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In vitro antibacterial phenolic extracts from 'sugarbag' pot-honeys of Australian stingless bees (*Tetragonula carbonaria*)

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ABSTRACT

Australian stingless bee honeys have been shown to exert antioxidant and *in vitro* antimicrobial properties; however their bioactive factors remained unidentified. This study investigated the antibacterial properties of phenolic extracts from *Tetragonula carbonaria* honeys.

Honeys were harvested from beehives in three sites of South East Australia. Liquid-liquid extractions yielded the phenolic concentrates, for analyses by liquid and gas chromatography mass spectrometry. Antibacterial assays were conducted against *Staphylococcus aureus* and *Klebsiella pneumoniae* by *in vitro* agar diffusion and broth dilution assays.

The phenolic extracts averaged to 5.87 mg/100 g of raw honeys, and constituents were 3-phenyllactic acid, lumichrome, di-glycosylflavonoids, norisoprenoids. The honeys did not contain methylglyoxal, dihydroxyacetone or phenolics characteristic of *Leptospermum* nectars. Hydrogen peroxide content amounted up to 155.8 μM in honeys. Beside the bactericidal effects of hydrogen peroxide at 760 μM, other antibacterial factors were the phenolic extracts of 'sugarbag' honeys that were active at minimum bactericidal concentrations of 1.2 - 1.8 mg/mL.

Keywords: flavonoids; norisoprenoids; phloroglucinols; propolis; Meliponini; *S.aureus*

INTRODUCTION

Nosocomial infections are a significant cause of morbidity, mortality and economic burden for prolonged hospitalization worldwide, and are often due to drug-resistant bacterial strains causing recalcitrant wounds [1]. In search for new antibiotics to overcome resistance, recent attention of main-stream medical research has re-investigated honey as a functional product. Honey has been used in medicinal preparations since ancient times and is still in use in alternative-complementary medicines worldwide, for instance as a topical antimicrobial agent in the treatment of burns and wounds [2].

Antimicrobial properties of honey have been associated to its high osmolarity, to acidic properties (pH of approximately 4.4 conferred by gluconic acid), and also to antimicrobial peptides such as bee-defensin that make the microbial colonisation less favourable [3]. One antimicrobial factor is the level of hydroxyl radicals being generated from peroxide in honeys [4]. Bees produce honey from digesting the nectars of flowers, and by doing so they enrich it with enzymatic secretions. Their salivary secretions contain glucose oxidases that produce gluconic acid and hydrogen peroxide (H_2O_2) in diluted honeys showing antimicrobial activity [4]. The high content of H_2O_2 in European cornflower honey and its associated antibacterial activity have proven effective in the treatment of digital dermatitis of cows [5].

Along with the peroxide content, methylglyoxal (MGO) levels have been quantified in medicinal-grade honey [6]. MGO is a metabolite of the glycolytic pathway and has been reported to contribute to the activity of honey against specific bacteria [6]. MGO has been detected in a specific honey variety called ‘mānuka’ from New Zealand, where bees forage on *Leptospermum scoparium* (Myrtaceae) nectars [7, 8]. Other marker components of mānuka honeys have been identified as the phenolic acids methyl syringate and its glycosidic derivative methyl syringate 4-O-β-D-gentiobiose (leptosin) as well as flavonoids and a pyrrole [8-11]. In Australia, the endemic *Leptospermum* flora comprises species such as *L. polygalifolium* and *L. liversidgii* from which honeybees (*Apis mellifera,* Apinae) can produce

a mānuka-like honey called 'jellybush' with levels of MGO comparable to the New Zealand variety [12]. The chemical composition of Australian jellybush honeys were recently identified to contain 2-methoxybenzoic acid, cis-linalool oxide and 3,4,5-trimethylphenol; these chemical markers were proposed to be used to differentiate jellybush honeys from mānuka and kanuka varieties [13] as well as other Australian sources.

In tropical and subtropical areas of the world, *Leptospermum* nectars are available also to native bee species, including Australian stingless bees (*Tetragonula carbonaria,* Meliponinae) that can produce multifloral honeys called 'sugarbag'[14]. *T. carbonaria* honeys have been investigated for their biological properties including antioxidant activities [15], and a broad-spectrum of *in vitro* antimicrobial effects have been reported as comparable to mānuka-like honeys *16, 17*. The physicochemical properties indicated a pH of 3.85, mean reducing sugar content of 54.2% (w/w) and mean water content of 25.4% (w/w) per 100g of honey *16*. However, no chemical constituents were specifically linked to the observed antimicrobial effects [12]. Previous chemical investigations identified total flavonoids and phenolic acids contents in *T. carbonaria* honeys [15], and flavonoid glycosides containing the aglycones quercetin, kaempferol or isorhamnetin were reported in *T. carbonaria* honeys from suburban areas of Queensland [18]. Whether these stingless bees collect *Leptospermum* nectars to produce 'jellybush-sugarbag' honeys was unknown.

The study of antimicrobial natural products in honeys is important to determine honey varieties with a medicinal potential and for clinical applications. Bioactive natural products found in honeys can be derived from plant nectars or other plant materials such as propolis [19]. Propolis is a resinous mixture that the bees produce by mixing their beeswax, salivary secretions and plant resins. Whether propolis can contribute to the composition of stingless bee honey was yet to be ascertained. Therefore, the natural products, MGO levels and peroxide content were investigated in this study to assess the contribution of these factors to the antimicrobial effects reported in *T. carbonaria* honeys [17].

Chemical investigations of Meliponini honeys require an appropriate sample preparation. The complex matrix rich in polar compounds including sugars and proteins can complicate the sample preparation and analysis [20]. Moreover, a high content of flavonoid glycosides that are not hydrolysed by the bee salivary β-glucosidases can be expected [19]. Therefore, a combination of methodologies of extraction techniques has proven successful in honey analyses including solid-phase extraction methods and liquid chromatography mass spectrometry to analyse non-volatile compounds in extracts [19, 21, 22]. Other analytical reports have indicated gas chromatography mass spectrometry to screen the volatiles with a chemotaxonomical relevance in honeys from Australian honeybees foraging on *Leptospermum* spp [23]. A combination of analytical techniques was applied to a dereplication approach for the characterisation of natural products in Australian *T. carbonaria* honeys in this study.

The aims were to characterise the natural products, levels of MGO and peroxide contents in *T. carbonaria* honeys, to determine if these honeys originate from *Leptospermum* nectars using known phytochemical markers, and to assess the *in vitro* antibacterial activities of their concentrated phenolic fractions and peroxide contents.

