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Antioxidant activity and constituents of propolis collected in various areas of China

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Abstract

Propolis is a resinous substance collected by honeybees from various plant sources. The composition of propolis depends on time, vegetation, and the area of collection. This study examined the antioxidant activity of propolis from various areas of China: Heilongjiang, Neimongol, Hebei, Shandong, Shanxi, Gansu, Henan, Hubei, Sichuan, Hunan, Yunnan and Hainan. Ethanol extracts of propolis (EEP) were prepared and evaluated for their antioxidant activities by β-carotene bleaching, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assays. Furthermore, the major constituents in EEP were identified by high-performance liquid chromatography (HPLC) analysis with a photodiode array (PDA) and mass spectrometric (MS) detection, and each component was quantitatively analyzed. All propolis samples except that from Yunnan had relatively strong antioxidant activity accompanied by high total polyphenol contents. Propolis with strong antioxidant activity contained large amounts of antioxidative compounds, such as caffeic acid, ferulic acid and caffeic acid phenethyl ester. On the other hand, propolis from Yunnan and Hainan had compounds not present in propolis from other areas.

Keywords: Antioxidant activity; Propolis; Free radical scavenging activity; PDA; China

1. Introduction

Propolis, a folk medicine employed in treating various ailments, is a resinous substance collected by honeybees from the bud and bark of certain trees and plants, and stored inside their hives. It has been used in folk medicine from ancient times in many countries and has been extensively studied in Eastern European countries (Bankova, Castro, & Marcucci, 2000; Castaldo & Capasso, 2002). Recently, it has been reported to possess various biological activities, such as antibacterial (Kartal, Yildiz, Kaya, Kurucu, & Topcu, 2003; Kujumgiev et al., 1999), antiviral (Amoros et al., 1994; Kujumgiev et al., 1999), anti-inflammatory (Strehl, Volpert, & Elstner, 1994; Wang, Mineshita, Ga, Shigematsu, & Matsuno, 1993), anticancer (Kimoto et al., 2001; Matsuno, 1995), antifungal (Kujumgiev et al., 1999; Murad, Calvi, Soares, Bankova, & Sforcin, 2002), and antitumoral (Ikeno, Ikeno, & Miyazawa, 1991) properties. 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 (Banskota, Tezuka, & Kadota, 2001; Burdock, 1998).

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EEP, ethanol extracts of propolis; MS, mass spectrometry; PDA, photodiode array; VE, α-tocopherol.

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Propolis has over 150 constituents, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids, and amino acids, but its composition varies qualitatively and quantitatively with the geographical and botanical origins (Bonvehí, Coll, & Jorda, 1994; Nieva Moreno, Isla, Sampieto, & Vattuone, 2000). Some of the observed biological activities might be attributed to the identified chemical constituents that partially stem from its high content of flavonoids. Because of the geographical differences, propolis samples from Europe, South America and Asia have different chemical compositions (Bankova et al., 1992; Kumazawa, Goto et al., 2004; Kumazawa, Hamasaka, & Nakayama, 2004; Marcucci, 1995; Velikova, Bankova, Sorkun, Popov, & Kujumgiev, 2001). Propolis from Europe and China contains many flavonoids and phenolic acid esters (Bankova et al., 2000). In contrast, the major components in propolis of Brazilian origin are terpenoids and prenylated derivatives of *p*-coumaric acids (Kumazawa et al., 2003; Marcucci & Bankova, 1999; Tazawa, Warashina, Noro, & Miyase, 1998, 1999). The biological activities of propolis samples also differ with the geographic area. Kujumgiev et al. (1999) reported that propolis samples from different geographic origins have different levels of antimicrobiological activity. Nieva Moreno et al. (2000) found a significant correlation between high total flavonoid contents and free radical-scavenging activity in propolis extracts from Argentina. The disease-preventing activity of propolis may be attributed to its antioxidant activity (Hamasaka, Kumazawa, Fujimoto, & Nakayama, 2004; Kolankaya, Selmanoğlu, Sorkun, & Salih, 2002; Kumazawa, Hamasaka et al., 2004; Ozen et al., 2004; Shimizu, Ashida, Matsuura, & Kanazawa, 2004).

Yamauchi, Kato, Oida, Kanaeda, and Ueno (1992) compared the propolis from China, Japan, Brazil, and United States against the inhibition of methyl linoleate autoxidation, and isolated benzyl caffeate as one of the antioxidants from the propolis collected in China. Banskota et al. (2000) reported that water extracts of Chinese and Brazilian propolis possessed strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity compared with the corresponding methanol extracts, whereas the methanol extracts of Netherlands and Peruvian propolis exhibited stronger DPPH free radical-scavenging activity than water extracts. Propolis from China contains many polyphenols such as flavonoids and phenolic acid esters, as described above. However, no studies on the geographic variation in the antioxidant activity and chemical constituents of Chinese propolis have been reported.

In this study, we investigated the in vitro antioxidant activity of the ethanol extracts of propolis (EEP) from various areas in China and analyzed the individual constituents in EEP. We used three assay systems for evaluating Chinese propolis: the inhibition of linoleic acid oxidation by β-carotene bleaching, the free radical-scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the scavenging activity on 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation. Furthermore, we identified and quantitatively analyzed several compounds in EEP by high-performance liquid chromatography (HPLC) analysis with a photodiode array (PDA) and mass spectrometric (MS) detection and quantitatively analyzed each component.

