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Component Analysis and Antiangiogenic Activity of Thailand Stingless Bee Propolis

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Abstract

Propolis is a natural resin produced by honey bees from certain plants, has gained popularity as a food and alternative medicine. However, to the best of our knowledge, few studies on native Thailand stingless bee propolis are available. Information on the chemical composition and biological activities of propolis is needed to investigate its potential utility. Recently we have reported the possible plant origin of Thailand stingless bee propolis, *Garcinia mangostana*. In this study, further component analysis, functional evaluation, and identification of the plant origin of Thailand stingless bee propolis are conducted. Nine xanthenes, including α -mangostin, garcinone C, γ -mangostin, cochinchinone T, β -mangostin, gartanin, 8-deoxygartanin, 9-hydroxycalabaxanthone, and mangostanol, were identified from the propolis. Comparative analysis of 70% ethanol extracts of Thailand stingless bee propolis (EEP) and the yellow resin from the fruit surface of *G. mangostana* (EEM) was performed using LC-MS, and similar chromatographic patterns were obtained. This result suggests that the plant origin of Thailand stingless bee propolis is confirmed to be the yellow resin from the fruit surface of *G. mangostana*. EEP and EEM were then tested for their ability to inhibit the tube formation of human umbilical vein endothelial cells, and both samples inhibited the tube formation of these cells in a concentration-dependent manner. This result indicates that Thailand stingless bee propolis may have future applications in the prevention and treatment of angiogenesis-related diseases.

Abstrak

Analisis Komponen dan Aktivitas Antiangiogenik dari Lebah Madu Tanpa Sengat Thailand. Propolis, campuran resin dari zat yang dikumpulkan oleh lebah madu dari tanaman tertentu, telah mendapatkan popularitas sebagai makanan dan obat alternatif. Namun, sejauh yang kami ketahui, hanya sedikit penelitian tentang propolis lebah madu tanpa sengat asli Thailand yang tersedia. Informasi tentang komposisi kimia dan aktivitas biologis propolis diperlukan untuk menyelidiki kegunaan potensialnya. Dalam penelitian ini, dilakukan analisis komponen, evaluasi fungsional, dan identifikasi asal tanaman dari propolis lebah madu tanpa sengat Thailand. Sembilan xanthone, termasuk α -mangostin, garcinone C, γ -mangostin, garcinone D, β -mangostin, gartanin, 8-deoxygartanin, 9-hydroxycalabaxanthone, dan mangostanol, diisolasi dari propolis. Analisis perbandingan dari 70% ekstrak etanol dari propolis lebah madu tanpa sengat Thailand (EEP) dan resin kuning dari permukaan buah *Garcinia mangostana* (EEM) dilakukan dengan menggunakan kromatografi cair fasa balik kinerja tinggi ditambah dengan spektrometri massa electrospray resolusi tinggi, dan pola-pola kromatografi serupa diperoleh. Hasil ini menunjukkan bahwa asal tanaman propolis lebah madu tanpa sengat Thailand adalah resin kuning dari permukaan buah *G. mangostana*. EEP dan EEM kemudian diuji kemampuannya untuk menghambat pembentukan tabung sel endotel vena umbilikal manusia, dan kedua sampel menghambat pembentukan tabung sel-sel ini dengan tergantung pada konsentrasi. Hasil ini menunjukkan bahwa propolis lebah madu tanpa sengat Thailand dapat memiliki aplikasi jangka panjang dalam pencegahan dan pengobatan penyakit terkait angiogenesis.

Keywords: Thailand stingless bee propolis, plant origin, Garcinia mangostana, angiogenesis, tube formation

1. Introduction

Propolis is a sticky material collected by honey bees from the bud exudates of plants. Humans in many re-

gions have used propolis as a folk medicine since ancient times. Propolis is generally known to have various chemical compositions depending on the plants surrounding bee hives [1]. The substance has been reported

to present various biological activities, including antioxidant [2], [3], antibacterial [4], [5], anti-inflammatory [6], and anticancer properties [7], [8]. For this reason, propolis is extensively used in food and beverages to improve health and prevent diseases, such as inflammation, diabetes, heart disease, and cancer [9].

