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Quality assessment and chemical diversity of Australian propolis from *Tetragonula carbonaria* and *Tetragonula hockingsi* stingless bees

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Stingless bee propolis has gained global attention as a novel source of bioactive compounds with potential applications in food and medicine to promote human health. However, research on Australian stingless bee propolis remains limited as much of propolis is discarded by Australian beekeepers due to a lack of understanding its value. This study aimed to assess and compare the quality and chemical diversity of propolis from two Australian stingless bee species *Tetragonula carbonaria* and *Tetragonula hockingsi* across Queensland and New South Wales. Chemical assays revealed that *T. hockingsi* propolis had significantly higher total phenolic and flavonoid contents than *T. carbonaria*. Australian stingless bee propolis also showed high phenolic but moderate flavonoid content compared to international propolis sources. The phenolic content displayed geographical dependence, decreasing from northern to southern regions. The analyses of uHPLC-QTOF MS and ^{1}H NMR profiling identified nine distinct propolis types, showcasing diverse chemical composition with the presence of flavan-3-ol, dihydroflavonol, flavanone, tannin, lignan, phenolic acid, cinnamic acid glycoside, chalcone, and alkyl-phenylketone compounds. These findings provide a foundation for future research into the value and therapeutic potential of Australian stingless bee propolis products, contributing to the development of the Australian propolis industry.

Keywords Australian propolis, Stingless bees, *Tetragonula carbonaria*, *Tetragonula hockingsi*, Quality assessment, Chemical diversity

Bees are the most important group of insect pollinators and approximately 20,000 species have been described[1]. Stingless bees, belonging to the tribe Meliponini within the family Apidae, are closely related to honeybees, orchid bees, and bumblebees, and represent the largest monophyletic group of eusocial bees with over 60 genera and 600 species[2]. Stingless bees are widely distributed in the warm, humid environments of tropical and subtropical regions across the Americas, Australia, Asia, and Africa[3]. The keeping of stingless bees (Meliponiculture) plays a significant role in environmental conservation by pollinating native plant species, subsequently helping reduce environmental degradation and deforestation[4]. Due to their atrophied stingers, stingless bees often rely on propolis, a resinous material made from various plant resins and beeswax, to physically and chemically deter intruders[5]. Additionally, they use propolis as the primary material for nest construction and to help maintain hive homeostasis, including temperature and humidity[6]. As a result, stingless bees normally produce propolis in greater quantity compared to honeybee species[7].

The use of propolis as a therapeutic agent for humans has been developed independently by various ancient cultures over millennia with Ancient Egyptians, Greeks, Persians, and Romans reportedly using propolis to treat ulcers and wounds[2]. Similarly, indigenous populations in India developed folk medicine that included stingless bee propolis for the treatment of ailments[8]. Modern research has confirmed the therapeutic benefits of stingless bee propolis, highlighting its antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, and anticancer properties[9]. As stingless bees collect resins and materials from various flora surrounding their hives, the chemical

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composition of their propolis varies depending on the local environment[10]. In general, its biological activities are largely attributed to the presence of small molecules belonging to polyphenol and terpenoid chemical classes[11]. However, research on stingless bee propolis is still in its infancy compared to that of honeybee propolis.

Most studies on stingless bee propolis have initially focused on American bee species. Since 2010, chemical composition and biological activity of propolis from 24 distinct bee species across eight genera, primarily in Brazil, have been investigated. These species include *Frieseomelitta longipes*[12], *Melipona beecheii*[13], *Melipona fasciculata*[14], *Melipona interrupta*[15], *Melipona marginata*[16], *Melipona mondury*[17], *Melipona orbignyi*[18], *Melipona quadrifasciata*[19,20], *Melipona quadrifasciata anthidioides*[21,22], *Melipona scutellaris*[23], *Melipona seminigra*[24], *Melipona subnitida*[25], *Nanotrigona testacularis*[24,26], *Plebeia droryana*[27], *Plebeia remota*[28], *Scaptotrigona bipunctata*[29], *Scaptotrigona depilis*[22], *Scaptotrigona jujuyensis*[30], *Scaptotrigona polysticta*[31], *Scaptotrigona postica*[32], *Tetragona clavipes*[33], *Tetragonisca angustula*[20], *Tetragonisca fiebrigi*[34], *Trigona spinipes*[35]. More recently, research on Asian stingless bee propolis has significantly increased, with studies conducted in Brunei, India, Indonesia, Malaysia, the Philippines, Thailand, and Vietnam on propolis from 21 bee species belonging to nine genera including *Geniotrigona thoracica*[36,37], *Heterotrigona itama*[36–38], *Lepidotrigona terminate*[39], *Lepidotrigona ventralis*[39], *Lisotrigona cacciae*[40], *Lisotrigona furva*[41], *Tetragonula biroi*[42], *Tetragonula fuscibasis*[43], *Tetragonula fuscobalteata*[43], *Tetragonula iridipennis*[44], *Tetragonula laeviceps*[45,46], *Tetragonula pagdeni*[39], *Tetragonula sapiens*[47], *Tetrigona apicalis*[48], *Tetrigona binghami*[37], *Tetrigona melanoleuca*[46], *Timia apicalis*[43], *Trigona incisa*[49], *Trigona minor*[50], *Trigona thoracica*[51], and *Wallacetrigona incisa* [52]. Approximately 87% of studies on the bioactivity of stingless bee propolis are in vitro, with only a limited number (13%) focusing on in vivo research[2].

In Australia there are 11 species of stingless bees which are part of the *Tetragonula* and *Austroplebia* genera[53]. These species mainly inhabit urban and forested areas of the east coast including Queensland and some northern regions of New South Wales, as well as northern areas of Western Australia and the Northern Territory[53]. Previous studies on Australian propolis produced by *T. carbonaria* bees demonstrated its therapeutic potential, including antibacterial[54], antioxidant[55], anti-inflammatory[55], and vasomodulation[56] activities. Stingless bee propolis from Australia is expected to contain uncommon bioactive compounds, since 84% of the flora is unique to the country[57]. The identification of tetragocarbone B (**1**)[58] and tomentosenol A (**2**)[59] (Fig. 1) in *T. carbonaria* propolis highlights the uniqueness of Australian stingless bee propolis. These compounds are trimethylated monomeric phloroglucinol derivatives that have not been found in propolis from other parts of the world. Tetragocarbone B has shown to possess an anti-inflammatory property[60] while tomentosenol A exhibited an anti-fibrotic activity[59].

Two species, *Tetragonula carbonaria* and *Tetragonula hockingsi*, play crucial roles in pollinating Australian native flora and horticultural crops[61]. These bees have recently gained interest as alternative pollinators in Queensland and part of New South Wales, particularly for crops such as macadamia, mango, lychee, avocado, and strawberry, following a *Varroa* mite outbreak in 2022[62]. Their predominant propagation for pollination services results in the massive production of propolis. However, much of the propolis produced by these bees is often discarded by Australian beekeepers due to a lack of understanding its value. This study aimed to assess the quality and chemical diversity of Australian stingless bee propolis produced by *T. carbonaria* and *T. hockingsi*, comparing both species and collection regions across two eastern states of Australia, Queensland and New South Wales (Fig. 2). Propolis extraction yield as well as total phenolic and flavonoid contents, which are key chemical quality control parameters of propolis[63,64], were assessed. The chemical diversity of stingless bee propolis was analysed through their ^{1}H nuclear magnetic resonance (NMR) spectra, high-performance liquid chromatography with diode array detection (HPLC–DAD), and ultra high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (uHPLC-QTOF MS) profiling. The findings of this study will establish a foundation for further investigations into the chemical composition and therapeutic properties of Australian *T. carbonaria* and *T. hockingsi* propolis, contributing to the knowledge of Australian propolis and the sustainability of both domestic and global propolis industries.

