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Anti-staphylococcal activity of *C*-methyl flavanones from propolis of Australian stingless bees (*Tetragonula carbonaria*) and

fruit resins of *Corymbia torelliana* (Myrtaceae)

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Abstract

Propolis of Australian stingless bees (*Tetragonula carbonaria*, Meliponini) originating from *Corymbia torelliana* (Myrtaceae) fruit resins was tested for its antimicrobial activities as well as its flavonoid contents. This study aimed at the isolation, structural elucidation and antibacterial testing of flavanones of *C. torelliana* fruit resins that are incorporated into stingless bee propolis. Flavanones of this study were elucidated by spectroscopic and spectrometric methods including UV, 1D and 2D NMR, EI-MS, ESI-MS and HR-MS. The results indicated known *C*-methylated flavanones namely, **1** (2*S*)-cryptostrobin, its regioisomer **2** (2*S*)- stroboponin, **3** (2*S*)- cryptostrobin 7-methyl ether, and **6** (2*S*)- desmethoxymatteucinol, and known flavanones **4** (2*S*)- pinostrobin and **5** (2*S*)- pinocembrin as markers for *C. torelliana* fruit resins and one propolis type. Ethanolic preparations of propolis were shown to be active against *Staphylococcus aureus* (ATCC 25923) and to a lesser extent against *Pseudomonas aeruginosa* (ATCC 27853). *C. torelliana* flavananones inhibited the growth of *S. aureus* therefore contributing to the antibacterial effects observed for Australian stingless bee propolis extracts.

Keywords: natural products; *Eucalyptus;* NMR spectroscopy; *Staphylococcus aureus;* antibacterial

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1. Introduction

Flavonoids are secondary metabolites widely distributed in the plant kingdom, and have been shown to have key functions in plant growth, UV filtering, chemical signalling and as phytoalexins [1]. Within the Myrtaceae family, flavonoids are found in the exudates of plant surfaces of *Eucalyptus, Corymbia, Syncarpia, Lophostemon* and *Angophora* [2]. Previous chemical investigations of Myrtaceae extracts included the identification of flavonoids sideroxylin, dimethyl-apigenin, angophorol and eucalyptin, a *C*-methylflavone from the leaf waxes of *Corymbia torelliana* F. Muell [2, 3]. The *C*-methylation of flavonoids is a typical feature of secondary metabolites found in Myrtaceae and therefore it has been suggested that these natural products can be used as markers for chemotaxonomy [2, 4]. Within this plant family, *Corymbia torelliana* produces resins in its woody fruiting capsules, or gumnuts [5]. However, limited chemical knowledge is available on the volatile portion of such fruit resins [6], and the presence of flavonoids has not been investigated yet.

Flavonoids have been reported as constituents of propolis of honeybees and stingless bees from temperate and tropical areas [7, 8]. Bee propolis is a mixture of resinous materials and bees secretions including beeswax [7-9]. In temperate areas of Australia, flavonoids of honeybee propolis included xanthorrhoeol, pterostilbene, sakuratenin and pinostrobin of Western Australian samples [10], and chalcones and flavonols in propolis originating from the stem exudates of *Acacia paradoxa* DC (Fabaceae) in South East Australia [11]. In tropical regions of eastern Australia, stingless bees *Tetragonula carbonaria* (Meliponini) interact with *C. torelliana* trees to collect fruit resins [5], and incorporate them into their cuticular profiles [6], suggesting that *C. torelliana* fruit resins can be incorporated into *T. carbonaria* propolis as a result of resin mixing with bees wax. Previous chemical analyses of stingless bee propolis by gas chromatography mass spectrometry resulted in the tentative identification of several volatiles including isoprenoids, gallic acid derivatives, hydrocarbons, esters and free fatty acids [6, 9, 12]. The chemical composition of

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propolis has been shown to depend upon the resin sources collected by bees [7]. Therefore, we hypothesised that *C. torelliana* fruit resins can contain characteristic flavonoids that can contribute to the final flavonoid content of the propolis mixture.

Stingless bees make extensive use of propolis during nest construction, for chemical defence [7], and possibly for the preservation of their honey from microbial spoilage [13]. The antimicrobial properties of propolis have been attributed to its high flavonoid content [14]. This study aimed at the isolation, structural elucidation and antibacterial testing of flavanones of *C. torelliana* fruit resins that are incorporated into stingless bee propolis. A combination of chromatography, spectroscopy and spectrometry techniques was used for the chemical analyses. Extracts of two chemotypes of propolis and isolated propolis constituents were tested against susceptible bacterial strains using two *in vitro* assays.

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2. Materials and methods

2.1 Chemicals

The solvents used for extraction and chromatographic analyses, absolute ethanol (EtOH), hexane (Hex), methanol (MeOH), acetonitrile (MeCN), deuterated solvent DMSO-d_6 were of analytical grade and purchased from Merck Pty Ltd (Kilsyth, Vic., Australia). Milli-Q water was obtained from an in-house Milli-Q Ultrapure Water System. Pinocembrin, pinostrobin, abietic acid, Muller-Hinton nutrient broth, 3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

2.2 Propolis and plant material

Wild colonies of *T. carbonaria* bees were transferred into clean beehives in July 2011. New top compartments were applied to each hive for the storage of propolis and honey. The bees were allowed to forage between September 2011 and March 2013. Multiple beehives (n= 14) were used across two experimental areas, where *C. torelliana* trees were present or absent. In the control apiary, *C. torelliana* trees were not present within 2 km of the beehives but other Myrtaceae species were found as resin sources for the control propolis. The propolis from replicate hives of the same apiary was combined into one sample batch to represent the two propolis chemotypes of this study. The fruit resins of *C. torelliana* were collected from fresh capsules in February 2012 and February 2013, and pooled in one sample for chromatography.

2.3 Extraction, purification and isolation

Fruit resins of *C. torelliana* capsules were dissolved in MeOH (5 % w/v) at room temperature in the dark (24 h) and stored at 4 °C for chromatography. The plant material was not sufficient for preparative chromatography, therefore compounds were purified from propolis for NMR characterisation.

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Propolis (5 g) from each site was extracted with hexane:MeOH (1:1) (1L). The MeOH extracts were evaporated to dryness to give the crude extracts. One type of propolis exposed to *C. torelliana* was fractionated by silica gel on an open column. Briefly, the MeOH dry extract (2 g) was dissolved in minimal warm hexane and loaded onto a flash column packed with silica gel (2% w/w) for fractionation, using a gradient elution from hexane to ethyl acetate. 55 fractions were recovered separately and analysed by thin-layer chromatography (silica gel 60F-254 eluted with hexane: ethyl acetate, 1:1) using iodine vapours for visualisation. Fractions 4 to 9 contained diterpenic acids and were pooled (200 mg) for further chromatography using a preparative column RP-C18 4 µm, 100 x 21.2 mm (Fusion Phenomenex). Fractions 16-23 contained the flavanones of interests, therefore they were grouped, and a portion (200 mg) was subjected to preparative chromatography. Preparative chromatography employed a gradient method with mobile phases of neutral Milli-Q water to acetonitrile from 100:0 to 0:100. Purified compounds were freeze dried to yield **1** (6.0 mg, 0.30% w/w), **2** (3.4 mg, 0.17% w/w), **3** (1.3 mg, 0.06% w/w), **4** (0.8 mg, 0.04% w/w), **5** (0.5 mg, 0.02% w/w), **6** (1.4 mg, 0.07% w/w), and **7** (1.5 mg, 0.07% w/w) (% crude w/w).

