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# Examination of the Phenolic Profile and Antioxidant Activity of the Leaves of the Australian Native Plant *Smilax glyciphylla*

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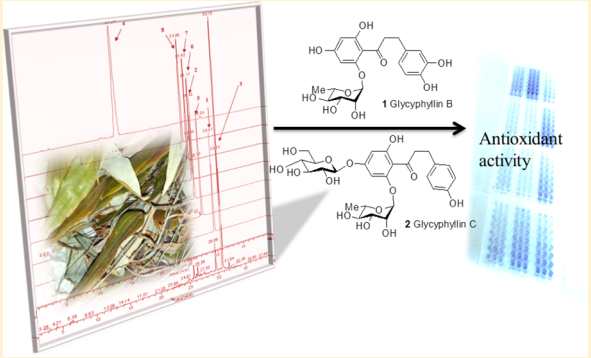
Supporting Information

**ABSTRACT:** Together with the sweet principle component glycyphyllin A (**3**), seven phenolic compounds including two new dihydrochalcone rhamnopyranosides, glycyphyllin B (**1**) and glycyphyllin C (**2**), and five known flavonoids, catechin (**4**), kaempferol-3-*O*-*β*-D-glucopyranoside (**5**), quercetin-3-*O*-*β*-D-glucopyranoside (**6**), kaempferol-3-*O*-*β*-neohesperidoside (**7**), and 2*R*,3*R*-dihydrokaempferol-3-*O*-*β*-D-glucopyranoside (**8**), have been isolated from the ethanolic extract of the leaves of *Smilax glyciphylla* for the first time. The structures of these compounds were characterized by spectroscopic methods including UV, MS, and 1D and 2D NMR. *In vitro* antioxidant capacity tests employing FRAP and DPPH assays indicated that **1**, **4**, and **6** exhibited potent antioxidant activity and are the key phenolics responsible for the antioxidant activity of the leaf extract of *S. glyciphylla*.




Polyphenols are renowned for their abilities to quench reactive oxygen species (ROS), whose free radical nature makes them unstable, short-lived, and highly reactive. ROS such as hydroxy (·OH), peroxy (·OOH), and superoxide ($O_2\cdot^-$) radicals can react readily with other molecules by hydrogen atom abstraction, which aids in their stabilization, but these processes result in the initiation of free radical chain reactions. Free radicals generated can form adducts with target molecules, resulting in modified products. These target substances can be small molecules such as lipids and coenzymes or large, biologically important molecules such as proteins and DNA, the damage of which is responsible for aging and disease in living organisms.[1−3] With increasing exposure to pollutants, radiation, drugs, inappropriate food processing, and cosmetics, substances in living organisms have become ever more susceptible to ROS. The need for preventative solutions to stop the generation of, or scavenge ROS, halt their propagation, and aid in minimizing potential possible damages to living organisms is of great importance. Polyphenols (e.g., flavonoids) are secondary metabolites produced in plants, vegetables, fruits, and some fungi and bacteria. Their wide availability and incorporation in food as edible sources render them superior substrates in battling ROS and ROS-associated problems for mankind.[4,5]

*Smilax glyciphylla*, also known as sweet sarsaparilla, is an Australian native plant growing on the eastern coast of New South Wales and Queensland. It is a climbing plant with black globose berries and lanceolate leaves. Featuring a unique bitter-sweetness on consumption of its leaves coupled with its medicinal usage by indigenous Australians, it was recommended as an alterative tonic and antiscorbutic by Sydney herbalists in the late 19th century.[6] The sweetness of this plant was found to be derived from glycyphyllin A (**3**), a rhamnoside of phloretin isolated and identified in 1886.[7] Interestingly, a later study revealed a distinct phytochemical and geographical relationship of *S. glyciphylla* where glycyphyllin A was found to be contained in the leaf samples of *S. glyciphylla* from New South Wales but absent in those from Queensland.[8] In place of glycyphyllin A (**3**), samples from Queensland contained the xanthone mangiferin as the major constituent. A recent study has shown that the hot water extract of *S. glyciphylla* leaves possesses antioxidant activity, but the authors did not specify which compounds led to this antioxidant activity, and the possibility that glycyphllin A (**3**) was a major contributor to this antioxidant activity was unsubstantiated.[9] Moreover, the high content of glycyphllin A in *S. glyciphylla* is a good source of phloretin upon hydrolysis, and the later substance has been found to be able to induce apoptosis in human leukemia, colon cancer, and breast tumor cells.[10−12] Consequently, extracts of this plant may have an underlying value that could be further developed to benefit the food, cosmetic, or pharmaceutical industries. We report herein an examination of the phenolic profile of the leaves of *S. glyciphylla* coupled with determination of the antioxidant activity of all major substances isolated, thus