Results and discussion

Propolis quality assessment

The pure resin yield from 170 propolis samples exhibited considerable variability, with a mean percentage yield of 51% (Table S1 in Supplementary Information), ranging from a minimum of 15% to a maximum of 86%

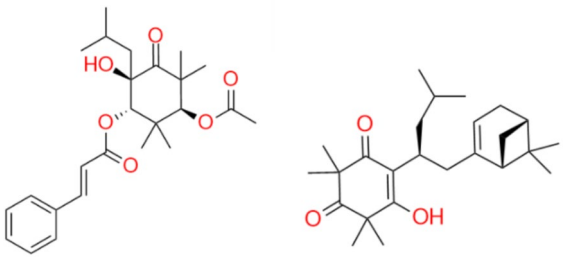
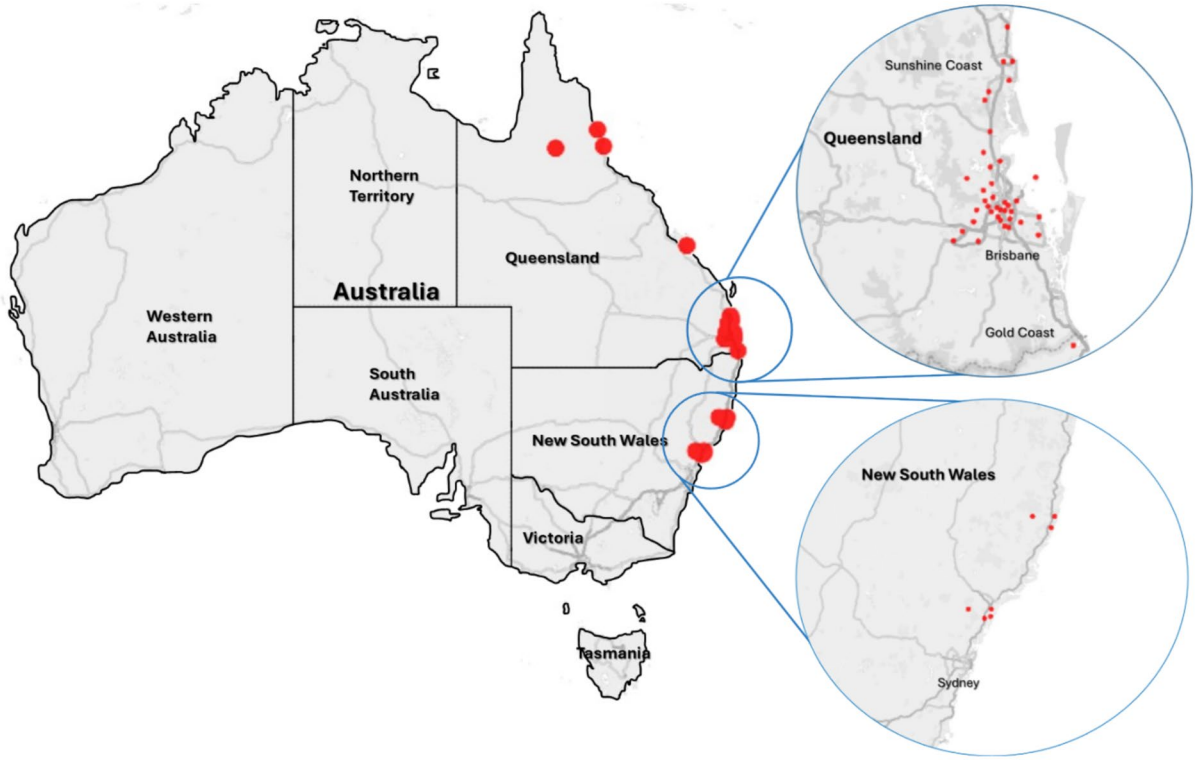


Fig. 1. Proposed structure of tetragocarbone B **(1)** and tomentosenol A **(2)** identified from *T. carbonaria* propolis. Created using ChemDraw and BioRender.com adapting previous compound illustrations[58,59].



State	Collection region	Number of *T. hockingsi* samples	Number of *T. carbonaria* samples
QLD	Far North QLD	3	0
QLD	Central QLD	9	9
QLD	Wide Bay Burnett	3	6
QLD	Sunshine Coast	4	11
QLD	Brisbane City	29	85
NSW	Sydney	0	11

Fig. 2. Map of Australia with stingless bee propolis collection regions. The map was created using Microsoft Excel and BioRender (https://www.biorender.com/).

(Figure S1A in Supplementary Information). Comparison of extraction yield between bee species revealed no significant differences between the propolis produced by *T. carbonaria* and *T. hockingsi* (Fig. 3A). However, the stingless bee propolis demonstrated double the extraction yield compared to that produced by honeybees (*Apis mellifera*) in our previous study[65].

The total phenolic content (TPC) of Australian stingless bee propolis extracts in this study ranged from 34.6 to 314.5 mg gallic acid equivalent (GAE)/g dry extract (Figure S1B in Supplementary Information). The results indicated that *T. hockingsi* propolis had a greater TPC than that of *T. carbonaria* propolis, with averaged values of 195.4 ± 2.2 mg GAE/g dry extract compared to 158.4 ± 1.8 mg GAE/g dry extract (Fig. 3B). Unpaired two-tailed t-tests indicated a statistically significant difference (*** $P \leq 0.001$) in TPC between propolis from *T. carbonaria* and *T. hockingsi*. Both types of propolis exhibited TPC levels approximately two to three times higher than that of *A. mellifera* propolis with an averaged TPC of 68 mg GAE/g dry extract (Table S1 in Supplementary Information).

In terms of total flavonoid content (TFC), Australian stingless bee propolis ranged from 2.1 to 27.1 mg quercetin equivalent (QE)/g dry extract (Figure S1C in Supplementary Information). A comparison of the TFC between the two Australian stingless bee species (Fig. 3C) revealed that *T. hockingsi* samples had a significantly higher TFC (14.6 ± 0.2 mg QE/g dry extract) compared to *T. carbonaria* samples (11.5 ± 0.2 mg QE/g dry extract). Overall, the TFC of propolis produced by these two stingless bee species is approximately two times lower than that of *A. mellifera* propolis (Table S1 in Supplementary Information).

