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Cerumen of Australian stingless bees (*Tetragonula carbonaria*): gas chromatography–mass spectrometry fingerprints and potential anti-inflammatory properties

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Abstract (248 words)

Cerumen, or propolis, is a mixture of plant resins enriched with bee secretions. In Australia, stingless bees are important pollinators that use cerumen for nest construction and possibly for colony's health. While extensive research attests to the therapeutic properties of honeybee (Apis mellifera) propolis, the biological and medicinal properties of Australian stingless bee cerumen are largely unknown. The chemical and biological properties of polar extracts of cerumen from Tetragonula carbonaria in South East Queensland, Australia were investigated using gas chromatography–mass spectrometry (GC-MS) analyses and in vitro 5-lipoxygenase cell-free assays. Extracts were tested against comparative (commercial tincture of Apis mellifera propolis) and positive controls (Trolox and gallic acid). Distinct GC-MS fingerprints of a mixed-diterpenic profile typical of native bee cerumen were obtained, with pimaric acid (6.31 ± 0.97%, w/w), isopimaric acid (12.23 ± 3.03%, w/w) and gallic acid (5.79 ± 0.81%, w/w) tentatively identified as useful chemical markers. Characteristic flavonoids and prenylated phenolics found in honeybee propolis were absent. Cerumen extracts from T. carbonaria inhibited activity of 5-lipoxygenase, an enzyme known to catalyse production of pro-inflammatory mediators (IC₅₀ 19.97 ± 2.67 µg/mL, mean ± SEM, n = 4). Extracts had similar potency to Trolox (IC₅₀ 12.78 ± 1.82 µg/mL), but were less potent than honeybee propolis (IC₅₀ 5.90 ± 0.62 µg/mL) or gallic acid (IC₅₀ 5.62 ± 0.35 µg/mL, P < 0.001). These findings warrant further investigation of the ecological and medicinal properties of this stingless bee cerumen, which may herald a commercial potential for the Australian beekeeping industry.

Keywords: propolis · resins · gc-ms · 5-lipoxygenase · honeypots · diterpenic acids
INTRODUCTION

Propolis is a resinous and waxy material that bees *Apis mellifera* produce by mixing plant resins and beeswax (Bankova 2009). Stingless bees also produce a mixture similar to propolis, the cerumen (Simone-Finstrom and Spivak 2010), and there is a suggestive evidence that stingless bees add head gland secretions during cerumen production (dos Santos et al. 2009). Stingless bees use the cerumen to mummify intruders and to preserve a sterile environment in the hive (Lehmberg et al. 2008; Patricio et al. 2002; Sawaya et al. 2004). In contrast to honeybees, which use propolis as internal layering around nest combs, stingless bees use large amounts of cerumen as a building material for nest elements such as honey pots, pillars and other structures in the nests (Roubik 2006). Although the terms cerumen and propolis are used interchangeably in literature on stingless bees (Patricio et al. 2002), in this article, cerumen refers to the product of stingless bees and propolis refers to the product of honeybees.

In honeybees, the chemical components and bioactive properties of propolis, bee foraging behaviour and resin preferences have been extensively studied (Simone-Finstrom and Spivak 2010). There is also an emerging body of literature on resin-foraging behaviour in stingless bees (Leonhardt and Blüthgen 2009; Nunez et al. 2008; Wallace et al. 2008; Wallace and Lee 2010; Wallace and Trueman 1995). Stingless bees are important pollinators for crop production and are selective in collection of plant resins (Heard 1999; Leonhardt et al. 2010a). For instance, the seed capsules of the eucalypt species *Corymbia torelliana* provides Australian stingless bees with a favourite source of resin, which promotes the seed dispersal activity (or melittochory) of the bees (Leonhardt et al. 2010b; Wallace et al. 2008; Wallace and Lee 2010). The chemical
composition of stingless bee cerumen depends on the botanical sources, the availability of plant resins to bees and the bees’ preferences (Leonhardt and Blüthgen 2009; Leonhardt et al. 2009; Leonhardt et al. 2010b). However, limited information exists on the chemical and bioactive properties of cerumen from Australian stingless bees. Tetragonula carbonaria bees store honey in honeypots that are made entirely of cerumen (T. Heard 1999). Previous studies on T. carbonaria honey indicated the antioxidant and antimicrobial properties (Oddo et al. 2008; Temaru et al. 2007). Temaru and coworkers (2007) also suggested that the non-peroxide activity behind the antimicrobial effect of stingless bee honey is possibly due to presence of phytochemicals derived from the cerumen. Hydrocarbons and fatty acids have been identified from non-polar extracts as the main constituents of T. carbonaria nest material (Milborrow et al. 1987). Recently, Leonhardt et al. (2010a) elucidated the chemical composition of non-polar and polar fractions obtained from T. carbonaria cuticular extracts. However, the analysis of polar extracts from the cerumen of this stingless bee is unreported.