2.4 GC-MS Analyses

Propolis MeOH extracts were analysed using a Perkin Elmer Clarus 580 GC-EI-MS fitted with Elite-5MS column (L= 30m, ID0.25, DF0.25, PerkinElmer) and 70 eV ionisation. The injection port was 260˚C, with a split ratio of 10:1. The oven program operated at 50˚C for 0.5 min, ramping at 40˚C/min until 100˚C, then ramping at 15˚C/min until 240˚C and held for 5 min, then ramped at 4˚C/min until 300˚C and held for 5 min for a total runtime of 36 min. The transfer line was set at 300°C. The MS scan range was 50–650 Da. A further protocol was employed for the retention indices: the oven was programmed from 50˚C to 315˚C at 3˚C/min, and then held for 5 min, for a total runtime of 83 min. GC retention indices of components were calculated against *n*-alkane standards (C_8-C_{40}).

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2.5 HPLC-ESI-MS and HRMS analyses

LR-ESI-MS were recorded using a Bruker Amazon X Ion Trap mass spectrometer coupled with DionexUltimate 3000 HPLC controlled by Hystar 3.2 software, or a Waters ZQ single quadrupole mass spectrometer connected with Waters 2790 Alliance HPLC controlled by MassLynx 4.1 software. Both instruments are equipped with Diode Array Detector (DAD) for acquisition at 200-400 nm. Mass spectra were acquired in both positive and negative ionisation modes. The flow rate was 1.2 mL/min and total runtime was 46 min. The LC separation was performed on a polar embedded RP-C18 column, 75 µm, 4.6 x 4 mm (Phenomenex Synergi Fusion).

Accurate mass measurements of **1-7** were obtained by High Resolution Time of Flight Mass spectrometry (HR-ToF-MS). HR-ESI-MS were conducted by direct infusion of the purified constituent into the Agilent 6520 LC-Q-TOF-MS 6520 (Santa Clara, USA) or into Applied BioSystems Mariner ESI-ToF mass spectrometer.

2.6 NMR spectroscopy

NMR analyses included ^{1}H ^{13}C and, 2D COSY, HSQC and HMBC spectroscopy acquired at 30 °C on either a Varian 500 or 600 MHz Unity INOVA spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks for DMSO-d_6 at δ_H 2.50 and δ_C 39.45, respectively (supplementary data). The atom numbering for flavonoids referred to Andersen and Markham's nomenclature [1].

2.7 Quantitation by RP-HPLC-DAD

Estimated quantities of propolis constituents were calculated as percentages of compound areas over total peak areas in propolis solutions (4.4 mg/mL) using relative abundances from the GC-MS analyses, and from HPLC-DAD detection at 205 nm on a HPLC Series 200 with Flexar DAD

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(Perkin Elmer).

Accurate quantitative analyses were carried out for two propolis fractions containing only flavanones **1-6** or other constituents from propolis *C. torelliana* type. Fraction 'flavanones' was quantitated as pinocembrin equivalents (%, w/w). Fraction 'others' pooled several compounds that were quantitated according to similarity of chemical class: diones of single UV_{max} were quantitated as abietic acid equivalents (%, w/w), and phenols of multiple UV_{max} (including phenolic acids and unknowns) as gallic acid equivalents (%, w/w). Signals were interpolated against standards of pinocembrin (0 – 3.3 mg/mL), abietic acid (0.5 – 3.7 mg/mL) and gallic acid (0.6 - 4.8 mg/mL) from a commercial source (Castle Hill, NSW, Australia). The HPLC-DAD parameters including method, mobile phase and flow rate were the same as used for the LC-ESI-DAD-MS analyses. Detection was set at 205 nm. Results are presented as means of two independent analyses.

2.8 Absolute configuration

Optical rotations were measured on a Jasco P1020 polarimeter with a 1 dm microcell at room temperature. Compounds were prepared in solution with analytical grade methanol (supplementary data).

2.9 Antimicrobial assays

Cultures of *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were prepared on nutrient Mueller-Hinton broth (MHB) for 18 h at 37 °C prior to the assays. After the replacement with fresh MHB, the inoculum growth was measured by turbidity using a UV-Vis spectrometer at 600 nm against a blank of MHB. The optical density was between 0.50 and 0.55 OD as equivalent to 10^8 CFU/mL of stock broth. Inocula were diluted with fresh media to the final concentration of 10^5 CFU/mL in plates. Placement of test solutions was randomized in plates. Two methods were used for the antibacterial tests.

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For the agar diffusion assay, a disposable 20 x 20 cm sterile plate was used to accommodate 250 mL of inoculated agar (10^5 CFU/mL) for one experiment. The samples were ethanolic preparations and 100 µL were dispensed into individual holes (8 mm in diameter) punched in the inoculated agar plate. Serial dilutions of propolis extracts were tested in the concentration ranging between 26 µg and 880 µg per 100 µL in each well. Two fractions of *C. torelliana* propolis were obtained from pooling either the six flavanones or the other constituents. Both mixtures were tested in the range of 12.5 µg to 100 µg per well as well as the six individual flavanones. The carrier (100% ethanol) was used as the negative control. The positive control was phenol standard in ethanol at 0.5%, 1%, 2%, 4% and 8% (w/v). The plates were incubated at 37° C for 16 – 18 h. Results of growth inhibition were reported as the mean zone of inhibition (mm) and standardised by values of phenol equivalents (% w/v, mean ± SEM, n= 3).

For the microbroth dilution assay and Minimum Inhibitory Concentration (MIC) determination, 170 µL of inoculated MHB (10^5 CFU/mL) were dispensed into each well of a 96 well microtitre plate. The negative controls were only MHB, inoculated MHB, and inoculated MHB with 5.6% ethanol (v/v). The phenol standard (positive control) was added (10 µL) from stock solutions 0.5 to 4% (w/v) to inoculated MHB. Samples were two propolis extracts, two fractions and six flavanones, and were dispensed (10 µL) from stock solutions of 3.28, 1.00, 0.50, 0.25 or 0.12 mg/mL to inoculated MHB. Microplates were incubated for 16 h at 37 °C. The MIC values (µg/mL) indicated the lowest concentration at which no growth was observed. To facilitate the evaluation of bactericidal activity, 20 µL of MTT ethanolic solution (5 mg/mL) were added to each well and then the plates were incubated for 20 min at 37 °C. A blue colouration indicated bacterial growth, while a yellow colouration indicated a bactericidal effect of the test solution(s). Aliquots (5-100 µL) of MICs were replated on fresh Mueller-Hinton agar plates then incubated for 24 h at 37 °C reducing 99.9% of initial *S. aureus* population. MIC values were reported as the highest value from three independent experiments replicated on different days using fresh MHB, inocula and

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MTT solutions.

