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Antioxidant activity of propolis of various geographic origins

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Abstract

Propolis is a resinous substance collected by honeybees from various plant sources. The antioxidant activities of propolis of various geographic origins, i.e., Argentina, Australia, Brazil, Bulgaria, Chile, China (Hebei, Hubei, and Zhejiang), Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, United States, and Uzbekistan were compared. Ethanol extracts of propolis (EEP) were prepared and evaluated for antioxidant activities of EEP samples by the β-carotene bleaching and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay systems. Major constituents were identified in EEP by HPLC analysis with photo-diode array (PDA) and mass spectrometric (MS) detection, and quantitatively analyzed. EEP from Argentina, Australia, China, Hungary and New Zealand had relatively strong antioxidant activities, and were also correlated with the total polyphenol and flavonoid contents. Propolis with strong antioxidant activity contained antioxidative compounds such as kaempferol and phenethyl caffeate.

Keywords: Antioxidant activity; Propolis; Free radical scavenging activity; LC/MS; PDA

1. Introduction

Propolis, a natural substance collected by honeybees from buds and exudates of certain trees and plants, is thought to be used in the beehive as a protective barrier against their enemies. Propolis has been used in folk medicines in many regions of the world (Ghisalberti, 1979) and has been reported to have various biological activities such as antibacterial (Kujumgiev, Tsvetkova, Serkedjieva, Bankova, Christov, & Popov, 1999), antiviral (Amoros, Lurton, Boustie, Girre, Sauvager, & Cormier, 1994), antiinflammatory (Wang, Mineshita, Ga, Shigematsu, & Matsuno, 1993), and anticancer (Kimoto et al., 2001; Matsuno, 1995) properties. For this reason, propolis is extensively used in food and beverages to improve health and prevent diseases such as inflammation, heart disease, diabetes, and cancer (Banskota et al., 2001a; Burdock, 1998).

Propolis usually contains a variety of chemical compounds, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids, and amino acids. The composition of propolis depends on the vegetation at the site of collection. Because of the geographical differences, propolis samples from Europe, South America, and Asia have different chemical compositions (Marcucci, 1995). Propolis from Europe and China contains many kinds of flavonoids and phenolic acid esters (Bankova, Castro, & Marcucci, 2000). By contrast, the major components in propolis of Brazilian origin are terpenoids and prenylated derivatives of *p*-coumaric acids (Marcucci & Bankova, 1999; Tazawa, Warashina, Noro, & Miyase, 1998; Tazawa, Warashina, & Noro, 1999). Due to the differences in their chemical compositions, the biological activities of propolis from different areas are also different. For example, Miyataka, Nishiki, Matsumoto, Fujimoto, Matsuka, and Satoh (1997) reported that Brazilian propolis and Chinese propolis differed in their abilities to inhibit hyaluronidase and to release histamine from rat peritoneal mast cells induced by compound 48/80 or concanavalin A (Miyataka et al., 1998). Hegazi, Abd El Hady, and Abd Allah (2000) found that German propolis possessed

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high antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, and Austrian propolis showed high activity against *Candida albicans*. Banskota et al. (2000) reported that the methanol extracts of propolis from The Netherland's and China possessed the strongest cytotoxicity toward murine colon 26-L5 carcinoma cells, while the activity in methanol extracts of propolis from Brazil varied with the sample.

There are a few comparative studies evaluating the antioxidant activities of propolis of different geographic origins. Yamauchi, Kato, Oida, Kanaeda, and Ueno (1992) compared the propolis from Japan, China, 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. Oyaizu, Ogihara, and Fujimoto (1999) examined the antioxidative activity of Chinese, Australian, New Zealand, and Japanese propolis extracts and found α-tocopherol (VE) to be present in almost all propolis samples and correlating with the antioxidative effect of propolis. Banskota et al. (2000) reported that the water extracts of Brazilian and Chinese propolis possessed stronger 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity than the corresponding methanol extracts, whereas the methanol extracts of Netherlands and Peruvian propolis exhibited stronger DPPH free radical scavenging activity.