Many plant-derived molecules present in propolis and cerumen are active in preserving bee health. In honeybees, studies on immune responses indicate that the level of social immunity is mainly responsible for the colony's health, and also support the need for propolis for defence purposes (Cremer and Sixt 2009, Simone et al. 2009). In stingless bees, activity of cuticular antimicrobial secretions increases with bee group size and within-nest genetic relatedness (Stow et al. 2007).

The healing properties of honeybee propolis have been known since ancient times, and propolis is still used extensively for its therapeutic properties as an
antimicrobial, anti-inflammatory and antioxidant agent in the preparation of herbal medicines (Banskota et al. 2001). Inflammatory conditions such as rheumatoid arthritis, psoriasis, ulcerative colitis and Crohn’s disease are mediated in part by leukotrienes that are produced following activity of lipoxygenase (LOX) enzymes (Rankin 2004). Therefore, molecules that exert LOX-inhibitory and antioxidant activities have potential anti-inflammatory activity (Polya 2003). The anti-inflammatory and antioxidant properties of the constituents of honeybee propolis were reviewed by de Almeida and Menezes (2002). Australian honeybee propolis has also been shown to have anti-inflammatory effects on wound healing in a rodent model of diabetes (McLennan et al. 2008).

Although the antimicrobial properties of stingless bee cerumen have been investigated (Farnesi et al. 2009), to date the chemical composition and other medicinal properties of the cerumen of Australian stingless bees remain unknown. Therefore, the aims of this study were to: (1) define the chemical composition of *T. carbonaria* cerumen from the subtropics (South East Queensland, Australia) using gas chromatography–mass spectrometry (GC-MS) techniques; and (2) investigate, using cell-free assays of cerumen extracts, the possible inhibitory (antioxidant and anti-inflammatory) effects on lipoxygenase activity. The chemical profiles and biological properties of the *T. carbonaria* cerumen were compared with those of standardized propolis from *Apis mellifera*, New Zealand.
Details of materials and methods used in the study are given in Online Resource 1. A summary of the methods is provided below.

**Sampling cerumen and propolis**

In 2008, stingless bee cerumen was sampled throughout the year 2008 from 40 nests of *T. carbonaria* colonies in South East Queensland, Australia. The raw samples were washed with water to remove debris and honey and then unified into a single homogeneous sample. Standardized tinctures in ethanol of commercial propolis (containing 172 mg/mL equivalents of New Zealand propolis from *A. mellifera*) were provided by Comvita New Zealand Ltd. All samples were stored in sealed plastic containers at 4°C, protected from light and air to minimize photosensitivity and possible oxidation or degradation.

**Solvents and reagents**

Reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and solvents used for extraction and chromatographic analyses were of analytical grade and purchased from Merck Pty Ltd (Kilsyth, Vic., Australia).

**Preparation of standards and samples for the chemical analyses**

Raw samples of *T. carbonaria* cerumen were weighed, then extracted to recover the balsam using methanol:hexane (1:1) for 2 h at room temperature. Solids and debris were removed by paper filtration. The hexane extracts were discarded and aliquots of the methanol extracts were used for further chemical and biological analyses. Aliquots of commercial propolis tinctures (*A. mellifera*) and of cerumen MeOH extracts (*T.*
carbonaria) were evaporated for solvent removal under a stream of nitrogen to yield the dry extracts immediately before the analyses.