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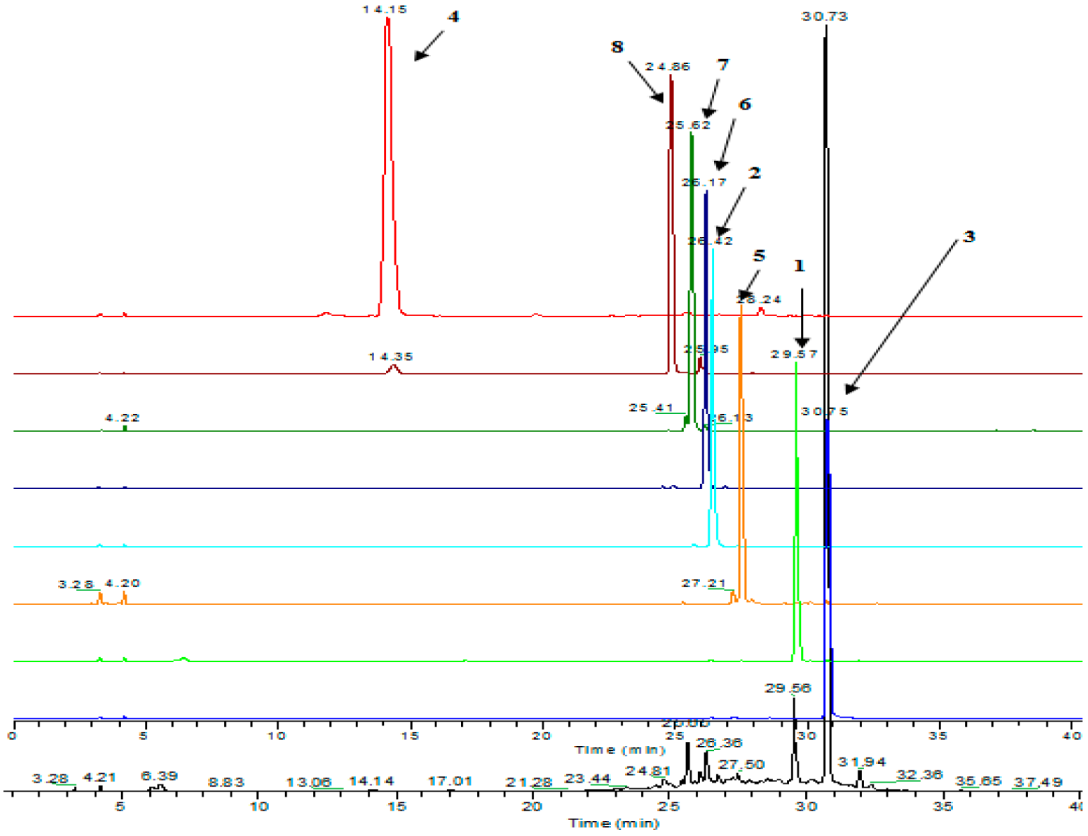


**Figure 1.** Overlaid chromatograms of the HPLC trace (280 nm) of the *n*-butanol fraction from the ethanolic extract of *S. glyciphylla* (bottom) with pure compounds **1**−**8** isolated.

providing a picture of the origin of the antioxidant activity of the leaf extract of *S. glyciphylla.*

## ■ RESULTS AND DISCUSSION

Samples of *S. glyciphylla* were collected from the Ku-ring-gai Council district in New South Wales, Australia, in September 2011. Raw samples were destemmed and leaves were air-dried upon arrival. Air-dried leaves were powdered before exhaustive extraction with 80% ethanol. Ethanolic extract concentrates were defatted with *n*-hexane followed by partitioning with *n*-butanol to obtain the polyphenol-rich *n*-butanol extract. Crude *n*-butanol extracts were separated with multilayer coil counter-current chromatography (MLCCC) using three different solvent systems successively according to the compounds' polarities. A total of eight polyphenols containing various sugar units were obtained by recrystallization and repeated silica and Sephadex LH-20 column chromatography (SCC). An overlaid chromatogram of the HPLC trace of the *n*-butanol fraction from the ethanolic extract of *S. glyciphylla* together with the pure compounds isolated is depicted in Figure 1. Pure compounds isolated were subjected to spectroscopic analysis including UV, LC-(HR)ESI-MS, and 1D and 2D NMR as well as antioxidant activity tests using FRAP and DPPH assays, and their structures are depicted in Figure 2.

Compound **1** was obtained as an amorphous yellow powder. It had an observed $[M + H]^+$ ion peak at $m/z$ 437.1433 upon HRESIMS analysis, leading to the generation of the formula $C_{21}H_{24}O_{10}$ (calculated for $C_{21}H_{24}O_{10}$, $m/z$ 437.1448). The $^{13}C$ NMR spectrum showed 21 carbon signals including one carbonyl ($\delta_{C\text{-}1}$ 206.0), one methyl ($\delta_{C\text{-}6''}$ 18.5), two methylene ($\delta_{C\text{-}2}$ 47.3, $\delta_{C\text{-}3}$ 31.0), and four aliphatic carbons attached to oxygen from $\delta_C$ 71.7 to $\delta_C$ 74.2 and 12 aromatic carbons. The $^1H$ NMR spectrum indicated the presence of a characteristic rhamnosyl moiety pattern with the anomeric and methyl protons at $\delta_H$ 5.45 and $\delta_H$ 1.27, respectively. Following HSQC correlation analysis, the connectivity between the carbons and protons was established as seven quaternary carbons, 11 CH (five from the rhamnosyl moiety), two $CH_2$ units, and one $CH_3$. COSY correlation further distinguished two sets of aromatic protons ($\delta_H$ 6.66/6.67/6.54 and 6.33/5.94) and two neighboring methylenes ($\delta_H$ 3.29/2.85) apart from the group of rhamnosyl protons. The coupling patterns and small coupling constants of H-3″ and H-5″ ($\delta_H$ 6.33, 5.94, respectively, d, $J_{3'',5''}$ = 2.4 Hz) both suggest a *meta* relationship, whereas the large coupling between H-6′ and H-5′ ($\delta_H$ 6.54, 6.66, respectively, dd, $^3J_{5',6'}$ = 7.2 Hz) and a small coupling between H-2′ and H-6′ ($\delta_H$ 6.65, 6.54, respectively, d, $^3J_{2',6'}$ = 1.8 Hz) of ring B indicated an *ortho* (H-5′, H-6′) and a *meta* (H-2′, H-5′/6′) relationship, respectively. The connectivity between the different moieties was further established based on HMBC correlation analysis (Figure 3). Methylene protons H-2 ($\delta_H$ 3.29) and H-3 ($\delta_H$ 2.85) both had HMBC correlations to the carbonyl carbon ($\delta_C$ 206.0) and C-1′ ($\delta_C$ 134.8). Additional HMBC correlations from H-3 to C-2′, C-5′, and C-6′ further established the direct connectivity between C-3 and C-1′. An HMBC correlation from the anomeric proton ($\delta_H$ 5.45) to C-2″ ($\delta_C$ 162.1) established **1** as a 2″-*O*-glycoside. Since there was no HMBC correlations from other protons to the carbonyl carbon except from the two neighboring methylene groups, C-1 was likely to be in close vicinity to the quaternary A-ring carbons. Therefore, C-2″, C-1″, and C-6″ were likely the neighboring aromatic carbons of C-1. This was evidenced by


