Phytochemistry 134 (2017) 87–97

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

A sedge plant as the source of Kangaroo Island propolis rich in prenylated *p*-coumarate ester and stilbenes

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ARTICLE INFO

Article history:
Received 10 February 2016
Received in revised form
10 November 2016
Accepted 16 November 2016
Available online 24 November 2016

Keywords:
Lepidosperma
Cyperaceae
Propolis
Honey bee
Prenylated hydroxystilbenes
Anticancer activity

ABSTRACT

Propolis samples from Kangaroo Island, South Australia, were investigated for chemical constituents using high-field nuclear magnetic resonance spectral profiling. A type of propolis was found containing a high proportion of prenylated hydroxystilbenes. Subsequently, the botanical origin of this type of propolis was identified using a beehive propolis depletion method and analysis of flora. Ligurian honey bees, *Apis mellifera ligustica* Spinola, were found to produce propolis from resin exuded by the Australian native sedge plant *Lepidosperma* sp. Montebello (Cyperaceae). The plants, commonly known as sword sedge, were found to have resin that matched with the propolis samples identified as the most abundant propolis type on the island containing *C*- and *O*-prenylated tetrahydroxystilbenes (pTHOS) in addition to a small amount of prenylated *p*-coumarate. The isolation of five pTHOS not previously characterized are reported: (*E*)-4-(3-methyl-2-buten-1-yl)-3,4′,5-trihydroxy-3′-methoxystilbene, (*E*)-2,4-*bis*(3-methyl-2-buten-1-yl)-3,3′,4′,5-tetrahydroxystilbene, (*E*)-2-(3-methyl-2-buten-1-yl)-3-(3-methyl-2-butenyloxy)-3′,4′,5-trihydroxystilbene, (*E*)-2,6-*bis*(3-methyl-2-buten-1-yl)-3,3′,5,5′-tetrahydroxystilbene and (*E*)-2,6-*bis*(3-methyl-2-buten-1-yl)-3,4′,5-trihydroxy-3′-methoxystilbene. A National Cancer Institute 60 human cell line anticancer screen of three of these compounds showed growth inhibitory activity. The large Australasian genus *Lepidosperma* is identified as a valuable resource for the isolation of substances with medicinal potential.

1. Introduction

Propolis is resinous material produced by honey bees, *Apis mellifera* L., to fill holes and cracks in the hive, reduce the size of the hive entrance, provide a sterile coating on the walls of the hive, or to embalm dead animals too large for removal from the hive. The material is collected as a sticky exudate from different parts of specific plant species (Bankova et al., 2006). Common plant parts from which bees collect are leaf or flower buds, leaf glands, exudates, wounds in the bark or stems of plants and exudates produced in response to microbial infection or insect attack. Bees remove the resinous material with their mandibles and transfer it to their hind leg 'pollen baskets' where they form it into a mass that typically appears as smooth, shiny, semi-transparent droplets. The resinous material is not ingested by bees, and it appears that bees do not alter the material other than mixing with beeswax in the hive (Daugsch et al., 2008; Park et al., 2004; Piccinelli et al., 2011; Simone-Finstrom and Spivak, 2010; Tran et al., 2012). For thousands of years propolis has been used for human medicinal purpose and many medicinal applications for propolis are supported by

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http://dx.doi.org/10.1016/j.phytochem.2016.11.005

scientific evidence. Bees are very selective in the material they collect to produce propolis. In Europe, bees collect from the leaf buds of poplar (*Populus* L.) and beech (*Fagus* L.) (Bankova et al., 2000). These sources are also foraged by bees in other parts of the world where these plants are available. Where the vegetation from which bees normally forage for propolis is not available, bees will forage from other resin producing plants. It has been found that propolis produced by honey bees from plants outside the traditional geographic location of honey bees generally has useful physical and antibiotic properties that effectively serve the purposes of bees. Propolis produced by bees in Europe typically contains polyhydroxyphenolic substances (Bankova et al., 2000) that have potent anti-oxidant and antibiotic properties. These substances include substituted cinnamates and flavonoids that may be methylated or prenylated, imparting them with a relatively non-polar property. The composition of propolis can be very variable as it depends on the plant source. Unique types of propolis have been found in many localities around the world. The vegetation from which they are sourced by honey bees has been identified and well characterized (Popova et al., 2007). For example, Brazilian propolis contains prenylated derivatives of *p*-coumaric acid and acetophenone (Park et al., 2002); Taiwanese propolis, also known as Pacific propolis, contains prenylated flavanones (propolins A-F) as major constituents (Chen et al., 2003); and Cuban propolis contains polyisoprenylated benzophenones as major components (Cuesta Rubio et al., 2002; Trusheva et al., 2004).

From a plant metabolism perspective, the prenylation of aromatic secondary metabolites plays a critical role in the biosynthesis of a wide range of molecules exerting valuable pharmacological effects across phylogenetically different classes of living organisms, from bacteria to mammals and plants. Anticancer potentials of prenylated hydroxystilbenes particularly those from peanut was recently reported. Arachidin-1, a prenylated piceatannol, isolated from germinating peanut seeds, inhibited human leukemia (HL-60) cells growth with an EC_{50} of approximately 4.2 μM, which is 4-fold more potent than resveratrol. That study showed that arachidin-1 induces programmed cell death in human leukemia HL-60 cells via both caspase-dependent and caspase-independent pathways (Huang et al., 2010).

Honey bees were introduced to Australia as part of agricultural practice more than 150 years ago and are now a well-established industry. Vast land areas and a flora largely unique to Australia have provided opportunity for bees to collect resins from non-traditional plant sources to produce propolis with different chemical composition and medicinal properties compared to propolis from other parts of the world. However, despite Australia's unique flora, no propolis of novel chemical constituent composition sourced from an Australian plant, defining both the chemical constituents profile and bee collection behaviour from the plant source, had been reported in detail. A study was undertaken to identify unique propolis types by chemical analysis of propolis samples collected in Australia and to select propolis types to track and characterize the vegetative source of the propolis. After preliminary studies, Kangaroo Island, South Australia, was identified as an area rich in relatively undisturbed endemic flora and as a source of honey bee propolis of unique chemical composition (Abu-Mellal et al., 2012). The variety of honey bee introduced to Kangaroo Island is *Apis mellifera ligustica* Spinola – the Ligurian honey bee, hereafter referred to as 'honey bee' or 'bee'.

Presented here are the isolation and characterization of five new and one known prenylated hydroxystilbenes (pTHOS) from Kangaroo Island propolis samples and the identification of the botanical origin of these propolis samples. Initial screening for inhibitory effect of the isolated prenylated hydroxystilbenes on the growth of cancer cells is also presented.

2. Results

2.1. Identification and characterization of five new prenylated stilbenes

As part of ongoing research into the medicinal properties of propolis, five prenylated stilbenes (Fig. 1), not previously characterized were isolated and characterized by 1D and 2D NMR, low- and high-resolution mass spectrometry. By evaluation of these data structures of **1**, **2**, **3**, **4**, and **5** (Fig. 1) were determined to have molecular formulae $C_{20}H_{22}O_4$, $C_{24}H_{28}O_4$, $C_{24}H_{28}O_4$, $C_{24}H_{28}O_4$ and $C_{25}H_{30}O_4$, respectively.

These compounds are systematically named as (*E*)-4-(3-methyl-2-buten-1-yl)-3,4′,5-trihydroxy-3′-methoxystilbene (**1**), (*E*)-2,4-bis(3-methyl-2-buten-1-yl)-3,3′,4′,5-tetrahydroxystilbene (**2**), (*E*)-2-(3-methyl-2-buten-1-yl)-3-(3-methyl-2-butenyloxy)-3′,4′,5-trihydroxystilbene (**3**), (*E*)-2,6-bis(3-methyl-2-buten-1-yl)-3,3′,5,5′-tetrahydroxystilbene (**4**) and (*E*)-2,6-*bis*(3-methyl-2-buten-1-yl)-3,4′,5-trihydroxy-3′-methoxystilbene (**5**).