**Solvent extraction and fractionation**

Aliquots of the methanol (MeOH) extracts of cerumen (T. Carbonaria) were further extracted with diethyl ether (Et₂O) and ethyl-O-acetate (EtOAc) to improve the separation of constituents. A portion of the MeOH extract was further dissolved in Et₂O: NaHCO₃ (2 % saturated) and partitioned into an ether extract and an aqueous extract. The aqueous extract was acidified and back-extracted with EtOAc. The liquid fractions in Et₂O and EtOAc were then rotavaped to remove these solvents, thus yielding the dry polar extracts for the chemical analyses.

**Derivatization of samples**

Crude polar extracts of stingless bee cerumen, honeybee propolis, and standards were converted to trimethylsilyl (Creaser et al. 1991; Greenaway et al. 1987; Markham et al. 1996; Online Resource 1). Compounds with no hydroxyl groups in their structures remained unchanged by the derivatization process. All samples were then submitted to chromatographic analysis.

**Gas chromatography–mass spectrometry analyses**

Gas chromatography–mass spectrometry (GC-MS) analyses were performed on the dry extracts of the cerumen and propolis reconstituted in a vehicle for the analyses. Available standards were chromatographed under similar experimental conditions. Substance identification was based on standards where possible, or on literature retention orders and mass spectra, and comparison with the MS database (NIST).

**Quantitation of compounds**
Hexadecane C16 (stock concentration of 0.668 mg/mL) was used as the internal standard (IS) for the quantitation of cerumen components. The content of the cerumen constituents was calculated from the relative peak areas that were compared to the IS peak area, and expressed as the percentage (%, w/w) of crude polar extracts. Response factors of one were assumed for all constituents.

The 5-lipoxygenase assay

The 5-lipoxygenase (5-LOX) assay was performed with a protocol modified from Anthon and Barrett (2001; Online Resource 1). Samples and standards were incubated with 5-LOX (2.25 µg/mL) and the substrate linoleic acid (LA, 125 µM), in the presence of the catalyst, haemoglobin (25 µg/mL) and the oxidatative coupling reactants, 3-methyl-2-benzothiazoline (MBTH, 0.1 mM) and 3-dimethylaminobenzoic acid (DMAB, 5 mM). The purple indamine dye produced by the reaction was measured by spectrophotometer at 598 nm. Controls were equal volumes of 70% EtOH (negative) and Trolox or pure gallic acid in EtOH solution (positives). Concentration-response curves were constructed for the determination of the concentration sufficient to obtain half of the maximum scavenging capacity (IC$_{50}$).

Data and statistical analyses

From the chemical analyses, qualitative data obtained were retention time ($R_t$) and mass ions ($m/z$). The components of the polar extracts of T. carbonaria cerumen (% w/w) were calculated (Online Resource 1). Results are reported as mean ± half range of two independent chemical analyses performed by extraction, fractionation, derivatization and chromatography.
From the bioassays, the percent inhibition of 5-LOX was calculated as reported by Kumaraswamy and Satish (2008; Online Resource 1). Concentration-response curves were constructed for *T. carbonaria* cerumen, *A. mellifera* propolis, gallic acid and Trolox (n = 3 or 4), and normalised to 70% EtOH controls. The IC$_{50}$ values were determined and expressed as mean ± SEM. Results were compared using a two-way ANOVA (not repeated measures).

RESULTS

Recovery, identification and quantification of cerumen and propolis constituents by GC-MS

The polar MeOH extracts of *T. carbonaria* cerumen were recovered with yields of 46.6 ± 2.1% (w/w). Different chromatographic profiles were obtained for the polar extracts of *T. carbonaria* cerumen and for the commercial tinctures of *A. mellifera* propolis. These two chromatographic fingerprints were used to distinguish the cerumen and propolis types (Fig. 1a, 1b). The Et$_2$O (Fig. 1c) and EtOAc (Fig. 1d) fractions of *T. carbonaria* cerumen were used to improve the separation of polar compounds, which simplified the interpretation of the eluted peaks and related mass spectra.