2. Materials and methods

2.1. Materials

Caffeic acid (**1**), *p*-coumaric acid (**2**), ferulic acid (**3**), and α-tocopherol (VE) were purchased from Sigma (St. Louis, MO, USA). 3,4-Dimethoxycinnamic acid (**4**), pinobanksin 5-methyl ether (**5**), pinobanksin (**6**), cinnamylideneacetic acid (**7**), and pinobanksin 3-acetate (**12**) were isolated from the ethanol extract of Uruguayan propolis (Kumazawa, Hayashi et al., 2002). Chrysin (**9**), pinocembrin (**10**), galangin (**11**) and tectochrysin (**14**) were purchased from Funakoshi (Tokyo, Japan). Butylated hydroxytoluene (BHT), β-carotene, linoleic acid, potassium persulfate, and Tween 40 were purchased from Kanto Chemicals (Tokyo, Japan). ABTS, caffeic acid phenethyl ester (**8**), gallic acid and DPPH were purchased from Wako Pure Chemicals Industries (Osaka, Japan).

Propolis samples were collected as the crude materials by beekeepers in various areas of China. Fig. 1 shows the collection sites of each sample. The crude propolis materials (100 mg each) were extracted with ethanol (3 ml) at room temperature for 24 h. The ethanol suspension was separated by centrifugation at 1000 rpm for 5 min at 25 °C, and supernatant was concentrated under reduced pressure to give EEP. EEP was stored under dry conditions at 4 °C until analyzed.

2.2. Total polyphenol and flavonoid contents

Total polyphenol contents in EEP were determined according to the Folin–Ciocalteu colorimetric method

Fig. 1. Collection sites of Chinese propolis. **a**, **b**, Heilongjiang; **c**, Neimongol; **d**, Hebei; **e**, **f**, Shandong; **g**, Shanxi; **h**, Gansu; **i**–**k**, Henan; **l**–**n**, Hubei; **o**, Sichuan; **p**, Hunan; **q**, **r**, Yunnan; **s**, **t**, Hainan.

(Kumazawa, Taniguchi et al., 2002; Singleton, Orthofer, & Lamuela-Raventos, 1999). EEP solution (0.5 ml) was mixed with 0.5 ml of the Folin–Ciocalteu reagent (Kanto Chemicals, Tokyo, Japan) and 0.5 ml of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h of incubation at room temperature. EEP samples were evaluated at the final concentration of 20 μg/ml. Total polyphenol contents were expressed as milligramms per gram of gallic acid equivalents.

Contents of flavonoid in EEP were determined according to the method of Woisky and Salatino (1998), with minor modifications. To 0.5 ml of EEP solution, was added 0.5 ml of 2% $AlCl_3$–ethanol solution. After 1 h at room temperature, the absorbance was measured at 420 nm. EEP samples were evaluated at a final concentration of 20 μg/ml. Total flavonoid contents were calculated as quercetin equivalents (milligrams per gram) from a calibration curve.

2.3. Antioxidant activity on linoleic acid oxidation

This experiment was carried out according to the method of Emmons, Peterson, and Paul (1999) with some modifications. β-Carotene (3 mg) was dissolved in 30 ml of chloroform, and 3 ml were added to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was removed under a stream of nitrogen gas. Then distilled water (100 ml) was added, and the solution was well mixed. Aliquots (3 ml) of the β-carotene/linoleic acid emulsion were mixed with 50 μl of EEP solution and incubated in a water bath at 50 °C. Oxidation of the emulsion was monitored spectrometrically by measuring absorbance at 470 nm over a 60 min period. The control sample contained 50 μl of solvent in place of the extract. The antioxidant activity was expressed as percent inhibition relative to the control after a 60 min incubation using the equation

$$AA = (DR_C - DR_S)/DR_C \times 100$$

where AA is the antioxidant activity, DR_C is the degradation rate of the control (=ln(a/b)/60), DR_S is the degradation rate in the presence of the sample (=ln(a/b)/60), a is the initial absorbance at time 0, and b is the absorbance at 60 min. EEP samples were evaluated at a final concentration of 10 μg/ml, and VE and BHT at 1 μg/ml were used as reference samples.

2.4. Free radical scavenging activity on DPPH

The reaction mixture contained 2 ml of ethanol, 125 μM DPPH and test samples. After a 1 h incubation at room temperature, the absorbance was recorded at 517 nm. Control solution contained only ethanol and DPPH. Results were expressed as percentage decrease with respect to control values (Okada & Okada, 1998; Chen & Ho, 1995). EEP samples were evaluated at a final concentration of 20 μg/ml, and VE and BHT at the same concentration were used as the reference samples.

2.5. Scavenging activity of ABTS radical cation

The ABTS radical cation ($ABTS^{\cdot+}$)-scavenging activity was measured according to the method described by Erel (2004) with some modifications. ABTS was dissolved in water to a 7 mM concentration. The ABTS radical cation was produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate (final concentration) in the dark at room temperature for 12–16 h to allow the completion of radical generation. This solution was then diluted with ethanol so that its absorbance was adjusted to 0.70 ± 0.02 at 734 nm. To determine the scavenging activity, 3 ml of diluted $ABTS^{\cdot+}$ solution were added to 20 μl of EEP solution, and the absorbance was measured at 734 nm 5 min after the initial mixing, using ethanol as the blank. The percentage inhibition was calculated by the equation

$$\%\text{inhibition} = (A_C - A_S)/A_C \times 100$$

where A_C is the absorbance of the control and A_S is the absorbance of the samples. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and VE were prepared as positive control samples. EEP samples were evaluated at a final concentration of 500 μg/ml, and trolox and VE, at 50 μg/ml, were used as the reference samples.