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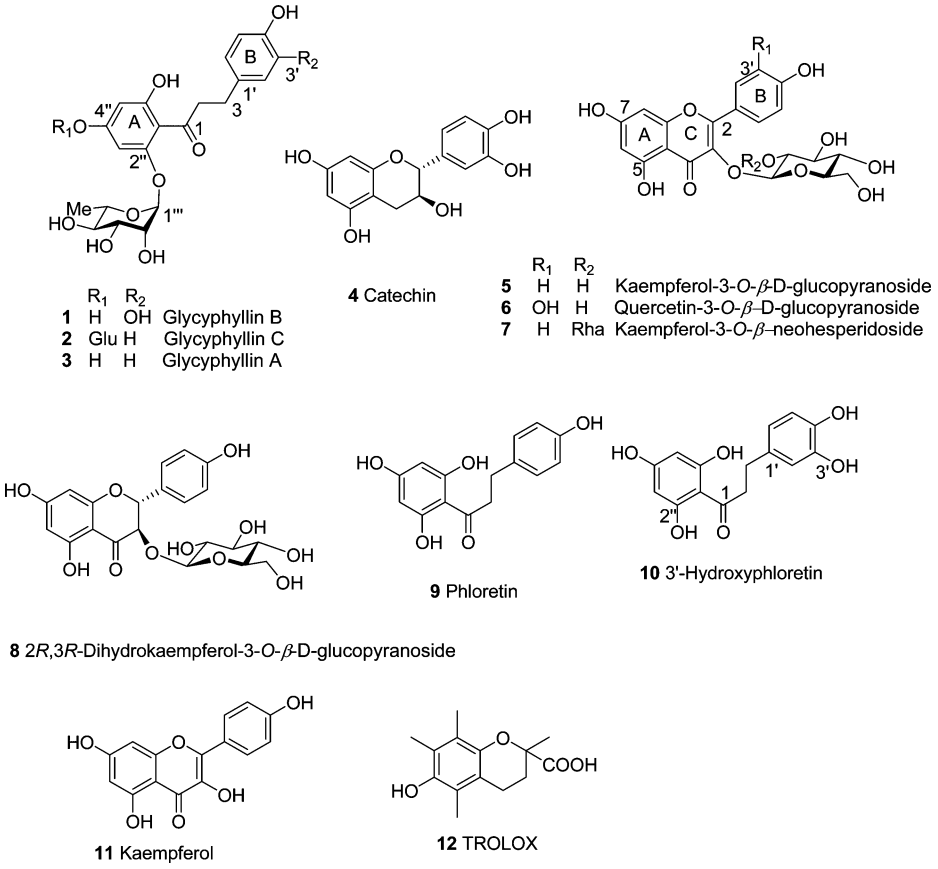