Mass spectra were interpreted to elucidate the fragmentation patterns of the TMS derivatives of *T. carbonaria* cerumen compounds. The probable mass ions were interpreted on their mass weight (Table 1). The overall chemical composition of *T. carbonaria* cerumen from South East Queensland showed 34 compounds with 31 possible identifications (Table 2). The constituents of *T. carbonaria* stingless bee cerumen were mainly gallic acid (compound 14) and diterpenic acids of pimaric (18, 19,
20 and 29) and abietic type (22, 23, 24 and 25). When components were grouped by chemical structural class, T. carbonaria cerumen was comprised of 29.2 ± 1.1% terpenic acids and 7.3 ± 0.9% phenolic acids. The constituents gallic acid (14), pimaric acid (20) and pimaric acid isomer (29) are suggested as chemical markers of T. carbonaria cerumen from South East Queensland.

The constituents of A. mellifera propolis were cinnamic acid (2), monosaccharide (6), gluconic acid (7), fructose (8), β-glucose (10), p-coumaric acid (12), and monosaccharide (15). These compounds were also found in cerumen of T. carbonaria. However, other propolis constituents were p-hydroxybenzoic acid (35), hydroxybenzoic ester (36), monosaccharide (37), monosaccharide (38), ferulic acid (39), caffeic acid (40), pentenyl ester iso-ferulic acid (41), pentenyl ester caffeic acid (42), pentenyl ester caffeic acid isomer (43), pinostrobin (44), pinocembrin (45), sterol (46), sterol (47), sterol (48), cinnamic acid ester (49), dihydroxy-2-methyl-anthraquinone (50), galangin (51) and they were not found in T. Carbonaria cerumen.

Inhibition of 5-lipoxygenase activity by cerumen and propolis

The solvent (70% EtOH) had only a negligible inhibitory effect on the 5-LOX assay. Inhibition of 5-LOX activity by T. carbonaria cerumen and A. mellifera propolis (Fig. 2a), and by the standards gallic acid and Trolox (Fig. 2b), were concentration dependent. The IC\textsubscript{50} values of the test and control solutions ranged from 5.62 to 19.97 µg/mL. Polar extracts of T. carbonaria cerumen inhibited 5-lipoxygenase with similar potency to the water-soluble vitamin E standard, Trolox (T. carbonaria, IC\textsubscript{50} = 19.97 ± 2.67 µg/mL, n = 4; Trolox, IC\textsubscript{50} = 12.78 ± 1.82 µg/mL), but was less potent than...
comparative honeybee propolis (5.90 ± 0.62 µg/mL) or the standard gallic acid (5.62 ±
0.35 µg/mL; $P < 0.001$).

DISCUSSION

In this study, we determined which bioactive compounds could be found in the polar
eXtracts of cerumen of stingless bees (T. carbonaria) from South East Queensland,
Australia. The methods of this study were suitable to determine the chemical and
biological properties of the Australian cerumen under investigation.

GC-MS fingerprints of T. carbonaria cerumen

We identified newly chromatographed fingerprints of Australian stingless bee cerumen,
showing that the composition of T. carbonaria cerumen from South East Queensland
differs from that of foreign types of stingless bee cerumen elsewhere reported (Patricio
et al. 2002; Sawaya et al. 2004; Velikova et al. 2000a). However, the chemical content
of T. carbonaria cerumen did not reveal the presence of any newly reported
constituents, while the predominance of diterpenic acids and gallic acids was
characteristic of a diterpenic-mixed profile (Bankova and Popova 2007; Velikova et al.
2000a). Pimaric and abietic acids were the main types of diterpenic acids found in T.
carbonaria cerumen, while other structural diterpenic types reported for foreign
cerumen were absent (Velikova et al. 2000b). Trace quantities of TMS-ethers of β-
amyrrins were tentatively identified as triterpenic acids of the ursane and oleanane types
in T. carbonaria cerumen. In contrast, these triterpenic acids are prevalent in foreign
tropical propolis (Pereira et al. 2002; Pereira et al. 2003; Teixeira et al. 2006).
When compared to GC-MS profiles of *A. mellifera* propolis, the Australian cerumen showed different chromatographic peaks (Fig.1) and none of the propolis phenolics were found in this cerumen. Our analyses on the standardized tinctures of propolis confirmed previous reports (Markham et al. 1996), and allowed us to develop methods that could be applied for the examination of the cerumen. The compounds gallic acid (14), pimaric acid (20) and pimaric acid isomer (29) were detected only in the cerumen extracts and were suggested as chemical markers for *T. carbonaria* cerumen from South East Queensland. Because the GC-MS fingerprints are unique for the analytes, the findings of our study can be used as a reference for further chemical analyses on cerumen.