MATERIALS AND METHODS

Chemicals

Methanol, acetonitrile, ethanol and ethyl acetate of analytical grade were purchased from Merck Pty Ltd (Kilsyth, Vic., Australia). Milli-Q water was obtained from an in-house Milli-Q Ultrapure Water System. Muller-Hinton nutrient broth (MHB) and agar (MHA) media, 3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyltetrazolium bromide (MTT), hexadecane, phenol standard (>99.5%), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine.HCl (PFBHA), hydroxyacetone (HA), methylglyoxal (MGO), dihydroxyacetone (DHA), hydroxymethylfurfural (HMF), gallic acid, quercetin

hydrate, 3-phenyllactic acid, o-dianisidine, horseradish peroxidase type IV and hydrogen peroxide were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Beehive products

Stingless bee *T. carbonaria* honeys were produced in beehives using wooden compartments (honeysupers) of 25 x 15 cm. Three sites were used for the apiaries (n=6 beehives each). Two sites contained plants of *Leptospermum polygalifolium* and *L.liversidgii* at high density in the coastal area of Tyagarah, NSW (site #1) and heathland of East Beerwah, Queensland (site #2). The third and control site was in heathland of West Beerwah, Queensland (site #3) and did not contain *Leptospermum* spp. The honeys were produced between September 2012 and April 2013 during *Leptospermum* nectar flow. The honeys from beehives within the same site were pooled to yield one representative batch per each site. The three batches were labelled as honeys#1, #2 and #3 (control).

The honeys were harvested from honeypots and sieved using a metal drainer to remove other hive materials including propolis, bees and pollen particles. Honey aliquots (200 mL) were centrifuged at 1000 rpm at room temperature for 5 min to recover the supernatants for later analyses. Propolis from the same honeypots of site #2 was extracted by maceration in abs. methanol (5% w/V) for 24 h at room temperature, filtered, and evaporated to dryness under vacuum to yield the propolis extract.

Liquid-liquid extraction for the phenolics concentrates

The raw honeys (ca. 50 g) were extracted in absolute ethanol (20 mL, 40% v/w) at around 60 °C and let cool at 24 °C for 2h, then at 4 °C for 2h then followed by the storage at -18 °C overnight. The supernatants were decanted and the sugar precipitates were extracted again following the same protocol. The combined supernatants were evaporated to dryness under reduced pressure. Three cycles of solvent extractions were repeated to precipitate the

carbohydrates of the honey matrices and to concentrate the phenolic fractions into ethanolic extracts. The last step of extraction used acetone: ethanol (1:1) followed by evaporation to dryness and freeze-drying to yield three honey phenolic concentrates (2.40, 2.70 and 3.70 mg). Aliquots of the three dry extracts were reconstituted in methanol for the liquid chromatography analyses, and in ethanol for the antibacterial assays.

A further extraction involved raw honeys (ca. 50 g) in deionised water:ethyl acetate (2:1) using a modified protocol [24]. The mixture was warmed at 50 °C in a water bath for 5 min and vortexed to homogeneity. The ethyl acetate extracts were transferred into clean test tubes and dried using anhydrous Na_2SO_4 (70 mg each) for 5 min. Aliquots of the final solutions were subjected to gas chromatography.

GC-MS analyses

Equipment was Perkin Elmer Clarus580 GC-EI-MS fitted with Elite-5MS column (L= 30m, ID0.25 mm, DF0.25 μm, PerkinElmer) and 70 eV ionisation. The ethyl acetate extracts (1.0 μL) were injected at 260 °C into the gas chromatograph, split ratio of 10:1, and oven program ramping from 50˚C to 315 °C at 5 °C/min, and held for 5 min, for a total run time of 47 min. A solution of *n*-alkane standards (C_8-C_{40}) was also analysed for the calculation of retention indices. The mass spectrometer acquisition was set in the range of *m/z* 50 to 650.

A further protocol required the analyses of trimethylsilyl (TMS) derivatives after BSTFA derivatisation of honey extract. The ethyl extracts were treated with BSTFA (50 μL) for 2h at 60 °C and analysed under the same GC-MS conditions.

UPLC-UV-ESI-HR-MS analyses

Samples of the three honeys and one propolis extract were reconstituted in MeOH (2 mg/mL) and injected onto an Eclipse Plus C18 UPLC column (2.1 x 100mm, 1.8um particle size). The equipment (Thermo Scientific, New Zealand) included a Dionex Ultimate 3000 UPLC pump

linked in series with eluent from the column into the photodiode array detector and then into the qExactive Mass Spectrometer (qEMS). The ESI module was heated ESI (HESI). The qEMS resolution was set at 140,000, and separate UPLC runs were done for positive and negative ionization modes. Mobile Phase A (MPA) consisted of 0.1% acetic acid in water and Mobile Phase B (MPB) consisted of 0.1% acetic acid in acetonitrile. The flow rate was 0.4 mL/min. The gradient was 2.5% MPB at T=0.5 min to 20% MPB at 15 min, and 100% MPB at 25 min, 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.

Quantitation of individual compounds was performed by HPLC-DAD at 205 nm on a HPLC Series 200 with Flexar DAD (Perkin Elmer) equipped with a RP-C18 column, 75 µm, 4.6 x 4 mm (Phenomenex Synergi Fusion), with a flow rate of 1.2 mL/min and the same analytical conditions of mobile phases and gradient method as indicated for the UPLC analyses. Accurate ethanolic solutions of the phenolic extracts were analysed against a standard calibration curve (R^2= 0.9984) of gallic acid (0 - 0.6 mg/mL), and signals were interpolated to yield gallic acid equivalents (GAE) per 100 mg of phenolic extract. Quantifications were performed at 205 nm, mean values, n= 2.

MGO protocols

The calibration of standards methylglyoxal (MGO), dihydroxyacetone (DHA) and hydroxymethylfurfural (HMF) was undertaken against the internal standard hydroxyacetone (HA) (3.0 mg/mL) known protocols *25, 26*. The internal standard HA (250 µL) was added to raw honey samples (ca. 0.3 g) and diluted with water (600 µL), before being mixed thoroughly. The solutions were derivatised with 1200 µL of PFBHA (0.02 g/mL in 0.1 M pH 4 citrate buffer) for 1 h at room temperature, then acetonitrile was added (2 mL). Solutions

were diluted with water (1 mL) and mixed. Aliquots(10 µL) were chromatographed by HPLC-DAD at 263 nm [25, 26]. This procedure was repeated in triplicate for batches harvested in 2013.

Antibacterial assays

Two bacterial strains were available at the University of the Sunshine Coast laboratory. Inocula of *Staphylococcus aureus* ATCC 25923 or *Klebsiella pneumoniae* ATCC 13883 were cultured in Mueller Hinton Broth (MHB) overnight at 37 ºC and used at lag phase as determined by optical density to be 0.500 - 0.550 OD. Fresh media were inoculated with culture suspensions to the final concentration of 10^5CFU/mL in plates. Controls were abs. ethanol and the phenol standard (>99.5% purity) solution in ethanol. The negative control showed no bacterial inhibition. The positive control phenol solution showed mean zones of inhibition of 9.78 – 28.55 mm used to construct the calibration curves for the concentrations of 0.5 – 8% (w/V) that were tested against the two bacterial strains. Samples were raw honeys or phenolic concentrates reconstituted in abs. ethanol. Two methodologies were applied for the assays.

In the agar diffusion assay, agar plates of 20 x 20 cm were prepared with Mueller Hinton Agar (MHA) inoculated media. Samples were dispensed to wells of 8mm diameter: aliquots (100 µL) of raw honeys or phenolic solutions (at final weights of 100 – 800 µg) were delivered to MHA plates. The plates were incubated at 37ºC for 16 h in a humidified incubator. Results of growth inhibition by the samples were reported as the mean zone of inhibition (mm) and calibrated by values of phenol equivalents in the range of 0 to 8% (w/V).

