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Component Analysis of Propolis from Papua New Guinea

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ABSTRACT

Propolis is an aggregate of functional components found in plant resins and has been reported to exhibit valuable biological activities. This study investigated the components and antioxidant activity of propolis from Papua New Guinea. In component analysis, seven known compounds, 6-deoxyhaplopinol (1), 5-formylguaiacol (2), *trans*-caffeic acid (3), *cis*-caffeic acid (4), *trans*-ferulic acid (5), *trans*-*p*-coumaric acid (6), and L-kaempferitrin (7), were isolated and identified from Papua New Guinean propolis. The structure of 1 was confirmed by comparing the ^{13}C NMR chemical shifts of the isolated and synthesized compounds. Based on component analysis, Papua New Guinean propolis may be a new type of propolis. The EtOH extracts of Papua New Guinean propolis exhibited antioxidant activity comparable to that of *Baccharis* and *Populus* propolis. This study demonstrated the potential of Papua New Guinean propolis in human health maintenance.

1. Introduction

Propolis is a resinous substance collected from the buds and exudates of certain trees and plants by the honeybee *Apis mellifera*. Propolis has various biological activities, including antibacterial, anti-inflammatory, antioxidant, and anticancer activities, and is used as a folk medicine in many regions around the world (Miyata *et al.* 2020; Rivera-Yañez *et al.* 2021). Generally, propolis is used in foods, beverages, and supplements to improve health and prevent conditions such as inflammation, heart disease, and cancer, as well as in cosmetics (Salantino *et al.* 2011; Sforcin and Bankova 2011).

Propolis usually contains a rich variety of chemical compounds, such as polyphenols, terpenoids, steroids, and amino acids, depending on the vegetation at the collection site. The geographical location greatly influences its chemical composition, thus imparting distinctive qualities. For example, green propolis from Minas Gerais State, Brazil, contains many terpenoids and prenylated derivatives of *p*-coumaric acid, particularly artepillin C and (*E*)-3-prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid, as the young leaves of *Baccharis dracunculifolia* are the source of the propolis (Kumazawa *et al.* 2003). In contrast, propolis from Europe and China contains many flavonoids and phenolic acid esters, such as chrysin, pinocembrin, and caffeic acid phenethyl ester, as the bud exudates of *Populus* species are the major source (Kumazawa *et al.* 2002, 2004b). Salantino *et al.* (2011) stated that although the focus of propolis research has centered mainly on Brazilian green propolis and poplar propolis, propolis collected from many other regions is also promising. Previously, we found that *Macaranga tanarius* is the source of propolis from Okinawa, which is the southernmost prefecture of Japan. Okinawan propolis contains many prenylflavonoids that exhibit strong antioxidant activities and are not present in propolis from other regions (Kumazawa *et al.* 2004a). Furthermore, differences in plant origins can affect other properties, such as texture, flavor, and color.

In this study, we aimed to examine propolis from Papua New Guinea. To the best of our knowledge, the components and biological activities of Papua New Guinean propolis have not been reported. Therefore, it would be interesting to investigate its components and biological activities. To assess its potential utility, seven compounds (**1**–**7**) were isolated from Papua New Guinean propolis, and their structures were determined by spectroscopic analysis. In addition, the antioxidant activity of Papua New Guinean propolis was evaluated.

2. Materials and Methods

2.1. General Experimental Procedures

Optical rotation values were measured using a DIP-1000 Digital Polarimeter (Jasco, Tokyo, Japan).

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1D and 2D NMR spectra were acquired with a Bruker BioSpin AVANCE-III (400 MHz) spectrometer (Rheinstetten, Germany), and chemical shifts were expressed in ppm. High-resolution electrospray ionization mass spectra (HRESIMS) were acquired using a Q-Exactive HRESI-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Silica gel column chromatography was performed using Silica Gel 60N (230–400 mesh size; Kanto Chemical, Tokyo, Japan). For reverse-phase high-performance liquid chromatography (RP-HPLC) separations with a recycling system, a PU-2086 Plus Intelligent Prep Pump (Jasco), a UV-970 detector (Jasco), a Capcell Pak C18 UG120 column (5 µm, 20 x 250 mm; Osaka Soda, Osaka, Japan), and HPLC-grade solvents were used. For analytical HPLC, a PU-980 Intelligent HPLC Pump (Jasco), a UV-2075 Plus Intelligent UV/VIS Detector (Jasco), an LG-2080-02 HPLC Ternary Gradient Unit (Jasco), and a DG-2080-53 3-Line Degasser were used. Data were analyzed using Chromato-PRO software (Lab Lab Company Co. Ltd., Tokyo, Japan).