2.6. HPLC analysis with PDA and MS detection

To identify and determine the constituents in EEP, we used HPLC with PDA and MS detection. EEP samples were dissolved in ethanol (2 mg/ml) and filtered with a 0.45 μm filter (German Sciences, Tokyo, Japan) prior to the injection of 5 μl into the HPLC system.

The HPLC system used was SI-1 (Shiseido, Tokyo, Japan) with a Capcell Pak ACR 120 (Shiseido Tokyo, Japan) C18 column (2 × 250 mm i.d., 5 μm). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was 20–80% B (0–60 min) at a flow rate of 1.0 ml/min. For analysis by PDA detection, UV spectra were recorded from 195 to 650 nm at a rate of 0.8 spectrum/s and a resolution of 4.0 nm.

MS was performed on an LCQ ion trap mass spectrometer (Thermo Electron, CA, USA) equipped with an electrospray ionization (ESI) source. The operating parameters were as follows: source voltages, 5 kV; ES capillary voltage, −10 V; capillary temperature, 260 °C. All MS data were acquired in negative ionization.

3. Results and discussion

3.1. Total polyphenol and flavonoid contents of various propolis samples

Table 1 shows the total polyphenol and flavonoid contents of EEP. The amounts of total polyphenol and flavonoid contents in Chinese propolis varied widely, ranging

Table 1
Collection sites, total polyphenol, and flavonoid contents of EEP

Propolis	Collecting site	Total polyphenol[a] (mg/g of EEP)	Flavonoids[b] (mg/g of EEP)
a	Heilongjiang	226 ± 5.4	76.2 ± 8.8
b	Heilongjiang	291 ± 7.3	81.1 ± 2.0
c	Neimongol	284 ± 5.9	159 ± 2.1
d	Hebei	302 ± 4.3	150 ± 2.4
e	Shandong	265 ± 3.3	133 ± 4.4
f	Shandong	296 ± 5.4	168 ± 7.8
g	Shanxi	283 ± 3.3	157 ± 9.7
h	Gansu	229 ± 7.1	188 ± 6.6
i	Henan	224 ± 3.3	155 ± 4.7
j	Henan	229 ± 7.1	142 ± 5.0
k	Henan	238 ± 3.7	130 ± 2.1
l	Hubei	225 ± 7.4	162 ± 6.5
m	Hubei	277 ± 5.5	138 ± 15.3
n	Hubei	212 ± 4.8	117 ± 2.7
o	Sichuan	166 ± 9.8	109 ± 1.6
p	Hunan	194 ± 2.9	155 ± 6.1
q	Yunnan	64.7 ± 1.5	43.5 ± 0.8
r	Yunnan	42.9 ± 0.8	8.3 ± 3.7
s	Hainan	246 ± 4.8	91.9 ± 1.6
t	Hainan	240 ± 5.9	93.8 ± 1.2

[a] Total polyphenol contents were determined by the Folin–Ciocalteu method. Each value is the mean ± standard deviation.
[b] Flavonoid contents were determined by $AlCl_3$ coloration. Each value is the mean ± standard deviation.

from 42.9 to 302 mg/g of EEP and from 8.3 to 188 mg/g of EEP, respectively. As shown in Table 1, the samples, other than that from Yunnan (**q**, **r**), had a total polyphenol content of 200–300 mg/g of EEP. Kumazawa, Hamasaka et al. (2004) previously reported that the polyphenol content of EEP from Europe (Bulgaria and Hungary) and China (Hebei, Hubei, and Zhejiang) was approximately 200–300 mg/g of EEP. However, the total polyphenol and flavonoid contents in EEP from Yunnan (**q**, **r**) were much lower than those from any other region.

Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity (Kahkonen et al., 1999). Propolis contains a wide variety of phenolic compounds, mainly flavonoids. Variation in the flavonoid content of propolis is mainly attributable to the difference in the preferred regional plants collected by honeybees. Contents of flavonoid and other phenolic substances have been suggested to play a preventive role in the development of cancer and heart disease (Kahkonen et al., 1999). The Folin–Ciocalteu method and the $AlCl_3$ coloration are currently used to determine the total polyphenol and flavonoid contents, respectively (Liu et al., 2004; Luximon-Ramma, Rahorun, Soobrattee, & Arioma, 2002). In the present study, we applied these methods to determine the total polyphenol and flavonoid contents of Chinese propolis samples. These physicochemical methods are useful for evaluating various propolis samples because propolis contains many phenolics. Woisky and Salatino (1998) also evaluated propolis using these methods.

3.2. Effects of various propolis samples on linoleic acid oxidation

Fig. 2 shows the antioxidant activity of various EEP samples determined by the β-carotene–linoleic acid system. The antioxidant assay using the discoloration of β-carotene is widely used, because β-carotene is extremely susceptible to free radical-mediated oxidation. β-Carotene is discoloured easily by the oxidation of linoleic acid, because its double bonds are sensitive to oxidation (Singh, Chidambara Murthy, & Jayaprakasha, 2002; Unten, Koketsu, & Kim, 1997).

