KANGAROO ISLAND PROPOLIS TYPES AND THEIR DISTRIBUTION

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Introduction:
Kangaroo Island, Australia, is sparsely populated and has been designated as a sanctuary for Ligurian honeybees which produce propolis sourced from the relatively intact native flora. Research so far has identified 5 distinct types of propolis of which 3 floral sources have been identified. The major propolis type was found to be rich in a prenylated cinnamate and a range of prenylated stilbene derivatives, not previously known in propolis. Distribution of the 5 propolis types is of significance in terms of efficient production of high quality single source propolis required for development of safe and effective medicinal products.

Methods:
Over 400 propolis samples from 72 apiary sites collected 2006 to 2013 were analysed by Nuclear Magnetic Resonance (NMR) spectra; Thin-Layer Chromatography (TLC); and High-Performance Liquid Chromatography (HPLC). For the NMR, TLC and HPLC analytical profiles pattern recognition was used to identify the less complex repeating patterns characteristic of propolis derived from a single floral source.

Results and discussion:
Sedge Type 1 propolis comes from a wetland sedge and distribution corresponds to locations that are wet and prone to flooding. The best locations for this propolis are in the southern and central to eastern part of the island. Sedge Type 2 propolis comes from a dryland sedge mainly in the northern elevated well drained areas. Kangaroo thorn propolis follows geographic distribution of the plant which is relatively invasive and is abundant in botanically disturbed areas. The remaining two propolis types are relatively uncommon and have not yet been linked to a particular plant source or geographic location. Specific apiary sites suitable for commercial production of single plant source propolis from Sedge Type 1 and kangaroo thorn were identified.

Introduction:

Kangaroo Island has been separated from mainland Australia for approximately 10,000 years and there is evidence that the island has been uninhabited for at least 6000 years (Robinson and Armstrong, 1999). The island is free from large predators such that it is well known for its dense population of kangaroos and wallabies and other marsupials. The isolation and heavy grazing has affected the species and varieties of plants. The island has been re-inhabited since the early 1800's but it is still sparsely populated with much of the native fauna and flora intact. European honeybees (*Apis mellifera* ligustica) were introduced in 1883 and since 1886 the island has been designated as a sanctuary for Ligurian honeybees (Oldroyd et al., 1992) and later protected by a quarantine on bees, bee products and used beekeeping equipment imported from the mainland. Consequently, Kangaroo Island is largely free from most honeybee diseases and pests. Under these conditions beekeeping has prospered with good markets and sales for honey, beeswax, propolis and live bees efficiently produced in a pristine environment.

In 2006, from a survey of Australian propolis by this research group, Kangaroo Island propolis was found to have a unique composition as it was found to consist mainly of prenylated cinnamates and prenylated hydroxystilbenes, many of which were novel chemical substances (Abu-Mellal et al., 2012). Also the propolis and many of the propolis substituents were found to have biological activities with high medicinal potential (Koolaji et al., 2013). Studies are in progress with Kangaroo Island propolis to explore the medicinal potential and the feasibility of commercial production of high quality medicinal grade propolis.

To date 6 main propolis types have been identified together with 3 floral sources; the other 3 floral sources remain to be identified. Identification of all the major floral sources will facilitate production of the main propolis type present to the extent of approximately 70% and consisting mainly of prenylated cinnamates and prenylated hydroxystilbenes.

Methods:

Propolis was collected to a small extent by researchers and to a large extent by Kangaroo Island beekeepers. Samples sizes were 5 g to 1 Kg, the 1 Kg samples typically destined for commercial production. Researchers collected propolis deposited in hive mats over 2 to 7 day periods, and beekeepers collected propolis deposited over 7 to 21 day periods. Propolis after collection was protected from sunlight and heat and stored in a freezer (-20°C). For hive preparation for floral source determination, 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. A 3 mm gap was created by placing bamboo satay sticks (or small flat wooden sticks) across the corners of the propolis mat then replacing the hive lid. Honey bees observed collecting from flora were captured in plastic tubes, capped and frozen. Sections of the bee hind legs holding propolis were cut and pooled.

For propolis analysis preparation, propolis survey samples (2.4 g) were mixed with ethanol (50 mL) and sonicated for 20 minutes, then allowed to cool to room temperature. The fine suspension was centrifuged (3000 rpm) and filtered with filter paper. A portion of the filtrate was evaporated under reduced pressure and the residue (65 – 70% yield for ethanol soluble portion) dried under vacuum and analysed by ^{1}H-NMR.

Extraction yield, NMR, ^{1}H and ^{13}C Nuclear magnetic resonance (NMR) analyses were carried out on Varian 400 MHz System with a SMS autosampler (Palo Alto, California, USA). NMR spectra were referenced to tetramethylsilane (TMS).