In all five new compounds (**1**–**5**) prenyl groups are clearly recognized from the two vinyl methyl groups appearing as broad singlets in the range 1.6–1.8 ppm and the olefinic hydrogen appearing as a broad triplet in the range 5.0–5.5 ppm (Tables 1 and 2). *O*- and *C*- prenylation are clearly distinguished in the ^{1}H NMR spectra by signals at approximately 4.5 and 3.4 ppm, respectively, for the prenyl CH_2 protons attached to phenolic *O* or aromatic ring *C*. The position of substitution is generally determined from chemical shifts of remaining A-ring aromatic protons and changes in symmetry of the A and B-ring.

^{1}H and ^{13}C NMR spectra for compound **1** revealed a stilbene, evident by the presence of unique doublets at δ_H 6.90 (H-β) and at δ_H 6.77 (H-α); the *E*-configuration is shown by the $J_{\alpha\beta}$ of 16.0 Hz. Similarly, compounds **2** to **5**, were all identified as structures based on an *E*-stilbene configuration.

In compound **1**, the 4 position for the *C*-prenyl group on the A-ring is shown by symmetry resulting in identical chemical shift and lack of coupling of the remaining 2 aromatic hydrogens at 6.47 ppm. The B-ring H-2′, at 7.08 ppm and H-6′ at 6.93 ppm are deshielded by the stilbene double bond and strongly shielded by the oxygen attached at the 3′ position, while H-5′ at 6.76 ppm is mainly affected by strong shielding from the oxygen attached at the 4′ position. The 2 Hz coupling shown by H-2′ indicates a four-bond coupling to H-6′. For H-6′, in addition to the 2 Hz four-bond coupling to H-2′, a three-bond coupling of 8.1 Hz to H-5′ was observed showing a 1′,3′,4′- trisubstituted pattern for the B-ring. Gradient Heteronuclear Multiple Bond Correlation (gHMBC) experiment cross-peaks between the *C*-prenyl CH_2 hydrogens and C-3, C-4 and C-5 indicated the prenyl group was attached to C-4 and the methoxy group was attached to C-3′ (Fig. 2).

Compound **2** was found to have un-symmetrical di-*C*-prenyl substitution on the A-ring from recognition of two sets of ^{1}H and ^{13}C NMR signals for the two prenyl groups and ^{1}H NMR singlet at 6.63 ppm for H-6. The 3,5-dihydroxy substitution on the A-ring was shown by the characteristic deshielded carbon signals at 154.38 and 154.82 ppm for C-3 and C-5, and also was shown by a gHMBC experiment correlation between the *C*-prenyl H-1″, 3.35 ppm, with C-1, C-2 and C-3, and *C*-prenyl H-1‴, 3.40 ppm, with C-4 and C-5 (Fig. 2). A 1′,3′,4′- trisubstituted pattern for the B-ring was determined from the ^{1}H NMR chemical shifts and coupling pattern as for compound **1**.

Compound **3** showed ^{1}H and ^{13}C NMR signals for *O*-prenyl and *C*-prenyl substitution on a piceatannol skeleton. The position of the *C*-prenyl group was established by the chemical shifts and coupling of the two remaining aromatic H on the A-ring at 6.62 ppm, H-6 and 6.32 ppm, H-4, both doublets, J = 2.3 Hz. The position of

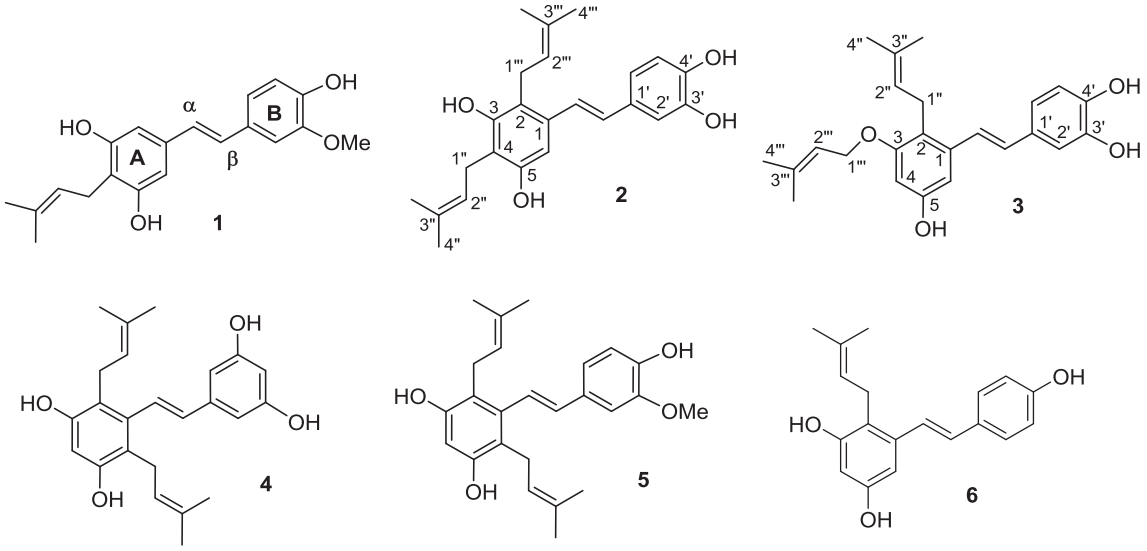


Fig. 1. Structures of prenylated tetrahydroxystilbenes identified in this study.

Table 1
^{1}H NMR spectroscopic data for compounds **1**, **2**, **3**, **4** and **5** (400 MHz, δ in ppm *J* values (Hz) in parentheses, measured in CD_3OD relative to TMS).

H	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5
1	–	–	–	–	–
2	6.47 *s*	–	–	–	–
3	–	–	–	–	–
4	–	–	6.32 *d* (2.3)	6.29 *s*	6.30 *s*
5	–	–	–	–	–
6	6.47 *s*	6.63 *s*	6.62 *d* (2.3)	–	
1′	–	–	–	–	–
2′	7.08 *d* (2.0)	6.94 *d* (2.0)	6.97 *d* (2.0)	6.73 *d* (1.0)	7.03 *d* (1.9)
3′	–	–	–	–	
4′	–	–	–	6.92 *bt*	
5′	6.76 *d* (8.1)	6.73 *d* (8.2)	6.74 *d* (8.2)	–	6.77 *d* (8.0)
6′	6.93 *dd* (8.1, 2.0)	6.78 *dd* (8.2, 2.1)	6.81 *dd* (8.2, 2.0)	6.73 *d* (1.0)	6.85 *dd* (8.2, 1.9)
α	6.77 *d* (16.0)	6.72 *d* (16.0)	6.78 *d* (16.1)	6.26 *d* (16.5)	6.33 *d* (16.6)
β	6.90 *d* (16.0)	7.05 *d* (16.0)	7.10 *d* (16.1)	6.81 *d* (16.5)	6.87 *d* (16.6)
1″	3.28 *bd* (7.1)	3.35 *bd* (6.9)	3.37 *bd* (6.5)	3.26 *bd* (6.6)	3.26 *bd* (6.0)
2″	5.24 *bt* (7.1)	5.21 *bt* (6.9)	5.06 *bt* (6.9)	5.12 *bt* (6.6)	5.14 *bt* (6.0)
3″	–	–	–	–	–
4″	1.77 *bs*	1.78 *bs*	1.79 *bs*	1.66 *bs*	1.66 *bs*
5″	1.66 *bs*	1.68 *bs*	1.66 *bs*	1.60 *bs*	1.60 *bs*
1‴	–	3.40 *bd* (6.9)	4.48 *bd* (6.5)	3.26 *bd* (6.6)	3.26 *bd* (6.0)
2‴	–	5.10 *bt* (6.9)	5.48 *bt* (6.6)	5.12 *bt* (6.6)	5.14 *bt* (6.0)
3‴	–	–	–	–	–
4‴	–	1.81 *bs*	1.79 *bs*	1.66 *bs*	1.66 *bs*
5‴	–	1.68 *bs*	1.75 *bs*	1.60 *bs*	1.60 *bs*
3′-OCH_3	3.90 *s*	–	–	–	3.88 *s*

substitution of both the *O*- and *C*-prenyl groups was established by a long-distance correlation experiment. *C*-Prenyl substitution at the 2-position was shown by gHMBC correlation between the *C*-prenyl H-1″, 3.37 ppm, with C-1, 139.53 ppm, C-2, 120.29 ppm and C-3, 159.05. *O*-Prenyl substitution on the oxygen at position 3 was shown by gHMBC correlation between the *O*-prenyl H-1‴, 4.48 ppm, with C-3, 159.05 ppm.