As shown in Fig. 2, EEP from Hainan (**s**, **t**) had a stronger antioxidant activity than those from other regions. Most of the EEP samples also had high antioxidant activity, over 60%. The contents of both total polyphenol and flavonoid were high in EEP from these samples. However, EEP from Yunnan (**q**, **r**), in which total polyphenol and flavonoid contents were low (Table 1), exhibited weak antioxidant activity. Phenolic compounds such as flavonoids are the types of antioxidant that possess a strong inhibitory effect against lipid oxidation through radical-scavenging. We previously examined the antioxidant activity and constituents of Korean propolis by geographic origin, and found polyphenol contents to be present in Korean propolis in correlation with the antioxidant effect of propolis (Ahn, Kumazawa, Hamasaka, Bang, & Nakayama, 2004). All EEP samples, except EEP from Yunnan (**q**, **r**),

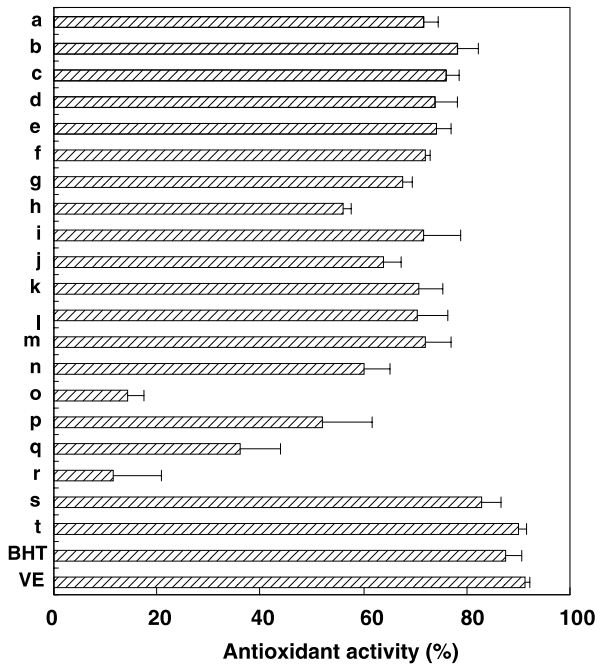

Fig. 2. Antioxidant activity of EEP (**a**–**t**) of Chinese propolis from various geographic origins in the β-carotene–linoleic acid system. **a**, **b**, Heilongjiang; **c**, Neimongol; **d**, Hebei; **e**, **f**, Shandong; **g**, Shanxi; **h**, Gansu; **i**–**k**, Henan; **l**–**n**, Hubei; **o**, Sichuan; **p**, Hunan; **q**, **r**, Yunnan; **s**, **t**, Hainan; VE, α-tocopherol; BHT, butylated hydroxytoluene. Measurements were carried out in triplicate. Means and standard deviations are indicated.

had high total polyphenol and flavonoid contents (Table 1), and the correlation between total polyphenol content and antioxidant activity was significant ($R^2 = 0.671$, data not shown). Flavonoids have been reported to be the most abundant and most effective antioxidant in propolis (Bonvehí & Coll, 1994; Isla, Nieva Moreno, Sampietro, & Vattuone, 2001; Martos, Cossentini, Ferreres, & Tomás-Barberán, 1997). The antioxidant activity of the ethanol extract of propolis has been attributed to the high content of flavonoids in propolis (Chen & Ho, 1995; Krol et al., 1990; Pascual, Gonzalez, & Torricella, 1994).

3.3. DPPH free radical-scavenging activity of various propolis samples

Because the free radical-scavenging activity of antioxidants is considered to be due to their hydrogen-donating ability, we used a method based on the reduction of DPPH, a stable free radical, to evaluate the antioxidant activity of various EEP samples (Chen & Ho, 1995; Tang, Kerry, Sheehan, & Buckley, 2002). DPPH has been widely used to test the free radical-scavenging activity of various samples (Hatano, Takagi, Ito, & Yoshida, 1997; Nagai, Inoue, Inoue, & Suzuki, 2003). We evaluated the free radical-scavenging activity of various EEP and the reference samples (VE and BHT) at a final concentration of 20 μg/ml (Fig. 3).

All propolis samples showed free radical scavenging activity. As shown in Fig. 3, the EEP samples from Neimongol (**c**), Hebei (**d**) and Hubei (**m**) had strong DPPH free radical-scavenging activities, over 70%. These EEP samples had high total polyphenol and flavonoid contents (Table 1). EEP samples from Neimongol (**c**), Hebei (**d**) and Hubei (**m**) showed strong antioxidant activity, also in the assay system using the discoloration of β-carotene (Fig. 2). EEP from Sichuan (**o**) and Yunnan (**q**, **r**), which had weak antioxidant activities in the assay system using the discoloration of β-carotene (Fig. 2), exhibited weak DPPH free radical-scavenging activity.

The DPPH free radical-scavenging activity shown in Fig. 3 seemed to correlate with the antioxidant activity shown in Fig. 2. The propolis with high antioxidant activity also had high DPPH free radical-scavenging activity (Fig. 2). The relationship between DPPH radical scavenging activity of various EEP and total polyphenol contents was examined, and a positive correlation between them was observed ($R^2 = 0.762$, data not shown). However, more detailed qualitative and quantitative analyses of the compounds with antioxidant activity will be necessary to fully elucidate the antioxidant activity of propolis.

