

Propolis Components and Biological Activities from Stingless Bees Collected on South Sulawesi, Indonesia

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ABSTRACT

Three new compounds, namely sulabiroins A (1) and B (2), and 2',3'-dihydro-3'-hydroxypapuanic acid (3), were isolated from the propolis of stingless bees (*Tetragonula aff. bironi*) collected on South Sulawesi, Indonesia. In addition, ten known compounds, (–)-papuanic acid (4), (–)-isocalolongic acid (5), isopapuanic acid (6), isocalopolyanic acid (7), glyasperin A (8), broussonflavonol F (9), (2*S*)-5,7-dihydroxy-4'-methoxy-8-prenylflavanone (10), isorhamnetin (11), (1'*S*)-2-*trans*,4-*trans*-abscisic acid (12), and (1'*S*)-2-*cis*,4-*trans*-abscisic acid (13) were identified. The structures of the new and known compounds were determined by spectroscopic analysis. The absolute configurations of sulabiroins A (1) and B (2) were determined by X-ray crystallography analysis and ECD calculation, respectively. The propolis from stingless bee (*Tetragonula aff. bironi*) collected on South Sulawesi contained compounds not present in propolis from other regions. Sulabiroin A (1) and isorhamnetin (11) were examined for xanthine oxidase (XO) inhibitory activity as one of biological activities; isorhamnetin (11) exhibited potent XO inhibitory activity, with an IC₅₀ value of 3.9 μM.

1. Introduction

Propolis is a resinous hive product collected from different plant sources by honeybees, *Apis mellifera*. Propolis has a variety of biological activities, including antibacterial, anti-inflammatory, antioxidant, and anticancer properties, and is used as a folk medicine in many regions of the world (Marcucci 1995; Bankova *et al.* 2000; Banskota *et al.* 2001). 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 (Lotfy 2006; 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. Geographical location has a bearing on the chemical composition, thus imparting distinctive qualities to propolis. 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 propolis source (Kumazawa *et al.* 2003). On the other hand, 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 propolis source (Kumazawa *et al.* 2002, 2004b). Salantino *et al.* (2011) have stated that although the focus of propolis research centered mainly on Brazilian green propolis and poplar propolis, propolis collected on many other regions are 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 prenyl flavonoids that exhibit strong antioxidant activities and are not present in the propolis from other regions (Kumazawa *et al.* 2004a). Furthermore, differences in plant origins also affect propolis properties, such as biological activity, texture, flavor, and color.

In this study, we aimed to examine the propolis from stingless bees. Stingless bees belong to the Meliponini tribe (Bankova and Popova 2007). There are more than 300 reported species in the Meliponini tribe, which are found in tropical regions of the world (Chinh and Sommeijer 2005). The nests of *Apis mellifera* honeybees are made from their beeswax,

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and their hives are coated with propolis as a sealant. On the other hand, the nests of stingless bees are constructed of propolis, since stingless bees do not produce hexagonal beeswax combs. The entire nests of stingless bees in tropical regions are referred to as “propolis” and used as the ingredients of soaps and mouthwashes. Despite this, the propolis from stingless bees has not been well studied.

With this in mind, the components of propolis from stingless bees (*Tetragonula aff. biroii*) collected on South Sulawesi, Indonesia, and their biological activities, were studied to assess their potential utility. Three new compounds (1–3) and ten known compounds were isolated, and their structures were determined by spectroscopic analysis. In addition, the absolute configurations of sulabiroids A (1) and B (2) were determined by X-ray structure analysis and electronic circular dichroism (ECD) calculation, respectively. Moreover, some of the isolated compounds were tested for xanthine oxidase (XO) inhibitory activity. Herein, we report the structures of the isolated compounds from the propolis, as well as XO inhibitory activity testing.