2.2. Biological Materials

The propolis sample was collected in June 2012 in Goroka, Eastern Highlands Province, Papua New Guinea. The bee species produced the propolis was *Apis mellifera*. The *Baccharis* and *Populus* types of propolis were collected in January 1999 in Minas Gerais, Brazil (Kumazawa *et al.* 2003), and in March 2000 in Montevideo, Uruguay (Kumazawa *et al.* 2002, 2004b), respectively. These propolis samples (crude materials) were stored at –18°C in a freezer until extraction and antioxidant activity evaluation.

2.3. Extraction and Isolation

The propolis sample (16 g) was extracted with EtOH (500 ml) under stirring at room temperature for 3 days, after which the solids were removed by filtration. The filtrates were concentrated at reduced pressure to obtain an EtOH extract (7.5 g). This extract was suspended in H_2O (300 ml) and successively partitioned with *n*-hexane (2 x 300 ml) and ethyl acetate (2 x 300 ml). The ethyl acetate-soluble fraction (2.7 g) was subjected to silica gel column chromatography (30 x 450 mm) with chloroform/MeOH gradient mixtures (10:0, 100 ml; 9.75:0.25, 10 ml; 9.5:0.5, 10 ml; 9:1, 10 ml; 8.5:1.5, 10 ml; 8:2, 10 ml; 7.5:2.5, 10 ml; 7:3, 10 ml; 1:1, 10 ml; 0:1, 175 ml) to yield 18 fractions (fr. 1, 10 mg; fr. 2, 2.0 mg; fr. 3, 1.6 mg; fr. 4, 3.1 mg; fr. 5, 7.5 mg; fr. 6, 8.8 mg; fr. 7, 25 mg; fr. 8, 41 mg; fr. 9, 100 mg; fr. 10, 495 mg; fr. 11, 788 mg; fr. 12, 230 mg; fr. 13, 420 mg; fr. 14, 417 mg; fr. 15, 229 mg; fr. 16, 141 mg; fr. 17, 62 mg; fr. 18, 24 mg). Fraction 9 was subjected to preparative RP-HPLC with H_2O-acetonitrile (67:33, 0.1% trifluoroacetic acid [TFA]) as the eluent to give **1** (3.0 mg). Fraction 11 was subjected to preparative RP-HPLC with H_2O-acetonitrile (80:20, 0.1% TFA) as the eluent to give **4** (1.3 mg) and **5** (2.1 mg). Fraction 13 was subjected to preparative RP-HPLC with H_2O-acetonitrile (80:20, 0.1% TFA) as the eluent to give **2** (1.1 mg). Fraction 15 was subjected to preparative RP-HPLC with H_2O-acetonitrile (84:16, 0.1% TFA) as the eluent to give **7** (14 mg), followed by **3** (1.1 mg) and **6** (1.7 mg) with H_2O-acetonitrile (80:20, 0.5% TFA) as the eluent. All preparative RP-HPLC separations were performed at a flow rate of 10 ml/min, and the compounds were detected at a wavelength of 270 nm.

2.4. HPLC Analysis

The HPLC conditions were as follows: Capcell Pak UG120 C18 column (4.6 x 250 mm, 5 µm, Osaka Soda, Osaka, Japan) using a mobile phase consisting of H_2O (A) and acetonitrile (B) containing both 0.1% TFA; flow rate 1.0 ml/min; linear gradient of 20–100% (B) in 45 min; detection wavelength, 280 nm.