A symmetrically substituted *E*-stilbene structure was revealed by the ^{1}H and ^{13}C NMR spectra for compound **4**. Di-*C*-prenyl substitution was shown H-1″/H-1‴ chemical shift of 3.26 ppm with the characteristic ^{1}H and ^{13}C NMR chemical shifts for two identical prenyl groups. 2,6-Diprenylation was shown by one remaining H-4, 6.29 ppm, on the A-ring showing gHMBC correlation of H-4 with C-2 and C-6, 119.82, also 2,6-di-*C*-prenyl substitution was shown by gHMBC correlation between the *C*-prenyl H-1″/H-1‴, 3.26 ppm, with C-1, C-2/C-6, and C-3/C-5 (Fig. 2). For the B-ring symmetric 3′,5′-dihydroxy substitution was shown H-4′ appearing as a weakly coupled triplet, coupling to H-2′/H-6′, appearing as a doublet, $J = 1$ Hz.

From ^{1}H and ^{13}C NMR spectra compound **5** was found to have a structure based on di-*C*-prenylated piceatannol. Symmetrical 2,6-di-*C*-prenylation was shown by identical chemical shifts for all of the H and C atoms of the *C*-prenyl groups and the chemical shift and lack of coupling of the remaining H-4 on the A-ring. The substitution pattern was confirmed by H-4, 6.30 ppm, on the A-ring showing gHMBC correlation with C-2 and C-6, 119.23, also 2,6-di-*C*-prenyl substitution was shown by gHMBC correlation between the *C*-prenyl H-1″/H-1‴, 3.26 ppm, with C-1, 140.95 ppm, C-2/C-6, 119.23 ppm and C-3/C-5, 154.65. The B-ring 1′,3′,4′-trisubstituted pattern was shown by H-2′, at 7.03 ppm and H-6′ at 6.85 ppm

Table 2
^{13}C NMR spectroscopic data for compounds **1**, **2**, **3**, **4** and **5** (100 MHz, δ in ppm, measured in CD_3OD relative to TMS).

C	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5
1	131.41	136.57	139.53	146.56	140.95
2	105.85	120.10	120.29	119.82	119.23
3	157.43	154.38	159.05	154.62	154.65
4	116.06	116.91	100.62	116.44	102.58
5	157.43	154.82	157.17	154.62	154.65
6	105.85	105.44	104.99	119.82	119.23
1′	137.70	132.11	131.59	140.99	131.70
2′	110.38	113.89	113.97	119.24	110.27
3′	149.33	146.43	146.66	146.31	149.26
4′	147.61	146.63	146.68	113.85	147.51
5′	116.47	120.15	116.54	146.31	116.42
6′	121.18	116.52	121.05	119.24	121.03
α	127.64	130.26	125.12	130.51	134.87
β	128.68	125.67	131.28	131.79	126.31
1″	23.46	23.91	26.12	27.18	26.12
2″	124.76	124.52	125.55	125.64	126.02
3″	131.25	131.34	131.09	126.21	130.41
4″	26.14	26.08	25.42	26.11	27.20
5″	18.07	18.13	18.33	18.33	18.37
1‴	–	26.05	66.45	27.18	26.12
2‴	–	125.53	121.72	125.64	126.02
3‴	–	131.85	138.36	126.21	130.41
4‴	–	26.13	26.03	26.11	27.20
5‴	–	18.33	18.39	18.33	18.37
3′-OCH_3	56.53	–	–	–	56.53

deshielded by the stilbene double bond and strongly shielded by the oxygen attached at the 3′ position, while H-5′ at 6.77 ppm was mainly affected by strong shielding from the oxygen attached at the 4′ position. The 1.9 Hz coupling shown by H-2′ indicates a four-bond coupling to H-6′. For H-6′, as well as the 1.9 Hz four-bond coupling to H-2′, a three-bond coupling of 8.1 Hz to H-5′ was observed consistent with a 1′,3′,4′- trisubstituted pattern. gHMBC long distance correlation experiment showed that the methoxy group is attached to C-3′.

Key long range couplings determined for these compounds **1** to **5** from gHMBC 2D NMR experiments are illustrated (Fig. 2).

2.2. Isolation and identification of 2-prenylresveratrol (*6*) and identification of compounds *7* to *14*

A minor constituent isolated from Kangaroo Island propolis was identified as, (*E*)-2-(3-methyl-2-buten-1-yl)-3,4′,5-trihydroxystilbene (**6**), a substance previously reported as a product from chemoenzymatic prenylation of resveratrol (Kumano et al., 2008).

Other compounds previously reported from Kangaroo Island propolis (Abu-Mellal et al., 2012), identified in this study by HPLC analysis (Tables 3 and 4, see Figs. 1S and 2S respectively for the chromatograms) include the major stilbenes (**7** to **13**) and methyl (*E*)-4-(4′-hydroxy-3′-methylbut-(*E*)-2′-enyloxy)cinnamate (**14**) (Fig. 3).

2.3. The vegetative source of the main Kangaroo Island propolis type

A beehive propolis depletion method, done by frequent removal of propolis, was adopted to increase the number of propolis foraging bees to assist in tracking bees to the plant source. At selected apiary sites in the western and central southern regions of Kangaroo Island, 76 beehives made up of 3, 10-frame boxes each fitted with a propolis mat under the hive cover lid were used to collect propolis samples. To further encourage bees to actively collect resins to produce propolis, the hive cover was raised leaving a 3 mm gap between the edges of the hive cover and the propolis mat. As a result, bees were found collecting propolis from the resin of a sedge type plant which was later identified as *Lepidosperma* sp. Montebello (voucher G.T. Plunkett 72, personal communication Plunkett, G. T., Duke, C. C., Duke, R. K., Tran, V. H., King, D. I., Barrett, R. L., Wilson, K. L., Bruhl, J. J.) located 200 m from the hives (Fig. 4). Chromatographic and NMR analyses of resinous material carried on the bees legs, propolis from the hives mats, and the resin from the base of stems of *L.* sp. Montebello showed the same chemical constituents in approximately the same proportions (Fig. 5, Table 3, see Fig. 1S for the chromatogram). Consequent to finding this plant as the botanical source of the main stilbene type in propolis, resins of similar plants of the same species were collected at different locations across Kangaroo Island and mainland Australia for NMR analysis, including some statistical clustering of spectra (Plunkett et al., 2013, 2014). Resins of these plants were brown-red in colour, and are mainly in spaces between the leaf overlap at the base of the plant and also in the finely serrated leaf margins. Resin on the leaf margins appeared to be oxidized to form dark red-brown materials.