3.4. Effects of various propolis samples on ABTS radical cation

Most of the EEP samples showed ABTS radical cation-scavenging activity (Fig. 4). The ABTS radical cation decolorization assay is a spectrophotometric method widely used for the assessment of antioxidant activity of

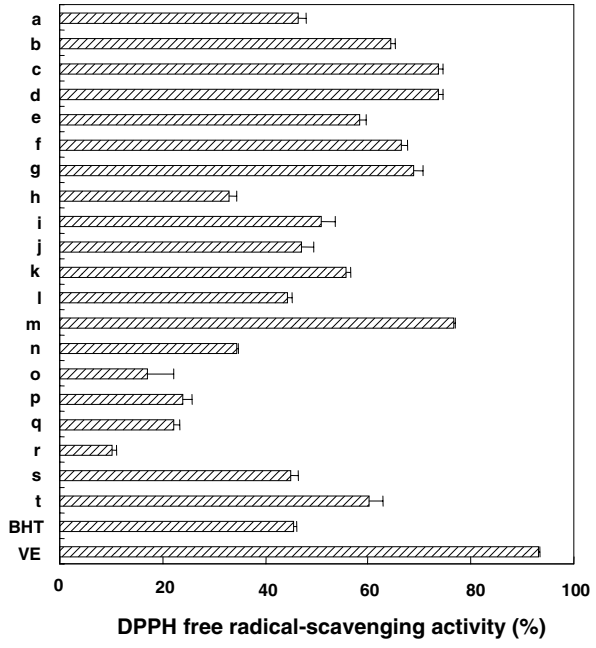

Fig. 3. DPPH radical-scavenging activity of EEP (**a**–**t**) of Chinese propolis from various geographic origins. **a**, **b**, Heilongjiang; **c**, Neimongol; **d**, Hebei; **e**, **f**, Shandong; **g**, Shanxi; **h**, Gansu; **i**–**k**, Henan; **l**–**n**, Hubei; **o**, Sichuan; **p**, Hunan; **q**, **r**, Yunnan; **s**, **t**, Hainan; VE, α-tocopherol; BHT, butylated hydroxytoluene. Measurements were carried out in triplicate. Means and standard deviations are indicated.

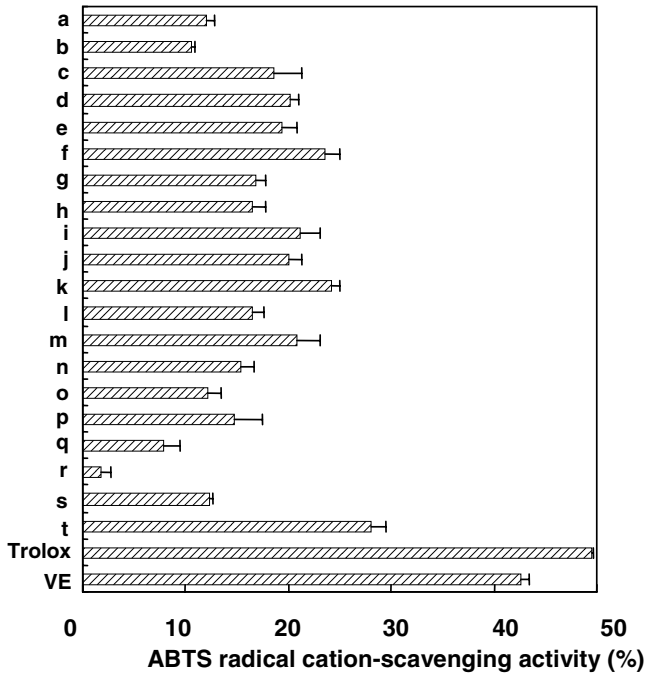

Fig. 4. ABTS radical cation-scavenging activity of EEP (**a**–**t**) of Chinese propolis from various geographic origins. **a**, **b**, Heilongjiang; **c**, Neimongol; **d**, Hebei; **e**, **f**, Shandong; **g**, Shanxi; **h**, Gansu; **i**–**k**, Henan; **l**–**n**, Hubei; **o**, Sichuan; **p**, Hunan; **q**, **r**, Yunnan; **s**, **t**, Hainan; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; VE, α-tocopherol. Measurements were carried out in triplicate. Means and standard deviations are indicated.

various substances (Erel, 2004). The $ABTS^{\cdot+}$ is generated by the oxidation of ABTS with potassium persulfate and is reduced in the presence of such a hydrogen-donating antioxidant. EEP samples were evaluated at a final concentration of 500 μg/ml for the assay. Trolox and VE were compared at 50 μg/ml under the same conditions.

As shown in Fig. 4, the EEP samples from Hainan (**t**) had the highest activities. The percentage inhibition of ABTS radical cation for all EEP samples, except that from Yunnan (**q**, **r**), was over 10%. EEP samples from Yunnan (**q**, **r**) showed weak antioxidant activity, also, in the assay system using the discoloration of β-carotene (Fig. 2) and DPPH free radical-scavenging activity (Fig. 3). The ABTS radical cation scavenging activity shown in Fig. 4 seemed to correlate with the antioxidant activity shown in Fig. 2. The relation between ABTS radical cation scavenging activity of various EEP and total polyphenol contents was examined, and a positive correlation between them was observed ($R^2 = 0.459$, data not shown). The propolis with high antioxidant activity also had high ABTS radical cation scavenging activity.