**Figure 2.** Structures of compounds **1**–**12**.

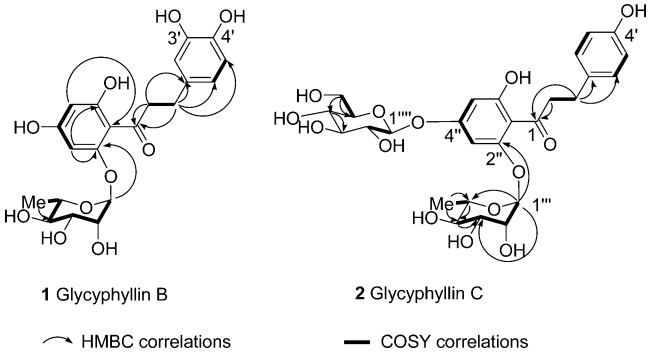


**Figure 3.** Key HMBC and COSY correlations of **1** and **2**.

the coalescence of the $^{13}$C chemical shifts of C-2″ ($\delta_C$ 162.1) and C-6″ ($\delta_C$ 168.1) of compound **1** at $\delta_C$ 166.6 in the $^{13}$C NMR spectrum of the aglycone **10** after acid hydrolysis. The structure of the aglycone was characterized as 3′-hydroxyphloretin (**10**), whose $^1$H NMR data (see Supporting Information) were in agreement with those reported previously.[13] The $\alpha$ orientation of *O*-rhamnoside was established based on the analysis of the coupling constants of rhamnosyl protons. The small $^3J_{1‴,2‴}$ = 1.8 Hz indicated an equatorial–equatorial relationship between anomeric proton H-1‴ ($\delta_H$ 5.45) and H-2‴ ($\delta_H$ 4.04). The larger coupling constants of H-3‴ ($^3J_{2‴,3‴}$ = 3.0 Hz, $^3J_{3‴,4‴}$ = 9.6 Hz) suggested H-3‴ ($\delta_H$ 3.83) was axial, while the large coupling constants for H-4‴ ($^3J_{4‴,3‴}$ = 9.6 Hz, $^3J_{4‴,5‴}$ = 9.0 Hz) indicated that H-4‴ and H-5‴ were both axially oriented. Hence, the methyl group ($\delta_H$ 1.27, $^3J_{5‴,6‴}$ = 6.0 Hz) was equatorial, indicating the sugar unit was an $\alpha$-rhamnose. The absolute configuration of **1** was also determined by LC-MS, which showed an identical retention time of the chiral derivative of its sugar unit after acid hydrolysis to that of the authentic L-rhamnose derivative. On the basis of the aforementioned analyses, the structure of **1** was established as 3′-hydroxyphloretin 2″-$\alpha$-*O*-L-rhamnopyranoside and named glycyphyllin B.

Compound **2** was obtained as a brown gum. It had an observed HRESIMS $[M + H]^+$ of *m*/*z* 583.2035, indicating a molecular formula of $C_{27}H_{34}O_{14}$ (calculated for $C_{27}H_{35}O_{14}$, *m*/*z* 583.2027). $^{13}$C NMR analysis showed 27 carbon signals including eight quaternary (including one carbonyl at $\delta_C$ 206.7), 16 methine, three methylene, and one methyl ($\delta_C$ 18.6) carbon based on $^1$H and $^{13}$C NMR and HSQC correlation analyses. The $^1$H and $^{13}$C NMR data of compound **2** resembled those of glycyphyllin A (**3**) with similar aromatic, methylene, methyl proton, and carbon signals except that **2** had one additional characteristic anomeric proton H-1‴ at $\delta_H$ 4.94, a slight shift of the other anomeric proton H-1⁗ from $\delta_H$ 5.46 (glycyphyllin A) to 5.50 ppm, and some additional saccharide-type signals in the $^1$H NMR spectrum, which indicated it was a diglycoside closely related to glycyphyllin A.[14,15] As a result of these close correlations of the aglycone moiety of **2** to those of glycyphyllin A, the aglycone unit of **2** was assumed to be phloretin, which was confirmed after further analyses of COSY, HSQC, and HMBC data. There were two groups of pyranosyl proton and carbon signals. HMBC correlations from the anomeric protons H-1‴ ($\delta_H$ 5.50, d, $^3J_{1‴,2‴}$ = 1.8 Hz) to C-2″ ($\delta_C$ 161.2) and from H-1⁗ ($\delta_H$ 4.94, d, $^3J_{1⁗,2⁗}$ = 7.2 Hz) to C-4″ ($\delta_C$ 165.2) were observed, suggesting the connectivity of the two pyranoses was to two different positions on the


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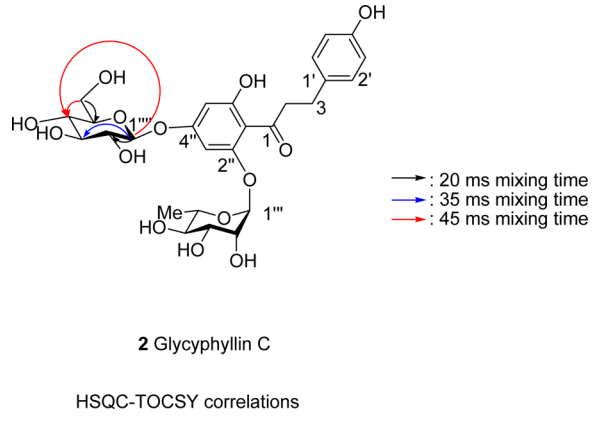
phloroglucinol A-ring as a 2″,4″-diglycoside. The additional pyranose connection to C-4″ was also supported by the $^{13}C$ NMR shift to $\delta_{C\text{-}4''}$ 165.2 from $\delta_{C\text{-}4''}$ 107.0 of glycyphyllin A (see Supporting Information). Other coupling constants of the protons ($^3J_{2''',3'''}$ = 3.0 Hz, $^3J_{5''',4'''}$ = 9.6 Hz, $^3J_{5''',6'''}$ = 6.0 Hz) indicated the presence of an $\alpha$-oriented rhamnose unit, which was confirmed by LC-MS showing an identical retention time of the chiral derivative of the sugar unit after acid hydrolysis of **2** to that of authentic L-rhamnose. The resolved coupling constants ($^3J_{1'''',2''''}$ = 7.2 Hz, $J_{6a'''',\,5''''}$ = 1.8 Hz, $J_{6b'''',\,5''''}$ = 5.1 Hz, $J_{6a'''',\,6b''''}$ = 12.0 Hz) on the second 4″-*O*-pyranose moiety suggest it to be a possible $\beta$-oriented glucose, which was confirmed by LC-MS showing an identical retention time of the chiral derivative of the sugar unit after acid hydrolysis of **2** to that of authentic D-glucose. Other coupling constants of the 4″-*O*-pyranose moiety were unable to be resolved due to overlapping. The connectivity of carbons in the 4″-*O*-pyranose unit was further determined by HSQC-TOCSY correlation analysis. Additional correlations from H-1″″ to C-3″″ ($\delta_C$ 78.4) when the spin mixing time was increased from 20 [H-1″″ to C-2″″ ($\delta_C$ 75.1)] to 30 ms and from H-1″″ to C-4″″ ($\delta_C$ 71.7) when the mixing time was increased from 30 to 45 ms established the connectivity of C-1″″; → C-2″″; → C-3″″; → C-4″″; on the 4″-*O*-pyranose unit. Methylene protons H-6a″″ and H-6b″″ also displayed additional HSQC-TOCSY correlations to C-5″″ ($\delta_C$ 78.9) and C-4″″ ($\delta_C$ 71.7), respectively, upon increasing the spin mixing time from 20 ms to 35 ms, which resolved the connectivity of C-6″″; → C-5″″; → C-4″″; and allowed the assignment of all carbons of the 4″-*O*-pyranose unit in **2**. The structures and absolute configurations of sugar moieties were confirmed and determined as D-glucose and L-rhamnose by acid hydrolysis followed by LC-MS analysis of their chiral derivatives, which had identical retention times to the chiral derivatives of authentic D-glucose and L-rhamnose. The structure of compound **2** was therefore determined as phloretin 2″-$\alpha$-*O*-L-rhamnopyranosyl-4″-$\beta$-*O*-D-glucopyranosyl diglycoside and named glycyphyllin C.