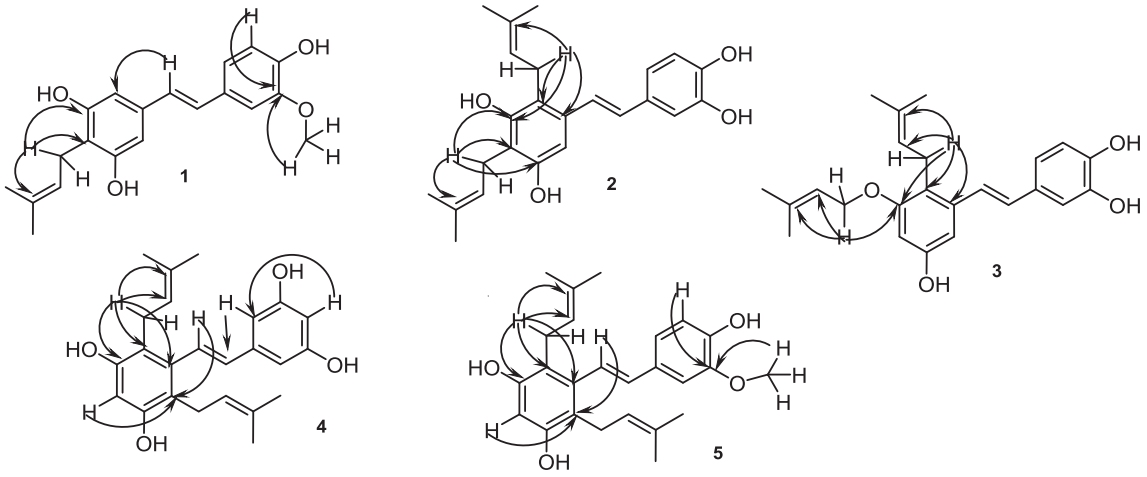


Fig. 2. Key heteronuclear multiple bond correlation (gHMBC) of compounds **1**, **2**, **3**, **4**, and **5**.

Table 3
Composition of propolis extract compared with extract from *Lepidosperma* sp. Montebello resin (weight %), determined by isocratic reversed-phase HPLC.

Compound	*RRt*[a]	Propolis[b]	Plant resin[b]
2	0.92	4.8	5.3
5	1.29	0.6	0.7
7	0.33	10.1	13.4
8	0.45[c]	4.4	4.8
9	0.37	0.9	1.6
10	1.34	1.8	1.7
11	0.56	16.0	15.0
12	1.00[d]	6.8	6.1
13	1.46	2.3	2.5
14	0.48[c]	9.6	13.8
Total		57	65

Average difference between duplicate analyses 0.45 wt%.
[a] Relative Retention Time.
[b] Location on Kangaroo Island: Intersection of Mt Taylor Road and South Coast Road.
[c] Reduced accuracy due to partial overlap of the peaks for compounds **8** and **14**.
[d] Reduced accuracy due to a small shoulder on the peak for compound **12**.

Table 4
Composition of propolis extracts from a range of locations in Kangaroo Island (weight %), determined by gradient reversed-phase HPLC.

Compound	*RRt*[a]	SW[b]	LS[b]	SB[b]	GR[b]	Average	STDev
2	0.92	6.4	8.0	8.3	6.6	7.3	1.0
5	1.06	1.3	1.7	1.6	1.1	1.4	0.3
7	0.60	7.4	4.9	13.3	6.2	8.0	3.7
8	0.76[c]	3.9	2.5	6.7	2.9	4.0	1.9
9	0.69	1.0	0.9	1.6	0.7	1.0	0.4
10	1.08	1.7	3.3	2.5	1.6	2.3	0.8
11	0.82	15.4	17.8	12.6	29.2	18.7	7.3
12	1.00[d]	10.2	14.6	8.2	14.6	11.9	3.2
13	1.29	1.3	1.3	1.3	2.4	1.6	0.6
14	0.80[c]	11.6	6.8	13.9	15.2	11.9	3.7
Total		60.2	61.7	70.0	80.4	68.1	
Average							2.3

Average difference between duplicate analyses 0.14 wt%.
[a] Relative Retention Time.
[b] Locations on Kangaroo Island: SW = south west; LS = Little Sahara; SB = Stun'sail Boom; GS = Gosse-Ritchie.
[c] Reduced accuracy due to partial overlap of the peaks for compounds **8** and **14**.
[d] Reduced accuracy due to a small shoulder on the peak for compound **12**.

2.4. ^{1}H NMR and HPLC analyses of plant and propolis samples

^{1}H NMR spectra of plant resin, bee leg resin and propolis (Fig. 5) showed signals that can be attributed to compound **14** (Abu-Mellal et al., 2012) as follows: 7.65 ppm, doublet, H-3; 7.46 ppm, doublet, H-2′/H-6′; 6.30 ppm, doublet, H-2; 5.77 ppm, broad triplet, H-2″; 4.63 ppm doublet, H-1″. Signals that may be attributed to stilbene compounds **1** to **13** are as follows: the unresolved aromatic signals at 6.4–7.3 ppm; the signal at 5.50 ppm for the *O*-prenyl olefinic H; 5.1–5.3 ppm *C*-prenyl olefinic H; 4.50 ppm, *O*-prenyl H-1; 3.8–4.0 ppm, *O*-methyl H; 3.41 ppm, *C*-prenyl H-1; 1.75–1.8 ppm, prenyl CH_3 groups. Variability in the composition of plant and propolis samples is visible in the ^{1}H NMR spectra as shown by the more prominent signals at 3.8–4.0 ppm in the propolis sample (Fig. 5c) indicating a higher degree of *O*-methylation. HPLC analyses (Tables 3 and 4, see Figs. 1S and 2S respectively for the chromatograms) showed a similar profile for resin from the plant and propolis from the hive. In order of abundance there was 3-*O*-prenylpiceatannol (**11**), followed by 3-*O*-prenyl-3′-*O*-methylpiceatannol (**12**) and the *p*-coumarate ester (**14**) in similar amounts, followed by compounds **2** and **7**, both in similar amounts. Minor compounds detected were compounds **5**, **8**, **9**, **10** and **13**. Compounds **1**, **3**, **4** and **6** were not detected with the HPLC conditions applied due to small quantity and similar retention characteristics to the major compounds. Analysis of propolis from a range of locations showed considerable variation in the amounts of the major compounds.

2.5. Growth inhibitory activity towards the NCI 60 human cell line anticancer screen

The prenylated tetrahydroxystilbenes showed structure dependent inhibition of cell growth of a range of tumor cell lines including leukemia, non-small cell lung cancers, colon cancers, CNS cancers, melanoma, ovarian cancers, renal cancers, prostate cancers and breast cancers (Table 5).

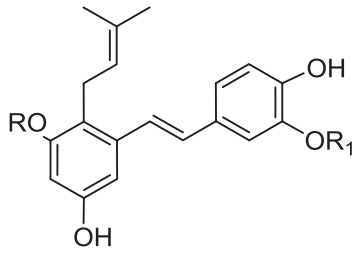
3. Discussion

3.1. Identification of the main Kangaroo Island propolis plant source as a species of Lepidosperma

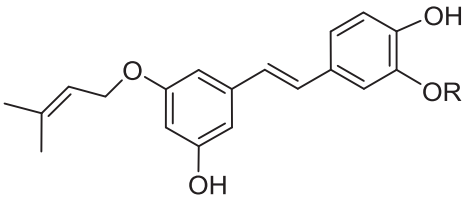
There have been very few reports of propolis sourced in Australia with composition substantially different from those reported in Europe and elsewhere despite the predominance of endemic flora. There is a report of propolis containing a stilbene (pterostilbene) sourced from species of *Xanthorrhoea* in Western Australia (Ghisalberti et al., 1978) but this association has not been firmly established. Preliminary studies in NSW by this group showed that the propolis generally had a composition consistent with bees foraging on introduced plants, in particular, poplar species. However, a sample of propolis from Kangaroo Island was found to have novel composition, containing mainly prenylated cinnamate and stilbenes and a small proportion of flavonoids (Abu-Mellal et al., 2012).