2. Materials and Methods

2.1. General Experimental Procedures

Optical rotation values were measured using a DIP-1000 digital polarimeter (Jasco, Tokyo, Japan). ECD spectra were obtained using J-600 and J-820 spectrometers (Jasco). IR spectra were recorded by a FT/IR-550 spectrometer (Jasco). UV-Vis spectra were acquired using a V-560 UV/VIS spectrophotometer (Jasco). HRESIMS spectra were acquired on a Thermo Fisher Scientific Q-Exactive HR-ESI-Orbitrap-MS (Thermo Fisher Scientific Inc., MA), while 1D and 2D NMR spectra were acquired on a Bruker Biospin AVANCE-III (400 MHz) spectrometer (Bruker BioSpin, Rheinstetten, Germany), with chemical shifts expressed in ppm. Silica-gel column chromatography was carried out using silica gel 60N (230–400 mesh, Kanto Chemical, Tokyo, Japan). For RP-HPLC separations with a recycling system, a PU-1586 Intelligent prep pump (Jasco), UV-8010 detector (Tosoh, Tokyo, Japan), CAPCELL PAK UG 120 C18 column (5 μ m, 20 x 250 mm, Shiseido, Tokyo, Japan), Shiseido CAPCELL PAK UG 120 C18 column (5 μ m, 10 x 250 mm), Shiseido CAPCELL PAK ACN C18 column (5 μ m, 20 x 250 mm), and HPLC-grade solvents were used. For qualitative analysis, an instrument equipped with a PU-980 intelligent HPLC pump (Jasco), UV-970 Plus intelligent UV/VIS detector (Jasco) and a CAPCELL PAK C18 UG 120 column (5 μ m, 4.6 x 250 mm, Shiseido) were

used. For XO inhibitory assay, an instrument equipped with a PU-2089 Plus quaternary gradient pump (Jasco Co., Inc.), MD-4017 photo diode array detector (Jasco), AS-4050 HPLC autosampler (Jasco) and a CAPCELL PAK C18 UG 120 column (5 μ m, 4.6 x 250 mm, Shiseido) were used.

2.2. Biological Material

Stingless bee propolis (*Tetragonula aff. biroii*) was collected in December 2015 in North Luwu, South Sulawesi Province, Indonesia. The stingless bee species was identified by Dr. Sih Kahono from Laboratorium Entomologi, Museum Zoologicum Bogoriense, Pusat Penelitian Biologi LIPI (Entomology Laboratory, Museum Zoologicum Bogoriense, Biology Research Center, The Indonesia Science Institute). A voucher sample of the propolis (1512RPPD01) studied in this paper has been deposited at PT RIN Biotek Indonesia, South Tangerang, Banten, Indonesia.

2.3. Extraction and Isolation

Stingless bee propolis (100 g) was extracted with 70% EtOH (1.3 l) with stirring at room temperature for 24 h, after which the solids were removed by filtration. The filtrates were concentrated at reduced pressure to give an EtOH extract (25 g). This extract was suspended in H₂O (300 ml) and successively partitioned with *n*-hexane (2 x 300 ml) and EtOAc (2 x 300 ml) to give *n*-hexane- (6.1 g), EtOAc- (4.1 g), and H₂O-soluble extracts, respectively. The *n*-hexane-soluble fraction (6.1 g) was subjected to silica-gel column chromatography (20 x 400 mm), with *n*-hexane/EtOAc-MeOH gradient mixtures (4:1, 850 ml; 3:1, 1,250 ml; 2:1, 200 ml; 1:1, 400 ml; 0:1, 200 ml; MeOH 400 ml) as eluents by 200 ml for each fraction, to yield 17 fractions (fr. 1, 799 mg; fr. 2, 2.44 g; fr. 3, 289 mg; fr. 4, 790 mg; fr. 5, 130 mg; fr. 6, 314 mg; fr. 7, 14 mg; fr. 8, 211 mg; fr. 9, 16 mg; fr. 10, 75 mg; fr. 11, 30 mg; fr. 12, 214 mg; fr. 13, 54 mg; fr. 14, 148 mg; fr. 15, 12 mg; fr. 16, 392 mg; fr. 17, 241 mg). The EtOAc-soluble fraction (4.1 g) was subjected to silica-gel column chromatography (30 x 500 mm), with *n*-hexane/EtOAc-MeOH gradient mixtures (4:1, 400 ml; 3:1, 500 ml; 2:1, 300 ml; 1:1, 300 ml; 0:1, 300 ml; MeOH 300 ml) as eluents by 200 ml for each fraction, to yield nine fractions (fr. 18, 226 mg; fr. 19, 1.03 g; fr. 20, 404 mg; fr. 21, 237 mg; fr. 22, 453 mg; fr. 23, 222 mg; fr. 24, 564 mg; fr. 25, 268 mg; fr. 26, 853 mg). Fraction 3 was subjected to preparative RP-HPLC with H₂O-MeCN (25:75, 0.1% TFA (trifluoroacetic acid)) as the eluent to give 7 (2.8 mg, Rt (Retention time):30 min). Fraction 4 was subjected to preparative RP-HPLC with H₂O-MeCN (25:75, 0.1% TFA) as the eluent to give 4 (39.7 mg, Rt:40