Other known compounds isolated were glycyphyllin A (**3**), catechin (**4**), kaempferol-3-*O*-$\beta$-D-glucopyranoside (**5**), kaempferol-3-*O*-$\beta$-D-glucopyranoside (**6**), quercetin-3-*O*-$\beta$-neohesperidoside (**7**), and 2*R*,3*R*-dihydrokaempferol-3-*O*-$\beta$-D-glucopyranoside (**8**). Characterization of all known compounds was based on detailed analysis of 1D and 2D NMR data (Supporting Information) and by comparison with reported data.[16−18]

*In vitro* antioxidant capacities of compounds **1**−**8** and three of their aglycones, phloretin (**9**), 3′-hydroxyphloretin (**10**), and kaempferol (**11**), were analyzed using FRAP and DPPH assays. Standard curves of all compounds tested using both FRAP and DPPH assays were plotted as shown in the Supporting Information. Compounds **2**, **7**, and **8** showed poor linearity in both assays due to their low or negligible antioxidant activities and the consequently magnifying matrix effects. Compounds **3**, **5**, and **9** exhibited low antioxidant activity with the same FRAP value of 0.33 and relatively larger standard deviations compared to the other compounds. Compounds **3**, **5**, and **9** also displayed poor linearity in the DPPH assay, as their poor radical scavenging capacities are so weak that they were dwarfed by the decomposition of the DPPH reagent. Potent antioxidant activities were observed for compounds **1**, **4**, **6**, **10**, and **11**, all of which showed good linearity in both FRAP and DPPH assays. Figures 5 and 6 display the FRAP and TE values for these compounds.




**2** Glycyphyllin C

HSQC-TOCSY correlations

**Figure 4.** Key HSQC-TOCSY correlations of **2**.

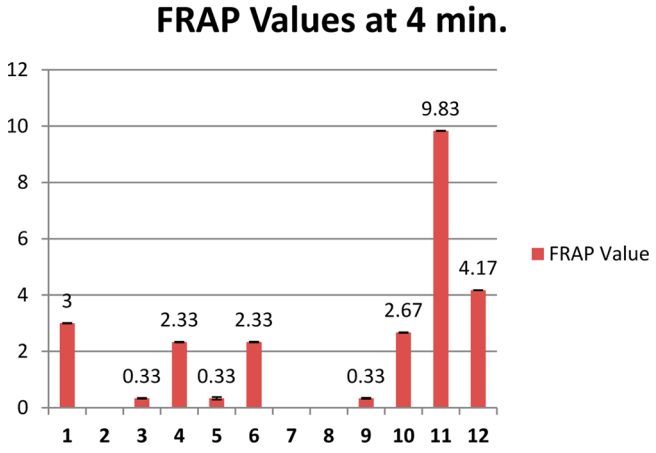



**Figure 5.** Antioxidant capacity of compounds **1**−**12** as determined by FRAP.

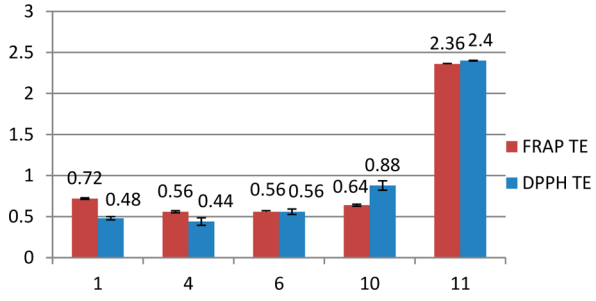



**Figure 6.** Comparison of TE values of compounds **1**, **4**, **6**, **10**, and **11** from both FRAP and DPPH assays.

Kaempferol (**11**) exhibited the most potent antioxidant activity among the five compounds, with TROLOX equivalent (TE) values of 2.36 in the FRAP assay and 2.4 in the DPPH assay (Figure 6). Due to the presence of a catechol moiety, quercetin-3-*O*-$\beta$-D-glucopyranoside (**6**) displayed good antioxidant activity with a TE value of 0.56 in both the FRAP and DPPH assays, comparable to that of catechin (**4**), which had TE values of 0.56 in the FRAP assay and 0.44 in the DPPH assay. The presence of a catechol moiety also imparts dihydrochalcone-type structures such as 3′-hydroxyphloretin (**10**) and its rhamnoside glycyphyllin B (**1**) antioxidant activities with TE values of 0.64 and 0.88 for **10** and 0.72 and 0.48 for **1** in the FRAP and DPPH assays, respectively. In contrast to **1** and **10**, glycyphyllin A (**3**) and its aglycone