Studies to characterize the propolis constituents showed that the main cinnamate and stilbene constituents were new chemical entities. Introduced plant species on Kangaroo Island (particularly towards the western end which was the main region of focus for this study) are far fewer in number and prominence compared with mainland Australia and Tasmania (Guerin et al., 2016; Robinson and Armstrong, 1999). Identification of the propolis constituents did not directly identify the vegetative source as the chemical constituent profiles of most Australian endemic plants have not been reported. A survey was carried out to analyse freshly deposited propolis samples either collected by researchers or donated by beekeepers from different locations across Kangaroo Island in all seasons. The samples were analysed by the ^{1}H NMR spectral profile method, mainly by comparison, with some use of statistical analysis by hierarchical clustering. For the entire island, one predominant type of propolis was identified and it was deduced to be derived from a single vegetative source.

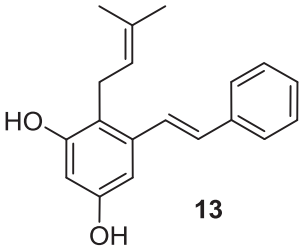
Attempts at identifying the plant source by analyzing the ^{1}H NMR spectral profiles of over 60 plant species were unsuccessful as the particular species later identified as the source was not initially collected. Unexpectedly, the plant source on which the bees were found foraging propolis was a sedge plant of the Cyperaceae family later identified as *Lepidosperma* sp. Montebello, an undescribed species widely distributed and abundant on Kangaroo Island (Fig. 4). The resin of this species is most abundant in the space between the overlapping leaves at the base of the plant, also spreading along the serrated leaf margins as the leaf emerges from the sheath, mostly on the lower half of the plant. There had been no previous reports of bees foraging for propolis on plants of the Cyperaceae family so these widespread plants had not been considered a likely source. Samples of plant exudates, resinous material carried on bee legs and freshly deposited propolis in the hive were analysed by TLC and ^{1}H NMR spectral profiling. Good



	R	R_1
Compound (**7**)	H	H
Compound (**8**)	H	CH_3
Compound (**9**)	CH_3	H
Compound (**10**)	CH_3	CH_3



	R
Compound (**11**)	H
Compound (**12**)	CH_3



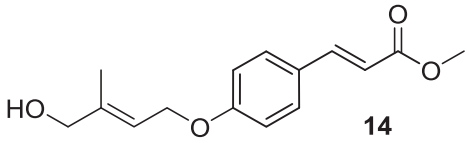


Fig. 3. Structures of prenylated hydroxystilbenes previously identified from Kangaroo Island propolis.

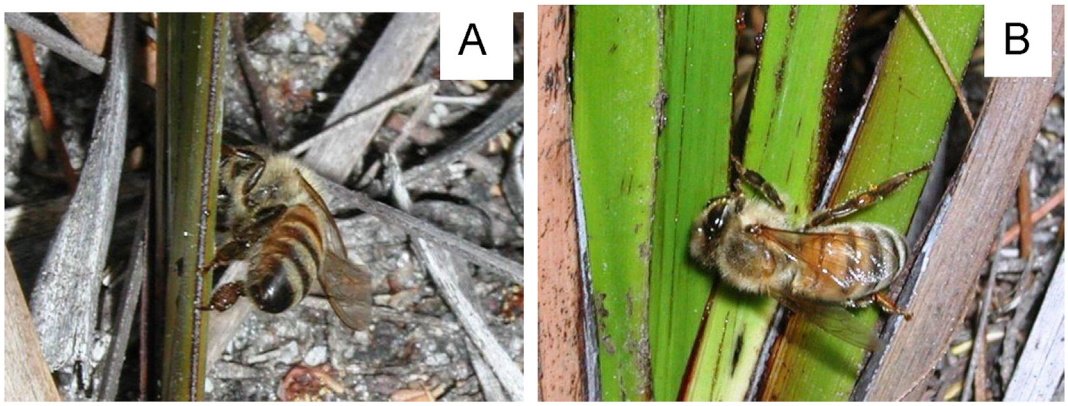


Fig. 4. ***Lepidosperma* sp. Montebello** leaf bases with honey bees carrying resin on hind legs (A) and collecting resin from leaf margins (B) *in situ*, Kangaroo Island.

statistical support was found, within a narrow range of variability for a match between the samples. The results showed that the main source of propolis from Kangaroo Island was *Lepidosperma* sp. Montebello.

A preliminary phytochemical survey found that Cyperaceae contain a unique range of flavonoids and related compounds (Harborne et al., 1985), making the family an excellent candidate for the discovery of novel compounds, including stilbenes (Dawidar et al., 1994; Abdel-Mogib et al., 2001). An ethanolic extract from *Lepidosperma viscidum* was shown to have antibacterial activity (Palombo and Semple, 2001, 2002). The L. *viscidum* species complex of which *Lepidosperma* sp. Montebello is a member, is undergoing chemotaxonomic re-appraisal through resin ^{1}H NMR spectral and morphological analyses (Plunkett et al., 2013). From a Kangaroo Island plant survey, it is reported that *L. viscidum s.l.* (i.e. the complex including the previously unrecognised *L.* sp. Montebello) is common throughout the whole island and makes up 0.5% of the flora, ranked 47th in abundance (Robinson and Armstrong, 1999). The genus *Lepidosperma* is only found in Australia and nearby regions. Recently its ecological importance (Barrett, 2013) and taxonomy (Barrett and Wilson, 2012) have been reviewed. Indigenous use of *Lepidosperma* for food and for basket weaving has been reported (Gott, 1993). However, no reports for indigenous medicinal use of *Lepidosperma* from Kangaroo Island were found.

3.2. Compounds isolated from the main Kangaroo Island propolis type

p-Coumarate and stilbenes constituents had been isolated from this main propolis type and fully characterized. The major *p*-coumarate (14) was an allylic hydroxy derivative of the parent compound (Abu-Mellal et al., 2012). The main stilbene constituents were piceatannol, prenylated and methylated derivatives of piceatannol, also prenylated resveratrol derivatives. Prenylated