Stingless honeybees are widely found in tropical and some subtropical regions all over the world, such as Thailand and Indonesia [10]. The bees are about 5 mm in length and play an important role in plant pollination in tropical regions; moreover, these bees produce propolis. In Thailand and India, stingless bee propolis is often applied to treat various maladies, such as acne, diabetes, and inflammation [11], [12]. The antioxidant and anti-tumor activities of several species of stingless bee propolis are well known [12], [13]. However, to the best of our knowledge, studies on native Thailand stingless bee propolis are limited. Therefore, information on the chemical composition, biological activities, and plant origin of Thailand propolis is needed to investigate its potential utility [14].

Angiogenesis refers to the formation of new blood vessels from preexisting ones. Folkman first observed in the early 1970s that angiogenesis is required for tumor growth [15]. Tumor-induced neovessels carry oxygen and nutrients to tumor tissues and function as the primary path of metastasis. Cutting off the blood supply of oxygen and nutrients to solid tumors represents a useful antiangiogenic strategy for tumors. Therefore, antiangiogenic treatment may be useful in the treatment and prevention of cancer progression [16]. Food factors capable of inhibiting angiogenesis, if found, would be useful to stop the progression of small cancers at an early stage.

Recently we have reported the possible plant origin of Thailand stingless bee propolis, *Garcinia mangostana* [17]. In the present study, we performed the further detailed component analysis of the propolis to confirm the plant origin. We also evaluated the effects of this propolis on angiogenesis *in vitro*.

2. Experimental

Materials. Medium 199 was purchased from Sigma (St. Louis, MO, USA). Endothelial Cell Growth Medium 2 was purchased from Promo Cell (Land Baden-Württemberg, Germany). Fetal bovine serum (FBS) was purchased from Moregate Biotech (Brisbane, Australia). Atelo Cell IPC-30 was obtained from Koken (Tokyo, Japan).

Sample preparation for HPLC analysis. Stingless bee propolis samples were collected from an orchard in Chanthaburi, Thailand in May 2016 as described previously [17]. The propolis was collected from different parts of three beehives and stored at 0–4 °C before testing. Yellow resin from the fruit surface of

Garcinia mangostana was collected in the same orchard in June 2017. Propolis and *G. mangostana* resin were extracted with 70% ethanol (EtOH) at room temperature for 24 hours to yield the corresponding EtOH extracts (propolis: EEP; resin: EEM). Each sample was dissolved in MeOH and filtered through a 0.22 µm membrane filter (Starlab Scientific, Shaanxi, China) before HPLC analysis.

Isolation of components. Thailand Stingless bee propolis (96.0 g) was extracted with 70% EtOH (1.2 L) at room temperature for 24 hours and filtered. All filtrates were concentrated under reduced pressure to yield 18.5 g of EtOH extract. The extract was suspended in H₂O (300 mL) and successively partitioned with *n*-hexane (2×300 mL) and ethyl acetate (EtOAc) (2×300 mL) to yield *n*-hexane (1.2 g), EtOAc (3.8 g), and H₂O (11.4 g) extracts. The EtOAc extract (3.8 g) was separated by column chromatography over silica gel 60N (230–400 mesh, Kanto Chemical, Tokyo, Japan). The column was sequentially eluted with gradient mixtures of *n*-hexane/EtOAc-MeOH to yield 26 fractions. We isolated nine compounds (**a-i**) from the fractions by preparative HPLC with an ODS column [17].

