Component Analysis and Antiangiogenic Activity of Thailand Stingless Bee Propolis

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**Recommended Citation**

Ishizu, Eriko; Honda, Sari; Ohta, Tosihro; Vongsak, Boonyadist; and Kumazawa, Shigenori (2019)  
"Component Analysis and Antiangiogenic Activity of Thailand Stingless Bee Propolis," *Makara Journal of Technology*  
Vol. 23 : No. 2 , Article 5.  
DOI: 10.7454/mst.v23i2.3703  
Available at: [https://scholarhub.ui.ac.id/mjt/vol23/iss2/5](https://scholarhub.ui.ac.id/mjt/vol23/iss2/5)

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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 xanthones, including α-mangostin, garcinone C, γ-mangostin, cochinchineone 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.

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 regions 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 antioxi-
dant [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 inflamma-
tion, 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 propo-
lis. 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 prop-
olis 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 antiangio-
genic strategy for tumors. Therefore, antiangiogenic
treatment may be useful in the treatment and prevention
of cancer progression [16]. Food factors capable of in-
hibiting 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 de-
tailed component analysis of the propolis to confirm the
plant origin. We also evaluated the effects of this propo-
lis 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
Promo Cell (Land Baden-Württemberg, Louis, MO, USA).
Endothelial Cell Growth Medium 2 was


ingredients.

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 spectrom-
eter (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,
Tokyo, Japan) 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 × 10⁴) 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 were supplemented with 8 nM/mL phorbol 12-myristate 13-acetate (PMA), together with various concentrations of the standard compounds, 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 dissolced 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 xanthones: α-mangostin (a), garcinone C (b), γ-mangostin (c), cochinchione T (d), β-mangostin (e), gartanin (f), 8-deoxygartanin (g), 9-hydroxycalabaxanthone (h), and mangoestanol (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 garcine D [17]. However, in the further detailed analysis, it was revealed that b is cochinchione T [28]. Cochinchione 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% ± 4.73%, 85.55% ± 3.03%, 63.55% ± 2.52%, and 69.28% ± 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

<table>
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<tr>
<th>Peak No</th>
<th>Rt HPLC (min)</th>
<th>Molecular formula</th>
<th>Experimental m/z [M–H]⁺</th>
<th>Theoretical m/z [M–H]⁺</th>
<th>Yield (mg)</th>
<th>Identification</th>
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<td>a</td>
<td>40.93</td>
<td>C₂₄H₂₆O₆</td>
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<td>C₂₃H₂₆O₇</td>
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<td>415.17513</td>
<td>4.10</td>
<td>garcinone C</td>
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<tr>
<td>c</td>
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<td>C₂₃H₂₆O₆</td>
<td>397.16336</td>
<td>397.16456</td>
<td>2.15</td>
<td>γ-mangostin</td>
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<tr>
<td>d</td>
<td>35.56</td>
<td>C₂₄H₂₈O₇</td>
<td>429.18967</td>
<td>429.19078</td>
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<tr>
<td>e</td>
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<td>C₂₃H₂₆O₆</td>
<td>425.19449</td>
<td>425.19586</td>
<td>5.00</td>
<td>β-mangostin</td>
</tr>
<tr>
<td>f</td>
<td>40.18</td>
<td>C₂₃H₂₆O₆</td>
<td>397.16309</td>
<td>397.16456</td>
<td>4.11</td>
<td>gartanin</td>
</tr>
<tr>
<td>g</td>
<td>38.20</td>
<td>C₂₃H₂₆O₆</td>
<td>381.16815</td>
<td>381.16965</td>
<td>5.74</td>
<td>8-deoxygartanin</td>
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<tr>
<td>h</td>
<td>45.36</td>
<td>C₂₄H₃₀O₇</td>
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<td>409.16456</td>
<td>9.22</td>
<td>9-hydroxycalabaxanthone</td>
</tr>
<tr>
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<td>C₂₄H₂₆O₇</td>
<td>427.17490</td>
<td>427.17513</td>
<td>1.77</td>
<td>mangostanol</td>
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</table>

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

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

<table>
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<th></th>
<th>mg/g of EEP</th>
<th>mg/g of EEM</th>
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<tr>
<td>γ-mangostin</td>
<td>9.31±0.07</td>
<td>146.55±6.43</td>
</tr>
<tr>
<td>gartanin</td>
<td>2.80±0.08</td>
<td>34.66±1.08</td>
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</table>

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 µg/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 µg/mL, EEP slightly reduced the width of the tubes. At 25 and 50 µg/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.

References