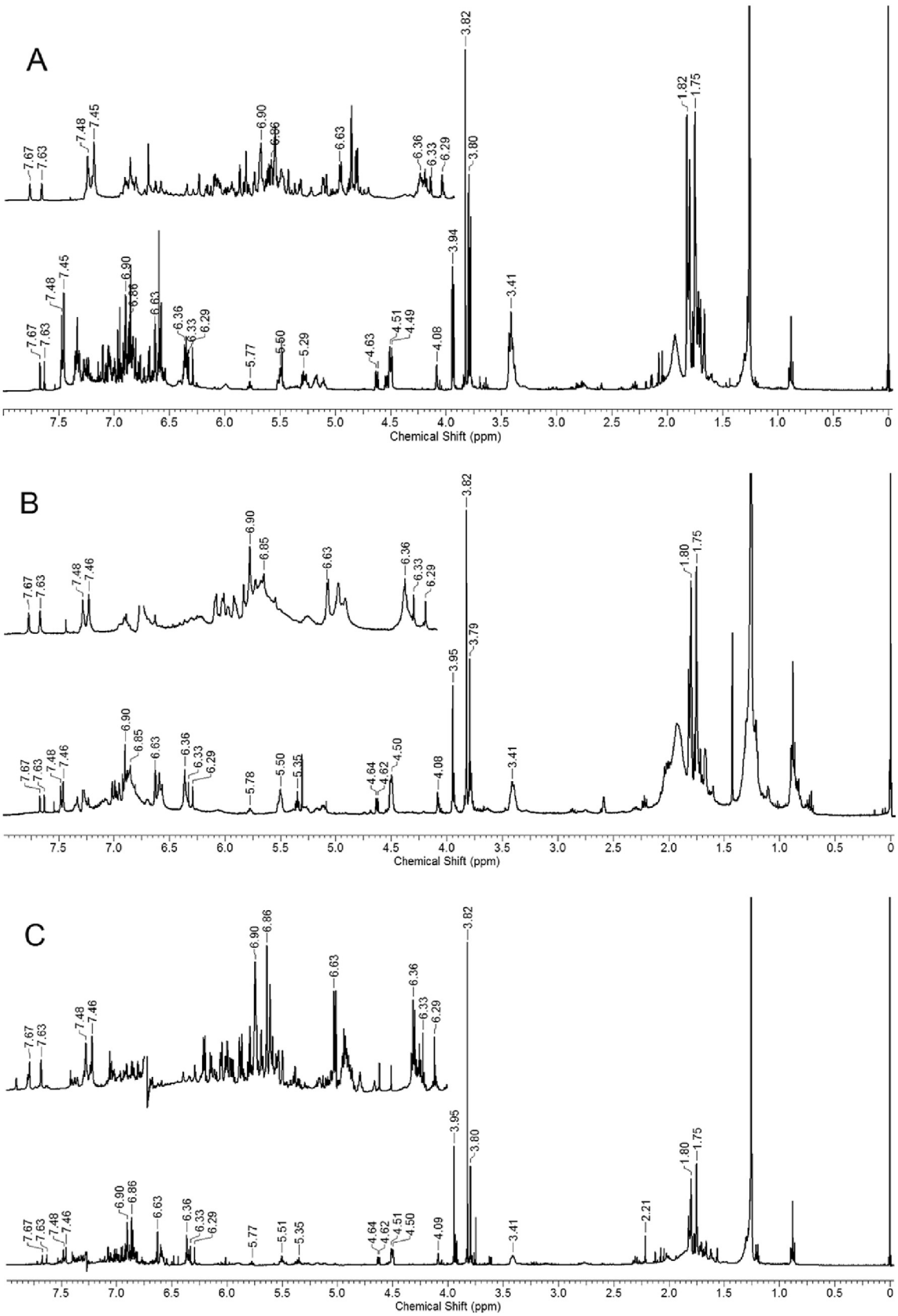


Fig. 5. ^{1}H NMR spectra of the resin of *Lepidosperma* sp. Montebello (A), honey bee hind legs (B) and of freshly deposited propolis from bee hives (C).

Table 5
Effect of prenylated hydroxystilbenes on cancer cells growth, compared with tamoxifen, expressed as GI_{50} (μM).

Compound[a,b]	1	2	3	10	Tamoxifen[c]
Cell lines					
Leukemia					
CCRF-CEM	6.56	2.73		0.291	3.84
HL-60 (TB)	2.55	2.39	4.06	0.0420	2.96
K-562	4.08	2.14	0.833	0.0385	2.58
MOLT-4	2.95	2.01	4.03	0.358	2.98
RPMI-8226	9.05	2.31	4.07	0.123	3.74
SR	2.80		0.670	0.0303	2.55
Non-Small Cell Lung Cancer					
A549/ATCC	4.01	1.88	4.33	0.400	5.08
EKVX	12.8	4.25	6.45	8.49	6.73
HOP-62	5.66	3.67	1.43	0.425	6.96
HOP-92	5.96	1.52	1.49	0.0415	3.24
NCI-H226	10.9	2.28	1.72	2.875	8.09
NCI-H23	5.66	1.81	2.92	0.204	5.51
NCI-H322M	5.90	5.29	12.5	5.98	6.73
NCI-H460	3.09	1.64	3.87	0.192	3.60
NCI-H522	2.13	1.61	0.750	0.0204	5.60
Colon Cancer					
COLO 205	15.6	1.79	14.6	4.42	3.23
HCC-2998	11.0	1.95	2.90	0.788	3.33
HCT-116	4.33	1.57	4.20	0.186	4.49
HCT-15	4.25	1.29	2.06	0.0516	4.06
HT29	17.8	3.64	14.0	4.66	2.84
KM12	3.15	1.93	3.74	0.287	3.73
SW-620	3.98	1.58	2.88	0.0365	3.64
CNS Cancer					
SF-268	6.17	2.26	4.65	0.166	5.77
SF-295	5.22	3.37	3.91	0.460	3.21
SF-539	7.32	2.21	2.96	0.0372	4.63
SNB-19	8.02	2.80	5.01	0.118	5.27
SNB-75	5.47	2.98	1.79	0.0294	4.53
U251	5.47	1.49	3.09	0.209	3.78
Melanoma					
LOX IMVI	4.48	1.71	1.36	0.0530	3.02
MALME-3M	5.53	1.97	3.14	1.76	4.11
M14	4.92	1.79	2.95	0.0373	3.22
MDA-MB-435	3.08	2.29	0.373	0.0211	3.79
SK-MEL-2	3.09	3.20	3.72	0.203	5.20
SK-MEL-28	5.64	3.65	3.05	10.5	4.31
SK-MEL-5	2.50	1.80	0.554	0.0462	3.14
UACC-257	5.35	2.18	10.2	2.93	3.87
UACC-62	5.30	2.80	2.61	1.25	5.74
Ovarian Cancer					
IGROV1	6.70	3.12	3.65	1.59	5.30
OVCAR-3	7.98	1.82	3.52	0.167	4.72
OVCAR-4	9.68	2.85	2.98	0.673	5.81
OVCAR-5	13.9	3.04	12.4	4.90	6.38
OVCAR-8	5.48	1.68	3.59	0.0789	6.79
NCI/ADR-RES	4.41	2.23	0.780	0.0298	4.97
SK-OV-3	7.96	5.98	2.85	0.120	8.21
Renal Cancer					
786–0	5.24	1.99	3.44	0.393	4.23
A498	3.88	11.1	13.0	2.09	5.16
ACHN	5.11	2.58	2.98	0.0614	6.40
CAKI-1	5.90	4.24	3.59	2.71	4.52
RXF 393	5.48	1.53	1.07	0.0442	3.04
SN12C	7.16	1.65	3.61	0.598	4.62
TK-10	6.98	3.62	3.79	2.96	4.81
UO-31	4.16	1.60	3.71	0.145	4.94
Prostate Cancer					
PC-3	6.36	2.02	6.65	0.596	3.75
DU-145	6.82	2.65	8.20	0.0491	6.31
Breast Cancer					
MCF7	0.68	2.10	2.74	0.172	1.61
MDA-MB-231/ATCC	1.63	1.49	3.15	0.153	5.60
BT-549			4.27	0.0677	6.26
HS 578T	2.40	1.84	2.71	0.0252	4.51
T-47D	2.92	1.88	2.71	1.40	2.87
MDA-MB-468	1.68	2.12	1.42	0.425	1.95
Average	**5.80**	**2.51**	**4.06**	**1.12**	**4.53**
STDev	**3.35**	**1.42**	**3.33**	**2.09**	**1.45**
Lowest	**0.68**	**1.29**	**0.37**	**0.020**	**1.61**
Highest	**17.8**	**11.1**	**14.6**	**10.5**	**8.21**

[a] Compound **4** showed no significant growth inhibition towards any of the 60 cell lines at 10 μM.
[b] Compound **5** was not tested.
[c] A drug used in chemotherapy (accessed from NCI-60 database).

stilbenes were of two types, *C*-prenylated and *O*–prenylated; di-*C*-prenylated derivatives were also identified. The majority of prenylated stilbene derivatives (Abu-Mellal et al., 2012) discovered in this propolis are built on the piceatannol molecule, a 3′-hydroxy derivative of resveratrol.