3.5. HPLC analysis of various propolis samples

We identified the major components in EEP samples by HPLC analysis, by PDA and MS detection. Fig. 5 shows the chemical structures of the compounds identified. Previously, Kumazawa, Hayashi et al. (2002) isolated and identified 33 compounds: 18 flavonoids, 4 aromatic carboxylic acids, and 11 phenolic acid esters from Uruguayan propolis. Concerning the compounds that could not be obtained from commercial sources, we used those isolated from Uruguayan propolis as authentic compounds to identify each component.

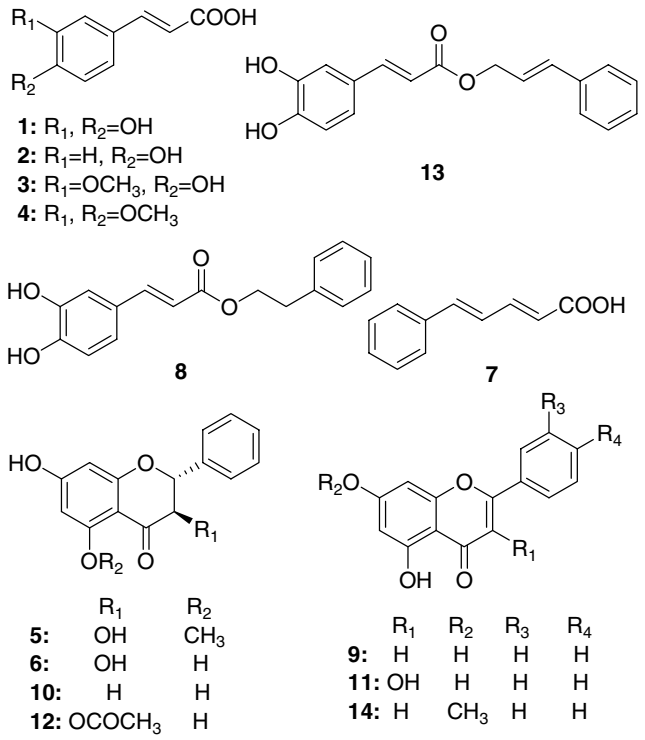

Fig. 5. Structures of the constituents identified from propolis. **1**, caffeic acid; **2**, *p*-coumaric acid; **3**, ferulic acid; **4**, 3,4-dimethoxycinnamic acid; **5**, pinobanksin 5-methyl ether; **6**, pinobanksin; **7**, cinnamylideneacetic acid; **8**, caffeic acid phenethyl ester; **9**, chrysin; **10**, pinocembrin; **11**, galangin; **12**, pinobanksin 3-acetate; **13**, cinnamyl caffeate; **14**, tectochrysin.

Fig. 6 shows the HPLC chromatograms of EEP samples **a**–**t**. The numbers **1**–**14** indicate the peaks identified by the HPLC analysis with PDA and MS detection. To identify each peak, UV spectra and the selected ion monitoring (SIM) of MS spectra of all peaks were compared with those of authentic standards.

Fujimoto, Nakamura, and Matsuka (2001) analyzed various propolis samples from all over the world by UV and HPLC and classified the propolis into two groups according to the difference of their components: one is a Brazilian-type (*Baccharis*-type), and the other is a European-type (poplar-type). Brazilian-type propolis is rich in *p*-coumaric acid derivatives and is found only in Brazil. On the other hand, European-type propolis is rich in flavonoids and is collected, not only in Europe but also in China and other countries (Fujimoto et al., 2001). The present study revealed that propolis samples from various areas of China were similar to poplar-type propolis.

The results of the quantitative analysis of all EEP samples are shown in Table 2. Values are expressed as means of triplicate analyses for each sample. EEP from Henan (**i**) contained the largest amount of caffeic acid (**1**) (32.2 mg/g of EEP). The content of *p*-coumaric acid (**2**) in EEP from Heilongjiang (**a**, **b**) were over 40 mg/g of EEP. EEP from Heilongjiang (**a**) had the highest content of ferulic acid (**3**) (5.3 mg/g of EEP). 3,4-Dimethoxycinnamic acid (**4**) was detected in the highest amount in EEP from Henan (**i**) (57.4 mg/g of EEP), but propolis from Gansu (**h**) did not contain **4**. Pinobanksin 5-methyl ether (**5**) was present in nearly all the EEP samples from Chinese propolis, but was not quantified. Pinobanksin (**6**) was detected in almost all EEP samples in large amounts. EEP from Hubei (**n**) had the highest content of **6** (76.5 mg/g of EEP) in all EEP samples. The content of cinnamylideneacetic acid (**7**) in EEP from Henan (**i**, **k**) was over 40 mg/g of EEP. EEP from Shanxi (**g**) had the highest content of caffeic acid phenethyl ester (**8**) (8.7 mg/g of EEP). Chrysin (**9**) is one of the representative flavonoids of propolis (Marcucci & Bankova, 1999), and was present in all EEP samples except EEP from Yunnan (**q**, **r**) and Hainan (**s**, **t**). EEP from Hubei (**l**) had the highest content of chrysin (**9**), while that from Gansu (**h**) had the highest contents of pinocembrin (**10**), galangin (**11**) and pinobanksin 3-acetate (**12**) among the EEP samples analyzed. Cinnamyl caffeate (**13**) was in large amount in that from Henan (**k**) (25.1 mg/g of EEP). EEP from Shanxi (**g**) had the highest content of tectochrysin (**14**) (42.7 mg/g of EEP). Furthermore, constituents of propolis from Yunnan (**q**, **r**) and Hainan (**s**, **t**) were apparently different from those from other areas and did not contain any compounds shown in Fig. 5 that had been detected