2.5. Synthesis of 1

Umbelliferone (1.0 g, 6.2 mmol, 1.0 equiv.) was dissolved in acetonitrile (20 ml) under a nitrogen atmosphere and cooled to 0°C. Sodium iodide (470 mg, 3.1 mmol, 0.5 equiv.), potassium carbonate (1.7 g, 12 mmol, 2.0 equiv.), and 1-chloro-3-methyl-2-butene (8.2 ml, 62 mmol, 10 equiv.) were added at 0°C, and the reaction mixture was slowly allowed to reach room temperature. After 16 h, the reaction mixture was quenched with H_2O and extracted with ethyl acetate (3 x 100 ml). The organic layers were dried over $MgSO_4$ and evaporated in vacuo. The residue was purified by flash column chromatography (*n*-hexane/ethyl acetate = 3:1) to give *O*-prenylumbelliferone (**8**, 1.3 g, 5.8 mmol, 95%) as a white powder.

O-Prenylumbelliferone (**8**); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.63 (d, *J* = 9.4 Hz, 1H), 7.36 (d, *J* = 8.4 Hz, 1H), 6.82–6.86 (m, 2H), 6.24 (d, *J* = 9.4 Hz, 1H), 5.47 (t, *J* = 6.8 Hz, 1H), 4.57 (d, *J* = 6.8 Hz, 1H), 1.81 (s, 3H), 1.77 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 162.1, 161.3, 155.9, 143.4, 139.3, 128.7, 118.6, 113.2, 113.0, 112.4, 101.6, 65.4, 25.8, 18.3.

O-Prenylumbelliferone (**8**, 400 mg, 1.8 mmol, 1.0 equiv.) was dissolved in dichloromethane (6 ml) and cooled to 0°C. Then, 70% *t*-butyl hydroperoxide solution (0.7 ml, 5.4 mmol, 3.0 equiv.) and selenium dioxide (100 mg, 0.9 mmol, 0.5 equiv.) were added at 0°C, and the reaction mixture was slowly allowed to reach room temperature. After 2 days, MeOH (4 ml) and sodium borohydride were added until foam formation, and the reaction was checked using KI/starch paper. The resulting solution was extracted with ethyl acetate (3 x 100 ml). The organic layers

were dried over $MgSO_4$ and evaporated in vacuo. The residue was purified by flash column chromatography (*n*-hexane/ethyl acetate = 1:1) followed by RP-HPLC (H_2O-acetonitrile = 70:30, 0.1% TFA) to afford **1** (150 mg, 35%) as a white solid.

6-Deoxyhaplopinol (**1**); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.64 (d, *J* = 9.5 Hz, 1H, H-4), 7.37 (d, *J* = 8.5 Hz, 1H, H-5), 6.85 (d, *J* = 8.5 Hz, 1H, H-6), 6.82 (s, 1H, H-8), 6.25 (d, *J* = 9.5 Hz, 1H, H-3), 5.77 (t, *J* = 6.4 Hz, 1H, H-2′), 4.67 (d, *J* = 6.4 Hz, 1H, H-1′), 4.11 (s, 2H, H-4′), 1.79 (s, 3H, H-5′), ^{13}C NMR (100 MHz, $CDCl_3$): δ = 161.9 (C-2), 161.3 (C-7), 155.9 (C-9), 143.4 (C-4), 140.9 (C-3′), 128.8 (C-5), 118.7 (C-2′), 113.2 (C-3), 113.1 (C-6), 112.6 (C-10), 101.6 (C-8), 67.6 (C-4′), 65.0 (C-1′), 14.1 (C-5′).

2.6 DPPH Free Radical Scavenging Assay

The effect of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging was evaluated using a modified method previously reported by Okumura (Okumura *et al.* 2016). The reaction mixture contained 0.15 mM DPPH/EtOH and the test sample, which was dissolved in a small amount of dimethylsulfoxide (total volume: 200 µl). After 1 h of incubation at room temperature in the dark, the absorbance was recorded at 517 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The DPPH radical scavenging rate was calculated using the following equation: Inhibition (%) = [(absorbance of the control experiment) − (absorbance of the sample experiment)] × 100/(absorbance of the control experiment). Vitamin E (α-tocopherol) was used as the positive control.