In the broth dilution assay, 96-well microplates were filled with 170 µL of inoculated MHB. Aliquots (10 µL) of raw honeys or phenolic solutions (at final concentrations up to 3.3 mg/mL) were added to wells of microplates. Abs. ethanol (10 µL) yielded a final concentration of 5.5% v/v. Phenol standard solution ranged between 0.02 and 0.2% (w/V). The microplates were incubated at 37ºC for 16 h in a humidified incubator. The MTT

tetrazolium dye was used to visualize the inhibition of bacterial growth, showing a yellow coloration for full inhibition. Minimum Inhibitory Concentrations (MIC) were replated using cotton swabs on fresh agar plates that were incubated for 24h at 37 ºC. The bactericidal effects were assessed for >99% lack of bacterial growth.

Peroxide quantitation

Quantitative concentrations of hydrogen peroxide in honey were determined from a modified protocol *3*. Briefly, 135 µL of a buffer containing 50 µg/mL of o-dianisidine and 20 µg/mL of horseradish peroxidase type IV (Sigma Aldrich, Australia) were dispensed to wells of a 96-well microplate. The negative control was deionised water. The positive control was a standard solution of hydrogen peroxide at the concentrations 1 µM to 1 mM used for calibration curves. The assay measured the product formation after horseradish peroxidase catalysis of hydrogen peroxide in the presence of o-dianisidine. The absorbance values were read at 540 nm at intervals of 5, 15, 30 and 60 min using Versa Max Tunable Microplate Reader (Associates of Cape Cod Int. Inc., Liverpool, UK). The enzymatic assays were conducted at 24 ºC. Standard and honeys were analysed in duplicate. Mean values (± standard error) were averaged for the three honeys to show common levels of hydrogen peroxide in undiluted and diluted stingless bee honeys.

Peroxide assays were undertaken using undiluted and diluted honeys to compare the accumulation of hydrogen peroxide. Aliquots (40 µL) of raw honeys were added to wells containing the reagents for 5 min, and the absorbances were read at the conditions indicated above. A further test employed honeys diluted in water (44%) for 1 h, and aliquots (40 µL) were transferred to wells containing the reagents for 5 min before monitoring the absorbance at the time intervals aforementioned.

Data and statistical analyses

GC-MS results were interpreted against NIST library, mass spectra of chemical references, and literature on norisoprenoids and other volatiles from honeys and nectars including *Leptospermum* sources *23, 27, 28*.

The quantitation of compounds was undertaken by the internal standard method using hexadecane C_{16} (stock 8.89 µg/ 100 mL) in the GC-MS analyses. The peak areas were standardised to hexadecane areas to the mass (µg/g) of individual components in honey. A response factor of one was assumed for all constituents.

The HR-MS data was analysed using Thermo Xcalibur (v. 3.0.63) .Accurate mass measurements and elemental compositions were used to generate chemical formula using http://www.chemcalc.org/ *29*. Constituents were dereplicated using SciFinder (https://scifinder.cas.org/) against available literature. Known natural products were searched using ESI-MS marker ions in the positive mode at *m/z* 550-700 for glycosil flavonoids. Other ESI-MS markers were searched at *m/z* 165 for 3-phenyllactic acid and *m/z* 243 for lumichrome [30]. *Leptospermum* markers of mānuka and 'jellybush' honeys were searched for methyl syringate (MYSR) *7*, leptosin [31], unedone [10] and MGO [8]. Propolis constituents were identified in honeys from the same honeypots by comparing retention times of individual peaks and their ESI-UV-MS against known data [32].

MGO, HMF and DHA were identified by comparison of their retention times and UV spectra against those of commercial standards (Sigma-Aldrich, NSW) showing distinct retention times and UV spectra for DHA (Rt: 9.82; UV_{max}: 201, 256), HA (Rt: 13.27; UV_{max}: 199, 259), HMF (Rt: 14.45; UV_{max}: 276) and MGO (Rt: 18.39; UV_{max}: 201, 242). Linear regression curves (MGO: $R^2 = 0.998$, n=3; DHA: $R^2 = 0.9999$; n=3; HMF: $R^2 = 0.9996$, n= 3) were used for quantitation.

Quantitative amounts of hydrogen peroxide were measured in undiluted (raw) and 44% diluted honeys by interpolating the absorbance readings of samples at 30 min of enzymatic

activity against linear regressions of standard peroxide ($R^2 = 0.9989$).

The antibacterial concentration curves from the agar diffusion assay against *S. aureus* were fitted with centred second order polynomial equations (quadratic curve-fits) using GraphPad Prism6.0. Phenol equivalents were calculated against calibrated curves of phenol standard solutions tested in the same plate (mean values, n= 3, standard error).

RESULTS

Glycosyl flavonoids and propolis phenolics

The UPLC-UV-HR-ESI-MS(+) analyses of the three phenolic extracts showed the high content of flavonoid glycosides **1** – **7** with MW of 596 – 626 (Table 1, Fig. 1). Their UV maxima were in the ranges of 220-264 nm for flavonoids that could be distinguished at subclass level between flavones for the band I's absorption at 335-350 nm or flavonols at 360-390 nm. The ESI-MS(+) fragmentation patterns of the protonated molecular ions showed the diagnostic mass losses of 162 Da for a hexosyl residue and/or 146 Da for a deoxyhexosyl (most likely rhamnose) residue. Therefore, **1**–**7** were identified to be *O*-glycosylated flavonoids containing two glycosidic residues (Table 1; Fig. 1). The ESI-MS(+) fragmentation of **2** showed a mass loss of 132 Da that was indicative of an internal cleavage of a *C*-pentosyl unit. The aglycones were determined from the protonated base peaks with accurate mass measurements at *m/z* 286 ($C_{15}H_{11}O_6$) for **4** and **6**, *m/z* 302 ($C_{15}H_{11}O_7$) for **1** - **3**, and at *m/z* 317 ($C_{16}H_{13}O_7$) for **5** and **7**: identifications were kaempferol, quercetin and isorhamnetin, respectively. The base peaks were detected as the deprotonated aglycones of compounds **2**-**4** and **6**-**7** (Fig. 1).

Comparison of UPLC-ESI(-)-MS analyses of the extracts obtained from honey #2 and propolis from the same honeypots showed some common constituents (Fig. S1). Trace amounts of flavonoid aglycones pinocembrin and cryptostrobin, and of two phloroglucinols were found in the late eluting regions of honey extracts (Table 1). The glycosyl flavonoids

were found only in honeys and eluted at 12 – 17 min by LC. Gallic acid (a phenolic acid) and a catechin (a polar flavonoid aglycone) were found only in propolis by LC.

Norisoprenoids

The LC-MS analyses detected three norisoprenoids **9** – **11** namely abscicic acid, unedone and 13-hydroxyabscicic acid by dereplicating the accurate mass measurements of their deprotonated MS ions (Table 1) for structural identification (Fig. 2). The *cis*, *trans* isomerism of **11** was identified by the diagnostic fragment ion at *m/z* 151 (Fig. 2A) as previously reported *33*. The GC-MS analyses of the ethyl acetate extracts of *T. carbonaria* honeys contained 3-oxo-α-ionone **13** and related norisoprenoids **14** – **15** that were tentatively identified against entries of NIST library (Table 2, Fig. 2).