Among the 6 compounds reported (**1** to **6**) in this study, compounds **3** and **4** are rather unusual in comparison with the main prenylated stilbenes that had been previously reported (Abu-Mellal et al., 2012). Compound **3** is comprised of both *C*- and *O*-prenyl units, whilst compound **4** is a derivative of 3,3′,5,5′-tetrahydroxystilbene which is not built on the piceatannol (3,3′,4′,5-tetrahydroxystilbene) or resveratrol structure as previously reported (Abu-Mellal et al., 2012) for the main stilbene constituents. Compounds **1** and **2** are mono- and di-*C*-prenylated derivatives of piceatannol and its 3′-*O*-methyl derivative respectively. The latter compound is an unsaturated derivative of gancoanin S which was previously isolated from Kangaroo Island propolis (Abu-Mellal et al., 2012) and also from liquorice, *Glycyrrhiza uralensis* (Leguminosae) (Fukai et al., 1991). Compound **5** is an unsaturated derivative of the previously reported compound gancaonin R (Fukai et al., 1991) that has been methylated at the 3′-OH group.

3.3. *C*- and *O*-prenylated phenolic phytochemicals

Both *C*- and *O*-pTHOS were found in this single source of plant material with a variety of *C*-pTHOS derivatives. For decades, *O*-prenylated secondary metabolites have been considered merely as biosynthetic intermediates of *C*-prenylated compounds (Epifano et al., 2007) and evidence showing these phytochemicals exerting interesting and valuable biological activities is emerging.

In the last decade, natural products containing a prenyloxy chain have been recognized as valuable biologically active phytochemicals. Prenylation is a chemical or enzymatic addition of a hydrophobic isoprenoid side chain to an accepting molecule such as a terpenoid molecule, an aromatic compound, or a protein, etc. As a result the molecule may be more effective than the parent compound from a pharmacological point of view. Prenylation results in greater lipophilicity of molecules, allowing them to more readily penetrate cell membranes. An increase in lipophilicity often correlates with increased biological activity within groups of compounds of similar structure (Sobolev et al., 2011). These natural products represent a new paradigm for the development of novel drugs (Alhassan et al., 2014).

3.4. Growth inhibitory activity towards cancer cells

The initial screening for cancer growth inhibitory activity of prenylated tetrahydroxystilbenes **1**, **2**, **3** and **10** against a panel of cancer cell lines is shown in Table 5. Other studies by our research group showed that the structurally related compound **10** had potent growth inhibitory activity towards many of the 60 cell lines screened (Duke et al., 2012) making it a useful compound for comparisons. Also shown for comparison on Table 5 is the growth inhibitory activity of tamoxifen, a drug used in chemotherapy.

Compound **1** showed the weakest average activity over all the cell lines. However, the GI_{50} of 0.68 μM towards MCF7 breast cancer was close to compound **10** with a GI_{50} 0.17 μM, with compounds **2** and **3** showing weaker activities with GI_{50} of 2.1 and 2.74 μM respectively. Compound **2** showed stronger average activity compared with **1** and **3** but had only about half the average growth inhibitory activity of **10**. The highest potency of **2** was towards HCT-15 colon cancer cells with a GI_{50} of 1.29 μM compared with a GI_{50} of 0.05 μM for **10**. Overall, compound **3** showed weaker average activity towards the 60 cell lines, comparable to the weakest average activity observed for compound **1**. However, the most potent activity for **3** observed towards MDA-MB-435 melanoma cells with a GI_{50} of 0.37 μM was considerably less than the GI_{50} of 0.02 μM observed for **10** on the same cell line.

Of particular interest is that these compounds showed a broad spectrum of inhibition of cell growth against a panel of 60 cancer cell lines. This indicates that they act on a pathway that is required for cell growth in many tumor types.

4. Conclusions

High-field nuclear magnetic resonance spectroscopy was shown to be an effective analytical tool to obtain fingerprints of the propolis samples for comparison of plant resin and propolis spectral profiles. As a result, a type of propolis was recognized and its botanical origin was subsequently identified. For the first time, propolis was found to be produced by honey bees from resin collected from *Lepidosperma* sp. Montebello, a plant species of the family Cyperaceae. Identification of the botanical origin of the propolis enables adaptation of beekeeping practice for the production of propolis from a single plant species, which would have better defined and more consistent composition. Furthermore, the discovery of the prenylated tetrahydroxystilbenes in this propolis may stimulate innovative research approaches to look for new chemical entities for use in drug development and as tools for biological investigation. Abundant resins are found in a number of *Lepidosperma* species, with colour ranging from pale yellow to blood red. Investigation of other species remains a priority to determine whether further new biochemically active chemical entities may be present in related sedges.

5. Experimental

5.1. Materials

Honey bees observed collecting from the sedge (*L.* sp. Montebello) were captured in plastic tubes, capped and frozen. Sections of the bee hind legs holding resinous material were cut and pooled. Resin from plants was collected and stored at −20 °C until analysis. The plant, bee hind leg and propolis samples were extracted before analysis (see Section 5.2). Voucher specimens of representative plants (G.T. Plunkett 72 and 74) are currently lodged at N.C.W. Beadle Herbarium, University of New England, Armidale, NSW, Australia. Seventy-six beehives made up of 3 10-frame boxes each fitted with a propolis mat under the hive cover lid were used to collect propolis samples. The individually numbered hives were used on location in the apiary sites situated in the western and central southern region of Kangaroo Island. The main sample from which compounds **1** to **6** were isolated came from the Little Sahara area in the central south of Kangaroo Island.

EtOH, EtOAc, CH_2Cl_2, MeOH, hexane and AcOH were purchased from Asia Pacific Specialty Chemical Ltd (APS). Deuterated methanol (d_4-methanol), deuterated dimethyl sulfoxide (d_6-dimethyl sulfoxide) and deuterated chloroform (d-chloroform) were purchased from Sigma Aldrich Pty Ltd.

5.2. General

^1H and ^{13}C Nuclear magnetic resonance (NMR) analyses were carried out on a Varian 400 MHz System with a SMS autosampler (Palo Alto, California, USA). NMR spectra were internally referenced to tetramethylsilane (TMS). Low resolution mass spectra were obtained from a ThermoFinnigan TSQ 7000 (LC-MS/MS system) and a Finnigan Polaris Ion Trap MS/MS system (Finnigan, San Jose, USA) using an Xcalibur 1.2 data system.

High resolution ESI-MS were measured on a Bruker Daltonics Apex Ultra Fourier Transform Ion Cyclotron Resonance 7 T Mass Spectrometer.

Hierarchical clustering analysis of spectroscopic data was performed for some samples using IBM SPSS Statistics v21 (Armonk, New York, USA).

5.3. High-performance liquid chromatography (HPLC)

Preparative HPLC was performed on a Shimadzu preparative gradient LC-8A system on a reversed-phase column (Grace, Alltima C18 5 μM 22 mm ID × 250 mm), injection volume of 500 μl, eluted with MeOH (75%) and H_2O at 10 ml/min and detected at 280 nm with a UV−Vis detector (Shimadzu SPD-20A).

Analyses were initially carried out on an isocratic HPLC system (Table 3, Fig. 1S) and subsequently done on a gradient HPLC system (Table 4, Fig. 2S). Isocratic analytical HPLC was performed on a Shimadzu UFLC, LC-20AD pump, SIL-20A HT autosampler, with a Hewlett-Packard Column, NUCLEOSIL 100 C_{18}, 5 μm, 4 mm × 125 mm, injection volume 20 μl, eluted at 1 ml/min and detected at 230 nm with a UV−Vis detector (Shimadzu SPD-20A). The column was eluted with MeOH-H_2O-AcOH (65:34.8:0.2). Standards for compounds **1** to **10** were prepared as stock solutions at 1 mg/ml in MeOH and diluted to 0.025 mg/ml with mobile-phase for determination of retention times. Concentrations for standard curves were 0.00625, 0.0125, 0.1875 and 0.025 mg/ml. Extracts were analysed and peak areas used to calculate the concentration of each compound. The weight % values in Tables 3 and 4 were calculated by dividing the concentration (mg/ml) of each compound by the total concentration of the extract and multiplying by 100.