Propolis obviously possesses antioxidant activity, as reported by many researchers. However, there are few studies on the relationship between the antioxidant activity and individual chemical constituents in propolis. Thus, in this study, we investigate the in vitro antioxidant activity of the ethanol extracts of propolis (EEP) of various geographic origins, and analysed the individual constituents in EEP. We use two assay systems of the inhibition of linoleic acid oxidation, by β-carotene bleaching and the free radical scavenging activity on DPPH. Several compounds in EEP are identified by HPLC analysis with a photo-diode array (PDA) and mass spectrometric (MS) detection. Further, we quantitatively analyse each compound, together with its DPPH free radical scavenging activity.

2. Materials and methods

2.1. Materials

Caffeic acid (**1**), *p*-coumaric acid (**2**), and α-tocopherol were purchased from Sigma (St. Louis, MO, USA). 3,4-Dimethoxycinnamic acid (**3**), pinobanksin 5-methyl ether (**5**), pinobanksin (**8**), cinnamylideneacetic acid (**9**), pinobanksin 3-acetate (**13**), phenethyl caffeate (**14**), and cinnamyl caffeate (**15**) were isolated from the ethanol extract of Uruguayan propolis (Kumazawa, Hayashi, et al. 2002). Apigenin (**6**), kaempferol (**7**), chrysin (**10**), pinocembrin (**11**), galangin (**12**), and tectochrysin (**16**) were purchased from Funakoshi (Tokyo, Japan). Butylated hydroxytoluene (BHT), β-carotene, linoleic acid, quercetin (**4**), and Tween 40 were purchased from Kanto Chemicals (Tokyo, Japan). Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) (**17**), DPPH, and gallic acid were purchased from Wako Pure Chemicals Industries (Osaka, Japan).

Propolis samples were supplied by the Japan Propolis Conference, Dr. Shigemi Tazawa (Api Corporation, Gifu, Japan) and Dr. Jun Nakamura (Tamagawa University, Tokyo, Japan) as crude materials or ethanol extracts. Table 1 shows the collection sites of each propolis sample. Propolis samples from Argentina, Brazil, Chile, China (Hebei, Hubei, and Zhejiang), Hungary, Thailand, Ukraine, Uruguay, and Uzbekistan were obtained as crude materials and, from Australia, Bulgaria, New Zealand, South Africa, and United States, as ethanol extracts. Crude propolis materials were extracted with ethanol at room temperature for 24 h. The ethanol suspension was separated by centrifugation, and the supernatant was concentrated under reduced pressure to give EEP.

2.2. Specific absorbance of UV spectrum

The specific absorbance of UV spectrum of each EEP sample was obtained according to the method of Miyataka et al. (1997). The specific absorbance at the wavelength of each absorption maximum (λ_{max}), from which $E^{1\%}_{1\ cm}$ value were calculated, were measured with a Hitachi U2000 spectrophotometer (Tokyo, Japan).

2.3. Total polyphenol and flavonoid contents

Total polyphenol contents in EEP were determined by the Folin-Ciocalteau colorimetric method (Kumazawa, Taniguchi et al., 2002; Singleton, Orthofer, & Lamuela-Raventos, 1999). EEP solution (0.5 ml) was mixed with 0.5 ml of the Folin-Ciocalteau 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 incubation at room temperature. EEP samples were evaluated at the final concentration of 20 μg/ml. Total polyphenol contents were expressed as mg/g (gallic acid equivalents).

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

2.4. Antioxidant activity on linoleic acid oxidation

This experiment was carried out by 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. Distilled water (100 ml) was added and mixed well. 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. Control sample contained 50 μl of solvent in place of the extract. The antioxidant activity is expressed as per cent inhibition relative to the control after a 60-min incubation using the following equation:

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

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 the final concentration of 10 μg/ml, and VE and BHT at 1 μg/ml were used as the reference samples.

2.5. Free radical scavenging activity on DPPH

The reaction mixture contained 2 ml of ethanol, 125 μM DPPH, and test samples. After 1 h incubation at room temperature, the absorbance was recorded at 517 nm. Results were expressed as percentage decrease with respect to control values (Chen & Ho, 1995; Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002). EEP and identified constituents were evaluated at the final concentration of 20 μg/ml, and VE and BHT at the same concentration 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 (5 mg/ml), filtered with a 0.45-μm filter (German Sciences, Tokyo, Japan) prior to 10 μl injected into the HPLC system.