Lumichrome and aromatics

Lumichrome (7,8-dimethylalloxazine) **12** was identified in trace amounts in honey #1 (Table 1). In the negative mode of ESI-MS, the accurate mass measurement of the deprotonated ion at *m/z* 241.0733 yielded the elemental composition of $C_{12}H_9N_4O_2$ and *m/z* 198.0667 ($C_{11}H_8N_3O$) due to the loss of HNCO (Fig. S3) and confirmed the interpretations reported previously *30*.

3-Phenyllactic acid **8** was found in the three honeys by UPLC-ESI-MS and also by GC-MS (Tables 1 and 2). It showed the same UV spectra with maxima at 224sh and 283 nm of the reference compound (Sigma-Aldrich, NSW). Its deprotonated fragmented ESI-MS ions (Fig. S4) were dereplicated against literature *30*.

In the attempt to determine whether *Leptospermum* nectars contributed to the composition of multifloral 'sugarbag' honeys, samples #1 and #2 were obtained from two sites where these plant species were predominant during *Leptospermum* nectar flow. Results indicated that no MGO or phenolics recommended as *Leptospermum* markers were found

306 within the *T. carbonaria* honeys analysed in this study.

307

308 **Peroxide content in honeys and antibacterial activity of standard**

309 Levels of hydrogen peroxide accumulated up to 155.8 ±10.0 µM and 91.5 ±11.3 µM (mean
310 values, standard error) in undiluted and 44% diluted honeys, respectively. The standard
311 peroxide showed to be bactericidal at 760 µM in broth dilution, and mean zones of inhibition
312 were at up to 43.86 mm against *S. aureus* ATCC 25923 and up to 42.63 against *K.*
313 *pneumoniae* ATCC 13883 at the concentrations of 0 – 5.5 mM in agar diffusion (Table 3).

314

315 **Antimicrobial effects of phenolic concentrates from honeys**

316 In agar diffusion, the raw honeys (100 µL) were active against both bacterial strains (Table 3;
317 Figure S5). However, the phenolic extracts (100 µg) inhibited only *S. aureus* growth with
318 diameters of inhibition ranging from 10.18 to 19.87 mm with phenol equivalents of 1.0 to
319 5.1% (w/V) (Table 3). The phenolic extracts up to 100 µg did not inhibit *K. pneumoniae*
320 ATCC 13883. The positive control phenol solution showed mean zones of inhibition of 10.00
321 - 10.75 mm at the concentrations of 1% (w/V) against the two strains (Table 3).

322 In broth dilution, the raw honeys and phenolic concentrates were active against both
323 strains at the dilution used. The raw honeys in MHB were used at the dilution of 5.5% (v/v)
324 showing bactericidal effects after 16 h incubation. The phenolic concentrates had bactericidal
325 MIC values ranging from 1.2 to 1.8 mg/mL. The control phenol standard yielded a MIC value
326 of 0.18% (w/V) against both bacterial strains (Table 3).

327

328 **DISCUSSION**

329 **Chemotaxonomical composition and significance**

330 The liquid-liquid extraction methodologies applied in this study proved successful in
331 recovering the phytochemicals, and to concentrate them in phenolic extracts for the

chromatographic analyses and the antibacterial assays. The soft ionisation of ESI coupled to MS interpretation allowed for the characterisation of the aglycone type, the number of sugar units and the type of *O*-glycosidic flavonoids. The high abundances of the mass-to-charge ions of the aglycone moieties indicated that the glycosidic residues were possibly attached on a single position of the flavonoid core *19, 34*. The sugar residues could be possibly positioned on C-3 or C-7 [18], and previous reports of *T. carbonaria* honeys were identified to be 3-*O*-glycosides [15, 18]. For instance, the ESI-MS in positive mode of compounds **2**-**4** and **6**-**7** showed the base peaks of the protonated aglycones after two glycosidic losses, and thus these compounds were identified to be flavonol *O*-diglycosides (Table 1, Figure 1). Compounds **1** and **5** showed low relative abundances of the aglycone ions (Table 1, Figure 1), as previously reported in flavonoid di-*O*-glucosides. *34*.

The first order ESI-MS technique of this study suggested a (1→2) interglycosidic linkage as it was identified based on the high relative abundance (10-35%) of the moieties containing one sugar-*O*-aglycone after the loss of one glucosidic (162 Da) or rhamnosyl (146 Da) unit [34]. Further tandem MS^n studies are warranted to carry out the sequential fragmentation of parent molecular ions and daughter ions to confirm the type of interglycosidic linkage of flavonoids of this study [19, 35]. Using ESI-tandem MS^n, the interglycosidic linkages on flavonoids were characterised previously *18, 19, 35*. For instance, the ESI-MS fragmentation in positive mode of a dihexoside flavonol with interglycosidic linkage 1→2 was reported in Argentinean honeybee honeys [36].

The presence of several *O*-glycosylated flavonoids **1** – **7** and of few flavonoid aglycones was consistent with previous reports describing the honey composition due to Meliponini bees lacking β-glucosidases [18, 19]. On the contrary, Australian honeys from honeybees were reported to be low in glycosidic flavonoids and high in flavonoid aglycones [21], especially myricetin, tricetin and luteolin that were detected in *Eucalyptus* honeys from Australia and Europe *37, 38*. These aglycones were not found in *T. carbonaria* honeys. Aglycones of

358 flavonols **1** – **7** matched previous identifications in 'sugarbag' pot-honeys [18].

359 Compounds **3**, **5** – **7** showed a rhamnosyl residue (loss of 146 Da) and were indicative of
360 floral nectars rich in flavonoid rhamnosides that are not hydrolysed by *T. carbonaria* bees
361 salivary enzymes. Similar observations were reported on flavonoid rhamnosides found in
362 European honeys of honeybees foraging on *Robinia pseudoacacia* [19]. Therefore, 'sugarbag'
363 honeys of *T. carbonaria* bees have a particular profile high in *O*-glycosidic flavonoids
364 including rhamnosides and lower in flavonoid aglycones than Australian *A. mellifera*
365 honeybees [18].

366 Some flavonoid aglycones and phloroglucinols were found in honey. These natural
367 products are widely distributed in plant materials, and are likely to have originated from floral
368 nectars converted into honeys. These compounds were also tracked in propolis extracts from
369 the same honeypots (Fig. S4), hence it is possible that they were obtained from propolis
370 during honey maturation and harvest [18]. However, a highly polar catechin of propolis was not
371 traced in honey (Fig. S4), and was expected to be transferred into the polar matrix of honey
372 from propolis. Therefore, the extent to which propolis can contribute to the phytochemical
373 profiles of honeys remains to be confirmed. Nevertheless, the presence of flavonoids and
374 phloroglucinols in honeys is of interest because these compounds were reported for their *in*
375 *vitro* anti-staphylococcal activities of stingless bee propolis extracts [32].