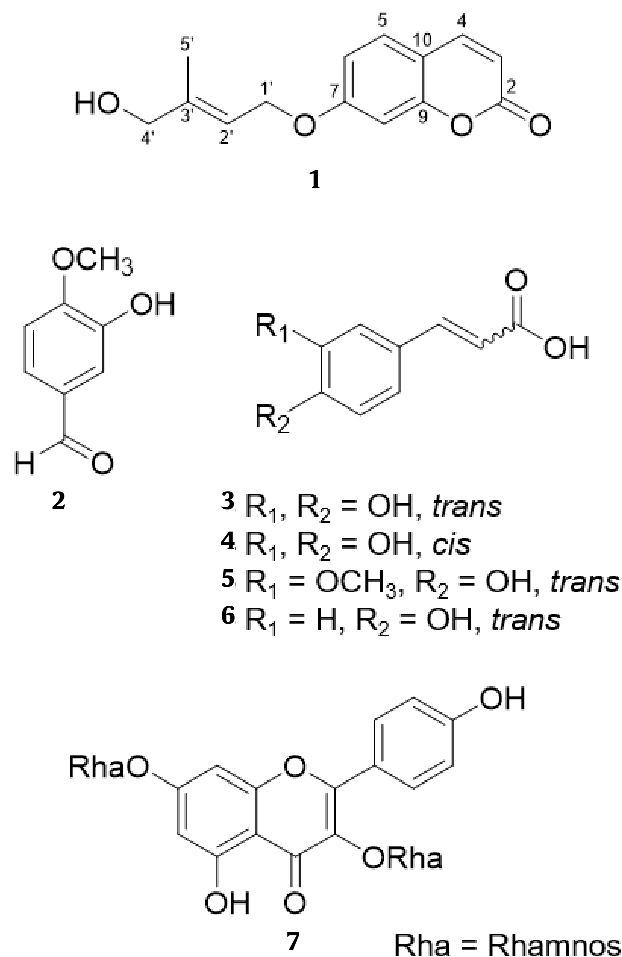


Figure 1. Structures of the compounds from Papua New Guinean propolis

3. Results

3.1. Component Analysis of Propolis from Papua New Guinea

Propolis from Papua New Guinea was extracted with ethanol by stirring at room temperature. The extract was suspended in H_2O and successively partitioned with *n*-hexane and ethyl acetate to yield *n*-hexane, ethyl acetate, and H_2O-soluble fractions. Further separation and purification of the ethyl acetate fraction led to the isolation of seven known compounds (Figures 1 and 2).

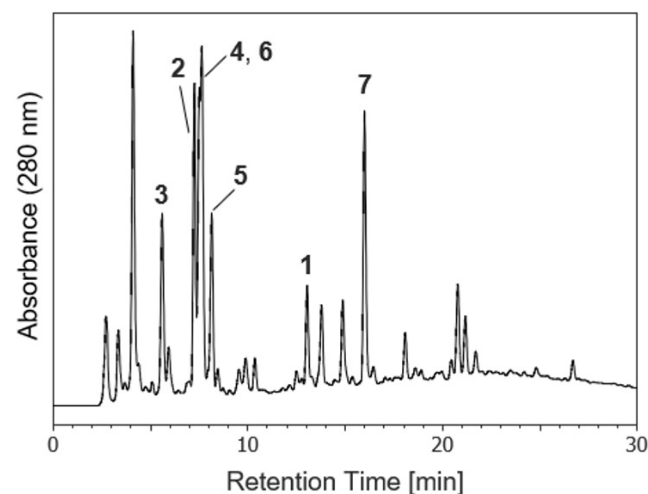


Figure 2. HPLC chromatogram of EtOH extracts of Papua New Guinean propolis

3.2. Antioxidant Activity of Propolis from Papua New Guinea

The antioxidant activity of the EtOH extracts of Papua New Guinean propolis was evaluated by DPPH assay. Brazilian *Baccharis* and Uruguayan *Populus* propolis samples were used as references. In comparison with both reference propolis samples, the EtOH extracts of Papua New Guinean propolis showed a similar level of antioxidant activity (Table 1).

Table 1. DPPH radical scavenging rate of EtOH extracts of propolis

Type of propolis	Radical scavenging rate (%)
Papua New Guinea	40.5±1.9
Baccharis	45.7±0.8
Populus	35.6±3.6

4. Discussion

Through comparison with published data, isolated compounds were identified as 5-formylguaiacol (**2**) (Imashiro *et al.* 1983), *trans*-caffeic acid (**3**) (Nakazawa and Ohsawa 1998), *cis*-caffeic acid (**4**) (Parrino *et al.* 2016), *trans*-ferulic acid (**5**) (Young *et al.* 1992), *trans-p*-coumaric acid (**6**) (Salum *et al.* 2010), and L-kaempferitrin (**7**) (Pauli 2000; Nakabayashi *et al.* 2009).