min), 5 (9.0 mg, Rt:25 min), and 6 (2.8 mg, Rt:35 min). Fraction 6 was subjected to preparative RP-HPLC with H₂O-MeCN (40:60, 0.1% TFA) as the eluent to give 10 (1.3 mg, Rt:15 min) and 1 (31.9 mg, Rt:10 min). Fraction 8 was subjected to preparative RP-HPLC with H₂O-MeCN (40:60, 0.1% TFA) as the eluent to give 8 (6.8 mg, Rt:50 min), 9 (6.8 mg, Rt:30 min), and 2 (3.1 mg, Rt:15 min). Fractions 11–13 were subjected to preparative RP-HPLC with H₂O-MeCN (40:60, 0.1% TFA) as the eluent to give 3 (2.3 mg, Rt:30 min). Fraction 22 was subjected to preparative RP-HPLC with H₂O-MeCN (75:25, 0.1% TFA) as the eluent to give 2 (17.0 mg, Rt:15 mg). Fraction 24 was subjected to preparative RP-HPLC with H₂O-MeCN (75:25, 0.1% TFA) as the eluent to give 12 (3.6 mg, Rt:20 min) and 13 (3.9 mg, Rt:25 min) followed by 11 (7.7 mg, Rt:15 min) with H₂O-MeCN (50:50, 0.5% TFA) as the eluent. All preparative RP-HPLC separations were performed at a flow rate of 10 ml/min and compounds were detected at a wavelength of 270 nm. The purity by HPLC of all isolated compounds at a wavelength of 270 nm is >98%.

2.4. X-ray Crystallographic Data for 1

Crystals of compound 1 were obtained from 2-propanol, and the absolute configuration of 1 was determined from data collected on a Rigaku XtalLAB Synergy-S diffractometer with Cu K α radiation ($\lambda = 1.54187$) at $T = 100.2(5)$ K. The structure was solved by the SHELXT method and refined based on full-matrix least-squares on F^2 using SHELXL (Sheldrick 2015). Crystallographic data for sulabiroin A (1): plates, colorless, crystal size 0.343 x 0.077 x 0.040 mm, C₂₂H₂₂O₇, $M = 398.39$, monoclinic space group, $P2_1$, $a = 11.01570(10)$ Å, $b = 6.75950(10)$ Å, $c = 13.03660(10)$ Å, $\beta = 106.3730(10)^\circ$, $V = 931.348(18)$ Å³, $Z = 2$, $D_{\text{calcd}} = 1.421$ g/m³, 37659 collected reflections ($7.068^\circ \leq 2\theta \leq 153.24^\circ$), $\mu(\text{Cu K}\alpha) = 0.885$ mm⁻¹, $R_1 = 0.0322$ for $I \geq 2\sigma(I)$, and $wR_2 = 0.0857$ (all data), $S = 1.081$, Flack parameter = $-0.09(5)$, Hooft parameter = $-0.09(9)$. Crystallographic data for 2 have been deposited at the Cambridge Crystallographic Data Centre (CCDC number 1850729).