376 The local flora included several *Eucalyptus* species that were visited by the *T. carbonaria*
377 bees of this study. The monoterpenoid eucalyptol (MW 154) was not found in the ethyl
378 acetate extracts of stingless bee honeys although it was reported as one abundant compound
379 of honeybee *Eucalyptus* honeys [39]. However, *Eucalyptus* as a source for nectars collected by
380 stingless bees was supported by the presence of norisoprenoids among the volatiles [23].
381 Norisoprenoids derive from carotenoid degradation [39]. Oxo-α-ionone **14** together with related
382 norisoprenoids **13** and **15** are chemical markers of honeys from *Eucalyptus* [39]. Other identified
383 norisoprenoids of Australian stingless bee honeys were unedone **10**, abscicic acid **12** and

384 derivative **9** and were previously described in *Apis* honeys originating from Sardinian
385 strawberry-tree (*Arbutus unedo* L., Ericaceae) that were used in medicinal preparations by
386 Ancient Greeks and Romans *33, 40*. Abscicic acids are plant hormones that help regulate
387 stressful conditions via control of stomatal closure [33]. In the subtropical areas of Eastern
388 Australia, water-stress conditions are likely to induce a production of abscisic acid and its
389 derivatives.

390 Lumichrome was found in trace amounts in *T. carbonaria* honeys. This compound was
391 reported in mānuka honey by German researchers Oelschlägel et al [10], as well as detected in
392 Mediteranean thistle (*Galactites tomentosa* Moench) honeys along with phenyllactic acid [30].
393 Lumichrome and its carboxyl derivative were indicated as characteristic floral markers of
394 cornflower (*Centaurea cyanus*) honeys from Romania and Germany [5]. Lumichrome is a
395 breakdown product of riboflavin and it is formed under photochemical-induced conditions, is
396 a plant-growth regulator and a constituent of unifloral honeys from Dalmatian sage (*Salvia*
397 *officinalis* L.) [41]. It was not found in previous analyses of *Eucalyptus* monofloral honeys of
398 honeybees, and therefore its botanical origin of Australian stingless bee honeys remains to be
399 determined.

400 3-Phenyllactic acid is a compound commonly found in honey, including *Leptospermum*
401 mānuka-varieties *10, 30*. This phenolic is also common in kanuka honey from New Zealand
402 *Kunzea ericoides* (Myrtaceae) [8] and in Australian leatherwood honeys (*Eucryphia lucida*) [27];
403 it was linked to the sensorial notes of thistle honey [30], and found across the three investigated
404 honeys although being a predominant marker in the honey #2.

405 *Leptospermum* varieties of 'jellybush-sugarbag' honeys were attempted using *T.*
406 *carbonaria* stingless bees in two sites of this study. However, no diagnostic chemical markers
407 of *Leptospermum* nectars were detected in *T. carbonaria* honeys: MGO and DHA levels [26]
408 were nil, and the phenolic markers MYSR [7], leptosin [31] and flavonoids pinobanksin, galangin,
409 chrysin were absent [9]. Traces of unedone were detected in the investigated honeys but this

410 norisoprenoid is a common phytochemical in honeys from Myrtaceae ie. *Eucalyptus,* and
411 therefore not specific to *Leptospermum* species [10]. When compared to previous chemical
412 analyses of Australian honeybee jellybush honey [13], the chemical markers reported for this
413 honey were not found in Australian stingless bee honeys of this study. It was concluded that *T.*
414 *carbonaria* stingless bees might not collect these nectars for their honey production.
415 Commercial honeys processed through heating or their improper storage can cause the
416 formation of the undesirable cyclic aldehyde hydroxymethylfurfural (HMF). HMF is
417 mutagenic and thus a limit of 40mg/kg is warranted for commercialisation to reduce the
418 health hazard to food consumers [25]. In stingless bee honeys of this study, HMF levels were nil,
419 therefore this bee product can suit future uses for food and medicinal purposes.
420
421 **Bioactive constituents of the extracts**
422 *T. carbonaria* raw honeys and their phenolic extracts showed *in vitro* antibacterial effects
423 (Table 3) that were within the reported ranges of 9.7 to 23.3 mm and 2.3 to 8.9% (w/V) of
424 phenol equivalents [16]. However, higher values of 26.3% (w/V) were indicated for other *T.*
425 *carbonaria* honeys, and compared to 18.0% for manuka honeys and 13.4% for other *A.*
426 *mellifera* honeys [12]. Nevertheless, *T. carbonaria* honey at 5.5% (v/v) inhibited the microbial
427 growth consistently with the reported dilution factor of <10% [16]. As the phenolic fractions
428 contributed to an average of 1.1% phenol equivalents against the Gram-positive
429 *Staphylococcus aureus*, the identified phytochemicals can be considered as one antimicrobial
430 factor linked to the non-peroxide effects of Australian stingless bee honeys [12, 17].
431 Different results were observed from the antibacterial testing with the two methods
432 used (Table 3). All raw honeys inhibited the bacterial growth when tested by agar diffusion,
433 but no bactericidal effects were observed in broth dilution at the concentrations used. The
434 broth dilution method indicated that the raw honeys were active via bacteriostatic effects on
435 the bacterial strains tested. This method also seemed more sensitive than the agar diffusion

436 method in detecting the bactericidal activity of the phenolic extracts against *K. pneumoniae.*
437 The phenolic extracts did not stop the bacterial growth in agar testing, possibly due to the
438 limited diffusion of phenolic extracts through the hydrophobic agar media. It is possible that
439 such limited diffusion could be affected by the high molecular weight of the glycosilated
440 flavonoids as well as the reduced hydrophylicity of less polar flavonoid aglycones. When
441 tested in broth dilution, the bactericidal effects of the phenolic extracts were attained possibly
442 from the direct exposure of the compounds to the bacterial cells, since there was no physical
443 limitation to the diffusion as experienced in the agar tests. The phenolic extracts proved to be
444 bactericidal against both strains, therefore indicating that the phenolic fractions contribute to
445 the total antibacterial activities of *T. carbonaria* honeys.

446 The peroxide content in *T. carbonaria* honeys accumulated up to 155 µM (Table 3) in
447 undiluted and diluted (44.4% v/v) honeys. However, a minimum concentration of 760 µM
448 H_2O_2 was required for bactericidal effects (Table 3). As a comparison, a higher content of 5.62
449 mM was reported in medical-grade honey of *A.mellifera* bees [6]. Therefore, the total
450 antimicrobial effects of *T. carbonaria* honeys were partly ascribed to the peroxide content and
451 that other factors could be accounted for the non-peroxide activity, including the presence of
452 phytochemicals as previously mentioned.

453 No MGO was detected in *T. carbonaria* honeys of this study, thus the non-peroxide
454 antimicrobial activity might be derived from other factors. For instance, one peptide called
455 bee defensin-1, also known as royalisin, was previously linked to the non-peroxide activity of
456 honeys [6]. This cationic peptide with bactericidal properties was reported in medical-grade
457 honeys [42], but not found in mānuka varieties [6]. It remains to be investigated in Australian
458 stingless bee honeys in future work.
459

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465

466 **Supporting Information Available:** full ESI-MS chromategrams of the three honeys'
467 phenolic extract and of propolis from the same honeypots; mass spectra of (11) abscisic acid,
468 (2) lumichrome and (8) 3-phenyllactic acid; dose-response curves of anti-staphylococcal
469 inhibition by the three phenolic extracts. This material is available free of charge via the
470 Internet at http://pubs.acs.org.