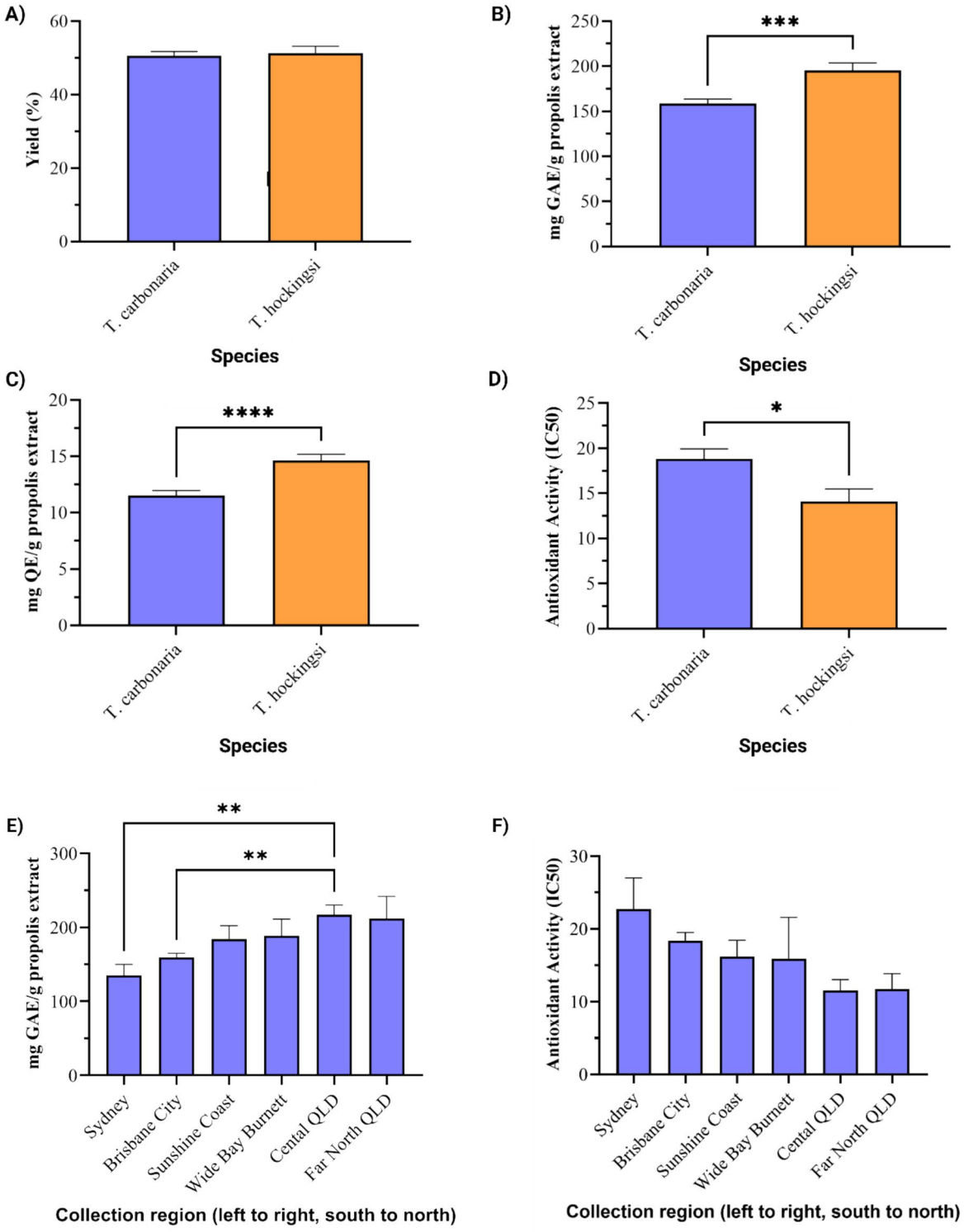


Fig. 3. Quality parameters of Australian stingless bee propolis. (**A**) Species comparison of extraction yield, (**B**) Species comparison of total phenolic content, (**C**) Species comparison of total flavonoid content, (**D**) Species comparison of antioxidant activity, (**E**) Comparison of total phenolic content based on collection region, (**F**) Comparison of antioxidant activity based on collection region. Asterisks indicate levels of statistical significance (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ **** $P \leq 0.0001$). Figure created using Biorender.com.

Australian propolis generally contains higher levels of phenolic and flavonoid contents than propolis from Indonesia, Thailand, and the Philippines (Table 1). The comparison of these parameters between Australian propolis and those from Argentina, Brazil and Malaysia is challenging due to differences in reference units (mg GAE per gram of extract *versus* per gram of raw material). However, it can be seen that most propolis produced by stingless bees exhibits TPC values that are over ten times greater than TFC. Notably, the ratio of TPC to TFC is 58 for *Tetragonula biroi* propolis in the Philippines and 39 for *Melipona quadrifasciata* propolis in Brazil. In term of Australian stingless bee propolis, this ratio is relatively comparable between two species, at approximately 13–14. Overall, these data suggest that stingless bees likely prefer foraging for plant resins which are rich in non-flavonoid compounds.

Testing propolis extracts at a concentration of 100 µg/mL revealed that *T. hockingsi* propolis exhibited higher antioxidant activity, with a free radical scavenging property ranging from 69 to 100% (Figure S1D in Supplementary Information). In contrast, *T. carbonaria* samples showed a broader range of scavenging activity, from 39 to 100% (Figure S1D in Supplementary Information). Samples having over 50% scavenging activity at 100 µg/mL were further tested to determine their IC_{50} values (with lower IC_{50} value indicating better antioxidant activity). Unpaired two-tailed t-tests indicated a statistically significant difference (* $P \leq 0.05$) in IC_{50} between propolis from *T. carbonaria* and *T. hockingsi*. The top 10% of both *T. hockingsi* and *T. carbonaria* propolis demonstrated IC_{50} values for free radical scavenging ranging from 4.9 to 7.8 µg/mL which are relatively comparable to the known antioxidant compound, vitamin C (ascorbic acid) having its IC_{50} value of 5.0 µg/mL (Table S2 in Supplementary Information). On average, *T. hockingsi* propolis demonstrated more potent antioxidant activity as evidence by its lower IC_{50} values than *T. carbonaria* propolis (14.1 µg/mL *versus* 18.8 µg/mL) (Fig. 3D). Ecologically, *T. hockingsi* bees are primarily found in the more tropical regions of northern Queensland, while *T. carbonaria* bees distribute in subtropical areas[67]. The results demonstrate that geographical distribution of these two species highly influences their total phenolic content and antioxidant activity.

Data analysis between TPC and collection region showed two comparisons of significance (** $P \leq 0.001$) between Central QLD, Brisbane City and Sydney (Fig. 3E). Lower antioxidant IC_{50} values (Fig. 3F) were found to correspond to further northern collections and positively correlate with the increase in TPC (Fig. 3E). These results indicate that stingless bee propolis collected in northern east-coast Australia has significantly higher antioxidant activity than samples collected further south. Similar trends were observed when analysing datasets for each bee species (Figure S2 in Supplementary Information). These findings suggest that propolis from warmer regions contains higher phenolic compounds and antioxidant activity compared to those from cooler areas. This phenomenon may be attributed to the fact that plants under thermal stress tend to accumulate more phenolic compounds[68,69]. Therefore, propolis produced by *T. hockingsi* bees exhibits higher TPC and superior antioxidant property compared to that of *T. carbonaria* bees.

Propolis chemical diversity assessment

Chemical composition of pure propolis has been known to consist of mainly polyphenolic and terpenoid compound classes[70], which can be confirmed by ^{1}H NMR profiles. Polyphenolic compounds exhibit aromatic protons in the range of 6.5 to 7.8 ppm, while terpenoids show saturated and olefinic methine and methylene protons in two separated regions from 1.0 to 2.5 ppm (saturated signals) and from 4.5 to 6.0 ppm (olefinic signals)[65]. The ^{1}H NMR analysis (Fig. 4) revealed that approximately 80% of the tested propolis samples were high in polyphenols, and 18% were rich in terpenoids. Additionally, about 2% of the samples contained a significantly high level of sugars which were indicated by ^{1}H signals between 3 and 5 ppm[65], suggesting potential contamination with honey when propolis was harvested.

Origin	Species	TPC (mg GAE/g dry extract)	TFC (mg QE/g dry extract)	TPC/TFC
Argentina	*Scaptotrigona jujuyensis* [30]	2.3 ± 0.5 [a]	0.8 ± 0.2 [b]	2.9
	Tetragonisca fiebrigi [30]	1.2 ± 0.2 [a]	0.8 ± 0.4 [b]	1.5
Brazil	*Melipona quadrifasciata* [20]	3.9 ± 0.3 [a]	0.1 ± 0.0[b]	39
	Tetragonisca angustula [20]	1.3 ± 0.2 [a]	0.2 ± 0.0[b]	6.5
Indonesia	*Heterotrigona itama*[66]	28.7 ± 10.7	2.6 ± 4.1	11
	Tetragonula biroi [66]	22.3 ± 0.5	3.4 ± 1.1	6.6
	Tetragonula laevicep [66]	15.3 ± 0.1	1.5 ± 0.3	10.2
Malaysia	*Trigona thoracica* [51]	1.8 ± 0.0 [a]	0.08 ± 0.0 [b]	22.5
Philippines	*Tetragonula biroi* [42]	308.0 ± 119.6	5.3 ± 1.4	58.1
Thailand	*Lepidotrigona terminate* [39]	21.6 ± 1.0	– [c]	– [c]
	Lepidotrigona ventralis [39]	31.5 ± 2.5	– [c]	– [c]
	Tetragonula pagdeni [39]	128.3 ± 7.2	– [c]	– [c]
Australia	*Tetragonula carbonaria*	158.4 ± 1.8	11.5 ± 0.2	13.8
	Tetragonula hockingsi	195.4 ± 2.2	14.6 ± 0.2	13.4

Table 1. A comparison of total phenolic and flavonoid contents in Australian stingless bee propolis *versus* propolis from stingless bees in other countries. [a] mg GAE per gram of raw propolis. [b] mg QE per gram of raw propolis. [c] Not determined. ± Standard deviation (S.D).