Instruments. High-resolution electrospray ionization mass spectra (HR-ESIMS) were recorded using an Accela LC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a quadrupole mass spectrometer (Q-Exactive; Thermo Fisher Scientific). 1D and 2D NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer (Bruker BioSpin, Billerica, MA, USA). Chemical shifts (δ) are reported in ppm, and coupling constants (J) are reported in Hz. Chemical shifts in the ¹H and ¹³C NMR spectra were corrected using the residual solvent signals. For RP-HPLC separation with a recycling system, a PU-2086 Plus intelligent prep pump (Jasco Co., Inc, Tokyo, Japan), UV-2075 Plus intelligent UV/VIS detector (Jasco Co., Inc.), CAPCELL PAK C18 UG 120 column (5 µm, 20×250 mm, Shiseido, Tokyo, Japan), and HPLC-grade solvents were used. For qualitative analysis, an instrument equipped with a PU-980 intelligent HPLC pump (Jasco Co., Inc.), UV-970 Plus intelligent UV/VIS detector (Jasco Co., Inc.), and CAPCELL PAK C18 UG 120 column (5 µm, 4.6×250 mm, Shiseido) were used. For quantitative analysis, an instrument equipped with a PU-2089 Plus quaternary gradient pump (Jasco Co., Inc.), MD-4017 photo diode array detector (Jasco Co., Inc.), AS-4050 HPLC autosampler (Jasco Co., Inc.), and CAPCELL PAK C18 UG 120 column (5 µm, 4.6 × 250 mm, Shiseido) were used. The mobile phases consisted of water with 0.1% TFA (A) and acetonitrile with 0.1% TFA (B). A linear gradient of 20%–100% B over 50 min followed by 100% B from 50 min to 60 min at a flow rate of 1 mL/min was applied. The injection volume was 10 µL.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were grown in HUVEC growth medium (Endothelial Cell Growth Medium 2 with 0.02 mL/mL fetal calf serum, 5 mg/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 20 ng/mL insulin-like growth factor, 0.5 ng/mL vascular endothelial growth factor 165, 1 µg/mL ascorbic acid, 22.5 µg/mL heparin, and 0.2 µg/mL hydrocortisone) (Promo Cell, Land Baden-Württemberg, Germany) and incubated at 37 °C under a humidified 95/5% (v/v) mixture of air and CO₂. The cells were seeded on plates coated with 0.1% gelatin and allowed to grow to subconfluence before the experimental treatments.

Tube formation assay. Capillary tube-like structures formed by HUVECs in collagen gel were prepared as previously described with slight modifications [18]. Collagen gels were made by Atelo Cell IPC-30 (type I collagen). Exactly 200 µL of collagen solution (0.21% in Medium-199) was poured into the wells of a 24-well plate, which was then incubated at 37 °C for 30 min to solidify the gels. HUVECs (6.0×10^4) in Endothelial Cell Growth Medium 2 with 10% FBS were seeded onto the collagen-coated wells and left at 37 °C in a 5% CO₂ incubator for 1 hour to attach to the collagen gel. After removal of the medium, 150 µL of the collagen solution was overlaid on the wells, and gelation was performed once more as described above. Next, 650 µL of Endothelial Cell Growth Medium 2 with 10% FBS supplemented with 8 nM/mL phorbol 12-myristate 13-acetate (PMA), together with various concentrations of the samples, was added to the wells and incubated for up to 48 hours. The resulting web-like capillary structures were viewed under a microscope, and images were captured using an Olympus E-620 digital camera (Olympus, Tokyo, Japan).

DPPH free radical scavenging activity. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was carried out to evaluate the antioxidant activity of propolis [19]. EEP and EEM were first dissolved in dimethyl sulfoxide (DMSO) to 100 mg/mL and then diluted with 50% EtOH at twice concentration. Aliquots of these solutions (100 µL) were added to 100 µL of 0.2 mM DPPH in EtOH. The final concentrations of EEP and EEM were 12.5, 25, 50, and 100 µg/mL. After incubation in the dark at room temperature for 30 minutes, the absorbance of the solutions was recorded at 517 nm. The control solution only contained EtOH and DPPH. The results are expressed as the percentage decrease in absorbance with respect to the control values.