2.10 Data and statistical analyses

NMR, UV and MS spectra, and elemental composition were analysed by ACD/LAbs v. 12.01, MestreNova v. 8.1.2., Bruker Daltonics DataAnalysis v. 4.0 and Agilent MassHunter Workstation Software B.02.00. Concentration curves and statistical analyses were calculated using Graph Pad Prism 6.0. The curve fitting was determined using centred second order polynomial equations (quadratic). Statistical differences between the two ethanolic preparations of propolis were measured using unpaired Student *t*-test.

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3. Results and Discussion

3.1 Characterisation of flavanones

This study shows the presence of flavonoids in *C. torelliana* fruit resins (Fig. 1 A). Flavanones **1-6** (Fig. 1 B) were isolated from Australian stingless bee propolis and their structures were elucidated by the interpretation of spectroscopic data and comparison with the published literature. Key features were a pyranone skeleton that is typical of flavanones attached to a non-substituted catecholic B-ring [15], and linked to a 2-hydroxyl-benzoyl A-ring showing *C*-methyl substituents.

The UV and MS spectra were useful to identify these flavanones as they showed absorption maxima within the 277-295 nm and 300-300 nm regions [16]. The B-ring unsubstitution of the six flavanones was confirmed by the presence of a fragment ion at *m/z* 77 by GC-MS. Fragment ions in the ESI-MS and EI-MS indicated an intense *m/z* 167 for the compounds suggesting that the *C*-methylation occurred on the 5,7-dihydroxylation on the A-ring. The elemental composition of the six flavanones was obtained from HRMS. However, the combination of UV and MS data allowed only the tentative identifications of these flavanones at subclass level, and it was not sufficient to distinguish between isomers with different *C*-methyl substitutions. The structure elucidation was therefore based on NMR spectroscopy. The general configuration of the flavanones showed the chromene C-ring to be saturated, with the characteristic diastereotopic methylene pair at H-3 exhibiting coupling constants of (1H, J= 17.1, 3.0 Hz, H-α) and (1H, J= 17.1, 12.9 Hz, H-β) respectively, as well as a methine resonance at position H-2 (1H, J=12.9, 3.3 Hz). For all six flavonones the unsubstituted phenyl B-ring was confirmed by five aromatic protons at 7.38-7.53 ppm. Therefore, the six flavanones of this study differed only in the patterns of substitution of the A-ring.

Compounds **1** and **2** (see Fig. 1) were two *C*-methyl-isomers, with nearly identical MS spectra and ^{13}C chemical shifts, and very close retention times in both GC and LC. NMR spectroscopy showed that the two flavanones were distinguished by one *C*-methyl on either position

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C-6 or C-8, respectively. The ^{1}H-^{13}C HMBC correlations indicated that the methyl group on C-8 (δ_H 1.9 ppm) correlated with the carbon carrying the hydroxyl on C-7 (δ_C 164.6 ppm) and the aromatic C-9 (δ_C 159.4 ppm) for cryptostrobin **1** (5, 7-Dihydroxy-8-methylflavanone). On the other hand, the methyl group on C-6 correlated to both hydroxyl carbons at C-5 (δ_C 160.6 ppm) and C-7 for strobopinin **2** (5, 7-Dihydroxy-8-methylflavanone). Chemical shifts were in agreement with previous reports [17, 18]. These *C*-methyl flavanones have been identified in other Myrtaceae species such as *Melaleuca quinquenervia* [3].

^{1}H-^{13}C HSQC and HMBC correlations showed that both flavanones **3** and **4** (Fig. 1) were methoxylated (δ_C 55.1-56.0 ppm, respectively) on the C-7 position (δ_C 165.9 and 167.9 ppm, respectively). However, compound **3** contained an additional methyl resonance that was not found in compound **4**.The long distance ^{1}H-^{13}C HMBC correlations showed that the methyl group was a singlet correlating to both aromatic C-9 (δ_C 159.2 ppm) and C-7, and therefore identifying cryptostrobin 7-methyl ether **3** (5-hydroxy-7-methoxy-8-methylflavanone) [19]. The ^{1}H NMR spectrum of compound **4** showed that the two aromatic methine protons (δ_H 6.11 and 6.15 ppm) were *meta*-oriented, and the structure was established to be pinostrobin (5-hydroxy-6-methoxy-2-flavanone) [2, 3, 20, 21]. The same splitting pattern was observed in compound **5** where two *meta*-oriented protons were shown as a two doublets (δ_H 5.91 and 5.95 ppm, J= 2.1 Hz) that were assigned to C-6 and C-8. In contrast to compounds **3** and **4**, the metabolite **5** had no methoxy resonance and was identified as pinocembrin [20, 22], a known dihydroxyflavanone of honeybee propolis and Australian *Leptospermum* honeys [23].

Flavanone **6** showed two methyl groups as singlets (δ_H 1.97 and 1.94 ppm) that were assigned to C-6 and C-8 from the long distance ^{1}H-^{13}C HMBC correlations (δ_C 104.1 and 103.8 ppm, respectively). Two hydroxyls were detected downfield as singlets (δ_H 12.35 and 12.05 ppm) and were assigned to C-5 and C-7. Therefore **6** was identified as desmethoxymatteucinol (5, 7-dihydroxy-6, 8-dimethyl-2-flavanone) in agreement with previous structural studies [24], and

isolates from myrtle species [18]. Flavanones **1**, **4** and **6** are known metabolites of myrtaceous species and were previously isolated from *Agonis spathulata* Schau [18].

The absolute configuration of all isolated compounds at C-2 was shown to be *S* by comparison with the published literature [17, 20]. This is the first report of the six known flavanones (**1**, **2**, **3**, **4**, **5** and **6**) being detected in *C. torelliana* fruit resins.

3.2 Chemotaxonomy significance of C. torelliana flavanones in stingless bee propolis

C-methylated flavanones of this study can identify one unusual type of tropical propolis. In fact, other studies reported hydroxylated flavonoids with an unsubstituted B-ring as common constituents of propolis from temperate areas [8]. In Australia, honeybee propolis was shown to contain similar *C*-methyl flavanones but their B-ring was substituted [11]. *Eucalyptus* (Myrtaceae) propolis from Turkey and Tunisia was characterised by phenolic acids including gallic acid and by dihydroxyflavonoids showing the unsaturated C-ring of flavones and flavonols [25, 26]. *Eucalyptus* genera are commonly distributed in Australia, and gallic acid was previously detected by GC-MS in Australian stingless propolis [9]. However, flavanones **1** – **6** were yet to be reported for Australian propolis and can be useful chemotaxonomical markers of *C. torelliana* fruit resins as one myrtaceous species providing resins to native stingless bees in tropical and subtropical areas of Eastern Australia.