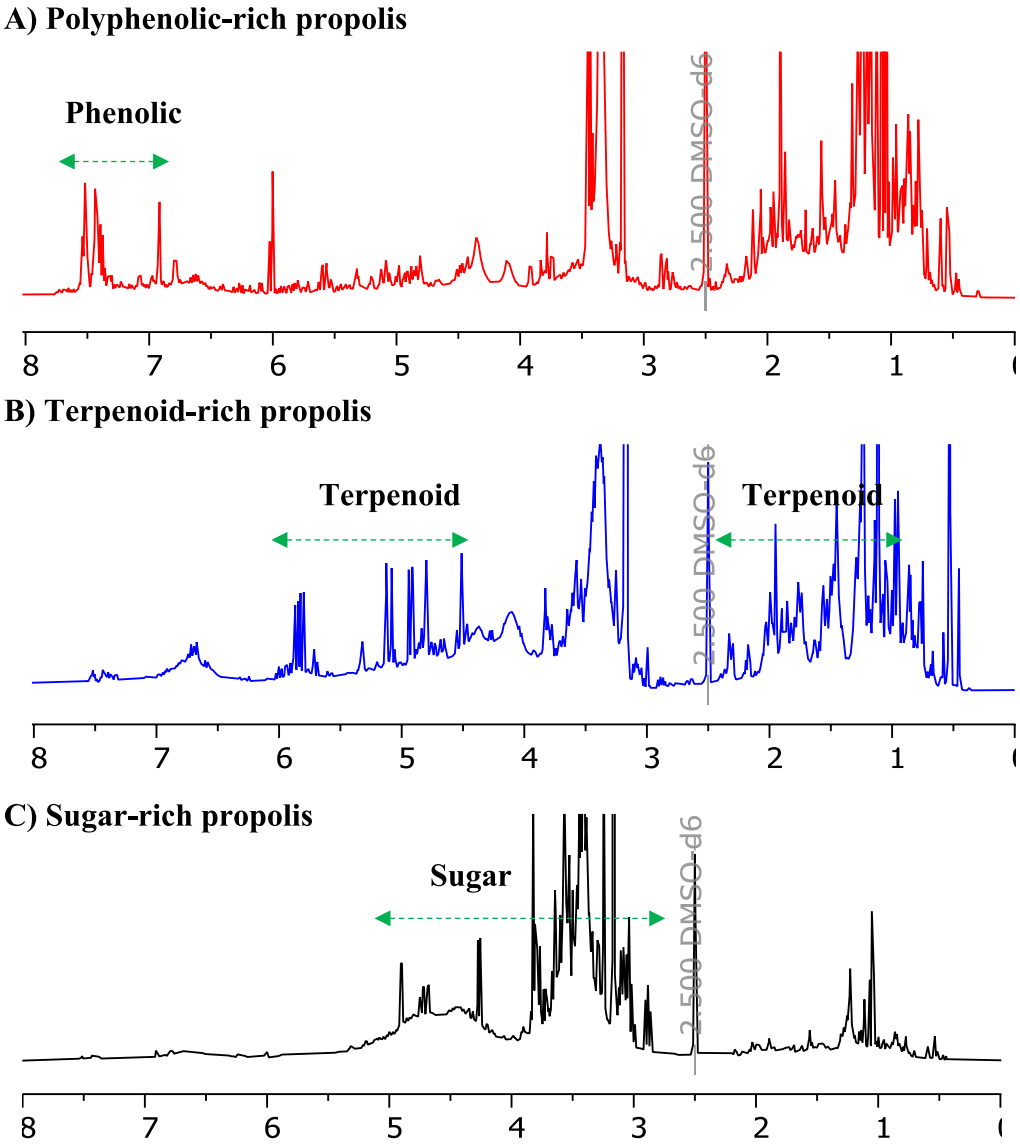


Fig. 4. ^{1}H NMR profiles of Australian stingless bee propolis representing (**A**) polyphenolic-rich propolis, (**B**) terpenoid-rich propolis, and (**C**) sugar-rich propolis.

The principal component analysis (PCA) for species comparison provides a graphical representation of the NMR peaks between the *T. carbonaria* and *T. hockingsi* propolis groups. The PCA for species comparison (Fig. 5A) revealed some differences between *T. carbonaria* and *T. hockingsi* propolis, with a maximum difference between species of over 30% composition of PC1 and a maximum difference between species of over 10% composition of PC2 for samples within the Hotelling's T^2 95% confidence ellipses. Additionally, the sparse partial least-squares discriminant analysis (sPLS-DA) for species comparison (Fig. 5B) further supported these findings by demonstrating a better separation of Hotelling's T^2 ellipses between *T. carbonaria* and *T. hockingsi* samples. This indicates a distinct difference in chemical composition of these two propolis groups.

The PCA for geographical location (Fig. 5C) showed that most samples overlapped, except for those collected from Sydney which had a higher PC2 (minimum of 10%) than samples from all other locations within their appropriate Hotelling's T^2 95% confidence ellipses. The sPLS-DA (Fig. 5D) indicated that samples from Brisbane showed consistent low variation in chemical composition and shared some similarities with other regions. The sPLS-DA results suggested that there was not much distinct propolis present in Sunshine Coast and Wide Bay Burnett compared to Brisbane. In contrast, Central QLD, Far North QLD, and Sydney displayed a large diversity in their compositions. Propolis samples from Sydney showed the highest variation with a range of 20% on component 2 with 95% confidence provided by Hotelling's T2 ellipse. The sPLS-DA also indicated some similarities between Central and Far North QLD propolis as demonstrated by overlapping Hotelling's T2 ellipse. The differences in chemical composition of propolis in Central and Far North QLD compared to Sydney are likely attributable to variations in flora, with Central and Far North QLD being more tropical while Sydney is more subtropical.