For TLC, thin layer chromatography sheets precoated with silica gel 60 F_{254} were purchased from Merck. TLC plates were developed with hexane/isopropanol 4:1 and visualized with a UVGL-58 mineral-light lamp multiband UV-254/366.
Analytical HPLC was performed on Shimadzu UFLC, LC-20AD pump, SIL-20A HT autosampler, with a Hewlett-Packard Column, NUCLEOSIL 100C18, 5 µm, 4 mm × 125 mm, injection volume 20 µL, eluted with methanol-water-acetic acid (70 : 29.8 : 0.2) at 1 mL/min and detected at 230 nm with a UV-Vis detector (Shimadzu SPD-20A).

NMR profiles were examined for complexity. Simple repetitive patterns are indicative of propolis derived from a single floral source. Probability of collecting propolis from a single floral source is greatly enhanced by collecting from a hive kept at the same location for a long period of time and scraped free of propolis and with a clean propolis mat. With a short collection period of 2 days the propolis collected was usually from a single floral source. Locations may be identified where all hives are producing the same type of propolis from a single floral source. The number of bees foraging for propolis may be increased greatly by removing propolis from the mat every 2 days and by leaving a 3 mm gap between the hive mat and hive lid. On Kangaroo Island the most suitable period to carry out this practice was during late spring to mid Autumn (November to April). Bees from the hives were observed to locate the floral source of the propolis.The floral source is confirmed by comparison of the plant resin/exudates with that carried on the bees hind legs and the propolis collected at the same time from the hives.
Where standards are established by ^{1}H-NMR analyses, TLC may be used to grade propolis to identify single floral source propolis or propolis from mixed floral sources.TLC is useful as it is technically not very demanding and can give moderately specific analytical profiles where multiple detection methods are used followed by colour development with a chemical reagent. Generally after mobile phase development TLC plate chromatographic profiles (patterns) were observed with visible light, then with UV light at 254 nm and at 366 nm, then finally after chemical reagent treatment. Each stage was photographically recorded to facilitate comparisons with standards, to check the grading classification and to provide a long-term record. The multiple detection profiles enable specific detailed comparison of test samples and standard established by the NMR method such that close matches with propolis type standards enables TLC identification of propolis derived largely from a single floral source.
The more technically demanding and slower HPLC analytical process was used to give accurate quantitative profiles of identified constituents that is required for production of high quality propolis extracts for medicinal products prepared under Australian Therapeutic Goods Administration Good Manufacturing Practice conditions.
^{1}H-NMR spectra of propolis samples provide the most information rich analyses as the spectra can be processed to chemical shift and peak intensity data that can be statistically evaluated to determine clustering/matching with standard propolis samples and plant resin/exudates samples, where floral source is known.

Results and Discussion:
^{1}H-NMR analysis of propolis samples from 64 apiaries showed that the main source of propolis on Kangaroo Island was from a wetland species of the *Lepidosperma viscidum* complex, making up approximately 50% as single floral source propolis samples and approximately 70% of all the propolis on Kangaroo Island (Table 1). This type of propolis has become known as “Sedge Type 1” propolis. Field observation of bees collecting resin from a

dryland species of the *Lepidospermum viscidum* complex, together with the analysis of the plant resin, bee hind legs resin and the hive propolis have established the floral origin of a minor propolis type (0.3%) named as Sedge Type 2 propolis. From a Kangaroo Island survey the *Lepidosperma viscidum* complex is reported to make up 0.5% of the flora ranked 47th in abundance and the plant complex is common thoughout the whole of the island (Robinson and Armstrong, 1999). By similar methods, the floral source of the second most common propolis type on Kangaroo Island was found to be propolis sourced by bees from *Acacia paradoxa*, commonly known as kangaroo thorn. This propolis was found to be composed mainly of flavonoids with 3 major chalcones and two major dihydroflavonol acetates (Tran et al, 2012). Kangaroo thorn makes approximately 0.9% of Kangaroo Island flora was ranked 10th in abundance (Robinson and Armstrong, 1999).

Table 1.