Analytical methods for the extraction of cerumen constituents

In this study, GC-MS proved a suitable technique to investigate the polar extracts of *T. carbonaria* cerumen. Analytical methods were developed for the extraction, the separation and the identification of polar volatile compounds found in raw cerumen from *T. carbonaria*. Mass spectra were informative for compound identification, based on their characteristic patterns of fragmentation (Table 1). Using available chemical standards, reference to the literature, retention order and the mass ions, the separation and identification of 34 constituents (Table 2) of *T. carbonaria* cerumen were attempted. However, these findings were not conclusive as similar mass spectra arise from related or similar structures (Pereira et al. 2003). Further investigations are needed to match the mass spectra of cerumen constituents with standards, possibly using tandem MS–MS, and for structural determination and conclusive identification of components of *T. carbonaria* cerumen.
Solvent extraction of bee cerumen and propolis was necessary to recover the constituents with potential activity. While ethanol:water is often the solvent system of choice for propolis extracts that can be included in medicinal preparations, in this study a methanol:hexane system was used to recover the polar constituents of the cerumen. The polar extracts of *T. carbonaria* cerumen showed good recovery from raw material, with yields of 46.6 ± 2.1% (w/w). Our dual solvent system was preferred to select for the cerumen polar fraction, while discarding the non-polars such as beewax components that would interfere with the chemical analyses. Further fractionation facilitated the separation into chemical classes of polars. The GC-MS profiles confirmed the consistency of our findings.

Extraction and purification procedures can potentially compromise the recovery of propolis constituents due to degradation and artefact generation (Piccinelli et al. 2009). Therefore, the stability of dry polar extracts of *T. carbonaria* cerumen was confirmed by the unaltered chromatographic profiles of GC-MS analyses that were repeated on the polar extracts after storage in the dark at 4°C for five months. The botanical origins of cerumen and propolis

The difference in the chemical constituents and chromatographic fingerprints between the cerumen of *T. carbonaria* from South East Queensland and the propolis of *A. mellifera* from New Zealand reflected the different botanical origins of these bee products. Propolis from *A. mellifera* had the characteristic profile of a phenolic poplar-like propolis (Creaser et al. 1991; Markham et al. 1996): terpenic acids were absent, while prevalent polyphenols were the flavanone pinocembrin, the flavonol galangin, and the phenolic acid derivatives of cinnamic acid and caffeic acid. The cerumen from
*T. carbonaria* in our study was sampled from hives that were mainly located in urban areas in South East Queensland, where stingless bees potentially forage on ornamental species (T. Heard unpubl. data). Although the investigation of the botanical sources of the *T. carbonaria* cerumen was beyond our scope of study, the chemical composition of the cerumen reported here could potentially be used to discriminate resin sources of cerumen in future studies. The predominance of terpenic compounds has been attributed to collection of specific plant resins, especially considering the short flying range of stingless bees (Leonhardt et al. 2010a, b; Milborrow et al. 1987; Roubik 2006). *T. carbonaria* bees were recently reported to forage on fruit resins of *C. torelliana* in Queensland (Wallace et al. 2008; Wallace and Lee 2010), and a resin chemical marker was found on bee surfaces thus indicating a forage preference of *T. carbonaria* bees for *C. torelliana* resins in Australia (Leonhardt et al. 2010). After comparison of the mass fragmentation patterns, we most likely determined the same unknown compound # 4 (Leonhardt S. unpubl. data). This provides some comparative information on the chemistry of Australian cerumen that can be representative of cerumen from East Australia where *C. torelliana* is distributed. The flora of Australia results from long geographic isolation, therefore, it is possible that the cerumen sampled in this study is unique to South East Queensland. Correlating the chemistry of the cerumen with its potential botanical sources would be necessary to elucidate the chemical diversity of cerumen produced in Australia.