Flavanones of *C. torelliana* (Fig. 2 A and 3 A) fruit resins were isolated from one chemotype of propolis (Fig. 2 B and 3 B). The non-substituted catechol B-ring is a known feature of flavonoids isolated from genera in the Myrtaceae family [27]. *C*-methylation has been used to determine the chemosystematics of surface exudates from Myrtaceae [15]. This substitution pattern is specific to some species, whereas others contain primarily *O*-methylated natural products; for instance, the leaf exudates of *Melaleuca* spp. are known to exude only *O*-methyl-flavonoids and not *C*-methyl-substitutes [3]. Our study confirmed that both methylation patterns were characteristic of *C.*

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torelliana fruit resins evidenced by the presence of *C*-methyl groups in flavanones **1, 2, 3** and **6** and one *O*-methyl group in **3** and **4**. From a quantitative perspective, *C. torelliana* flavanones **1 – 6** were found mainly in propolis of this chemotype (Table 1 and 2). The chromatographic profiles (Fig. 2 A and 3 A) of *C*-methyl flavanones can identify one chemotype of Australian propolis.

3.3 Other constituents of C. torelliana fruit resins and propolis

Some apolar constituents of *C. torelliana* resins warrant further investigations. In this study, these compounds could not be isolated by repeated preparative chromatography of propolis or in a sufficient purity for NMR spectroscopy. One dione with chromophore at UV_{max}= 259-270 nm eluted in the late region by reverse phase LC-UV analyses. Apolar phenolics with multiple UV absorption maxima were possibly alkylated given that no loss of isoprenyl units (68 Da) was found by ESI-IT-(+)-MS. *C. torelliana* resins analysed by GC-MS contained trace amounts of unknowns of MW 182, 386 and 338 and one abundant volatile of MW 250 (Table 1). The latter (*Rt* 8.53 min, Fig. 3) was reported to be a chemical marker of *C. torelliana* fruit resins and propolis sources [6, 9], and is not a unique secondary metabolite of *C. torelliana* fruit resins.

In both propolis types, other compounds were tentatively identified as flavonoids of low polarity and they did not originate from the fruit resins. Especially the control propolis (Figure 2 C) was rich in apolar flavonoids that were possibly prenylated, as per a loss of 68 Da from the protonated molecular ion at *m/z* 339 (Table 1).

Compound **7** was isolated from propolis and showed an absorption maximum of 234 nm, deprotonated molecular ion at *m/z* 301.2936 and molecular formula of $C_{20}H_{29}O_2$ by HR-MS. The elution times by chromatography and the UV-MS spectra matched those of the standard abietic acid obtained from a commercial source. Abietic acid was not a component of the fruit resins but is a known plant metabolite of *Eucalyptus*-type propolis [26].

We observed that the flavanones and diterpenoids of propolis had similar properties of

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volatility as they eluted closely by GC and therefore their detections overlapped within the same range of retention time (Fig. 3 B). In fact, the GC-MS spectra showed mixed fragments from both compounds **1** and **7**. Therefore, an efficient separation of these chemical types can be obtained by LC-DAD-ESI-MS. The *C. torelliana* flavanones eluted before the diterpenics of propolis by LC (Fig. 2A and B). The UV and MS spectra were distinctive for the two chemical classes. The combination of both chromatographic techniques by GC and LC is recommended in future routine analyses of stingless bee propolis chemotypes for the correct identification of the two types of constituents.

No diterpenes or triterpenes were found in *C. torelliana* fruit resins of this study while a previous report detected them by GC-MS [6]. However, one related compound was tentatively identified to be a meroterpenoid (KI= 2020) from the fruit resins analysed by GC-MS and against NIST library. In stingless bee propolis, diterpenic and triterpenic acids were found after sample silylation and GC-MS analyses [9]. Therefore, these plant metabolites are common constituents of Australian stingless bee propolis and originated from alternative plant sources than *C. torelliana* fruit resins.

3.4 Antibacterial activity

Australian stingless bee propolis inhibited the growth of *Staphylococcus aureus* ATCC 25923 *in vitro* (Fig. 4A and Table 3), while limited effects were exerted against *Pseudomonas aeruginosa* ATCC 27853 (data not shown). This reduced antimicrobial activity against Gram-negative strains was previously reported for assays testing *Eucalyptus*-type propolis [26] and Meliponini propolis [26]. Effects of the anti-staphylococcal tests of this study were investigated for two propolis crude extracts, six purified flavanones and two fractions obtained from propolis *C. torelliana*.

Both propolis *C. torelliana* and control types were active against *S. aureus* with similar inhibitory effects in the agar assay (t= 2.025, df= 7.447 P= 0.6015) and bactericidal activities at the

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MIC value of 6.94 μg/mL (Table 3). Propolis' MIC value was similar to the minimum bactericidal concentrations reported for Taiwanese propolis extracts [28], and lower than MICs of Brasilian sources [29]. In the agar diffusion assay, 52 μg (per 100 μL in well) of propolis inhibited the microbial growth by 16.5 and 18.5 mm of mean zone diameters (Fig. 4). Previous reports on Kenyan propolis extracts rich in geranyl-phenolics were similarly active at the higher concentration of 400 μg (per 100 μL in well) [30]. The standardised effects of propolis were 4.1 and 5.1 phenol equivalents (% w/w) (Table 3), within the range of inhibitory activity by stingless bee honeys which composition might be affected by propolis constituents [13].

The isolated flavanones **1-6** were tested individually against *S. aureus* (Fig. 4 B and Table 3). In the agar assay **1**, **2**, **4** and **5** were active starting from 12.5 μg per 100 μL in well (Fig. 4 B). The structural patterns favourable to the inhibition of *S. aureus* growth are proposed to be the hydroxylation at 5- and 5,7- positions, the unsaturation at C-2 and C-3, and the 4-carbonylic region of these flavanones [31, 32]. Conversely, a minimal activity was observed for **3** and **6** at 328 μg in the agar plate. This reduced activity can be ascribed to the methyl group positioned at C-8. In the microbroth dilution assay, the bactericidal effects of **2**, **4** and **5** were at MIC values of 13.89, 6.94 and 27.78 μg/mL, respectively (Table 3). For compounds **1** and **3**, bactericidal effects were observed at the high concentrations of 127.78 and 182.21 μg/mL, respectively. Compound **6** was tested to be ineffective up to 182.21 μg/mL. Former reports indicated limited or absent antibacterial effects for **1** and **2** [33, 34], but in this study stroboponin **2** showed strong antibacterial action against *S. aureus*. Its structure contains a methyl group at position C-6 but not substituent at C-8, and these structural features can be ascribed to its different bactericidal action when compared to **1**. The anti-staphylococcal activities by propolis extracts rich in flavonoids **4** and **5** have been documented [14, 31], and findings of this study confirm them as bactericidal flavanones**.** No reported data was available for **3**. The minimal effects of **6** against *S. aureus* were in agreement with a low antimycobacterial activity previous reported [34], while this compound showed strong *in vitro*

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antimalarial effects [33].