Quantification analysis of γ -mangostin and gartanin. Quantification of γ -mangostin and gartanin, which are known to have antioxidant activity, was performed using HPLC. γ -Mangostin standard was purchased from Sigma-Aldrich (St. Louis, MO, USA), while gartanin standard was purchased from Fujifilm Wako Pure

Chemical Corporation (Osaka, Japan). The calibration curve was produced for each standard compound. Analyses of EEP and EEM were conducted thrice, and the standard deviation was calculated. A recovery test was conducted using the standard addition method.

3. Results and Discussion

Chemical composition. The chemical profile of 70% EtOH extracts of propolis was studied by HR-ESIMS and NMR. The chromatographic patterns of Thailand stingless bee propolis differed from those of standard propolis patterns Brazilian [20] and Uruguayan [21]. Thus, isolation and identification of the compounds in stingless bee propolis is necessary to reveal its specific chemistry and plant origin.

The ethyl acetate (EtOAc) fraction of the 70% EtOH extract (EEP) was subjected to repeated chromatographic separation, and nine compounds were isolated and identified (Figure 1, Table 1) [22]-[24], including some prenylated xanthenes: α -mangostin (**a**), garcinone C (**b**), γ -mangostin (**c**), cochinchinone T (**d**), β -mangostin (**e**), gartanin (**f**), 8-deoxygartanin (**g**), 9-hydroxycalabaxanthone (**h**), and mangostanol (**i**). Most compounds were previously isolated from the pericarps of *G. mangostana* [25], [26]. The pericarp has long been used in Thai indigenous medicine to treat trauma, diarrhea, and skin infections [27]. In the previous studies, we concluded that **b** is garcinone D [17]. However, in the further detailed analysis, it was revealed that **b** is cochinchinone T [28]. Cochinchinone T is the first isolated from propolis as far as we know.

Identification of the plant origin of propolis. We focused on *G. mangostana* (mangosteen) after our chemical composition studies. Comparative analysis of EEP and EEM were performed using RP-HPLC coupled with HR-ESIMS. The extracts showed similar chromatographic patterns (Figure 2). Thus, *G. mangostana* may be the plant origin of Thailand stingless bee propolis [17].

We further compared the DPPH radical scavenging activities of the two extracts together with the EtOH extracts of Brazilian [20] and Uruguayan [21] propolis. The plant origins of Brazilian and Uruguayan propolis are *Baccharis dracunculifolia* and poplar species, respectively [21], [29]. At 100 µg/mL, all extracts exhibited potent radical scavenging activity. The activities of EEP, EEM, and Brazilian and Uruguayan propolis EtOH extracts were $46.84\% \pm 4.73\%$, $85.55\% \pm 3.03\%$, $63.55\% \pm 2.52\%$, and $69.28\% \pm 3.25\%$, respectively, as we previously described [17]. The contents of γ -mangostin and gartanin in EEP and EEM were subsequently quantified by HPLC; these two compounds have been reported to possess antioxidant activity [14], [25]. Our results are presented in Table 2. Recovery tests indicated a percentage recovery of 86.2%.