2.5. ECD Calculations for 2

3-Dimensional (3D) structure generation and conformational analysis of stereoisomers for 2 were carried out by use of a shell script previously reported (Ishikawa 2013). Briefly, 300 energy-minimized 3D structures of the stereoisomers were generated from the 2-dimensional chemical structures by Open Babel and Balloon (Vainio and Johnson 2007; O'Boyle *et al.* 2011; Sforcin and Bankova 2011). The single-point energy of each conformer was calculated with the PM7 Hamiltonian by MOPAC2016 (Stewart 2013). The several low-energy conformers were geometrically optimized with B3LYP/6-31G(d,p) level of density

functional theory (DFT) in gas phase by Gaussian 09 (Frisch *et al.* 2013). The theoretical calculation of ECD was conducted with B3LYP/6-31G(d,p) level of time-dependent density functional theory (TDDFT) in MeOH using the conductor-like polarizable continuum model (CPCM) by Gaussian 09. The calculated ECD data were processed and visualized by GaussView 5.

2.6. Qualitative Analysis of the Ethanol Extracts of Propolis

The mobile phases consisted of H₂O with 0.1% TFA (A) and MeCN with 0.1% TFA (B). A linear gradient of 20–100% B over 50 min followed by 100% B from 50 to 60 min at a flow rate of 1 ml/min was used. The injection volume was 10 μ l. The HPLC chromatograms are shown at 270 nm.

2.7. XO Inhibitory Assays

Allopurinol, xanthine, and xanthine oxidase were purchased from Sigma-Aldrich (St. Louis). The XO inhibitory assays were carried out following a slightly modified, previously reported method (Honda *et al.* 2017). The reaction medium, composed of 10 μ l of 1 mM xanthine in DMSO, and 160 μ l of 12.5 mM phosphate buffer (pH 7.4) was pre-incubated at 37°C for 5 min. XO buffer solution (0.020 units/ml, 20 μ l) was added to the solution. After incubation at 37°C for 10 min, 3% aqueous HClO₄ (25 μ l) was added to terminate the reaction. To quantify the amount of uric acid produced, an aliquot (20 μ l) of the solution was injected onto an HPLC column under the following conditions: column, 5 μ m, 4.6 x 250 mm, Shiseido CAPCELL PAK UG120 C18; flow rate, 1.0 ml/min; eluent, 0.1% phosphoric acid in H₂O-MeOH (96:4, v/v); detection, 290 nm. Percent inhibition was calculated according to the following equation: inhibition (%) = [(peak area of uric acid in the control experiment) – (peak area of uric acid in the sample experiment)] x 100 / (peak area of uric acid in the control experiment).

3. Results

Propolis from the stingless bee, *Tetragonula aff. biroi*, was extracted with 70% EtOH by stirring at room temperature. The extract was suspended in H₂O and successively partitioned with *n*-hexane and EtOAc to yield *n*-hexane-, EtOAc-, and H₂O-soluble fractions, respectively. Further separation and purification of the *n*-hexane and EtOAc fractions led to the identification of three new compounds (1–3) and ten known compounds (4–13) (Figure 1 and 2). The structures of all isolated compounds were identified by 1D and 2D NMR, MS, ECD, and optical-rotation spectra.