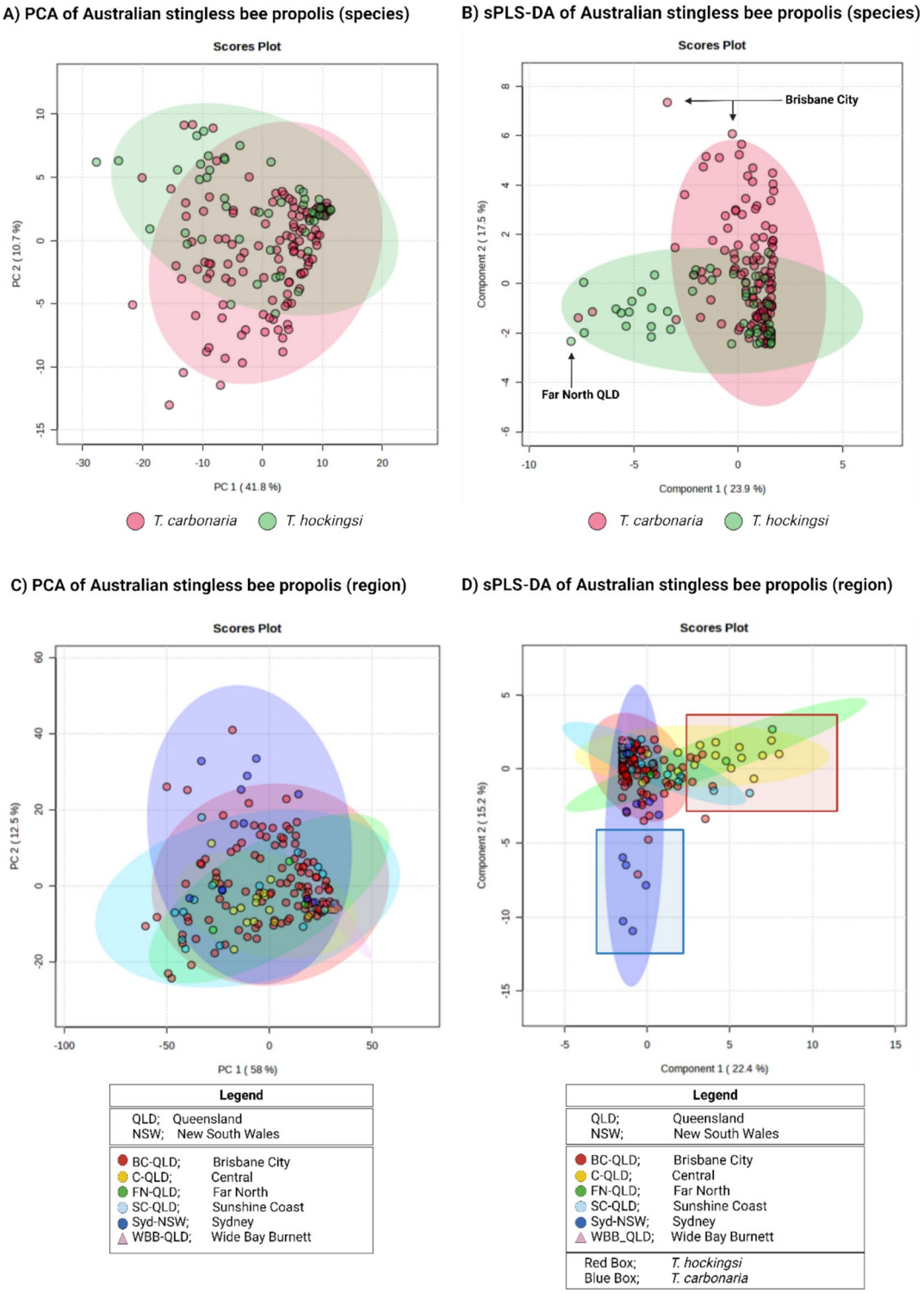


Fig. 5. 2D score PCA **(A)** and sPLS-DA **(B)** plots of Australian stingless bee propolis based on bee species; 2D score PCA **(C)** and sPLS-DA **(D)** plots of Australian stingless bee propolis based on collection region. Samples in the Hotelling's T2 ellipses show a 95% confidence level. Samples outside any Hotelling's T2 ellipses are labelled with collection region. Created using BioRender.com with chemometric analysis from MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/).

Propolis classification and compound identification

Through the analysis of HPLC–DAD profiles, propolis samples in this study were classified into nine groups (Table 2). A representative chromatogram for each group underwent comprehensive UV and MS/MS analyses to identify their major components (Table 3). Of the 26 compounds detected, 17 compounds were successfully identified, and their tentative chemical structures are shown in Fig. 6. Twelve of them belong to the flavonoid family while the remaining five are classified as non-flavonoid phenolic compounds. The identified flavonoid compounds are classified into four classes: flavan-3-ol (one compound), dihydroflavonol (three compounds), flavanone (six compounds) and chalcone (two compounds). The identified non-flavonoid phenolic compounds belong to five classes: tannin, lignan, phenolic acid, cinnamic acid glycoside, and alkyl-phenylketone (one compound for each class). The identification of diverse non-flavonoid phenolic compound classes as major chemical components in stingless bee propolis further supports the high level of total phenolic content.

Among the 17 compounds identified, six compounds including padmatin (**E**), pinoresinol (**F**), 1-O-cinnamoyl-6-O-p-coumaroyl-β-D-glucopyranose (**I**), 3-methyl-1-(2,4,6-trihydroxyphenyl)-1-butanone

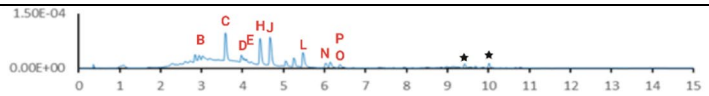
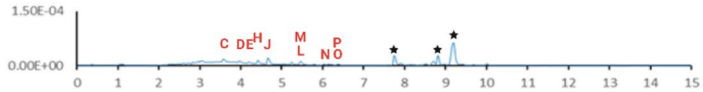
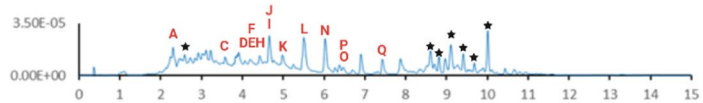
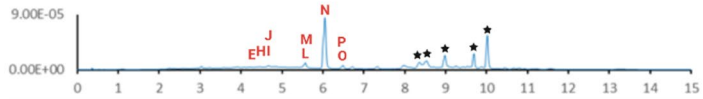
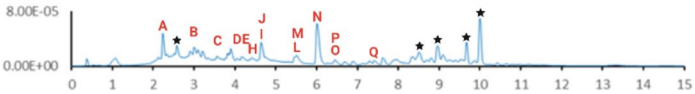
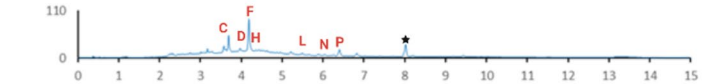
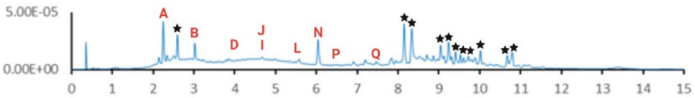
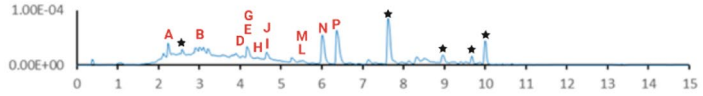
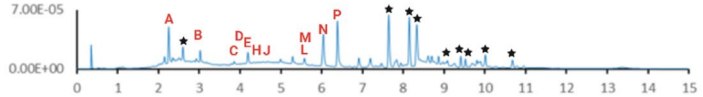
Propolis Type	Bee species (Region)	HPLC-DAD profile (λ = 280 nm)
1	*T. hockingsi* (Far North QLD)	
2	*T. hockingsi* (Far North QLD)	
3	*T. carbonaria* *T. hockingsi* (Central QLD)	
4	*T. carbonaria* *T. hockingsi* (Brisbane, SC, WBB)	
5	*T. carbonaria* *T. hockingsi* (Brisbane, SC, WBB)	
6	*T. carbonaria* (Brisbane)	
7	*T. carbonaria* (Brisbane)	
8	*T. carbonaria* (Brisbane)	
9	*T. carbonaria* (Sydney)	

Table 2. HPLC–DAD profiles of nine Australian stingless bee propolis types with major compounds labelled with letters for identified compounds and stars for unidentified compounds.
QLD: Queensland, SC: Sunshine Coast, WBB: Wide Bay Burnett, stars indicate compounds that were not determined (n.d).