The HPLC system used was a 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 0.08% formic acid in acetonitrile (B). The gradient was 20–30% B (15 min), 30% B (15–35 min), 30–80% B (35–60 min) at a flow rate of 0.2 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 (ThermoElectron, CA, USA) equipped with an electrospray ionization (ESI) source. The operating parameters were as follows: source voltage 5 kV; ES capillary voltage −10 V; capillary temperature 260 °C. All MS data were acquired in the negative ionization.

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

Propolis	Collection site	Specific absorbance[a] ($E^{1\%}_{1\,cm}$ value)	Total polyphenol[b] (mg/g of EEP)	Flavonoids[c] (mg/g of EEP)
a	Argentina	253 (292)	212±9.2	130±5.5
b	Australia	322 (291)	269±16.3	145±6.5
c	Brazil	210 (294)	120±5.6	51.9±2.4
d	Bulgaria	246 (291)	220±2.5	157±8.9
e	Chile	260 (291)	210±11.1	116±9.3
f	China (Hebei)	353 (293)	298±8.7	147±9.3
g	China (Hubei)	353 (291)	299±0.5	158±10.8
h	China (Zhejiang)	277 (292)	262±12.6	136±17.4
i	Hungary	298 (292)	242±0.2	176±1.7
j	New Zealand	298 (292)	237±6.0	152±12.6
k	South Africa	102 (290)	99.5±4.4	50.8±0.8
l	Thailand	5 (275)	31.2±0.7	2.5±0.8
m	Ukraine	208 (291)	255±7.4	63.7±3.2
n	Uruguay	313 (292)	187±8.5	168±6.4
o	United States	367 (290)	256±15.7	122±6.2
p	Uzbekistan	149 (292)	174±6.7	94.2±6.8

[a] The values were measured at the wavelength of each absorption maximum (nm) shown in parentheses.
[b] Total polyphenol contents was determined by the Folin-Ciocaltau method. Value is mean±standard deviation.
[c] Flavonoid contents were determined by $AlCl_3$ coloration. Value is mean±standard deviation.

3. Results and discussion

3.1. Total polyphenol and flavonoid contents of various propolis samples

Propolis is commercially available as tinctures or tablets made from ethanol extracts in many countries. We therefore examined the effect of the ethanol extracts from various propolis samples as shown in Table 1. All propolis samples, except for that from Thailand, had a pleasant odour and were light yellow to dark brown in colour. The colour of propolis from Thailand was brown but it was odourless.

The $E^{1\%}_{1\ cm}$ value of UV absorption is the one of the physicochemical parameters used to evaluate propolis (Fujimoto, 1992; Miyataka et al., 1997), because it has been believed that various pharmacological activities of propolis are attributed to phenolics, such as flavonoids and caffeic acids in it. As shown in Table 1, the $E^{1\%}_{1\ cm}$ values of most EEP samples were 200–350. These values were near to those previously reported by Miyataka et al. (1997, 1998). However, the $E^{1\%}_{1\ cm}$ of EEP from South Africa (**k**), Thailand (**l**), and Uzbekistan (**p**) showed small values compared with other EEP samples. Particularly, the $E^{1\%}_{1\ cm}$ value of EEP from Thailand (**l**) was extremely small.

Table 1 shows the total polyphenol and flavonoid contents of EEP, besides their $E^{1\%}_{1\ cm}$ values. Total polyphenol contents of EEP from South Africa (**k**), Thailand (**l**), and Uzbekistan (**p**), whose $E^{1\%}_{1\ cm}$ values were small, showed low values. The total polyphenol contents of EEP from Brazil (**c**) was lower than those of EEP from Europe (**d** and **i**) and China (**f**, **g**, and **h**), suggesting that the main components of Brazilian propolis are different from those of European and Chinese propolis. The main components in Brazilian propolis are prenylated derivatives of *p*-coumaric acid, flavonoids not seen in European propolis (Marcucci & Bankova, 1999; Tazawa, Warashina, & Noro, 2000). The flavonoid content of EEP from Brazil (**c**) was low. On the other hand, the $E^{1\%}_{1\ cm}$ and total polyphenol of EEP from Ukraine (**m**) showed high values, but its flavonoid contents were not high. This means that EEP from Ukraine (**m**) contains non-flavonoid phenolics. Not only the $E^{1\%}_{1\ cm}$ value, but also total polyphenol and flavonoid contents, of EEP from Thailand (**l**) were the lowest values in all EEP samples.