The antimicrobial activity of propolis has been linked to the synergism of flavonoids and sesquiterpenes [28]. During *C. torelliana* fruiting season, stingless bees incorporated copious amounts of the yellow fruit resins into their propolis that contained the flavanones **1** – **6** up to 31.3 % of propolis crude extracts (w/w, Table 1). Beyond **1** – **6**, propolis *C. torelliana* type contained other constituents (Table 1) such as flavonoids of relative low polarity and isoprenoids including **7** that might contribute to the overall inhibitory activity. The control propolis was rich in prenylated flavonoids, a phytochemical class known for its anti-staphylococcal activity [32]. Fractions of propolis *C. torelliana* were pooled in two groups to assess the activity levels in the presence or absence of *C. torelliana* flavanones. Fraction 'flavanones' contained **1 - 6** as 11.8 % pinocembrin equivalents (at 205 nm, w/w, n= 2). Fraction 'others' did not contain these flavanones but comprised only the remaining propolis constituents (including **7**) as 25.6 % of abietic acid equivalents, 8.4 % of pinocembrin equivalents and 42.8 % of gallic acid equivalents (at 205 nm, w/w, n= 2). Both fractions inhibited the growth of *S. aureus* (Fig. 4 C) with MIC values of 6.94 and 13.89 µg/mL (Table 3).

C. torelliana flavanones **1 - 6** showed bacteriostatic/bactericidal activity after being tested individually, collectively (within one fraction) and as part of the propolis mixture. Therefore, these flavanones contributed to the anti-staphylococcal activity of propolis, but a further inhibitory activity was also retained by the other components including known bacterio-lytic natural products such as abietic acid [35], sesquiterpenes [28] and prenylated flavonoids and phenolics [30]. Further research is warranted on the mechanisms of action of stingless bee propolis flavonoids, and whether xenobiotic effects might occur [32].

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4. Conclusion

C-methylated flavanones are reported as chemomarkers of *C. torelliana* fruit resins and propolis from Australian stingless bees (*T. carbonaria*) by a combination of spectroscopic and chromatographic techniques. Propolis crude extracts and *C*-methylated flavanones inhibited the growth of *S. aureus in vitro* but limited inhibitory activity was observed on *P. aeruginosa* growth at the concentrations used. *C. torelliana* flavanones would be useful in the phytotherapeutic strategy against *S. aureus,* and further work is needed to assess the bactericidal and bacteriostatic potential of propolis extracts and constituents against antibiotic resistant strains of *S. aureus*.

Supplementary data

MS and NMR data of **1-7** are available on line at http://www.sciencedirect.com/.

Acknowledgments

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Declaration of ethical standards

The authors declare that the experiments described in this article comply with the current laws for the conduct of scientific research in Australia.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author Contributions

Conceived and designed the experiments: CFM PB. Performed the experiments: CFM. Analyzed the data: CFM MK TG HV PB. Contributed reagents/materials/analysis tools: RJQ CC MMH HMW PB. Wrote the manuscript: CFM MK TG HV RJQ TAH CC MMH HMW PB.

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Fig.1. The yellow fruit resins (arrows) were harvested from (A) capsules of *Corymbia torelliana* (Myrtaceae) and contained the (B) six flavanones of this study. These compounds were isolated from stingless bee propolis for structural characterisation and comparative chromatography.

Fig.2. LC-DAD analyses. The flavanones **1** – **6** were found in the fruit resins of *Corymbia torelliana* (A) and isolated from one type of propolis of Australian stingless bees (B). The control propolis (C) was not exposed to *C. torelliana* resin flow. The diterpenic acid **7** was found only in one chemotype of propolis. Chromatograms were acquired at 200-400 nm.

Fig.3. GC-MS profiles. (A) The fruit resins from capsules of *Corymbia torelliana* (Myrtaceae) contained the flavanones **1-6** among other volatiles. (B) Propolis containing these flavanones and the diterpenoid **7**. The (C) control propolis was unexposed to *C. torelliana* resin flow.

Fig. 4. Antibacterial activity. Australian stingless bee propolis inhibited the growth of *Staphylococcus aureus* ATCC 25923 by the agar diffusion method. (A) The methanolic extracts of two propolis chemotypes were tested (● propolis *C. torelliana,* ⊟ propolis control). (B) The isolated flavanones were tested individually (● cryptostrobin **1**, ⊟ strobopinin **2**, ▲ cryptostrobin ether **3**, ■ pinostrobin **4**, ◆ pinocembrin **5**, ⊖ desmethoxymatteucinol **6**). (C) Propolis *C. torelliana* (●) was fractionated into two mixtures containing either flavanones (⊟) or other constituents (✱).Fractions 'flavanones' and 'others', and compounds **1** – **6** were tested in the range of 12.5 to 100 µg in well. Samples were prepared in abs. ethanol, and the negative control ethanol did not inhibit *S. aureus* growth (8 mm diameter, baseline axis). Mean ± SEM (n= 3).

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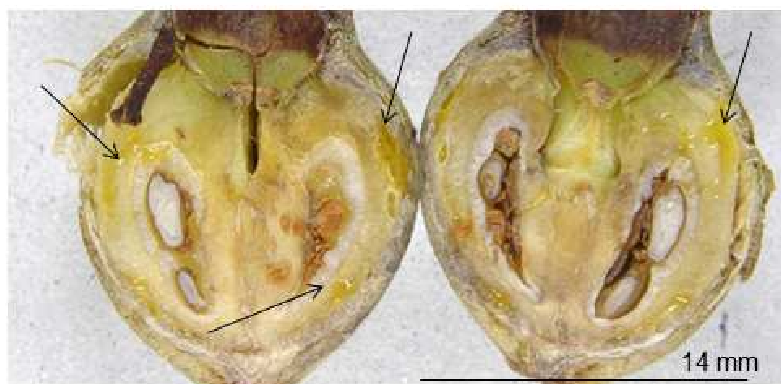
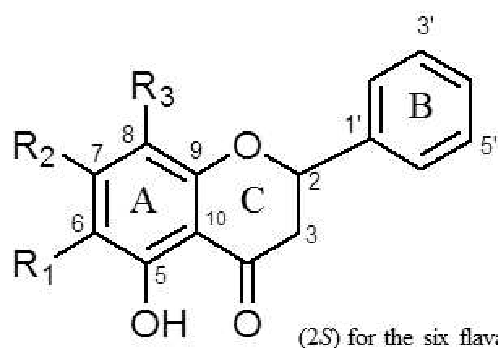


(A)

(B)



