative HPLC. Preparative chromatography was performed on an RP-C18 4 μ m, 21.2 \times 100 mm column (Fusion Phenomenex). Mobile Phase A was 95:5 water:ACN and Mobile Phase B was 10:90 water:ACN. The flow rate was 2 mL/min, gradient elution from Mobile Phase A to Mobile Phase B was 90/10 \rightarrow 0/100, total run-time was 194 min, and detection was at 205, 260, 290 and 340 nm wavelength.

3.3.3. UPLC-HR-MS Analysis

Samples from three *C. torelliana* kino samples were reconstituted in MeOH (2 mg/mL) and injected onto an Eclipse Plus C18 UPLC column 1.8 μ m, 2.1 \times 100 mm. Mobile Phase A consisted of 0.1% acetic acid in water and Mobile Phase B consisted of 0.1% acetic acid in ACN. The flow rate was 0.4 mL/min. The gradient was 2.5% Mobile Phase B at 0.5 min to 20% Mobile Phase B at 15 min and 100% Mobile Phase B at 25 min, then held for 2.5 min. A photodiode array detector was coupled to the LC and set at 205, 260, 290 and 340 nm. The spray voltage was set to 3.5 kV with the source temperature at 100 °C. The ESI module was Heated ESI (HESI) connected onto a qExactive Mass spectrometer (qEMS). The qEMS resolution was set at 140,000 and separate UPLC runs were performed for positive and negative ionization modes.

3.3.4. Spectroscopic Analysis

NMR spectra were recorded on either a Varian 500 or 600 MHz unity INOVA spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^1H and ^{13}C -NMR chemical shifts were referenced to the solvent peaks for DMSO- d_6 at δ_{H} 2.50 and δ_{C} 39.43, for CD $_3$ OD- d_4 at δ_{H} 3.35 and δ_{C} 49.05 and for CDCl $_3$ - d_1 at δ_{H} 7.25 and δ_{C} 77.0, respectively. Chemical shifts (δ) are given in ppm and coupling constants (J) in Hz (Tables 1–3).

4. Conclusions

We identified seven flavanones for the first time from kino exudate of the ecologically and ethnobotanically significant eucalypt tree, *Corymbia torelliana*. These compounds have a 4',5-dihydroxyflavanone core structure that is further substituted with hydroxyl, methoxy and/or methyl groups. Three of these flavanones have been reported previously from kino exudates of other *Corymbia* species, but we are reporting four other flavanones for the first time from eucalypt kino. The antimicrobial activity of these flavanones, and their potential role in the attractiveness of *C. torelliana* fruits to stingless bees, are the subjects of further study.

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Author Contributions

Motahareh Nobakht and Peter R. Brooks conceived and designed the experiments. Motahareh Nobakht and Tanja Grkovic performed the experiments. Motahareh Nobakht, Tanja Grkovic, Peter R. Brooks and Ronald J. Quinn analysed the data. Peter R. Brooks, Tanja Grkovic, Stephen J. Trueman, Helen M. Wallace and Mohammad Katouli contributed reagents, materials or analysis tools. Motahareh Nobakht, Peter R. Brooks, Tanja Grkovic, Stephen J. Trueman, Helen M. Wallace, Mohammad Katouli and Ronald J. Quinn wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of compounds **1–7** are available from the authors.

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