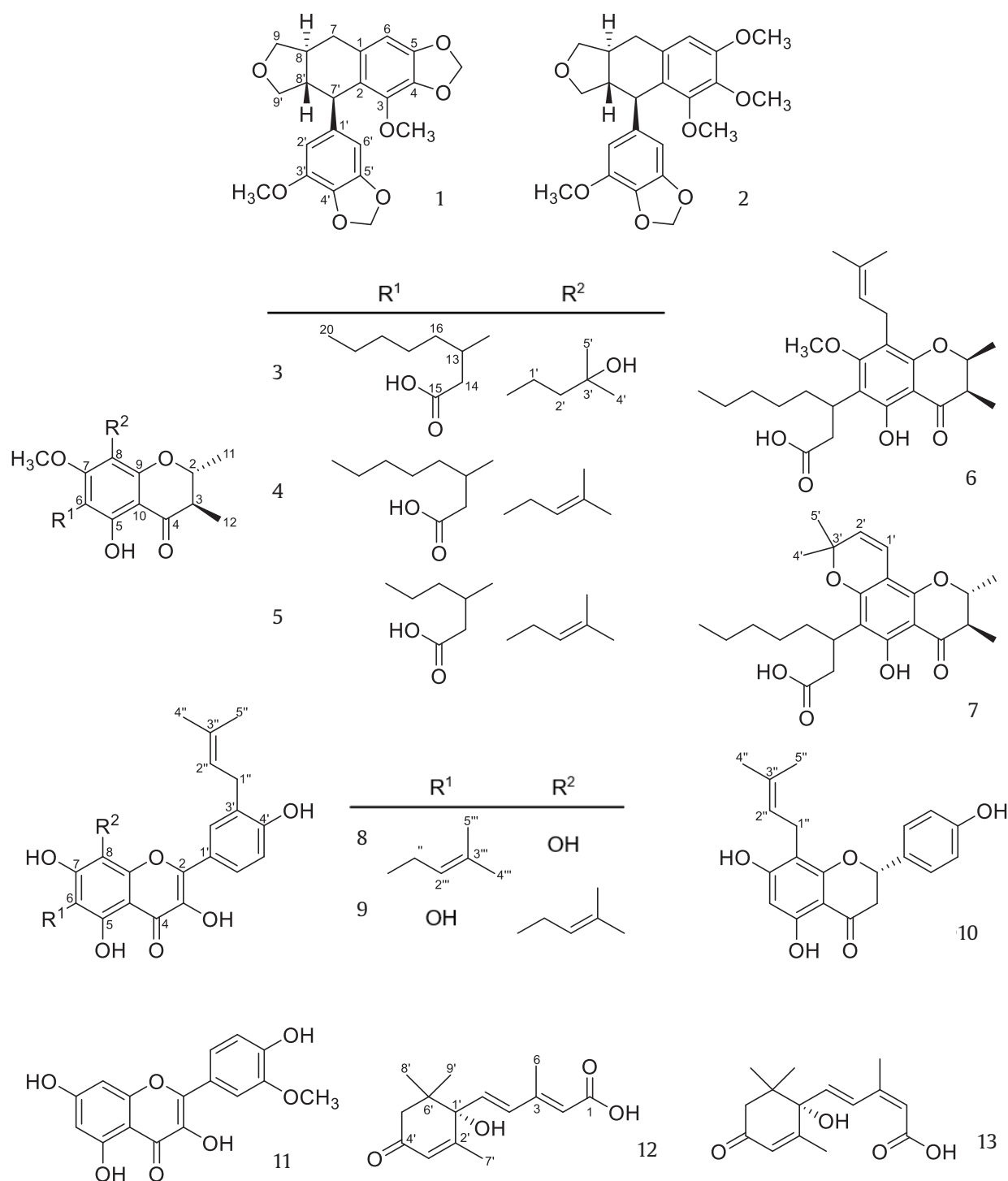


Figure 1. Structures of the compounds from stingless bee propolis collected on South Sulawesi, Indonesia

Compounds 1 and 2 were determined to be new podophyllotoxin derivatives. Compound 1 was obtained as a white powder. Its molecular formula was determined to be $C_{22}H_{22}O_7$ by HRESIMS (calcd. for $C_{22}H_{23}O_7$; 399.1444 [M+H]⁺). The absolute configurations of the stereocenters in 1 were also

determined as 8*S*, 7'*R*, 8'*S* with a Flack parameter of $-0.09(5)$ by single-crystal X-ray diffraction with Cu K α radiation. Based on the spectroscopic and crystallographic analyses, 1 was determined to be a new podophyllotoxin derivative and assigned the name "sulabiroidin A".