**Inhibition of 5-lipoxygenase activity by *T. carbonaria* cerumen**

In this study, polar extracts of *T. carbonaria* cerumen were tested with the pro-inflammatory enzyme, 5-lipoxygenase, and showed antioxidant and/or 5-lipoxygenase-
inhibitory properties against controls. The 5-LOX-inhibitory activity of these assays occurred by suppressing the catabolism of linoleic acid via the lipoxygenase pathway. Therefore, the bioactivity of this cerumen suggests a potential for preventing the lipid oxidation of linoleic acid, thus protecting the integrity of cell membranes.

While the phenolics of propolis were confirmed to exert the antioxidant and 5-LOX-inhibitory activities in vitro as reported in the literature (Kaneko et al. 2001; Kortenska et al. 2002; Sud’ína et al. 1993), a correlation between the mixed-diterpenic profile of *T. carbonaria* cerumen and the observed bioactivities could not be established due to the lack of commercial standards. For this reason, we were not able to distinguish among synergistic/antagonistic effects of cerumen components. Gallic acid and diterpenic acids could possibly exert LOX-inhibitory activity as already reported for other molecules of similar structural classes (Banskota et al. 2001; Ulusu et al. 2002; Urzúa et al. 2008). Future investigations could examine the potential of diterpenic acids of the pimaric and abietic types to inhibit 5-LOX. The mechanisms of structural interaction between cerumen constituents and biological targets remain unknown. While previous studies have examined the ability of natural products containing gallic acid (Akula and Odhay 2008) and diterpenic acids (Pferschy-Wenzig et al. 2008) to inhibit 5-LOX-mediated leukotriene B4 production, similar studies have not yet been carried out with cerumen extracts. Further cell-based assays could examine whether the polar compounds of this cerumen prevents such antiinflammatory signalling pathways.

**Molecular reactivity of cerumen polar constituents**

Propolis and cerumen have a complex matrix containing compounds that show different degrees of polarity and lipophilicity, depending on the solvent used for the extraction.
When comparing the bioactivities of the standardized extracts of propolis and cerumen, the phenolic compounds in propolis scavenged the peroxides and/or inhibited LOX activity with lower IC$_{50}$ values than cerumen polar constituents ($P < 0.001$). The activity of cerumen polars was lower similar to that of gallic acid and lower than Trolox which were both water-soluble molecules used as the positive control. As previously reported, it is likely that the presence of a lipophilic matrix in propolis extracts render the propolis phenolics molecular reactive with lipid radicals during incubation in assays, because of the improved stability of linoleic acid (Suđ'ina et al. 1993). These findings suggest that cerumen ethanolic extractions could be attempted to increase the lipophilic matrix for the active compounds to enhance the observed antioxidant and anti-LOX activities. However, our preliminary work on ethanolic extracts of T. carbonaria cerumen showed that wax compounds interfered with the chemical analyses (unreported data). Therefore, the dual solvent system (methanol:hexane) was used to select for the cerumen polars. Further work on ethanolic and hydroalcoholic extractions of cerumen will need to be optimised for medicinal preparations, while discarding the inert compounds of the beeswax.

Commercial potential of T. carbonaria cerumen

Commercial use of stingless bee cerumen in herbal products and medicinal preparations would depend on beekeepers' production and on analytical solvent extraction. In honeybee keeping, the honey production engages the bees to produce less propolis therefore beekeepers can use removable clean frames to increase propolis yields (Inoue et al. 2007). In stingless bee keeping (or meliponiculture), the cerumen can be harvested from honey pots while collecting honey for other commercial purposes. Our samples of
T. carbonaria cerumen were obtained mainly from the top honey storage section of the hives, where honey pots and thick masses of cerumen, pillars and structures are used by stingless bees to form a temporary surface while honey is ripened (T. Heard unpubl. data). Thus, with optimised sampling and protocols for solvent extraction in cerumen, substantial yields of cerumen constituents for commercial purposes could possibly be obtained.

CONCLUSION

This study identified new GC-MS fingerprints for cerumen from the stingless bee T. carbonaria in South East Queensland, Australia, and showed that this cerumen inhibited 5-LOX activity in vitro. While structural characterisation of T. carbonaria cerumen components is required, this study raises the possibility that polar extracts of this cerumen may have some therapeutic potential. Investing in production technologies for native bee cerumen could also be an economic stimulus for the Australian beekeeping industry.