	Retention time	Experimental mass (+) m/z (-) m/z	Theoretical mass (+) m/z (-) m/z	Mass error (ppm) (+) m/z (-) m/z	Predicted formula	Fragments	Compound class	Propolis type [a]	Name
1	2.35	291.0869 289.0725	291.0863 289.0718	2.1 2.4	$C_{15}H_{14}O_6$	139.0391, 123.0441 125.0245, 123.0453, 109.0294	Flavanol	**3**,**5**,**7**,**8**,**9**	Catechin (**A**)
2	2.67	471.0201 469.0050	471.0194 469.0049	1.5 0.2	$C_{21}H_{10}O_{13}$	- -	n.d	**5**,**7**,**8**,**9**	n.d
3	3.07	303.0137 300.9993	303.0135 300.9990	0.7 1.0	$C_{14}H_6O_8$	285.0033, 275.0187, 257.0087 245.0091	Tannin	1,**5**,**7**,8,**9**	Ellagic acid (**B**) (Gallogen)
4	3.66	289.0715 287.0571	289.0707 287.0561	2.8 3.5	$C_{15}H_{12}O_6$	153.0183, 149.0235, 107.0493 259.0608, 177.0555, 125.0245	Dihydroflavonol	**1**,2,3,5,**6**,9	Katuranin (**C**) (Aromadendrin)
5	4.04	289.0712 287.0564	289.0707 287.0561	1.7 1.0	$C_{15}H_{12}O_6$	163.0390, 153.0182 151.0036, 135.0452, 107.0137	Flavanone	1,2,**3**,**5**,6,7,8,9	Eriodictyol (**D**)
6	4.28	319.0816 317.0671	319.0812 317.0667	1.3 1.3	$C_{16}H_{14}O_7$	245.0812, 167.0344, 163.0394 152.0114, 125.0242	Dihydroflavonol	1,2,3,4,5,**8**	Padmatin (**E**) (Taxifolin-7-methyl ether)
7	4.24	359.1489 -	359.1489 -	0.0 -	$C_{20}H_{22}O_6$	270.0886, 137.0599 -	Lignan	3,**6**	Pinoresinol (**F**)
8	4.30	149.0597 -	149.0597 -	0.0 -	$C_9H_8O_2$	131.0492, 103.0540 -	Phenylpropanoid	8	Cinnamic Acid (**G**)
9	4.52	273.0768 271.0612	273.0757 271.0612	4.0 0.0	$C_{15}H_{12}O_5$	153.0190, 147.0446 151.0037, 119.0505, 107.0137	Flavanone	**1**,2,3,4,5,6,8,9	Naringenin (**H**)
10	4.72	- 455.1358	- 455.1348	- 2.2	$C_{24}H_{24}O_9$	161.0609, 163.0402, 145.0300 145.0300	Cinnamic acid glycoside	**3**,4,**5**,7,8	1-O-Cinnamoyl-6-O- P-Coumaroyl-B-D- Glucopyranose (**I**)
11	4.79	303.0870 -	303.0863 -	2.3 -	$C_{16}H_{14}O_6$	229.0858, 167.0336, 163.0385 -	Dihydroflavonol	**1**,2,**3**,4,**5**,7,8,9	Folerogenin (**J**) (Aromadendrin-7-methyl ether)
12	5.08	211.0968 209.0828	211.0965 209.0819	1.4 4.3	$C_{11}H_{14}O_4$	155.0340, 151.0392, 123.0441 165.0919, 125.0244, 81.0344	Alkyl-phenylketone	3	3-Methyl-1-(2,4,6- Trihydroxyphenyl)-1- Butanone (**K**)
13	5.61	287.0922 285.0778	287.0914 285.0768	2.8 3.5	$C_{16}H_{14}O_5$	167.0342, 147.0442, 119.0482 165.0194, 119.0504	Flavanone	1,2,**3**,4,5,6,7,8,9	Sakuranetin (**L**)
14	5.53	261.1132 259.0987	261.1121 259.0976	4.2 4.2	$C_{15}H_{16}O_4$	- -	n.d	2	n.d
15	5.676	257.0812 255.0661	257.0808 255.0663	1.6 -0.8	$C_{15}H_{12}O_4$	205.0494, 153.0184, 131.0494 213.0555, 171.0450, 151.0039	Flavanone	2,4,5,8,9	Pinocembrin (**M**)
16	6.16	271.0972 269.0830	271.0965 269.0819	2.6 4.1	$C_{16}H_{14}O_4$	167.0340, 131.0492 165.0197, 121.0295	5-Hydroxyflavonoid	1,2,**3**,**4**,**5**,6,**7**,**8**,**9**	Cryptostrobin (**N**)
17	6.51	315.1232 313.1082	315.1227 313.1081	1.6 0.3	$C_{18}H_{18}O_5$	195.0658, 147.0443 -	Chalcone (Flavonoid)	1,2,3,4,5	4-Hydroxy-2',4',6'- trimethoxychalcone (**O**)
18	6.55	285.1129 283.0984	285.1121 283.0976	2.8 2.8	$C_{17}H_{16}O_4$	181.0497, 131.0492 241.0873, 179.0350, 135.0453	Flavanone	1,2,3,4,5,7,**8**,**9**	Demethoxymatteucinol (**P**)
19	7.03	287.1284 285.1145	287.1278 285.1132	2.1 4.6	$C_{17}H_{18}O_4$	- -	n.d	1,**2**,**3**,4,5,7,**8**,**9**	n.d
20	7.52	317.1396 315.1250	317.1384 315.1238	3.8 3.8	$C_{18}H_{20}O_5$	105.0701, 91.0543 285.0778, 257.0826	2-Hydroxy-dihydrochalcone	**3**,5,7	2'-Hydroxy-3',4',6'- Trimethoxydihydrochalcone (**Q**)
21	7.93	473.3640 471.3556	- -	- -	n.d	- -	n.d	9	n.d
22	8.25	507.2370 505.2254	507.2377 505.2232	-1.4 4.4	$C_{30}H_{34}O_7$	- -	n.d	**7**	n.d

Continued

	Retention time	Experimental mass (+) m/z (-) m/z	Theoretical mass (+) m/z (-) m/z	Mass error (ppm) (+) m/z (-) m/z	Predicted formula	Fragments	Compound class	Propolis type [a]	Name
23	8.430	521.2552 519.2390	521.2534 519.2388	3.5 0.4	$C_{31}H_{36}O_7$	- -	n.d	4,5,7,8,**9**	n.d
24	9.126	387.2905 385.2754	387.2894 385.2748	2.8 1.6	$C_{25}H_{38}O_3$	- -	n.d	1,2,3,**4**,5,7,8,9	n.d
25	9.784	521.2529 519.2408	521.2534 519.2388	-1.0 3.9	$C_{31}H_{36}O_7$	- -	n.d	9	n.d
26	10.12	387.2908 -	- -	- -	n.d	- -	n.d	1,2,3,**4**,5,6,7,8,9	n.d

Table 3. Compounds identified from the uHPLC-QTOF MS analysis of Australian stingless bee propolis. [a] Bold numbers are major components detected by UV. n.d: Not determined.

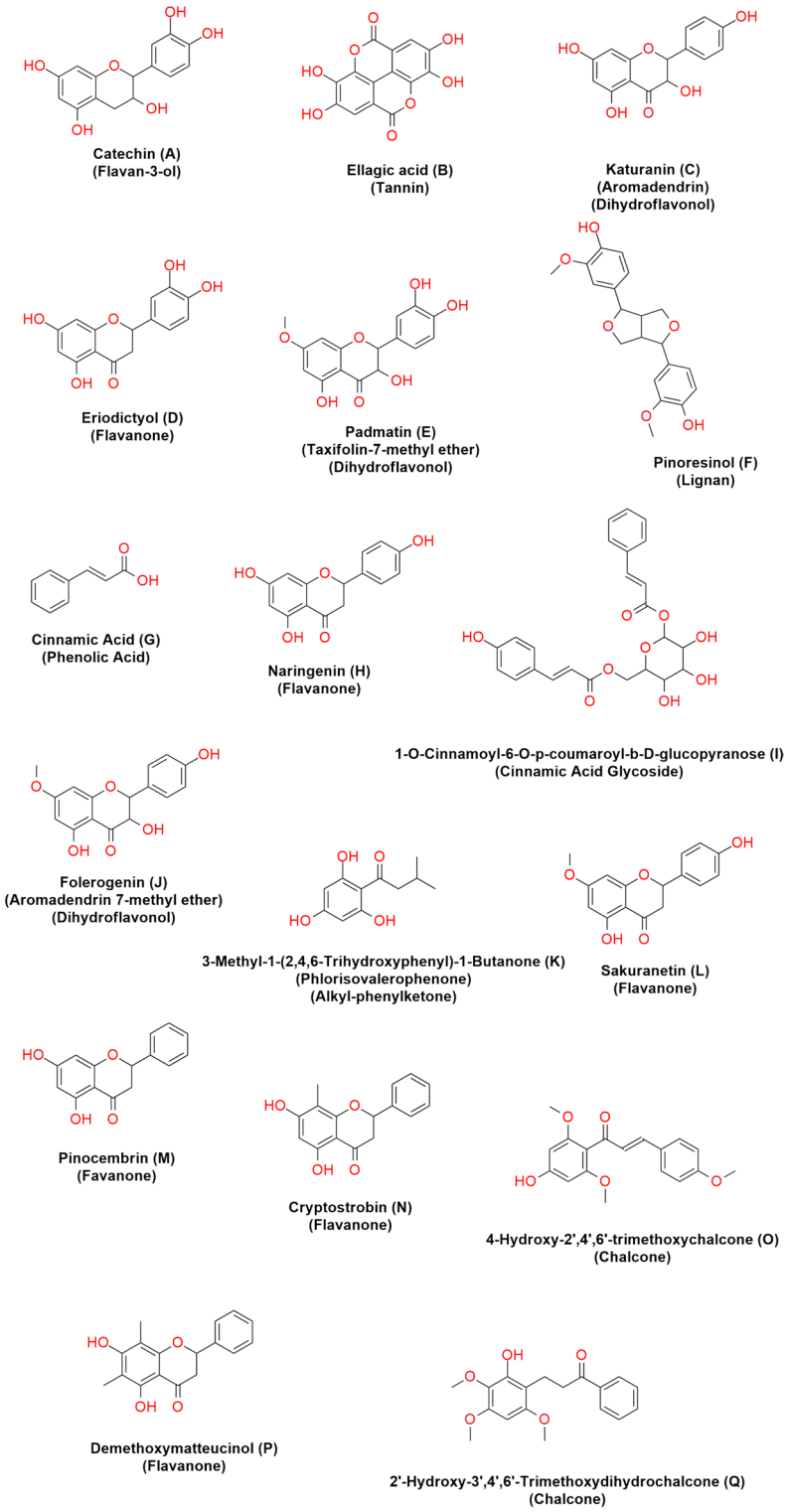


Fig. 6. Chemical structures of the major compounds **A**-**Q** present in each propolis type.