471

472 **Declaration of Ethical Standards**

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

475

476 **Conflict of Interest**

477 The authors declare that they have no conflict of interest.

478

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Figure Captions

Figure 1. Full mass spectra of the glycosylated flavonoids (1) quercetin-6,8-di-*O*-glucoside; (2) quercetin-3-*O*-glucosyl-*O*-pentose; (3) tricetin-3-*O*-rhamnose-*O*-glucoside; (4) kaempferol-3-*O*-diglucoside; (5) isorhamnetin-3-*O*-rhamnose-*O*-glucoside; (6) luteolin-3-*O*-rhamnose-*O*-glucoside, as identified in honey #2 by UPLC-ESI-(+)-MS. Characteristic fragmentation patterns showed the diagnostic mass losses for hexose moieties of 162 Da for a glucose unit, 146 Da for a rhamnose unit and 132 Da for the internal cleavage of a *C*-pentosyl unit in **2**.

Figure 2. Structures of dereplicated honey constitutents: (8) 3-phenyllactic acid, (12) lumichrome, (9) 13-hydroxyabscicic acid, (11) abscicic acid, (13) 6-hydroxy-3-oxo-alpha-ionone, (14) 3-oxo-α-ionone, (15) oxabicycloheptanyl.

Table 1. Phenolic Extracts of Honeys by LC-UV-HR-ESI-MS.

#	R_t (min)	Identification	UV_{max} (nm)	Formulas for $[M-H]^-$		Other HR-MS fragments (*m/z*)	Abundances (%)[d]		
							#1	#2	#3
8[c]	10.91	3-Phenyllactic acid	288	$C_9H_9O_3$	165.0551	(-) 147 $[M-H_2O]$, 119	3.7	12.7	5.9
1	12.48	Quercetin-6,8-di-*O*-glucoside	210, 263, 347	$C_{27}H_{29}O_{17}$	625.1423	(+) 627.1562 $[M+H]^+$, (+) 465.1032 $[M+H-162]^+$ (+) 303.0501 (aglycone) $[M+H-162-162]^+$	tr	tr	2.3
2	13.39	Quercetin-3-*O*-glucosyl-*O*-pentose	248, 292, 362	$C_{26}H_{27}O_{16}$	595.1319	(+) 597.1453 $[M+H]^+$, (+) 465.1031 $[M+H-132]^+$ (+) 303.0500 $[M+H-162]^+$	tr	tr	2.2
3	13.62	Tricetin-3-*O*-rhamnose-*O*-glucoside	224, 289	$C_{27}H_{29}O_{16}$	609.1474	(+) 611.1609 $[M+H]^+$, (+) 465.1031 $[M+H-146]^+$ (+) 303.0500 (aglycone) $[M+H-146-162]^+$	tr	1.1	3.0
4	13.76	Kaempferol-3-*O*-diglucoside	250, 285, 348	$C_{26}H_{27}O_{17}$	609.1476	(+) 611.1268 $[M+H]^+$, (+) 450.1116 $[M+2H-162]^+$ (+) 287.0552 (aglycone) $[M+H-162-162]^+$	3.1	2.8	6.6
5	14.56	Isorhamnetin-3-*O*-rhamnose-*O*-glucoside	260, 290, 340	$C_{28}H_{31}O_{16}$	623.1626	(+) 625.1768 $[M+H]^+$, (+) 479.1189 $[M+H-146]^+$ (+) 317.0658 ($C_{16}H_{13O7}$) $[M+H-146-162]^+$	1.6	43.7	22.6
6	14.87	Luteolin-3-*O*-rhamnose-*O*-glucoside	299	$C_{26}H_{27}O_{16}$	595.1319	(+) 595.1661 $[M+H]^+$, (+) 449.1082 $[M+H-146]^+$ (+) 287.0551 (aglycone) $[M+H-146-162]^+$	tr	1.1	12.7
9	15.12	13-Hydroxyabscicic acid	260	$C_{15}H_{19}O_5$	279.1242	(-) 168.0787 ($C_9H_{12}O_3$), 139.0758 ($C_8H_{11}O_2$)	-	-	tr
7	15.30	Isorhamnetin-3-*O*-rhamnose-*O*-glucoside isomer	248, 295, 358	$C_{28}H_{31}O_{16}$	623.1628	(+) 625.1767 $[M+H]^+$,(+) 479.1188 $[M+H-146]^+$ (+) 317.0657 (aglycone) $[M+H-146-162]^+$	-	0.3	tr
10[c]	15.50	Unedo	nd	-	-	(+) 241.1436 ($C_{13}H_{21}O_4$) $[M+H]^+$, 197.0010 $[M+H-CO_2]^+$	-	-	tr
12[c]	16.65	Lumichrome	260, 340	$C_{12}H_9N_4O_2$	241.0733	(-) 223 $[M-H-H_2O]^-$, (-) 198.0667 ($C_{11}H_8N_3O$) $[M-H-HNCO]^-$ (+) 243.0879 $[M+H]^+$	tr	-	-
11[c]	19.20	Abscicic acid (*cis*,*trans*)	260	$C_{15}H_{19}O_4$	263.1293	(-) 219.1390 ($C_{14}H_{19}O_2$) $[M-COOH]^-$, 151.0758 ($C_9H_{11}O_2$)	-	tr	-
-	19.54	Methylflavanon	260, 290	$C_{15}H_9O_7$	301.0358	(-) 285.0409 $[M-H-CH_3]^-$	-	-	tr
-	20.17	Flavonol	260, 340	$C_{15}H_{11}O_5$	271.0618	(+) 273.0760 $[M+H]^+$	tr	-	-
-	20.69	Flavonol	290, 340	$C_{16}H_{13}O_6$	301.0724	(+) 303.0865 $[M+H]^+$	tr	-	-
-	21.61	Flavonol	290, 340	$C_{17}H_{15}O_5$	299.0931	-	tr		-
-	22.03	Pinocembrin[a,b]	290	$C_{15}H_{11}O_4$	255.0666	-	-	tr	-
-	22.78	Cryptostrobin[a,b]	290, 340	$C_{16}H_{13}O_4$	269.0824	(+) 271.0966 $[M+H]^+$	1.6	1.7	0.1
-	26.88	Acylphloroglucinol[b]	260	$C_{25}H_{37}O_3$	385.2757	(+) 387.2893 $[M+H]^+$	tr	tr	tr
-	27.42	Phloroglucinol-flavanone[b]	nd	$C_{31}H_{35}O_7$	519.2399	(-) 471, 339, 325, 255, 181	tr	tr	tr

[a]matched with references
[b]identified in propolis from the same honeypots of honey #2
[c]see structures in Fig. 2
[d]Quantitative amounts were measured as mg/100 mg of gallic acid equivalents at 205 nm. Mean values, n= 2.

Legend: 'nd' not determined

Table 2. GC-MS Analyses of the Ethyl Acetate Extracts of Honeys.