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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Fig. 1 The GC-MS profiles of (a) methanolic (MeOH) extracts of *Tetragonula carbonaria* cerumen from South East Queensland, Australia and (b) a commercial tincture of *Apis mellifera* propolis from New Zealand. The *T. carbonaria* MeOH extracts were fractionated in (c) diethyl ether (Et₂O) and (d) ethyl-O-acetate (EtOAc) solutions to improve the separation of polar compounds and simplify the interpretation of the eluted peaks. Differences between the chromatographic profiles of polar extracts of *T. carbonaria* cerumen and the tincture of *A. mellifera* propolis are evident. The compounds gallic acid (14), pimaric acid (20) and its isomer (29) were detected only in the *T. carbonaria* cerumen from South East Queensland and are suggested as chemical markers for this cerumen. Constituents of *A. mellifera* propolis (b): cinnamic acid (2), monosaccharide (6), gluconic acid (7), fructose (8), β-glucose (10), p-coumaric acid (12), and monosaccharide (15) were also found in cerumen of *T. carbonaria*; however, p-hydroxybenzoic acid (35), hydroxybenzoic ester (36), monosaccharide (37), monosaccharide (38), ferulic acid (39), caffeic acid (40), pentenyl ester iso-ferulic acid (41), pentenyl ester caffeic acid (42), pentenyl ester caffeic acid isomer (43), pinostrobin (44), pinocembrin (45), sterol (46), sterol (47), sterol (48), cinnamic acid ester (49), dihydroxy-2-methyl-anthroquinone (50), galangin (51) were not found in *T. carbonaria* cerumen.

Fig. 2 Concentration-response curves for inhibition of 5-lipoxygenase (5-LOX) activity by (a) extracts of *Tetragonula carbonaria* cerumen from South East Queensland, Australia (IC₅₀ = 19.97 ± 2.78 µg/mL; n=4) and *Apis mellifera* propolis from New Zealand.
Zealand (IC$_{50}$ = 5.90 ± 0.62 µg/mL; n=4); and (b) standards Trolox (IC$_{50}$ = 12.78 ± 1.82 µg/mL; n=3) and gallic acid (IC$_{50}$ = 5.62 ± 0.35 µg/mL; n=3). The percent inhibition (mean ± SEM) was calculated after plotting absorbance at 598 nm against the log concentration of the test solutions. Absorbance of vehicle (70% ethyl alcohol) was subtracted from all data.
Table 1 Molecular ions from the mass spectra of TMS-constituents of polar extracts of *Tetragonula carbonaria* cerumen.

<table>
<thead>
<tr>
<th>Compounds&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TMS derivatives</th>
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<tbody>
<tr>
<td><strong>#</strong></td>
<td><strong>Chemical name</strong></td>
</tr>
<tr>
<td>2</td>
<td>Cinnamic acid</td>
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<td>3</td>
<td>Phenolic acid</td>
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<tr>
<td>34</td>
<td>Sterol</td>
</tr>
</tbody>
</table>

<sup>a</sup> = individual constituents were tentatively identified according to patterns of fragmentation and molecular ions; TMS = trimethylsilylether; MW = mass weight; [M] = molecular ion mass; [M – 15] = loss of methyl group (CH<sub>3</sub>); [M – 89] = loss of trimethylsilyloxy group [OSi(CH<sub>3</sub>)<sub>3</sub>]; [m/z] = other characteristic mass ions; nd = not defined; BP = base peak ions showing relative abundance ratio of 100%
**Table 2** Constituents of polar extracts of *Tetragonula carbonaria* cerumen.