(2*S*) for the six flavanones

	R_1	R_2	R_3
1.	H	OH	Me
2.	Me	OH	H
3.	H	OMe	Me
4.	H	OMe	H
5.	H	OH	H
6.	Me	OH	Me

Figure 1

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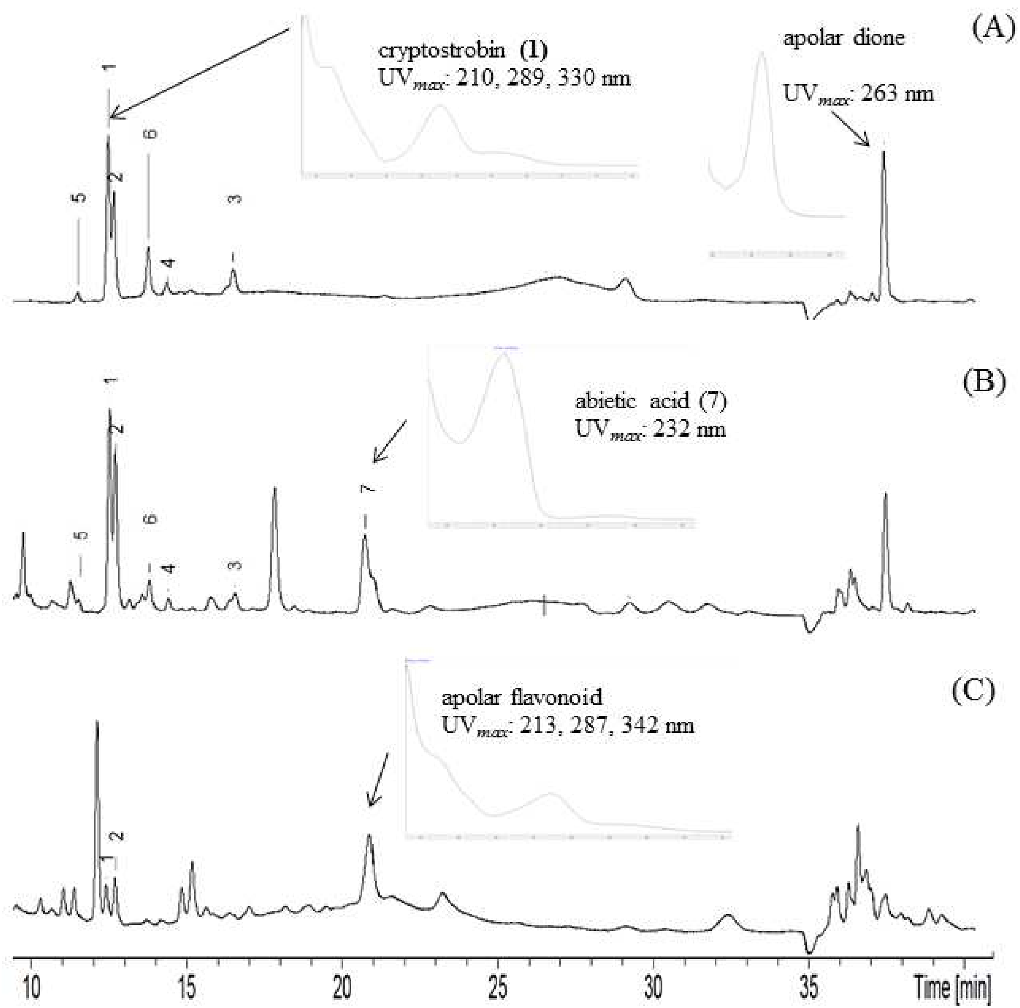

Figure 2

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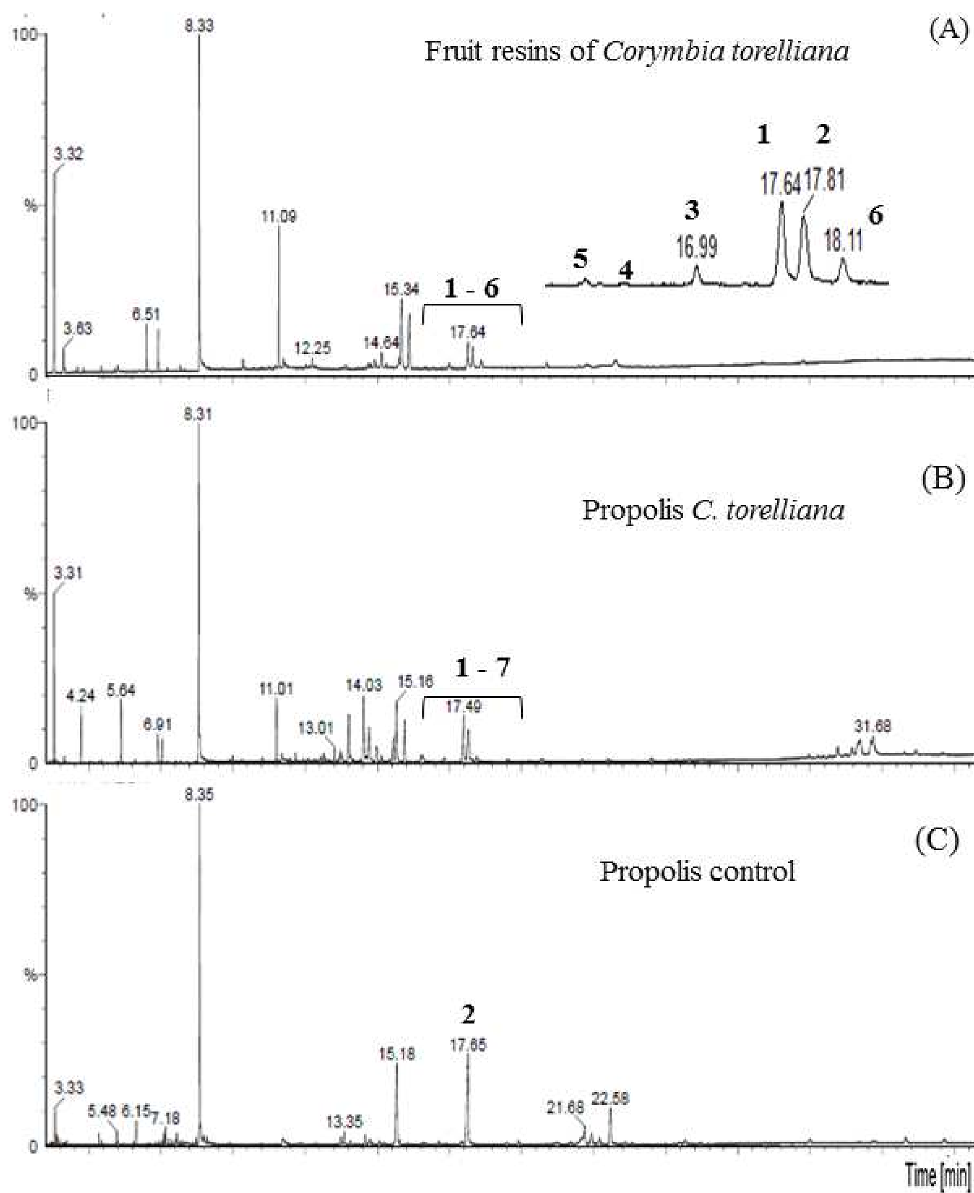