For **1**, the structure was presumed to be 6-deoxyhaplopinol based on the MS, 1D, and 2D NMR spectra. However, the chemical shift of ^{13}C NMR at the 3′ position was inconsistent with published data. In a previous report, the chemical shift at the 3′ position of **1** was δ_C 121.8 (Torres *et al.* 2006). In contrast, the ^{13}C NMR spectrum of **1** exhibited a shift of δ_C 140.9. The signals of δ_C 121–122 were not observed in the ^{13}C NMR spectrum of **1**. To determine the accuracy of the structure of **1**, we attempted to synthesize **1** and compare each spectrum. As shown in Scheme 1, **1** was synthesized using a previously reported method (Ibe *et al.* 2019). The chemical shift at the 3′ position of the synthesized **1** was δ_C 140.9. As demonstrated by the synthesis of **1**, the chemical shift for **1** was different from that in the previous study. The NMR data of **1** were re-assigned as shown in the Materials and Methods section. Compound **1** was identified as 6-deoxyhaplopinol.

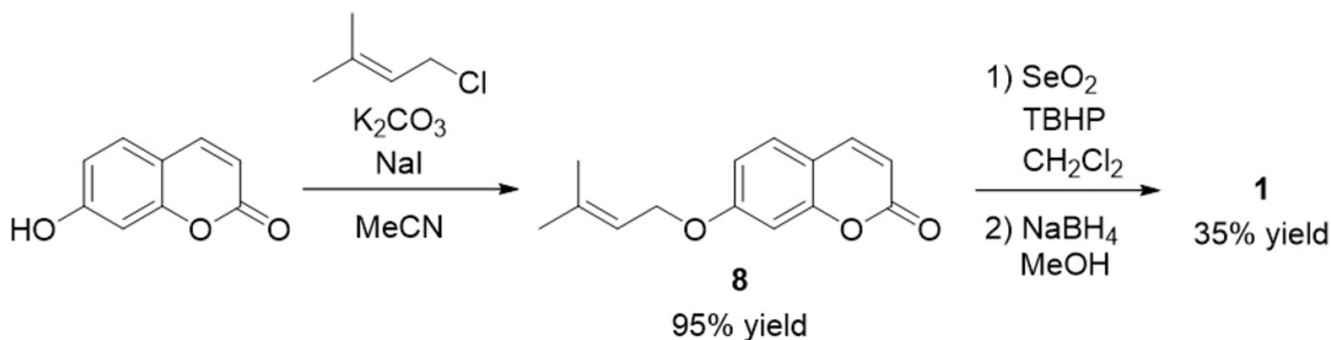
In component analysis, seven known compounds were isolated and identified from Papua New Guinean propolis. In this study, 6-deoxyhaplopinol (**1**) and L-kaempferitrin (**7**) were isolated from propolis for the first time. Therefore, Papua New Guinean propolis may be a new type of propolis. Compound **1** has been isolated from the twigs of *Melicope lunu-ankenda* (Ito *et al.* 2017). As *Melicope* plants are found in tropical regions, including Oceania (Hartley 2001), one of the sources of Papua New Guinean propolis might be a plant of the *Melicope* genus.

The antioxidant activity of Papua New Guinean propolis was also evaluated. The EtOH extracts of Papua New Guinean propolis showed antioxidant activity at levels equivalent to those of the Brazilian *Baccharis* and Uruguayan *Populus* propolis. Considering that *trans*-caffeic acid (**3**) and *cis*-caffeic acid (**4**) have antioxidant properties (Toreti *et al.*, 2013), it is likely that these compounds contribute to the antioxidant activity of Papua New Guinean propolis. Although the antibacterial and anti-inflammatory activities of Papua New Guinean propolis were also evaluated, there was no notable activity (data not shown).

This study shed light on the components and antioxidant properties of Papua New Guinean propolis. Papua New Guinean propolis is currently not used effectively. Nevertheless, Papua New Guinean propolis was found to have antioxidant activity in this study; thus, it may be used to help maintain human health.

Conflicts of Interest

No potential conflict of interest is reported by the authors.



Scheme 1. Synthesis scheme of **1**

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