<table>
<thead>
<tr>
<th>Peak Label</th>
<th>R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>Compound</th>
<th>Content&lt;sup&gt;a&lt;/sup&gt; (%)&lt;sup&gt;, w/w&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.60</td>
<td>acetophenone</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>5.46</td>
<td>cinnamic acid</td>
<td>0.20 ± 0.00</td>
</tr>
<tr>
<td>3</td>
<td>5.56</td>
<td>phenolic acid</td>
<td>0.90 ± 0.09</td>
</tr>
<tr>
<td>4</td>
<td>6.33</td>
<td>unknown compound</td>
<td>2.54 ± 0.32</td>
</tr>
<tr>
<td>5</td>
<td>8.27</td>
<td>monosaccharide</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>6</td>
<td>9.61</td>
<td>monosaccharide</td>
<td>1.79 ± 0.45</td>
</tr>
<tr>
<td>7</td>
<td>9.73</td>
<td>gluconic acid</td>
<td>0.91 ± 0.32</td>
</tr>
<tr>
<td>8</td>
<td>9.83</td>
<td>fructose</td>
<td>2.18 ± 1.40</td>
</tr>
<tr>
<td>9</td>
<td>10.48</td>
<td>alpha glucose</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>10.99</td>
<td>beta glucose</td>
<td>3.52 ± 0.81</td>
</tr>
<tr>
<td>11</td>
<td>11.17</td>
<td>monosaccharide</td>
<td>0.91 ± 0.09</td>
</tr>
<tr>
<td>12</td>
<td>11.32</td>
<td>p-coumaric acid</td>
<td>0.44 ± 0.00</td>
</tr>
<tr>
<td>13</td>
<td>11.65</td>
<td>ether glucitol</td>
<td>2.55 ± 0.68</td>
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<tr>
<td>14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.80</td>
<td>gallic acid</td>
<td>5.79 ± 0.81</td>
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<tr>
<td>15</td>
<td>12.39</td>
<td>monosaccharide</td>
<td>2.88 ± 0.38</td>
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<tr>
<td>16</td>
<td>12.62</td>
<td>unknown</td>
<td>0.77 ± 0.18</td>
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<tr>
<td>17</td>
<td>16.05</td>
<td>sterol</td>
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</tr>
<tr>
<td>18</td>
<td>16.52</td>
<td>pimaric acid isomer</td>
<td>0.87 ± 0.23</td>
</tr>
<tr>
<td>19</td>
<td>16.64</td>
<td>pimaric acid isomer</td>
<td>1.22 ± 0.43</td>
</tr>
<tr>
<td>20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.03</td>
<td>pimaric acid</td>
<td>6.31 ± 0.97</td>
</tr>
<tr>
<td>#</td>
<td>Rt</td>
<td>Component</td>
<td>Value (± Range)</td>
</tr>
<tr>
<td>---</td>
<td>-----</td>
<td>----------------------------</td>
<td>---------------------</td>
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<tr>
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<td>17.18</td>
<td>sterol</td>
<td>5.25 ± 0.41</td>
</tr>
<tr>
<td>22</td>
<td>17.46</td>
<td>abietic acid</td>
<td>0.37 ± 0.14</td>
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<tr>
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<tr>
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<td>17.94</td>
<td>dehydroabietic acid</td>
<td>0.54 ± 0.05</td>
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<tr>
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<td>2.69 ± 1.15</td>
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<tr>
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<td>18.50</td>
<td>sterol</td>
<td>7.34 ± 0.84</td>
</tr>
<tr>
<td>27</td>
<td>19.27</td>
<td>sterol</td>
<td>1.96 ± 0.33</td>
</tr>
<tr>
<td>28</td>
<td>19.96</td>
<td>sterol</td>
<td>14.27 ± 1.50</td>
</tr>
<tr>
<td>29</td>
<td>20.52</td>
<td>pimaric acid isomer</td>
<td>12.23 ± 3.03</td>
</tr>
<tr>
<td>30</td>
<td>20.89</td>
<td>unknown</td>
<td>2.64 ± 0.39</td>
</tr>
<tr>
<td>31</td>
<td>26.69</td>
<td>unknown isomer (of 30)</td>
<td>4.80 ± 1.24</td>
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<tr>
<td>32</td>
<td>29.20</td>
<td>beta-amyrin</td>
<td>0.99 ± 0.37</td>
</tr>
<tr>
<td>33</td>
<td>29.62</td>
<td>beta-amyrin isomer</td>
<td>0.90 ± 0.34</td>
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<tr>
<td>34</td>
<td>29.77</td>
<td>sterol</td>
<td>2.37 ± 0.85</td>
</tr>
</tbody>
</table>

2. *a* = mean ± half range of two independent experiments, using the internal standard method with hexadecane; *b* = compounds proposed as chemical markers; R<sub>t</sub> = retention time of individual components eluting from the column.
Figure

(a)

(b)
Supplementary Material

Click here to download Supplementary Material: Massaro-2010-ESM-REV 1.doc