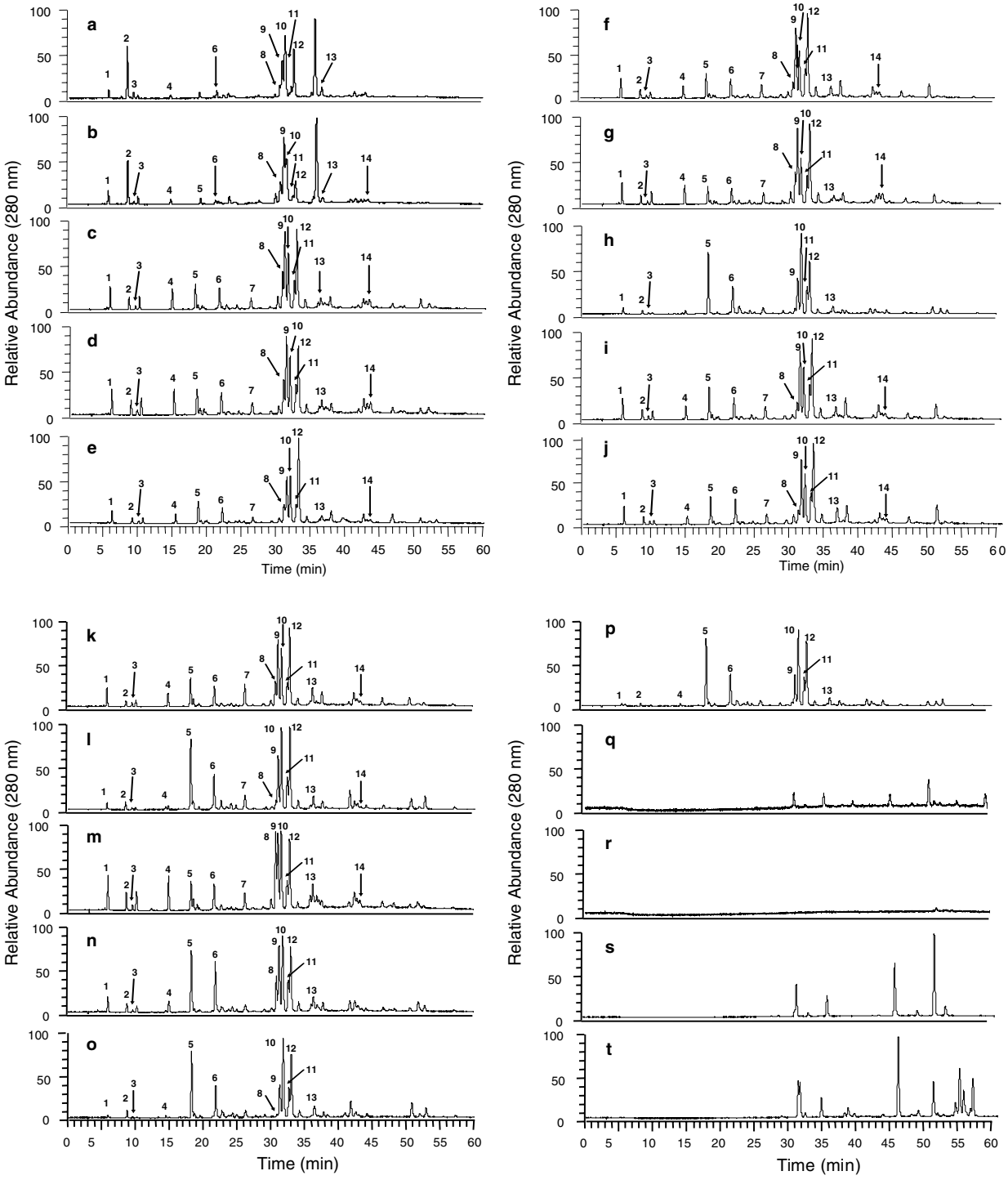

Fig. 6. HPLC chromatograms of EEP (**a**–**t**) of Chinese propolis from various geographic origins. **a**, **b**, Heilongjiang; **c**, Neimongol; **d**, Hebei; **e**, **f**, Shandong; **g**, Shanxi; **h**, Gansu; **i**–**k**, Henan; **l**–**n**, Hubei; **o**, Sichuan; **p**, Hunan; **q**, **r**, Yunnan; **s**, **t**, Hainan. The numbers in panels **a**–**t** represent the same compounds. **1**, caffeic acid; **2**, *p*-coumaric acid; **3**, ferulic acid; **4**, 3,4-dimethoxycinnamic acid; **5**, pinobanksin 5-methyl ether; **6**, pinobanksin; **7**, cinnamylideneacetic acid; **8**, caffeic acid phenethyl ester; **9**, chrysin; **10**, pinocembrin; **11**, galangin; **12**, pinobanksin 3-acetate; **13**, cinnamyl caffeate; **14**, tectochrysin.

in propolis in the past. EEP from Yunnan had relatively weak antioxidant activity, which was consistent with the low total polyphenol contents, as mentioned above. The propolis from Hainan, however, had high antioxidant and radical-scavenging activities. Thus, the active components in propolis from Hainan may be different from those from other areas. Research on the active components in propolis from Hainan is in progress.