Propolis from 64 Apiary Sites Analysed by ^{1}H-NMR

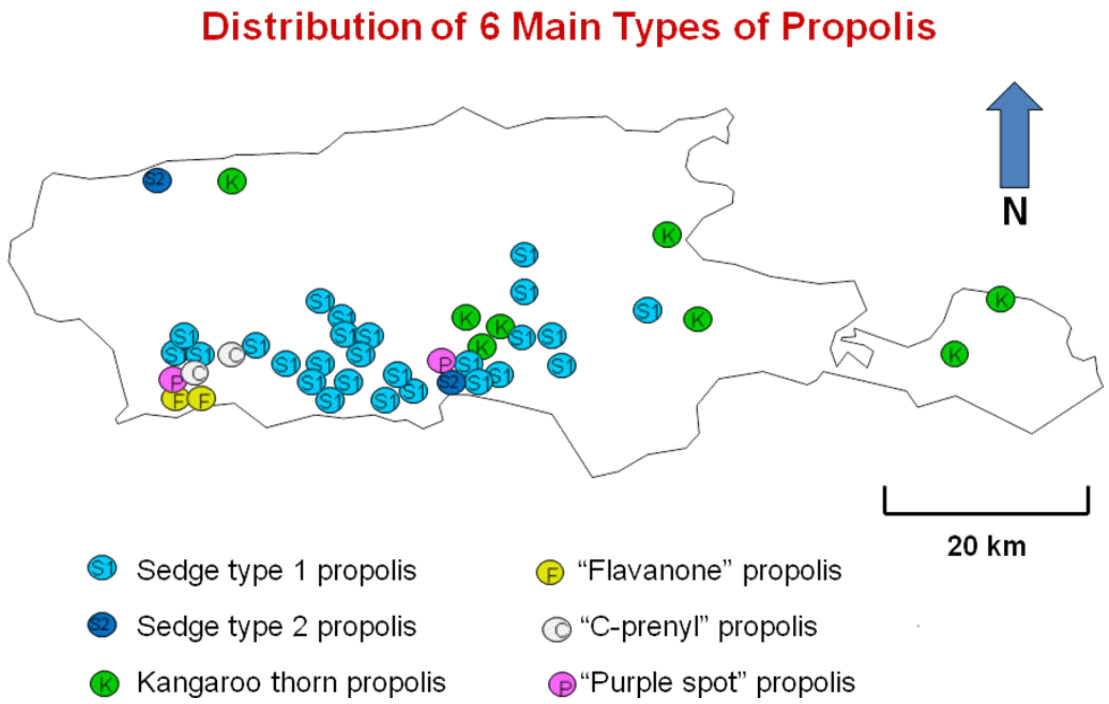
Propolis Type	# Sites	Purity	# Samples	%TOTAL
Sedge Type 1	33	80-100%	157	48.3
		70 to 90%	25	7.7
Sedge Type 2	2	80-100%	1	0.3
		70 to 90%	1	0.3
Kangaroo thorn	8	80-100%	16	4.9
		70 to 90%	3	0.9
"Flavanone"	8	80-100%	9	2.8
		70 to 90%	4	1.2
"C-Prenyl"	3	80-100%	6	1.8
		70 to 90%	1	0.3
"Purple spot"	2	80-100%	2	0.6
		70 to 90%	1	0.3
Mixed propolis	43		99	30.5
TOTAL			**325**	**100.0**

Another 3 single source propolis types are proposed based on ^{1}H-NMR and TLC analysis results and studies are in progress to identify their floral sources. Of the six propolis types identified, only the “Flavanone” type propolis has been found on the Australian mainland in the south eastern area of South Australia, in the vicinity of the eastern end of Kangaroo Island. To date, propolis from *Lepidosperma* species has not been identified or reported on the Australian mainland and the *Lepidospermum viscidum* complex variants on Kangaroo Island appear to be distinct from variants found on the mainland (Plunkett et al., 2013). Kangaroo thorn is found in disperse locations throughout south east Australia, however, no propolis with ^{1}H-NMR signals characteristic for kangaroo thorn propolis have been found so far by this research group except for propolis sourced from Kangaroo Island.

Studies so far indicate that production of large quantities of Sedge Type 1 propolis is feasible on Kangaroo Island. Localities producing kangaroo thorn propolis have been identified, however, production of commercial quantities is yet to be established.

The sampling was not even or random in terms of localities sampled and frequency of sampling as the location of apiaries depends largely on honey production and permission from landholders for sites. Also, as *Apis mellifera* is not native to Australia, beekeeping is restricted or excluded from conservation and wilderness area and from National Parks. Bias was also introduced by commercial production of Sedge Type 1 propolis as beekeepers provided more samples from sites known to produce this type of propolis.

Figure 1. Distribution of 6 main propolis types on Kangaroo Island



The results indicate that the best areas for production of Sedge Type 1 propolis are the central south to south west area of Kangaroo Island (Fig. 1). This area is less developed in terms of habitation and agriculture with much wetland and sedgeland that is relatively intact. The western end of the island is all National Park and inaccessible for beekeeping. The central north and north west of the island is elevated and well drained, generally, a more suitable habitat for the *Lepidosperma viscidum* complex dryland variant. This dryland variant produces copius quantities of resin but is not highly preferred by bees. Where both dryland and wetland sedge are available to bees, bees show a strong preference for wetland sedge such that propolis will be almost exclusively consist of Sedge Type 1 propolis. In the areas where there is only dryland sedge, there is a tendency to produce mixed propolis as other resin sources such as kangaroo thorn are preferred by bees.
The central east area has been longer term habituated with more intense agriculture. *Lepidosperma* sedges have been largely eliminated from these areas. This area has prolific stands of the invasive kangaroo thorn and is possibly suitable for production of kangaroo thorn propolis. The eastern peninsula, Dudley peninsula, has a mixed habitat and moderate agricultural development. Good stands of both dryland and wetland sedges are found but there is insufficient sampling to give an accurate measure of distribution. In conclusion, bees were found to show preference for particular resin types and from individual hives show a strong preference to collect one resin type over short time intervals. Our results indicate that on Kangaroo Island there is a strong potential to produce commercial quantities of single source propolis from *Lepidosperma* sedge and possibly from kangaroo thorn.

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