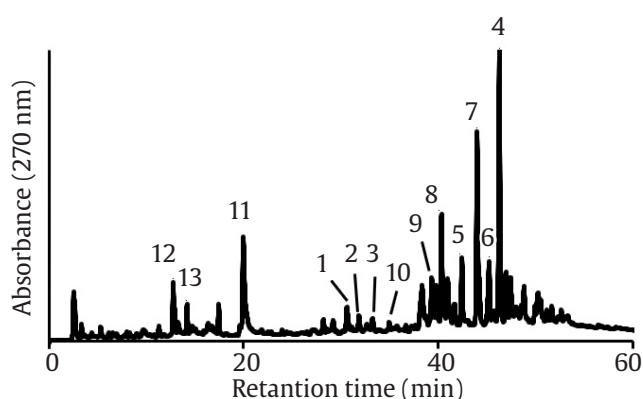


Figure 2. HPLC profiles of ethanol extracts of the yellow resin from surface of propolis from Indonesian stingless bees (*Tetragonula aff. biroi*); for peak assignment numbers, refer to Figure 1

Compound 2 was obtained as a colorless oil. Its molecular formula was determined to be $C_{23}H_{26}O_7$ by HRESIMS (calcd. for $C_{23}H_{27}O_7$:415.1757 $[M+H]^+$). The experimental ECD spectrum of 2 showed one large positive cotton effect at 220 nm, and its λ_{max} and band shape were almost identical with those of 1. Furthermore, the calculated ECD spectrum of the (8*S*,7'*R*,8'*S*) diastereoisomer of 2 was in good accordance with the experimental one. Hence, the absolute configuration of 2 was established as (8*S*,7'*R*,8'*S*). Based on the various spectroscopic analysis, 2 was also determined to be a new podophyllotoxin derivative and assigned the name "sulabiroin B".

Compound 3 was obtained as a brown oil. Its molecular formula was determined to be $C_{25}H_{38}O_7$ by HRESIMS (calcd. for $C_{25}H_{37}O_7$:449.2539 $[M-H]^-$). To determine the relative configuration between C-2 and C-3 of 3, the proton coupling constant between these protons was compared with those of calopolyanic acid (*cis*-configured C-2 and C-3) and isocalopolyanic acid (*trans*-configured C-2 and C-3), which have the same 2,3-dimethylchroman-4-one skeleton as 3 (Wang *et al.* 2010). Since the proton coupling constant between H-2 and H-3 in 3 ($J = 11.2$ Hz) was closer to that of isocalopolyanic acid ($J = 11.6$ Hz) than that of calopolyanic acid ($J = 3.1$ Hz), the relative configuration between C-2 and C-3 in 3 was determined to be a *trans*. Based on the various spectroscopic analyses, 3 was determined to be a new chromanone derivative, and assigned the name "2',3'-dihydro-3'-hydroxypapuanic acid".

The 1H NMR spectra of 4, 5, 6, and 7 revealed proton coupling constants (J) between H-2 and H-3 of 11.4 (4), 11.3 (5), 3.3 (6), and 13.9 Hz (7). The relative configurations between C-2 and C-3

in the 2,3-dimethylchroman-4-one skeletons were determined to be 4:*trans*, 5:*trans*, 6:*cis*, and 7:*trans* from the above-mentioned proton coupling constants between H-2 and H-3.