figure 3

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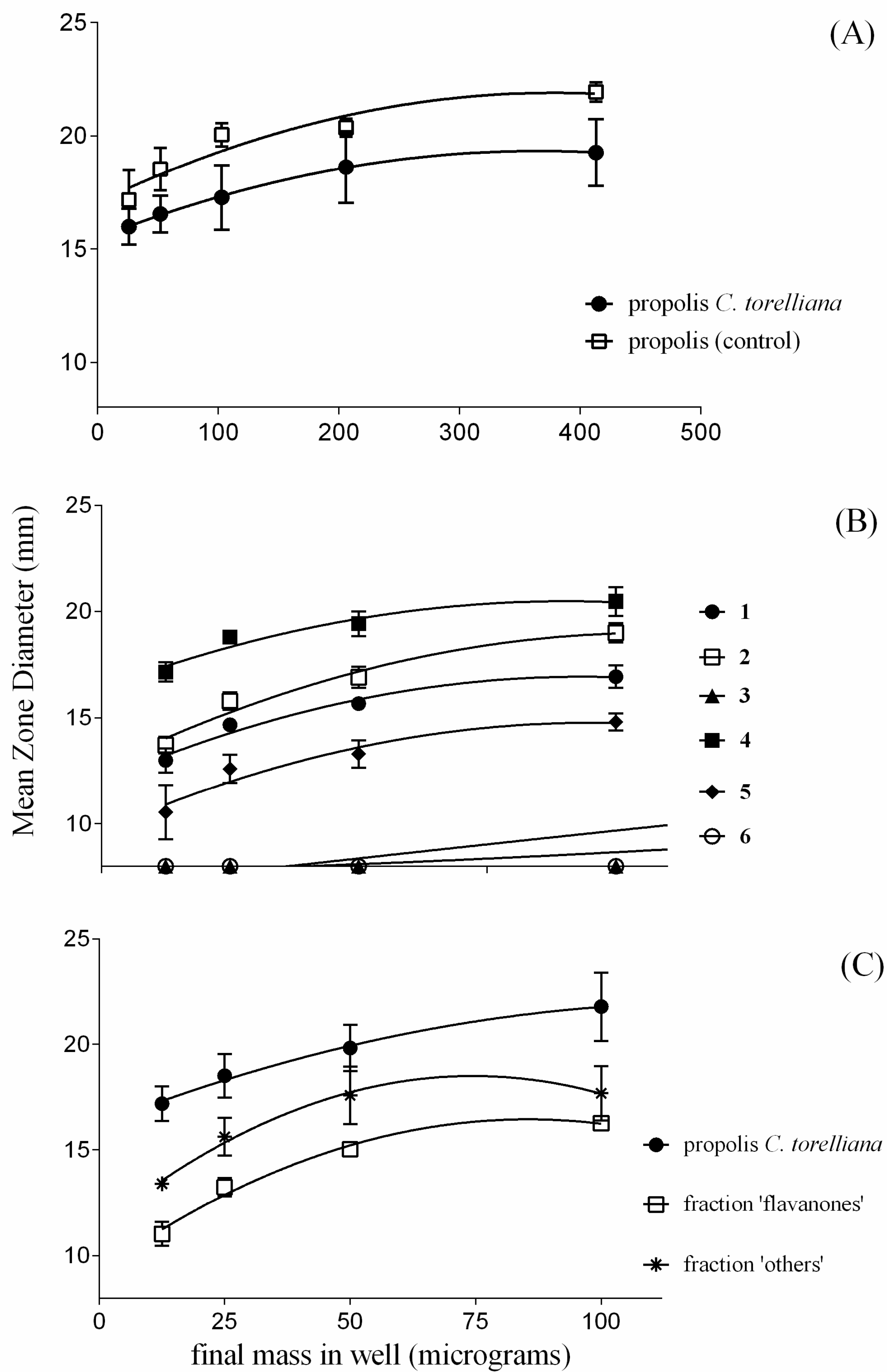

Figure 4

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Table 1

Analyses by LC-DAD-ESI(-)-MS. The extracts of (A) fruit resins of *C. torelliana* contained flavanones **1** – **6**, isolated from one (B) chemotype of propolis that was exposed to the fruit resins. The diterpenoid **7** was isolated and detected only in this propolis. The (C) control propolis was not exposed to the *C. torelliana* resin flow. Diagnostic UV-MS data enabled the identification at chemical class level. Quantitative amounts were calculated from the relative abundances of compound area over total peak areas (%, w/w). Quantities less than 1% were indicated as traces 'tr'.

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R_t (min)	$[M+H]^+$ (m/z)	Description	UV_{max}	Fruit resins (A)	Propolis *C. torelliana* (B)	Propolis control (C)
-	-	gallic acid[a]	216, 269	-	4.0	-
-	-	phenolic acid	207, 261	5.0	4.7	23.6
-	-	phenolic acid	213, 275	-	15.0	-
9.8	-	phenolic acid	220, 283	-	3.3	-
10.2	261	flavonoid	201, 230, 278	-	-	1.9
11.0	-	phenolic acid	225, 284	-	1.8	-
11.2	305	flavonoid	217, 335	-	-	1.2
11.2	257	flavanone **(5)**[a]	209, 230sh, 285	3.7	1.4	-
12.6	271	flavanone **(1)**[b]	210, 289, 330	30.4	11.5	1.1
12.8	271	flavanone **(2)**	229, 285, 333	16.6	12.3	3.1
14.7	285	flavanone **(6)**	217, 287, 344	4.1	2.1	tr
14.9	271	flavanone **(4)**	212, 287	2.5	3.3	tr
16.2	287	flavonoid	211, 233, 287	-	-	1.3
16.8	285	flavanone **(3)**	212, 230,290	2.7	0.7	-
17.4	-	flavonoid	204, 254, 274	-	3.8	-
17.6	-	flavonoid	218, 283	-	-	2.3
20.5	303	abietane	230	-	4.5	-
21.0	303	abietic acid **(7)**[a, b]	232	-	1.9	-
21.1	-	flavonoid [b]	205, 219, 275	-	-	2.5
21.1	-	abietane	234	-	3.1	-
23.2	339	prenylflavonoid	213, 287, 342	-	-	26.5
28.0	521	unknown	225sh, 279	11.4	11.9	-
29.4	-	unknown	228, 261, 297, 343	9.7	2.0	-
33.8	407	flavonoid	220, 270	-	-	3.2
36.6	-	unknown	211, 258	-	2.7	-
37.4	387	dione [c]	263	7.8	4.1	-
38.9	-	dione	270	-	-	4.1
39.1	-	dione	259	-	-	2.1
	Total flavanones **1** – **6**			60.0	31.3	4.2
	Total other flavonoids			-	3.8	35.7
	Total phenolics and unknowns			26.1	45.4	23.6
	Total isoprenoids (including **7)** and diones			7.8	10.5	6.2

[a] matched with standards
[b] UV spectra shown in Fig. 2

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Table 2

Volatiles of *C. torelliana* fruit resins by GC-MS. Compounds **1 - 6** were tracked into the extracts of (A) fruit resins of *C. torelliana* that did not contain **7**. Two chemotypes of propolis were (B) exposed to these fruit resins or (C) not exposed (control). Kovats indices 'KI' were calculated against a solution of *n*-alkanes (C_2-C_{40}). Tentative identifications were carried out against NIST library. Quantitative amounts were calculated from the relative abundances of compound area over total peak areas (%, w/w). Quantities less than 0.1% were indicated as traces 'tr'. Legend: 'M*': molecular ion. 'n.d.': not determined.