#	R_t (min)	Identifications	EI-MS (*m/z*)	M*	KI	µg/g		
						#1	#2	#3
	6.65	B-pinene	93 (BP), 136	136	980	4.9	0.7	6.9
-	18.06	Lactic acid diTMS	73, 147 (BP), 189, 233, 261, 303	318	1476	1,241.5	682.1	1,398.9
8[c]	18.73	3-Phenyllactic acid[b]	65, 91 (BP), 103, 148, 166	166	1508	-	25.3	-
-	20.69	Alkylated phloroglucinol[c]	70 (BP), 137, 179, 207, 250	250	1605	0.8	-	-
-	21.21	2-Hydroxyisocaproic, diTMS[d]	73, 147 (BP), 189, 275, 03	360	1631	0.8	0.8	0.9
14[c]	21.46	3-Oxo-α-ionone	73, 180, 150, 172	206	1644	3.5	1.3	3.4
-	23.38	Succinate, diTMS[d]	73 (BP), 147, 289	346	1743	44.8	11.7	11.9
13[c]	24.01	6-Hydroxy-3-oxo-alpha-ionone	75, 124 (BP), 149, 166, 207	222	1777	4.4	0.9	9.3
15[c]	25.73	Oxabicycloheptanyl	93(BP), 123, 137, 153, 166	222	1880	0.8	0.8	0.9
-	27.06	Hexadeconoic acid[b]	73 (BP), 129, 171213, 227, 256	256	1961	17.3	5.1	6.5
-	28.01	3-Phenyllactic, diTMS[d]	147(BP), 73, 189, 309	310	2015	20.6	45.2	22.8
-	29.79	Linoleic acid[b]	67 (BP), 81, 95, 109, 123, 280	280	2133	0.7	8.4	5.5
-	29.89	α-Linoleic acid[b]	67, 79 (BP), 95, 108, 222, 280	280	2140	0.7	8.4	5.5
-	33.68	Acylphloroglucinol	69, 91, 135, 191, 245 (BP), 386	386	2302	10.0	2.3	9.2
-	35.04	Hydroxybenzoic, diTMS ester[d]	73 (BP), 193, 439, 480	496	2504	4.7	0.8	6.4
-	35.39	Cryptostrobin[a]	69, 138 (BP), 166, 193, 270	270	2531	5.2	3.5	44.2
-	35.53	Stroboponin[a]	69, 138, 166 (BP), 193, 270	270	2542	4.5	0.8	0.9
-	37.28	Docadosene	55 (BP), 67, 97, 308	322	2581	0.8	0.8	0.9
-	40.85	Lupeol	69, 207 (BP), 281, 341, 408	426	2886	15.2	0.3	6.7

[a]identified against purified references
[b]identified only in underivatised, non-silylated extracts
[c]see structures in Fig. 2
[d]BSTFA was used in derivatising for trimethylsilyl (TMS) groups.
[e]Compounds were quantitated in honey (µg/g) using peak areas against the internal standard hexadecane in underivatised and silylated extracts. Mean values, n= 2.

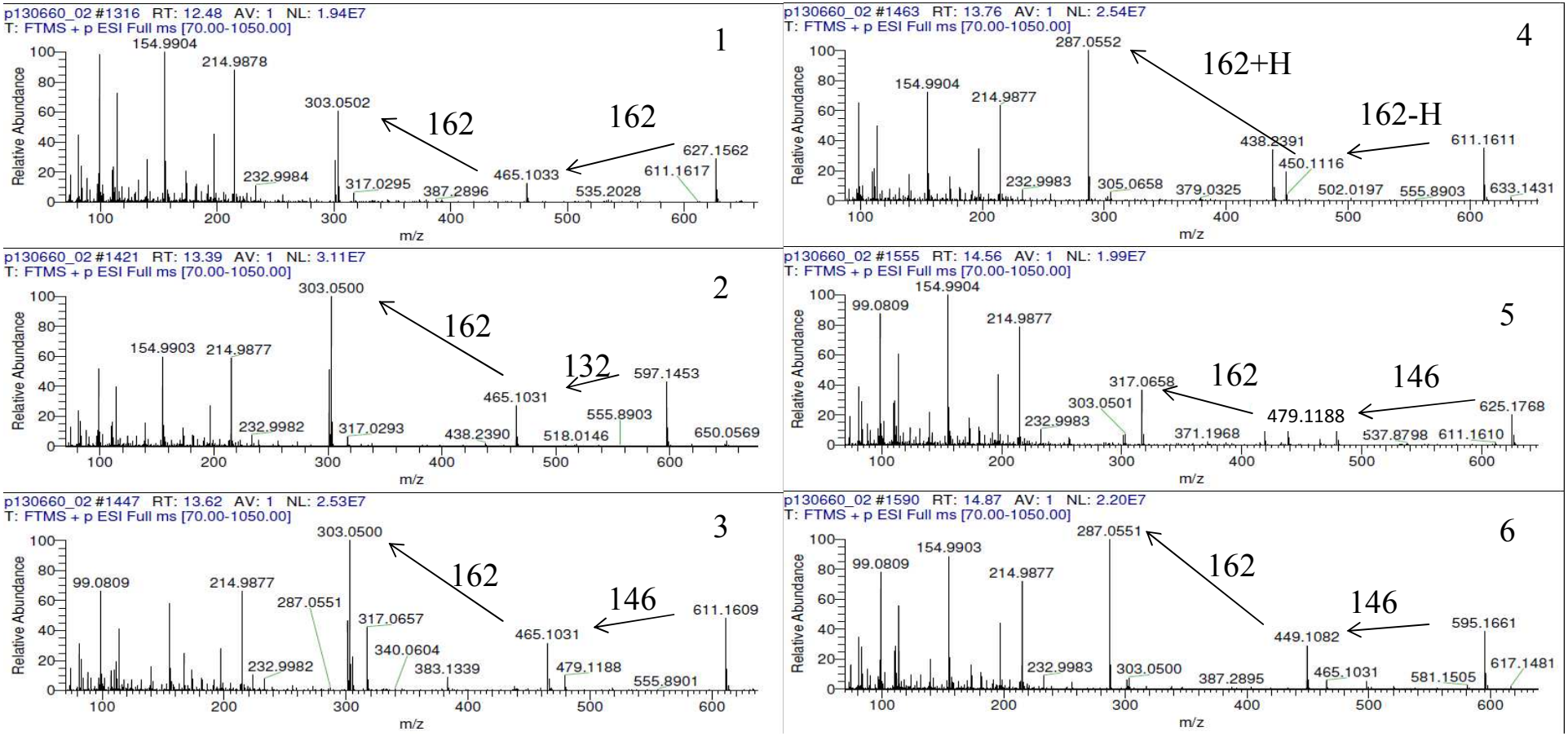
Legend: 'M*': molecular ion, 'tr': traces less than 1%, ‘BP’: base peak, 'KI': Kovats indices.

Table 3. Antibacterial Effects of Honeys and their Phenolic Extracts.