(**K**), 4-hydroxy-2',4',6'-trimethoxychalcone (**O**), and 2'-hydroxy-3',4',6'-trimethoxydihydrochalcone (**Q**) have not been previously reported in stingless bee propolis. Catechin (**A**), ellagic acid (**B**), katuranin (**C**), cinnamic acid (**G**), naringenin (**H**), and pinocembrin (**I**) have been identified in the propolis of various Melipona species, including *M. quadrifasciata*[19,71], *M. quadrifasciata anthidioides*[72], *M. fasciculata*[73,74], *M. scutellaris*[73] and *M. subnitida*[75]. Folerogenin (**J**) and sakuranetin (**L**) have been reported as a component of *Trigona spinipes* propolis[35]. Sakuranetin (**L**) has also been found in *Tetragonula carbonaria* propolis together with eriodictyol (**D**), cryptostrobin (**N**) and demethoxymatteucinol (**P**)[54,76]. Eight compounds (**A**-**H**) have exhibited strong

antioxidant activity[77–84], which may contribute to the potent antioxidant effect of propolis extracts observed in this study. In addition, these compounds, along with other flavonoids **J**, **L**, **M** and **N** have also demonstrated anti-inflammatory property[77–88]. Compounds **F**, **G**, and **H** have been known for their neuroprotective effects[82–84]. The remaining identified compounds **I**, **K**, **O**, **P** and **Q** have been sparsely studied in the literature regarding their potential bioactivities. Understanding the biological activities of these major compounds in Australian propolis could guide future developments in using this natural resource to advance human health.

No distinctly observable difference in chemical profiles of propolis produced by *T. carbonaria* and *T. hockingsi* bees was identified as evidenced by the profiles of samples collected in Central Queensland, Wide Bay Burnett, Sunshine Coast and Brisbane regions (propolis types 3–5). The chemical profiling clearly highlighted the unique composition of Far North Queensland propolis (types 1 and 2), which displayed distinct profiles compared to those from Sydney (type 9). Unique chemical profiles were also found in Central Queensland (type 3) and some regions in Brisbane (type 6). Some samples shared common compounds across a wide geographical distribution. Notably, a flavan-3-ol catechin (**A**) was detected as a major component in propolis from Central QLD to Sydney (propolis types 3, 5, and 7–9) while a flavanone naringenin (**H**) was universally present as a major or minor constituent in propolis from Far North Queensland to Sydney (propolis types 1–6, and 8–9). Only sakuranetin (**L**) and demethoxymatteucinol (**P**) was found to be present in all nine propolis types with the UV absorbance peaks being visible across all nine types. Additionally, cryptostrobin (**N**) was found to be a major constituent in propolis from Central QLD to Sydney. These results suggests that bees are likely able to find similar resins across multiple regions for their propolis production and compounds **A**, **H**, **L** and **P** may play a key role for bees' defensive system.

The co-presence of compounds in both propolis types 4 and 5 indicates that these types share a common botanical source, potentially found from Wide Bay Burnett to Brisbane. Interestingly, propolis type 9 appears to be a mixture of types 7 and 8 which were collected from the southern regions of Brisbane as they share similar compounds. This indicates that these propolis type were likely produced from similar plant species, distinct from those in the northern regions. *Corymbia torelliana* is likely one of the resin sources used by the bees as compounds **M**, **N**, and **P** have previously been identified in the resin of this plant species[54]. These chemical profiles serve as a valuable resource for identifying the origins of these propolis samples in the future.

Conclusion

In conclusion, this is the first comprehensive study to overview the quality and chemical diversity of Australian propolis produced by stingless bees *T. carbonaria* and *T. hockingsi*. The results indicate that Australian stingless bee propolis possesses potent antioxidant activity due to its high level of polyphenol compounds consisting primarily of non-flavonoid phenolics. The antioxidant capacity of propolis suggests that it could be used as an ingredient in the preparation of functional foods and dietary supplements.

Chemical analysis by ^{1}H NMR and uHPLC-QTOF MS profiles facilitated the classification of nine propolis types and shed a light into the chemical diversity of propolis collected from the northern to southern regions of Eastern coast Australia. Although not all compounds were determined from the uHPLC-QTOF MS data mining, the identification of multiple compound classes suggested that Australian stingless bee propolis has a diverse chemical composition. Further investigation into the undetermined compounds in this study may lead to the discovery of novel bioactive compounds that could serve as candidates for pharmaceutical development. The findings of this study will enhance awareness of Australian stingless bee propolis in both domestic and global markets and contribute to the growth of propolis industry in Australia.

Methods

Solvents and reagents

All solvents including ethanol (EtOH), methanol (MeOH), acetonitrile (MeCN), water and formic acid used for extraction and chromatography are LC–MS grade and were purchased from Merck. Dimethylsulfoxide-d_6 (DMSO-d_6) for NMR analysis was purchased from Merck. Gallic acid, Folin-Ciocalteu reagent, quercetin, potassium acetate (CH_3COOK), aluminium nitrate ($Al(NO_3)_3$), used for colorimetric assays were purchased from Merck. 2,2-diphenyl-1-picrylhydrazyl (DPPH) used for antioxidant assay was purchased from Merck.

Propolis collection and extraction

In this study, 170 raw propolis samples including *T. carbonaria* propolis (122 samples) and *T. hockingsi* propolis (48 samples) were collected across the eastern coast of Australia from Queensland (QLD, 159 samples) and New South Wales (NSW, 11 samples) by local beekeepers from 2022 and 2023 (Fig. 2). Sample details were provided in Table S3 (Supplementary Information). The raw propolis was frozen and ground into a powder before 0.5 g of the powdered propolis was transferred into a 15 mL falcon tube. After which 5 mL of 70% ethanol was added to each tube and briefly vortexed to mix. The samples were left in tubes and incubated in a 65 °C water bath for 30 min in darkness and were vortexed every 10 min. After the 30-min incubation, the samples were agitated through sonication for 2 min, vortexed for 2 min and then placed back into the ultrasonic water bath for 3 min, for a total of 5 min sonication time. The solution was then centrifuged at 4,000 rpm for 15 min at 4 °C. Once centrifuged, the supernatant was transferred into a 15 mL test tube. The supernatant was then dried overnight through centrifugal evaporation in a GeneVac using the low boiling point method. The extract was then dried further in a vacuum desiccator for 30 min. Once sufficiently dried, the extract was finally weighed to obtain the extraction yield and kept in darkness at -20 °C for NMR and uHPLC-QTOF MS sample preparation.

Determination of total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu colorimetric method as previously described[65]. In brief, 80 µL of the Folin–Ciocalteu solution was added to 410 µL of H_2O, followed by the addition of 10 µL of propolis extracts (10 mg/mL) or 10 µL of gallic acid standard solution in MeOH and then 500 µL of 10% (m/v) aqueous sodium carbonate. MeOH was used as a blank sample (negative control). The samples in Eppendorf tubes were incubated in darkness at room temperature for 60 min before being plated to a 96-well plate (200 µL/well). Finally, the absorbance at 760 nm was measured on a Perkin Elmer Enspire microplate reader. All measurements were performed in triplicates, the mean values were interpolated in a gallic acid calibration curve and the total phenolic content was expressed as mg Gallic Acid Equivalents (GAE) per gram of dry extract.