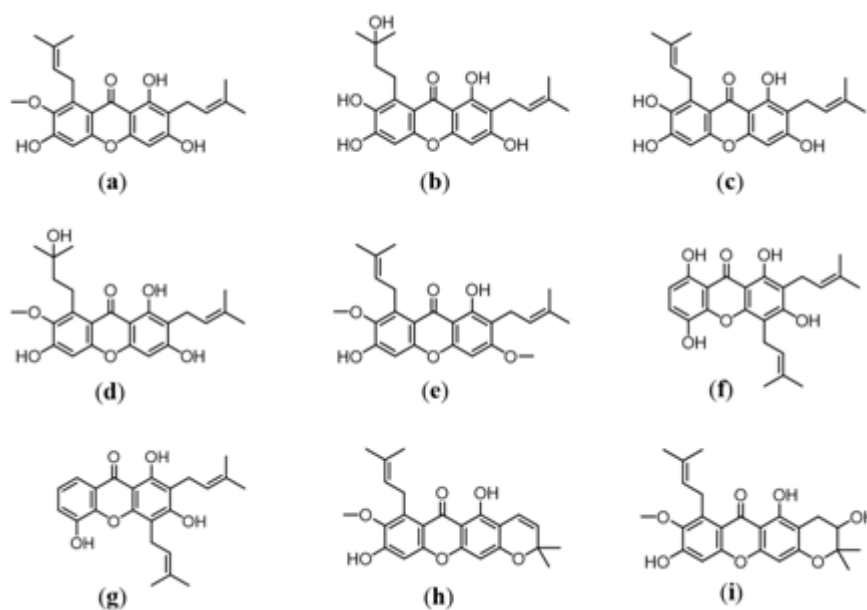


Figure 1. Chemical Structures of the Compounds Found in Propolis: (A) γ -Mangostin; (B) Garcinone C; (C) γ -Mangostin; (D) Cochinchinone T; (E) γ -Mangostin; (F) Gartanin; (G) 8-Deoxygartanin; (H) 9-Hydroxycalabaxanthone; (I) Mangostanol

Table 1. Retention Times, MS Data, and Yields of Nine Compounds Identified in Propolis

Peak No	Rt HPLC (min)	Molecular formula	Experimental m/z $[M-H]^+$	Theoretical m/z $[M-H]^+$	Yield (mg)	Identification
a	40.93	C ₂₄ H ₂₆ O ₆	411.17950	411.18021	24.51	α -mangostin
b	29.58	C ₂₃ H ₂₆ O ₇	415.17352	415.17513	4.10	garcinone C
c	37.58	C ₂₃ H ₂₄ O ₆	397.16336	397.16456	2.15	γ -mangostin
d	35.56	C ₂₄ H ₂₈ O ₇	429.18967	429.19078	2.12	Cochinchinone T
e	47.49	C ₂₅ H ₂₈ O ₆	425.19449	425.19586	5.00	β -mangostin
f	40.18	C ₂₃ H ₂₄ O ₆	397.16309	397.16456	4.11	gartanin
g	38.20	C ₂₃ H ₂₄ O ₅	381.16815	381.16965	5.74	8-deoxygartanin
h	45.36	C ₂₄ H ₂₄ O ₆	409.16354	409.16456	9.22	9-hydroxycalabaxanthone
i	33.41	C ₂₄ H ₂₆ O ₇	427.17490	427.17513	1.77	mangostanol

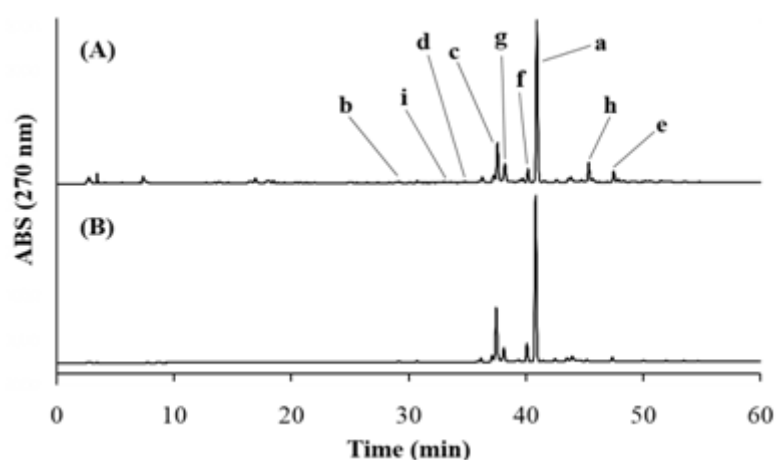


Figure 2. HPLC Profiles of 70% Ethanol Extracts of Propolis (A) and the Yellow Resin from the Surface of *G. mangostana* (B). Peak Assignments are Identical to those in Figure 1. The Retention Times of all Compounds, Including those of Minor Constituents (b, d, and i), were Confirmed