We identified 14 compounds from 20 Chinese propolis and determined the quantitative value of each compound

Table 2
Contents of the constituents in EEP samples

	Content[a] (mg/g of EEP)																			
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t
Caffeic acid (**1**)	11.1	23.1	20.8	21.2	18.8	22.0	24.6	3.5	32.2	19.8	18.9	6.5	19.4	7.0	4.0	3.3	–	–	–	–
p-Coumaric acid (**2**)	42.3	52.2	7.5	9.2	6.0	6.4	7.0	2.4	11.3	6.6	4.7	4.0	8.9	2.8	7.9	2.3	–	–	–	–
Ferulic acid (**3**)	5.3	3.0	2.7	2.7	2.8	2.5	1.9	0.6	3.7	2.5	2.8	1.2	3.8	1.0	0.7	–	–	–	–	–
3,4-Dimethoxycinnamic acid (**4**)	13.1	15.5	47.8	50.4	33.6	35.9	55.5	–	57.4	25.9	34.8	10.4	53.2	8.1	6.2	5.8	–	–	–	–
Pinobanksin 5-methyl ether (**5**)	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
Pinobanksin (**6**)	15.4	5.8	36.6	32.6	40.8	41.4	27.7	64.6	62.5	55.9	39.0	61.3	36.6	76.5	60.0	65.2	–	–	–	–
Cinnamylideneacetic acid (**7**)	–	–	24.6	20.2	23.1	33.2	21.2	–	45.4	26.8	50.0	27.8	25.2	–	–	–	–	–	–	–
Caffeic acid phenethyl ester (**8**)	4.0	6.3	5.6	4.7	5.6	3.0	8.7	–	4.5	3.1	4.5	1.8	7.8	1.2	0.6	–	–	–	–	–
Chrysin (**9**)	6.6	3.9	12.9	5.3	11.8	17.1	16.2	19.5	13.5	12.2	10.0	23.3	7.6	10.1	9.4	12.9	–	–	–	–
Pinocembrin (**10**)	11.5	4.7	14.3	16.2	17.4	18.8	11.6	35.7	12.8	13.4	14.4	21.2	3.9	16.2	15.7	22.2	–	–	–	–
Galangin (**11**)	5.6	3.8	6.6	8.3	6.5	8.2	7.2	12.8	6.8	5.9	8.4	7.1	5.3	6.6	7.2	7.5	–	–	–	–
Pinobanksin 3-acetate (**12**)	18.2	8.3	11.4	14.1	24.1	21.0	18.2	24.3	15.6	12.8	15.4	18.1	9.0	11.0	16.2	18.6	–	–	–	–
Cinnamyl caffeate (**13**)	19.2	14.1	12.5	11.0	13.4	17.2	12.6	10.0	20.9	21.8	25.1	12.5	20.2	8.5	10.8	11.2	–	–	–	–
Tectochrysin (**14**)	–	33.8	33.1	35.4	18.6	25.3	42.7	–	35.9	14.9	13.2	3.8	15.5	–	–	–	–	–	–	–

–: not detected.
+: constituent present, but not quantified.
[a] Each value is the mean of triplicate analyses for each sample.

in the present study. Bonvehí and Coll (2000) analyzed the composition, bacteriostatic and ROO·-scavenging potential activity of the propolis from China and Uruguay, and found significant differences ($P \leqslant 0.05$) in the contents of polyphenols, flavonoids and active components between fresh and aged propolis. The resinous excretions of the buds of the poplar tree are mentioned as the main sources of propolis from Europe, North and South America, and Western Asia (Bankova et al., 1992, 2000; Tomás-Barberán, García-Viguera, Vit-Olivier, Ferreres, & Tomás-Lorente, 1993). Fujimoto et al. (2001) reported that the characteristic compounds of the propolis from China, Hungary, Bulgaria, Uruguay and Argentina are pinocembrin, chrysin, galangin and tectochrysin and that the source plant is *Populus* spp. (poplar). Because we detected these compounds from the EEP samples from areas other than Yunnan and Hainan, poplar may be one of the main source plants of these propolis samples. All Chinese propolis samples, except that from Yunnan, had relatively strong antioxidant activity accompanied by high total polyphenol contents, as mentioned above. Propolis with strong antioxidant activity contained large amounts of antioxidative compounds, such as caffeic acid (**1**), ferulic acid (**3**), and caffeic acid phenethyl ester (**8**). Flavonoids and phenolic acid esters, especially caffeic acid and ferulic acid, are known for their antibacterial, antiviral and antioxidant activity (Pietta, 2000; Rao, Desai, Kaul, Amin, & Reddy, 1992; Tapia et al., 2004). On the other hand, propolis from Yunnan and Hainan had compounds not present in propolis from other areas. Kumazawa, Hamasaka et al. (2004) reported that compounds, such as caffeic acid (**1**), quercetin, kaempferol, caffeic acid phenethyl ester (**8**) and cinnamyl caffeate (**13**), exhibited strong DPPH free radical-scavenging activity.

In this study, we found that the Chinese propolis samples had relatively similar antioxidant activities and constituents, except those from Yunnan and Hainan, which are located in southern China, distant from the other sample areas. Further studies on the constituents and biological activities of Chinese propolis are underway.

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