Gradient analytical HPLC was performed on a Shimadzu Nexera X2 LC-30AD system, SIL-30 autosampler, with a Hewlett-Packard Column, NUCLEOSIL 100 C_{18}, 5 μm, 4 mm × 125 mm, injection volume 10 μl, eluted at 1 ml/min and detected at 320 nm with a SPD-M30A UV−Vis diode array detector. The column was eluted with a gradient system made up of MeOH (phase A), and MeOH: H_2O: AcOH (40:59.8:0.2) (phase B). A solvent gradient was applied as follows: 0−2 min: 32−50% A, 2−9 min: 50−90% A, 9−12 min: 90−100% A, 12−14 min, 100% A, and finally 14−15 min: 100−32% A, maintained over 5 min before each new injection. Standards for compounds **1** to **10** were prepared as stock solutions at 1 mg/ml in methanol and diluted to 0.1 mg/ml with mobile-phase B.

5.4. Determination of the ^1H NMR chemical profiles of the plant and propolis specimens

Resin samples from the base of the plant (0.1 g), bee hind leg (0.01 g) and beehive propolis (1.0 g) were extracted with EtOAc at room temperature for 15 min. The extracts were filtered, dried under reduced pressure and analysed by ^1H NMR and HPLC. For ^1H NMR analyses samples (approximately 10 mg) were dissolved in a mixture of deuterated chloroform (720 μl) and deuterated dimethyl sulfoxide (80 μl). Samples from these three sources were found to contain prenylated hydroxystilbenes and *p*-coumarate as major constituents. ^1H NMR spectra of these samples are shown (Fig. 5).

Propolis samples were subsequently selected for isolation of the components.

5.5. Isolation of prenylated hydroxylstilbenes – general method

Propolis (50 g) was extracted with EtOAc (200 ml) at room temperature with stirring for 1 h. The extract was filtered, dried under reduced pressure and purified using normal-phase short-column vacuum chromatography. A step-wise gradient of mobile phase (2 × 100 ml) consisting of CH_2Cl_2 and EtOAc at 0, 1, 2, 4, 8, 10, 15, 20, 50 and 100% was employed to elute the stilbenes. The eluents were analysed by TLC and NMR. Further purification of the compounds, if required, was subsequently carried out using similar normal-phase short-column vacuum chromatography with a different mobile phase system such as hexane and EtOAc and/or reversed-phase preparative HPLC. In addition to the major prenylated hydroxystilbenes reported previously (Abu-Mellal et al., 2012), five prenylated tetrahydroxystilbenes were isolated from the propolis through a reversed-phase column with methanol: water (75:25 v/v)as mobile phase at ambient temperature. Structures and identities of these purified compounds were characterized by ^1H and ^{13}C NMR and mass spectrometry. Detailed structural analyses of the compounds were carried out by 2D NMR using Gradient Heteronuclear Multiple Bond Coherence (gHMBC) method (Fig. 2).

5.6. Identification of prenylated hydroxystilbenes

5.6.1. Compound **1**

$C_{20}H_{22}O_4$, (*E*)-4-(3-methyl-2-buten-1-yl)-3,4′,5-trihydroxy-3′-methoxystilbene. Yellowish liquid, 16 mg. For ^1H & ^{13}C NMR spectroscopic, data see Tables 1 and 2. **HRESIMS:** 325.14453 $[M - H]^-$, (calculated 325.14399).

5.6.2. Compound **2**

$C_{24}H_{28}O_4$ (*E*)-2,4-bis(3-methyl-2-buten-1-yl)-3,3′,4′,5-tetrahydroxystilbene. Colourless liquid, 26 mg. For ^1H & ^{13}C NMR spectroscopic data, see Tables 1 and 2. **HRESIMS:** 379.19148 $[M - H]^-$, (calculated 379.19094).

5.6.3. Compound **3**

$C_{24}H_{28}O_4$, (*E*)-2-(3-methyl-2-buten-1-yl)-3-(3-methyl-2-butenyloxy)-3′,4′,5-trihydroxystilbene. Colourless liquid, 12 mg. For ^1H & ^{13}C NMR spectroscopic data, see Tables 1 and 2. **HRESIMS:** 379.19148 $[M - H]^-$, (calculated 379.19094).

5.6.4. Compound **4**

$C_{24}H_{28}O_4$, (*E*)-2,6-bis(3-methyl-2-buten-1-yl)-3,3′,5,5′-tetrahydroxystilbene. Yellowish liquid, 9 mg. For ^1H & ^{13}C NMR spectroscopic data, see Tables 1 and 2. **HRESIMS:** 379.19149 $[M - H]^-$, (calculated 379.19094).

5.6.5. Compound **5**

$C_{25}H_{30}O_4$, (*E*)-2,6-bis(3-methyl-2-buten-1-yl)-3,4′,5-trihydroxy-3′-methoxystilbene. Yellowish liquid, 5 mg. For ^1H & ^{13}C NMR spectroscopic data, see Tables 1 and 2). **HRESIMS**: 417.20363 $[M + Na]^+$, (calculated 417.20418 for $C_{25}H_{30}O_4Na$).

5.6.6. Compound **6**

(*E*)-2-(3-methyl-2-buten-1-yl)-3,4′,5-trihydroxystilbene. Light yellow gum, yield 24 mg (0.2% of propolis extract) (Kumano et al., 2008) ^1H NMR: (CD_3OD, 400 MHz) δ 7.30 (2H, d, J = 8.6 Hz, H-2′, H-6′), 7.10 (1H, d, J = 16.1 Hz, H-α), 6.80 (1H, d, J = 16.1 Hz, H-β), 6.75 (2H, d, J = 8.6 Hz, H-3′, H-5′), 6.53 (1H, d, J = 2.5 Hz, H-6), 6.21 (1H, d, J = 2.5 Hz, H-4), 5.09 (1H, br t, J = 6.9 Hz, H-2″), 3.35 (2H, d, J = 6.9 Hz, H-1″), 1.78 (3H, s, H-4″), 1.66 (3H, s, H-5″).

5.7. Determination of cytotoxicity of prenylated tetrahydroxystilbenes against human tumor cell lines

Prenylated tetrahydroxystilbenes were evaluated for inhibition of cell growth against the 60 cell lines utilized at the National Cancer Institute (NCI), USA, as part of the Developmental Therapeutics Program (Shoemaker, 2006). Details of the NCI-60 Screening Methodology can be found on-line at https://dtp.cancer.gov/discovery_development/nci-60/publications.htm.

The database from which the tamoxifen NCI-60 GI_{50} data was accessed is called "GI_{50} Data (June 2016)", author is Daniel Zaharevitz, publisher NCI, publishing date June 2016, accessed 2/11/16. The database can be found on-line at https://wiki.nci.nih.gov/display/NCIDTPdata/NCI-60+Growth+Inhibition+Data.

Acknowledgments

This research was supported by an Australian National Health and Medical Research Council Complementary and Alternative Medicine Project Grant (CAM 512679). The support from Mr. Graham Carmichael (Ildoura Wild Foods, Parndana, Kangaroo Island) and Mrs. Betty McAdam (JH & E McAdam, Hog Bay Apiary, Penneshaw, Kangaroo Island, South Australia) in providing access to apiary sites and preparing beehives for propolis sampling is very much appreciated. The initial identification of plants on Kangaroo Island by Mrs Beverly Overton is gratefully acknowledged. The authors wish to thank Mr Bruce Tattam for his expertise and assistance with the mass spectral analyses. Funding for G.T. Plunkett was from the Australian Biological Resources Study, an Australian Postgraduate Award, a Noel Beadle Scholarship, and the School of Environmental and Rural Science, UNE.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.11.005.

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