Determination of total flavonoid content

To determine total flavonoid content, the aluminium chloride colorimetric method was used as previously described[65] with some modifications. The aluminium chloride colorimetric method was completed by adding 20 µL of 10% $Al(NO_3)_3$ in H_2O and 20 µL of 1 M CH_3COOK in water to 950 µL of MeOH. 10 µL of 10 mg/mL propolis extract in MeOH or quercetin in MeOH was then added. The samples in Eppendorf tubes were then incubated at room temperature for 45 min in darkness before being plated in duplicate onto a 96 well plate (200 µL per well). The absorbance was then measured through spectrophotometry at 415 nm on a Perkin Elmer Enspire microplate reader. Quercetin was used as a chemical equivalent for quantification in this assay.

Evaluation of antioxidant activity using DPPH free radical scavenging assay

To determine the antioxidant activity, the DPPH free radical scavenging assay was used with propolis extracts and different concentrations as previously described[65]. Briefly, the DPPH solution was prepared on the day of measuring at a concentration of 100 µM in MeOH. The propolis extracts (200 µL) at different concentrations were added to 600 µL of DPPH solution in Eppendorf tubes. The mixtures were kept in the dark at room temperature for 20 min before being plated to a 96-well plate (200 µL/well) and measured at 518 nm using a Perkin Elmer Enspire microplate reader. All evaluations were performed in triplicates. Gallic acid and MeOH were used as positive and negative controls. The % inhibition of the DPPH radical for each sample was normalised and calculated using the following formula:

$$\%Inhibition = \left[1 - \frac{(A_S - A_P)}{(A_B - A_P)}\right] \times 100$$

where A_S is the absorbance of the sample, A_P is the absorbance of the positive control and A_B is the absorbance of the blank sample (negative control).

An IC_{50} curve for each extract was generated using GraphPad Prism 10.4.1 (https://www.graphpad.com/) with a 95% confidence interval. The IC_{50} values were determined as the concentration required to inhibit 50% of DPPH free radicals.

^{1}H NMR analysis and processing

To prepare samples for NMR, dry propolis extract was dissolved in 500 µL of Dimethylsulfoxide-d_6 (DMSO-d_6) to produce a 50 mg/mL propolis sample which was then transferred to a 10 mm NMR tube. The NMR data acquisition was conducted on a Bruker Ascend 400 MHz NMR instrument equipped with a 5 mm room temperature probe housed at The University of the Sunshine Coast. The acquisition method consisted of obtaining ^{1}H NMR spectra at 300 K. The data obtained from ^{1}H NMR was then analysed using Bruker Topspin 4.4.0 software. The spectrum was recorded using a standard pulse sequence with a 90° pulse length of 9.61 µs, 64 scans, a spectral width of 16 ppm, a relaxation delay of 5 s, and an acquisition time of 3.75 s. The spectra were referenced to the DMSO residual solvent signal at 2.50 ppm. Metabolomic analysis for chemical profile development was conducted using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/).

Chemometric analysis

^{1}H NMR acquisition data from 0 to 8 ppm was exported to MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca/) [89] in a comma-separated value (CSV) format. PCA[90] and sPLS-DA[91] were conducted to demonstrate the variations in sample composition depending on their geographical location and stingless bee species.

uHPLC-QTOF MS analysis

To prepare samples for uHPLC-QTOF MS, dry propolis extract was dissolved in methanol to produce a sample concentration of 10 mg/mL and stored in darkness at -20 °C in 2 mL Agilent LC–MS vials before uHPLC-QTOF MS analysis. The analysis was conducted on an Agilent 1290-uHPLC-6546-QTOF MS equipped with a DAD detector and was injected (2 µL) onto a Zorbax Eclipse plus C_{18} column (2.1 × 50 mm, 1.8 µm) with a guard column. The LC-QTOF method was completed in 1D and was completed in reverse phase utilising a gradient consisting of H_2O (solvent A, 0.1% formic acid) and MeCN (solvent B, 0.1% formic acid) with a sample flow rate of 0.4 mL per minute. uHPLC analysis utilised a DAD with 8 wavelengths being analysed, consisting of 200 nm, 260 nm, 280 nm, 300 nm, 320 nm, 360 nm, 420 nm, and 460 nm. The LC gradient consisted of a 15-min sample run time at a constant sample flow rate of 0.4 mL/min. The gradient program started at 2% solvent B for 0.5 min, increased to 100% solvent B for 9 min, kept at this level for the next 3 min, then reduced to 2% solvent B for 1 min, and finally re-equilibrated at 2% solvent B for 1.5 min.

The QTOF MS analysis was completed in positive and negative ion modes and was ionised with a Dual Agilent Jet Stream (Dual AJS) electrospray ionisation (ESI) ion source set to follow an ionisation energy formula

described by $\left(5 \times \frac{m/z}{100}\right) + 10$, with the MS scan to be completed in tandem MS (MS^2, MS/MS) mode. The TOF–MS fragmentor and skimmer were set at 120 V and 45 V, respectively. Acquisition was completed in positive and negative polarity modes with the analysed mass range being from 100 to 1,050 Da. The carrier gas source used in MS was nitrogen at 250 °C, with a flow rate of 12 L per minute. Analysis of QTOF-MS spectra was conducted using SIRIUS Version 6.0.7 (https://bio.informatik.uni-jena.de/software/sirius/).

Compound identification

The prediction of compounds in propolis samples was performed using DAD uHPLC-QTOF MS, controlled by Agilent MassHunter Workstation Software Version B.08.00 (https://www.agilent.com/en/promotions/masshunter-mass-spec) and SIRIUS Version 6.0.7 (https://bio.informatik.uni-jena.de/software/sirius/). Through DAD chromatograms, at least five major peaks were relatively identified for each propolis type by comparing their signals at eight different wavelengths: 200, 260, 280, 300, 320, 360, 420 and 460 nm. After identifying these major UV peaks, initial classification of compound family (flavonoid or non-flavonoid phenolics) was determined by considering the number of carbon atoms, unsaturated degrees from each compound's molecular formula and their UV absorbance signals[94,95]. Compound identification was conducted using MS/MS data in both positive and negative modes utilising the molecular formula identifier (MFI), fingerprint predictor (CSI:FingerID), and compound class predictor (CANOPUS) within the SIRIUS software with MS/MS mass accuracy threshold ≤ 10 ppm. The positive and negative MS^2 data for each major compound were searched using the online database searching (CSI:FingerID) feature within SIRIUS across 21 libraries including Biocyc, Blood exposome, CHEBI, COCONUT, FooDB, GNPS, HMDB, HSDB, KNApSAcK, LipidMaps, Maconda, MeSH, MiMeDB, NORMAN, Plantcyc, PubChem (bio and metabolites, drug, food, safety and toxic)[92], SuperNatural, TeroMOL, YMDB, and LOTUS[93]. Compounds with a confidence value < 80% were deemed not determined (n.d). MS^1 and MS^2 data of compounds **A**-**Q** were detailed in Figures S3-S5 (Supplementary Information).

Statistical analysis

Statistical analysis was completed using GraphPad Prism Version 10.4.1 (https://www.graphpad.com/). Unpaired two-tailed t-tests were conducted to compare the means of the yield, total phenolic content, and antioxidant activity between two species groups. One-way ANOVA with post-hoc Tukey's test was used to compare the means of each collection region. Statistical significance was expressed using asterisks. All error bars present are in the form of standard error mean (SEM).

Data availability

All data generated or analysed during this study are included in this paper and its Supplementary Information file.

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Author contributions

T.A.H., H.M.W. and T.D.T. contributed to project conceptualisation. T.A.H and T.D.T. contributed to sample collection. D.C.W. and M.A.O. contributed to experiments and data interpretation. T.D.T. supervised and administered the project. D.C.W. and T.D.T. wrote the original draft and all authors contributed to the review and editing.

Competing interests

The authors declare no competing interests.

Additional information

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