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Table 1. NMR Data (600 MHz for $^1$H and 150 MHz for $^{13}$C, Methanol-$d_4$) for Glycyphyllins B (1) and C (2)

| | glycyphyllin B | | | glycyphyllin C | | |
|---|---|---|---|---|---|---|
| position | $\delta_C$, type | $\delta_H$ ($J$ in Hz) | HMBC | $\delta_C$, type | $\delta_H$ ($J$ in Hz) | HMBC |
| 1 | 206.0, C | | | 206.7, C | | |
| 2 | 47.3, $CH_2$ | 3.29, m | 1, 1′, 3 | 47.7, $CH_2$ | 3.30, m | 1, 3, 1′ |
| 3 | 31.0, $CH_2$ | 2.85, t (7.2) | 1, 2, 1′, 2′, 5′, 6′ | 31.0, $CH_2$ | 2.89, t (7.2) | 1, 2, 1′, 2′, 6′ |
| 1′ Ph′ (B) | 134.8, C | | | 133.9, C | | |
| 2′ | 117.0, CH | 6.65, d (1.8) | 3, 1′, 3′, 4′, 6′ | 130.8, CH | 7.03, d (9.0) | 2′, 3′, 4′, 5′, 6′ |
| 3′ | 146.5, C | | | 116.8, CH | 6.68, d (9.0) | 1′, 3′, 4′, 5′ |
| 4′ | 144.9, C | | | 157.0, C | | |
| 5′ | 116.9, CH | 6.66, d (7.2) | 3, 1′, 3′, 4′, 6′ | 116.8, CH | 6.68, d (9.0) | 1′, 3′, 4′, 5′ |
| 6′ | 121.3, CH | 6.54, dd (7.2, 1.8) | 2′, 4′, 5′ | 130.8, CH | 7.03, d (9.0) | 2′, 3′, 4′, 5′, 6′ |
| 1″ Ph″ (A) | 166.5, C | | | 109.1, C | | |
| 2″ | 162.1, C | | | 161.2, C | | |
| 3″ | 95.9, CH | 6.33, d (2.4) | 1″, 2″, 4″, 5″ | 96.6, CH | 6.57, d (2.4) | 1″, 2″, 4′′, 5″ |
| 4″ | 107.0 C | | | 165.2, C | | |
| 5″ | 98.6, CH | 5.94, d (2.4) | 1″ | 99.4, CH | 6.27, d (1.8) | 1″, 3″, 4″, 5″ |
| 6″ | 168.1, C | | | 167.1, C | 3.30 | |
| 1‴ Rha | 101.6, CH | 5.45, s | 2″, 3‴, 4‴, 5‴ | 101.6, CH | 5.50, d (1.8) | 2′′, 3‴, 5‴ |
| 2‴ | 72.2, CH | 4.04, dd (3.0, 1.8) | | 72.1, CH | 4.02, dd (3.0, 1.8) | 4‴ |
| 3‴ | 73.1, CH | 3.83, dd (9.6, 3.0) | | 73.0, CH | 3.77, dd (9.0, 3.0) | |
| 4‴ | 74.2, CH | 3.47, dd (9.6, 9.0) | | 74.2, CH | 3.45, m[a] | 1‴, 2‴, 3‴, 6‴ |
| 5‴ | 71.7, CH | 3.60, dq (9.0, 6.0) | | 71.8, CH | 3.57, dq (9.6, 6.0) | 4‴ |
| 6‴ | 18.5, $CH_3$ | 1.27, d (6.0) | 2‴, 4‴ | 18.6, $CH_3$ | 1.25, d (6.0) | 4‴, 5‴ |
| 1⁗ Glu | | | | 101.8, CH | 4.94, d (7.2) | 4″ |
| 2⁗ | | | | 75.1, CH | 3.45, m[a] | 1⁗, 2⁗, 3⁗ |
| 3⁗ | | | | 78.4, CH | 3.45, m[a] | 1⁗, 2⁗, 3⁗ |
| 4⁗ | | | | 71.7, CH | 3.40, m | 3⁗ |
| 5⁗ | | | | 78.9, CH | 3.45, m[a] | 1⁗, 2⁗, 3⁗ |
| 6⁗ | | | | 62.8, $CH_2$ | 3.89, dd (12, 1.8) | 4⁗ |
| | | | | | 3.70, dd (12, 5.1) | 5⁗ |

[a]Overlapping.

phloretin (**9**), both of which were devoid of catechol groups, showed weak antioxidant activity, with a FRAP value of 0.33 and no linearity in the DPPH assay.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** MLCCC separation was carried out with an MK5 LabPrep 1000 machine (AECS QuikPrep) coupled with two LC1110 HPLC pumps (GBC) and a FRAC-100 fraction collector (Phamacia Fine Chemicals). Silica column chromatography was performed using either LC60A 40−63 μm silica (Grace Davison) or silica gel 60 (0.015−0.040 mm) from Merck. Sephadex LH-20 was purchased from Amersham Pharmacia Biotech and packed and performed on a flash column. TLC was performed with TLC silica gel 60 $F_{254}$ plates (Merck KGaA) using standard vanillin stain for visualization. UV spectra were recorded on a Cintra 40 UV−visible spectrometer (GBC). LC-MS was performed with a Surveyor LCQ DECA XP Plus system (Thermo Finnigan) with an Alltima $C_{18}$ 5 μm column (250 × 2.1 mm, 5 μm, 100 Å, Grace). NMR spectra were recorded with a Varian-Inova 600 MHz spectrometer. Absorbances for FRAP and DPPH assays were recorded on a TECAN Infinite 2000 PRO plate reader utilizing 96-well plates. Solvents for preparative chromatography were all AR grade. HPLC grade solvents were used for LC-MS analysis. Phloretin and kaempferol (>90% for HPLC), which were employed in the antioxidant assays, were purchased from Sigma-Aldrich. L-(+)-Rhamnose (BDH), D-(+)-glucose (Sigma), and L-(−)-glucose (Sigma-Aldrich) were used as standards for determining the absolute configuration of the sugar units of **1** and **2**.