Table 2. Contents of γ -Mangostin and Gartanin in EEP and EEM

	mg/g of EEP	mg/g of EEM
γ -mangostin	9.31 \pm 0.07	146.55 \pm 6.43
gartanin	2.80 \pm 0.08	34.66 \pm 1.08

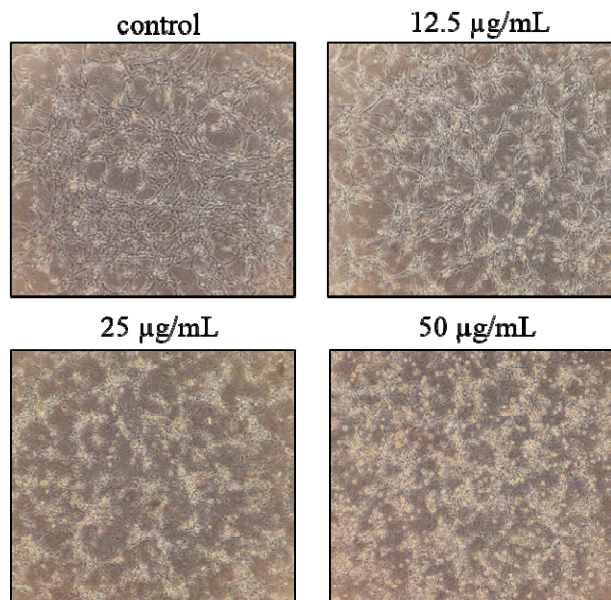


Figure 3. Inhibitory Effects of Thailand Stingless Bee Propolis on the Tube Formation of HUVECs. HUVECs were Sandwiched between Two Layers of Collagen Gel and Induced to Form Blood Vessel-Like Tubes. HUVECs were Treated with 12.5, 25, or 50 μ g/ml EEP and Observed After 48 Hours

We reported the similar results in the previous report but the contents of γ -mangostin and gartanin in EEM were much higher than those of EEP [17]. Propolis may contain secretions originating from honey bees, but whether these secretions possess antioxidant activity has not been reported. Thus, the DPPH radical scavenging activity of EEM may be higher than that of EEP because of differences in the antioxidant compound contents of the samples. Mangosteen resin is yellow, whereas propolis resin ranges in color from dark green to black. Judging from this difference in color, the antioxidants in the resin could have decomposed after collection.

Antiangiogenic activity of EEP and EEM *in vitro*. We examined the effects of EEP and EEM on angiogenesis *in vitro* using a tube formation model of HUVECs cultured in a 2D system. After induction of tube formation, the endothelial cells formed a network of capillary-like tubes composed of multiple cells that gathered together and adhered to each other. Figure 3 shows the inhibitory effects of EEP on the tube formation of endothelial cells. At 12.5 μ g/mL, EEP

slightly reduced the width of the tubes. At 25 and 50 μ g/mL, EEP completely inhibited the formation of capillary networks. EEM was evaluated in the same manner and showed inhibition of the elongation of HUVECs at all tested concentrations.

Our results suggest that Thailand stingless bee propolis exhibits antiangiogenic activity *in vitro*. However, the propolis sample used in this study had been stored for a long period since its collection. If freshly collected propolis is used for evaluation, EEP may exhibit antiangiogenic activity equivalent to that of EEM. These findings further extend the potential pharmacological effects of Thailand stingless bee propolis and could demonstrate its usefulness in cancer prevention and treatment.

4. Conclusions

To investigate the potential utility of Thailand stingless bee propolis, we analyzed the components and evaluate the effects of the propolis in angiogenesis. The plant origin of Thailand stingless bee propolis is likely the resin from the surface of *G. mangostana* from the comparative analysis. Ethanol extracts of this propolis exhibited antiangiogenic activity. This result indicates that Thailand stingless bee propolis may have future applications in the prevention and treatment of angiogenesis-related diseases.

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