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Description	M*	KI	Fruit resins (A)	Propolis *C. torelliana* (B)	Propolis control (C)
β-pinene	136	974	19.7	14.5	5.8
α-phellandrene	136	1005	-	-	0.9
terpinen-4-ol	154	1177	-	1.2	1.8
unknown	182	1261	0.6	0.2	2.2
eixene	204	1300	-	0.2	3.8
δ-elemene	204	1328	tr	-	0.3
α-copaene	204	1372	4.7	0.5	0.6
β-caryophyllene	204	1400	-	2.2	0.7
4,8-α-epoxy caryophyllene	204	1414	tr	0.2	2.0
unknown	250	1599	33.3	26.6	53.6
cyclohexadione[a]	356	1796	14.3	6.1	-
meroterpenoid	270	2020	1.1	2.2	-
totarol	286	2292	-	5.2	-
6,7-dihydro-ferruginol	286	2316	-	tr	-
unknown	386	2326	0.4	-	1.3
unknown	386	2341	tr	-	2.1
trans-ferruginol	286	2341	-	5.2	0
unknown	386	2344	tr	tr	tr
unknown	386	2354	0.6	2.7	0.0
unknown	386	2388	1.6	1.4	0.5
unknown	386	2389	-	1.1	12.8
unknown	386	2405	+	5.4	0
pinocembrin **(5)**	256	2452	tr	tr	0.6
pinostrobin **(4)**	270	2471	tr	tr	-
cryptostrobin methyl ether **(3)**	284	2482	0.6	0.6	-
cryptostrobin **(1)**	270	2519	2.6	5.4	-
podocarpatrienedione	328	2520	-	tr	-
abietic acid **(7)**	302	2524	-	tr	-
strobopinin **(2)**	270	2526	2.2	2.7	1.4
desmethoxymatteucinol **(6)**	284	2543	0.8	1.1	-
sugiol	300	2604	-	0.4	-
unknown	406	2754	-	-	2.9
cinnamic acid derivative	n.d.	2812	-	-	5.8
cinnamic acid derivative	n.d.	2819	-	-	tr
unknown	338	2810	0.7	0.2	-
unknown	338	2810	tr	tr	-
lupenone	424	3230	-	3.6	-
β-amyrin	426	3314	-	0.5	-
lupeol	426	3332	-	0.8	-
α-amyrin	426	3362	-	0.8	-
ursane ester	468	3435	-	tr	-
lupeol isomer	446	3499	-	tr	-
total flavanones **1** – **6**			6.2	9.8	2.0
total isoprenoids (including **7**)			39.8	43.6	15.9
total phenolics and unknowns			37.2	37.6	81.2

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Table 3

The *in vitro* growth inhibition of *Staphylococcus aureus* ATCC 25923 (10^5 CFU/mL) was tested using two methods. Effects from the agar diffusion assay were standardised to phenol (positive control) equivalents (%, w/v) for two propolis methanolic extracts, two fractions of propolis *C. torelliana* and six purified flavanones (mean ± SEM, n= 3). Minimum Inhibitory Concentrations (MIC) values were assessed in the microbroth dilution assay using the MTT dye (n= 3). The carrier was ethanol (100% in agar plate and 6.25% v/v in microplate) did not affect the bacterial growth.

Samples against *S. aureus*	Phenol equivalents (%, w/v) [a]	MIC (µg/ml) [b]
Propolis control	5.1 ± 1.0	6.94
Propolis *C. torelliana*	4.1 ± 1.0	6.94
Fraction ‘flavanones’	3.1 ± 0.4	13.89
Fraction ‘others’	4.8 ± 1.5	6.94
1	4.1 ± 0.4	127.78
2	5.4 ± 0.8	13.89
3	inactive [a]	182.21
4	6.6 ± 1.4	6.94
5	3.0 ± 0.3	27.78
6	inactive [a]	inactive [c]

[a] at 52 µg in well (0.52 mg/mL)

[b] bactericidal effects for this concentration reducing 99.9% of initial *S. aureus* population

[c] at 182.21 µg/mL

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Chemical compounds studied in this article (PubChem CIDs and CAS registry numbers):

1 (2*S*)-cryptostrobin (PubChem CID 15953986; CAS 55743-21-0)

2 (2*S*)- strobopinin (PubChem CID 442520; CAS 11023-71-5)

3 (2*S*)- cryptostrobin 7-methyl ether (CAS 55869-77-7)

4 (2*S*)- pinostrobin (PubChem CID 73201; CAS 480-37-5)

5 (2*S*)- pinocembrin. (PubChem CID 25200438; CAS 480-39-7)

6 (2*S*)- desmethoxymatteucinol (CID 180550; CAS 56297-79-1)

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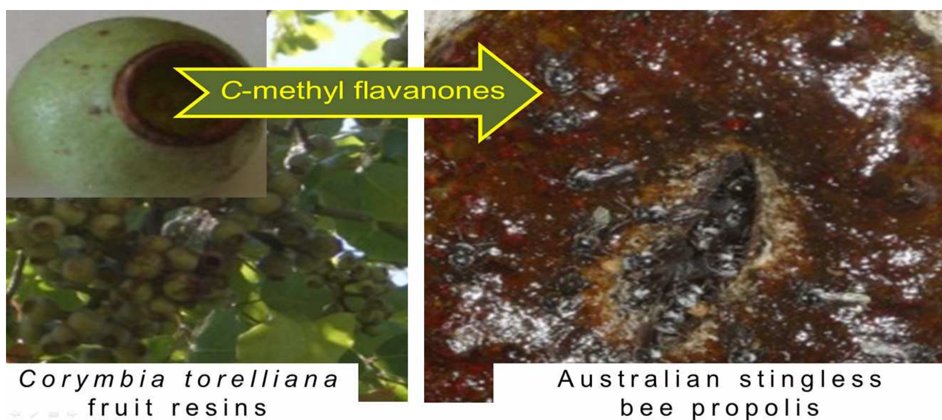

Graphical abstract

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