**Plant Materials.** Samples were collected from the Lane Cove catchment within the Ku-ring-gai Council district in New South Wales, Australia, in September 2011 and identified by Lindy Williams (Bushcare Officer, Ku-ring-gai Council). A voucher specimen (NSW972831) has been deposited in the National Herbarium of New South Wales, NSW, Australia.

**Extraction and Isolation.** Samples were destemmed and leaves were air-dried upon arrival. Dried leaves were powdered with a mechanical blender. Approximately 150 g of dried leaf powder was extracted exhaustively with 80% ethanol (1.5 L/day for 3 days). Ethanolic extracts were filtered and concentrated to approximately 300 mL under reduced pressure at a temperature below 60 °C. Combined extract concentrates were defatted by partitioning with *n*-hexane (3 × 250 mL). Defatted aqueous extracts were partitioned with *n*-butanol (5 × 150 mL), which upon solvent removal *in vacuo* and further drying under high vacuum yielded 23.7 g of a brown solid, which was sealed under nitrogen and stored at 4 °C before separation. The brown solid was separated using MLCCC combined with other column chromatography as detailed: A net loaded amount of 3.8 g of brown solid was roughly separated by solvent system 1 ($CHCl_3$/MeOH/$H_2O$ = 7:13:8, ascending mode) to afford four main fractions. Fraction 1 contained ca. 90% pure glycyphyllin A (**3**) and ca. 85% glycyphyllin B (**1**), as shown by $^1$H NMR spectra. Fraction 2 contained mainly **5**. Fraction 3 contained **4**, **6**, and **8** as a mixture. Fraction 4 contained **2** and **7** as a mixture. Pure glycyphyllin A (2.1 g) and glycyphyllin B (350 mg) were obtained after repeated recrystallization of the relatively pure fractions from hot water. Fraction 2 was subjected to a second MLCCC separation employing solvent system 2 ($CHCl_3$/MeOH/$H_2O$/*n*-BuOH = 10:10:6:1, ascending mode) followed by silica column chromatography using ultrafine silica (0.015−0.040 mm) and eluting with $CHCl_3$/MeOH (98:2, 96:4, 94:6, 92:8, 90:10, 88:12, 80:20) to furnish 7.2 mg of pure **5** as a pale yellow solid. Fractions 3 and 4 were further separated using MLCCC with solvent system 3 (EtOAc/*n*-BuOH/$H_2O$ = 1:4:5, ascending mode). Purified fractions from fractions 3 and 4 were combined correspondingly and further


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separated by SCC [eluting with MeOH/$CH_2Cl_2$ (92:8, 88:12, 86:14, 85:15, 83:17, 75:25)] to yield 1.1 mg of pure **4** and 3.4 mg of pure **2** as well as relatively pure (ca. 90%) **6**, **7**, and **8**. Recystallization of **6** (ca. 88% purity) from hot water furnished 5.6 mg of pure **6**. Pure **7** and **8** were obtained in 16.4 mg and 2.5 mg yields, respectively, by repeated chromatography with Sephadex LH-20 (MeOH/$CH_2Cl_2$, gradient elution from 10:90 to 30:70) and ultrafine silica (15−40 μm) using a gradient of MeOH/$CH_2Cl_2$ (93:7, 92:8, 90:10, 86:14, 84:16, 82:18, 75:25, 70:30) for elution.

**LC-ESIMS Analysis.** The crude brown solid from the *n*-butanol fraction and the isolated pure compounds were analyzed by LC-ESIMS. The analyses were performed with an Alltima $C_{18}$ 5 μm column employing a solvent system of 0.5% aqueous HCOOH (A) and 0.5% HCOOH in MeCN (B) as eluents. Eluting gradients were as follow: 0−10 min, 90% A to 85% A; 10−20 min, 85% A to 65% A; 20−30 min, 65% A to 10% A; 30−35 min, 10% A; 35−36 min, 10% A to 90% A; 36−46 min, 90% A. The flow rate was set at 0.2 mL/min, and the injection volume was 10 μL. Both diode array (200−600 nm) and MS were used as detectors to monitor the run. The eluting gradients for the analysis of derivatized monosaccharides by LC-MS were 0−25 min, 85% A to 65% A; 25−30 min, 65% A to 10% A; 30−35 min, 10% A; 35−36 min, 10% A to 80% A; 36−46 min, 80% A. The flow rate was 0.2 mL/min, and the injection volume was 10 μL.

*2-[3-(3,4-Dihydroxyphenyl)propanoyl]-3,5-dihydroxyphenyl α-L-rhamnopyranoside (**1**):* yellow powder; $t_R$ = 29.73 min; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 279 (3.97); $^1$H and $^{13}$C NMR, Table 1; HRESIMS *m/z* 437.1433 $[M + H]^+$ (calcd for $C_{21}H_{25}O_{10}$, *m/z* 437.1448).

*5-(β-D-Glucopyranosyl)-3-hydroxy-2-[3-(4-hydroxyphenyl)-propanoyl]phenyl α-L-rhamnopyranoside (**2**):* brown gum; $t_R$ = 26.73 min; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 285 (3.12); $^1$H and $^{13}$C NMR, Table 1; HRESIMS *m/z* 583.2035 $[M + H]^+$ (calcd for $C_{27}H_{35}O_{14}$, *m/z* 583.2027).