From the spectroscopic analysis and comparisons with literature data, the known compounds were identified to be isopapuanic acid (6) (Stout *et al.* 1968), isocalopolyanic acid (7) (Wang *et al.* 2010), glyasperin A (8) (Zeng *et al.* 1992), brousoflavonol F (9) (Zheng *et al.* 2008), (2*S*)-5,7-dihydroxy-4'-methoxy-8-prenylflavanone (10) (Parsons *et al.* 1993), isorhamnetin (11) (Lee *et al.* 2008), (1'*S*)-2-*trans*,4-*trans*-abscisic acid (12) (Ferrerres *et al.* 1996; Kikuzaki *et al.* 2004), and (1'*S*)-2-*cis*,4-*trans*-abscisic acid (13) (Ferrerres *et al.* 1996; Kikuzaki *et al.* 2004).

Since 1 and 11 were isolated as in high yields, they were evaluated for XO inhibitory activity. As a result, 1 exhibited little XO inhibitory activity, whereas 11 showed potent XO inhibitory activity, with an IC_{50} value of 3.9 μM . This IC_{50} value is close to that of allopurinol, which is used as anti-gout drug (IC_{50} :1.0 μM).

4. Discussion

As for the absolute configurations of chromanone derivatives, the optical rotations of (–)-papuanic acid (4) and (–)-isocalolongic acid (5) did not agree with the previously reported optical rotations, as described below. The originally isolated papuanic acid was reported to have an $[\alpha]_D$ of $+175^\circ$ (c 0.191, EtOH, $[\phi]_{589}^{+350^\circ}$ converted using the followed equation: $\alpha = \phi/2$) (Stout *et al.* 1968), while $[\alpha]_D^{25}$ of our (–)-papuanic acid (4) was -39.4° (c 0.191, EtOH). In addition, the originally isolated isocalolongic acid was reported to have an $[\alpha]_D$ of $+28^\circ$ (c 1.9, $CHCl_3$) (Guerreiro *et al.* 1973), while the $[\alpha]_D^{25}$ of our (–)-isocalolongic acid (5) was -72.4° (c 0.70, $CHCl_3$). The $[\alpha]_D$ of apetalic acid, with the same chromanone skeleton, was originally reported to be $+28.4^\circ$ (c 2.56, $CHCl_3$) (Govindachari *et al.* 1968), while the later-isolated apetalic acids were reported to have $[\alpha]_D^{25}$ values of -29.4° (c 0.15, $CHCl_3$) (Hay *et al.* 2003) and -53.0° (c 1.50, $CHCl_3$) (Huerta-Reyes *et al.* 2004). Because the configuration of the C-13 of the chromanone derivatives was neglected, these data suggest that these compounds have different absolute chromanone structures.

To the best of our knowledge, the chromanone derivatives have not been isolated from any other propolis (Figure 3). Component analysis of Indonesian propolis from stingless bees (*Tetragonula aff. biroi*) collected on South Sulawesi suggested that the plant origin of the propolis was a new type.

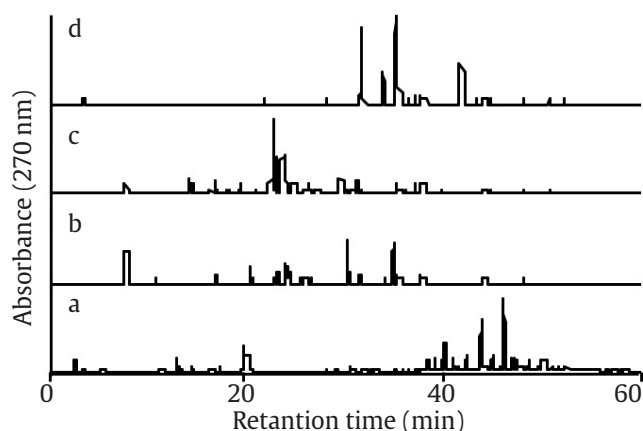


Figure 3. Comparison of HPLC chromatograms for each ethanol extract from propolis. (a) Indonesian stingless bees, (b) *Baccharis dracunculifolia* type (Brazil), (c) *Populus* type (Uruguay), and (d) *Macaranga tanarius* type (Okinawa, Japan)

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