Samples	*S. aureus* ATCC 25923		*K. pneumoniae* ATCC 13883	
	MID[a] (phen equiv.[e])	MIC	MID[a] (phen equiv.[e])	MIC
Raw honey #1	19.87 ± 0.02[a] (5.1[e])	-	17.39 ± 0.01[a] (4.0[e])	-
Raw honey #2	17.45 ± 0.44[a] (4.0[e])	-	15.41 ± 0.01[a] (3.3[e])	-
Raw honey #3	15.15 ± 0.02[a] (2.8[e])	-	15.42 ± 0.07[a] (3.3[e])	-
phenolics #1	10.60 ± 0.05[a] (1.2[e])	1.2[c]	-[a]	1.2[c]
phenolics #2	10.18 ± 0.03[a] (1.0[e])	1.8[c]	-[a]	1.2[c]
phenolics #3	10.27 ± 0.15[a] (1.1[e])	1.2[c]	-[a]	1.2[c]
H_2O_2 standard	12.37 ± 0.01[b] (1.8[e])	760.0[d]	11.20 ± 2.31[b] (1.6[e])	760.0[d]
Phenol standard	10.75 ± 0.01[f] (1.0[e])	0.18[e]	10.00 ± 0.0[f] (1.0[e])	0.18[e]

[a] MID (mm) for samples at 100 µg in a 100 µL. Mean values ± standard error (n=3)
[b] at 300 µM
[c] mg/mL
[d] µM
[e] phenol equivalents % (w/v)
[f] 1 % (w/v)

Legend: 'MID': Mean Inhibition Diameters, 'MIC': Minimum Inhibitory Concentrations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162
162
627.1562
611.1617
232.9984
317.0295
387.2896
465.1033
535.2028
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162+H
154.9904
214.9877
162-H
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611.1611
450.1116
232.9983
305.0658
379.0325
502.0197
555.8903
633.1431
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162
154.9903
214.9877
132
597.1453
465.1031
555.8903
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518.0146
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99.0809
214.9877
317.0658
162
146
303.0501
232.9983
479.1188
625.1768
371.1968
537.8798
611.1610
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162
99.0809
214.9877
287.0551
317.0657
146
611.1609
340.0604
465.1031
232.9982
383.1339
479.1188
555.8901
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T: FTMS + p ESI Full ms [70.00-1050.00]
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162
99.0809
214.9877
146
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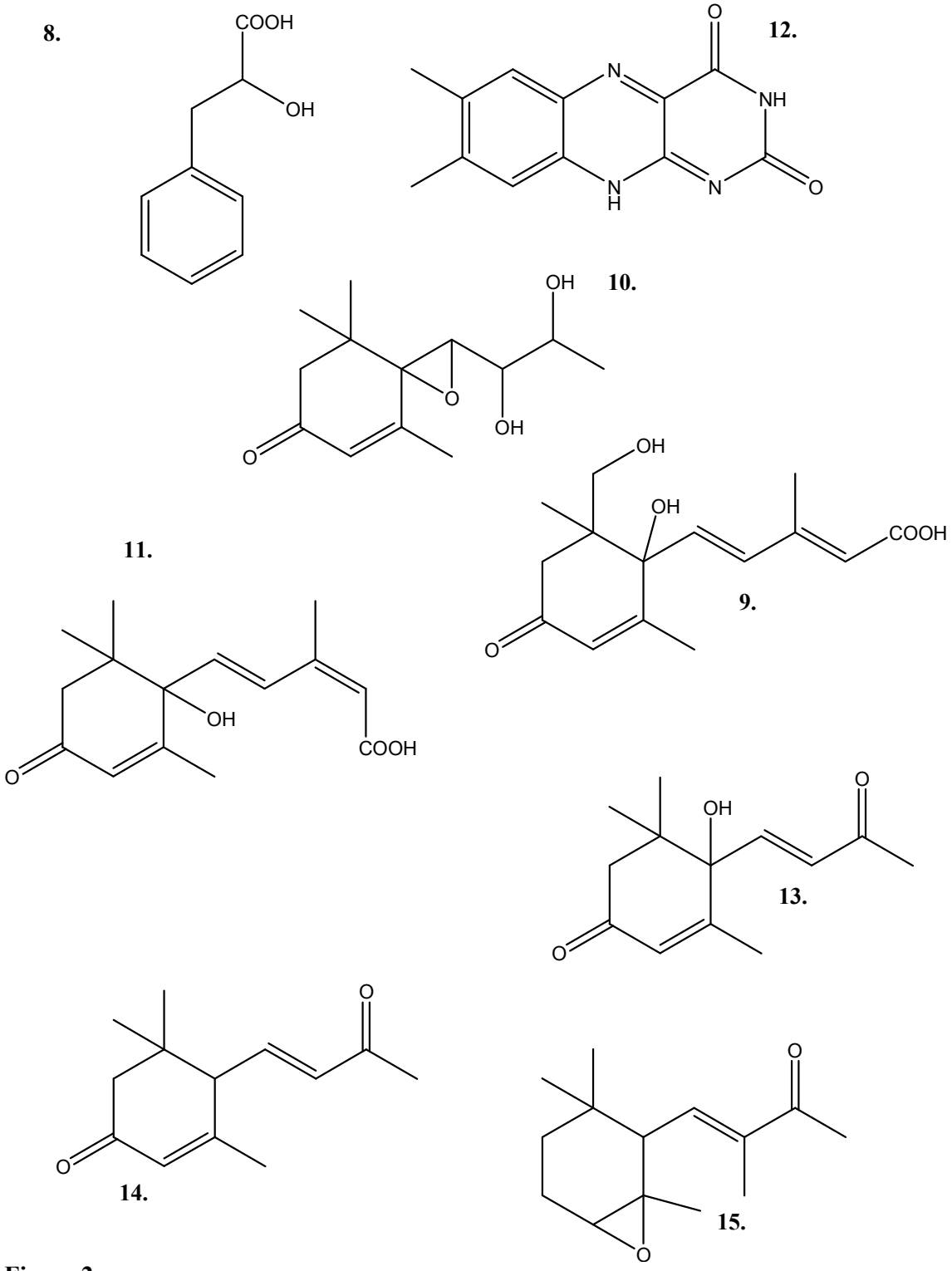
387.2895
465.1031
581.1505
Relative Abundance
m/z

Figure 1.


8.
COOH
OH
12.
O
N
NH
N
H
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OH
OH
COOH
9.
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OH
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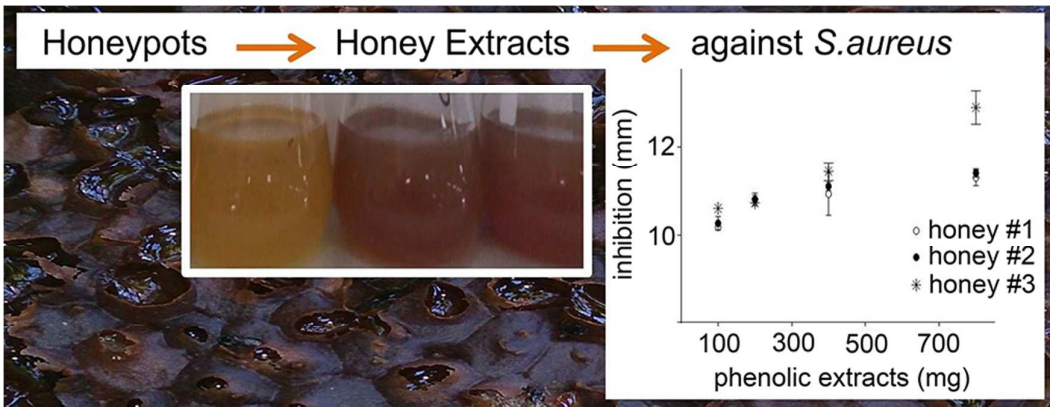
O
O
OH
13.
O
O
O
14.
O
15.
O

Figure 2.

TOC graphic


Honeypots
Honey Extracts
against S.aureus
inhibition (mm)
12
10
100
300
500
700
phenolic extracts (mg)
honey #1
honey #2
honey #3