**Acid Hydrolysis of Compounds 1 and 2.** To a stirred solution of 4 mg of glycyphyllin B (**1**) in 1 mL of MeOH was added 1 mL of 10% aqueous HCl. The solution was heated under reflux under $N_2$ overnight until TLC indicated that the starting material had been consumed. Hydrolyzed products were extracted with EtOAc (3 × 2 mL). Combined extracts were concentrated *in vacuo* and further purified by recrystallization from hot water to furnish compound **10**. The aqueous layer was concentrated *in vacuo* (<60 °C) and further dried under high vacuum. TLC analysis of the aqueous layer showed identical $R_f$ to authentic L-(+)-rhamnose when using the lower layer of MLCCC solvent system 2 as developing solvent. The absolute configuration of the sugar moiety was further determined by LC-MS using the derivatization method developed by Tanaka with minor modification.[19] L-Cysteine methyl ester hydrochloride (1.4 mg) was mixed with the dried aqueous residue from acid hydrolysis of **1** in 0.1 mL of dry pyridine (dried over KOH followed by fractional distillation). The mixture in the vial was capped and heated to 65 °C in an oven for 60 min. Phenyl isocyanate (ca. 1.5 μL) was added, and the mixture further heated to 65 °C for 2 h. The reaction mixture was diluted 100 times in pyridine and analyzed by HPLC-MS, which showed an identical retention time at 33.20 min to authentic L-rhamnose derivative prepared in the same manner. Following the same procedure, 3.2 mg of glycyphyllin C (**2**) was hydrolyzed followed by derivatization with 1 mg of L-cysteine methyl ester hydrochloride and 1 μL of phenyl isocyanate. The sugar moieties of **2** were determined to be D-glucose and L-rhamnose by LC-MS, which showed identical retention times to D-glucose (26.97 min) and L-rhamnose (33.20 min). The L-glucose derivative has a retention time of 26.41 min using the same method.

**FRAP Assay.** Ferric reducing ability of plasma (FRAP) is a facile method for evaluating the scavenging capacity of substrates toward ROS. Compounds **1**−**12** were subjected to a FRAP assay based on methods already developed with minor modification.[20,21] Reagents were prepared as follows: (A) pH 3.6 acetate buffer was prepared by adjusting the pH of a 300 mM aqueous NaOAc solution to 3.6 with HOAc/distilled $H_2O$; (B) 10 mM 2,4,6-trispyridyl-*s*-triazine (TPTZ) was prepared by making a solution of 10 mM TPTZ in 40 mM HCl; (C) 20 mM $FeCl_3 \cdot 6H_2O$ FRAP reagent was prepared by mixing solutions A, B, and C in a ratio of 10:1:1. Stock solutions of pure compounds **1**−**12** in MeOH were all diluted with MeOH to a scale of eight concentrations (9.38, 12.50, 18.75, 25.00, 37.50, 50.00, 75.00, and 100.00 μM) on 96-well plates, respectively. Substrates (25 μL/well) with the above concentrations in triplicate were mixed with FRAP reagents (175 μL/well, prepared freshly and prewarmed to 37 °C). The lowest concentration in one of the triplicates was left blank (25 μL of methanol without sample) in order to obtain the initial FRAP reagent absorbance at 593 nm. The absorbance of the reaction mixtures at 593 nm in 96-well plates were monitored from 4 min and every 2 min afterward. Absorbance changes at different concentrations of each compound tested were plotted as a standard curve. FRAP value was calculated using the linear slope of standard curves of compounds obtained at 4 min versus the linear slope of ferrous sulfate standard curve (conducted in the same manner as other samples) obtained at 4 min. In order to compare the FRAP assay with the DPPH assay, the FRAP value was also expressed in TROLOX equivalents using the linear slope of the compounds tested versus that of TROLOX (**12**).

**DPPH.** DPPH assays were also conducted to test the free radical scavenging ability of the compounds isolated. The protocol developed here was adapted to 96-well plates based on the Brand-Williams method.[22] 2,2-Diphenyl-1-picrylhydrazyl (DPPH, 20 mg) was dissolved in 100 mL of MeOH, stored at −20 °C, and employed as a stock solution. A working DPPH solution was prepared by diluting the stock solution with MeOH to obtain an absorbance of ca. 1.0 at 515 nm (dilution ratio was ca. 2:5). Stock solutions of the pure compounds being tested were diluted to concentrations in the range 0−100 μM (9.38, 12.50, 18.75, 25.00, 37.50, 50.00, 75.00, and 100.00 μM) on 96-well plates. Substrates (50 μL/well) with the above concentrations in triplicate were mixed with freshly prepared DPPH solution (150 μL/well). The lowest concentration in one of the triplicates was left blank (50 μL of MeOH without sample) in order to obtain the initial DPPH absorbance at 515 nm. The 515 nm absorbance of the reaction mixtures in the 96-well plates were monitored at 4 min and every 2 min afterward. Absorbance changes at different concentrations of each compound tested were plotted as a standard curve. DPPH value was expressed in TROLOX equivalents and calculated using the linear slopes of standard curves obtained at 4 min versus the linear slope of the TROLOX standard curve obtained at 4 min.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Copies of 1D and 2D NMR spectra for compounds **1** and **2**, NMR data in tables for known compounds **3**−**8**, and plots of standard curves for FRAP and DPPH assays. This information is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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