The determination of total polyphenol and flavonoid contents are important in various food materials. The Folin-Ciocalteau method and the $AlCl_3$ coloration, to determine the total polyphenol and flavonoid contents, respectively, are currently used (Liu, Li, Weber, Lee, Brown, & Liu, 2002; Luximon-Ramma, Rahorun, Soobrattee, & Aruoma, 2002). In the present study, these methods were applied to determine total polyphenol and flavonoid contents of propolis samples. These physicochemical methods are useful for evaluating various propolis samples because propolis contains many kinds of phenolics. Bonvehí and Coll (1994) and Woisky and Salatino (1998) also used the same methods in order to evaluate propolis.

3.2. Effects of various propolis samples on linoleic acid oxidation

Fig. 1 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 discolorized easily by the oxidation of linoleic acid, due to its double bonds being sensitive to oxidation (Singh, Chidambara Murthy, & Jayaprakasha, 2002; Unten, Koketsu, & Kim, 1997). EEP samples were evaluated at the final concentration of 10 μg/ml for

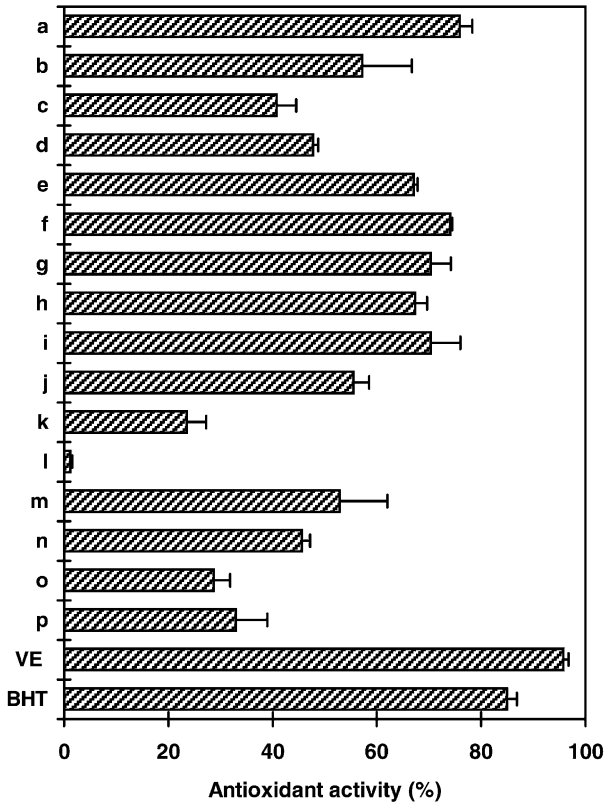


Fig. 1. Antioxidant activity of EEP (**a**–**p**) of various geographic origins in the β-carotene–linoleic acid system. **a**, Argentina; **b**, Australia; **c**, Brazil; **d**, Bulgaria; **e**, Chile; **f**, China (Hebei); **g**, China (Hubei); **h**, China (Zhejiang); **i**, Hungary; **j**, New Zealand; **k**, South Africa; **l**, Thailand; **m**, Ukraine; **n**, Uruguay; **o**, United States; **p**, Uzbekistan; VE, α-tocopherol; BHT, butylated hydroxytoluene. Each EEP sample was used for the assay at the final concentration of 10 μg/ml. VE and BHT were used at the final concentration of 1 μg/ml. Measurements were carried out in triplicate. Means and standard deviations are indicated.

the assay, and VE and BHT were compared at 1 μg/ml under the same conditions.

As shown in Fig. 1, EEP from Argentina (**a**), Chile (**e**), China (**f**, **g**, and **h**), and Hungary (**i**) had strong antioxidant activity, over 60%. EEP from South Africa (**k**) and Uzbekistan (**p**), whose $E^{1\%}_{1\,cm}$ values and flavonoid contents were small (Table 1), exhibited weak antioxidant activity. EEP from Thailand (**l**) had hardly antioxidant activity. Yamauchi et al. (1992) reported that propolis from China had stronger antioxidant activity against the autoxidation of methyl linoleate than those from Brazil and the United States. As with their results, we also observed that propolis from China had strong antioxidant activity.

The antioxidant activity shown in Fig. 1 seemed to correlate with total flavonoid contents of EEP. Flavonoids are reported to be the most abundant and most effective antioxidant in propolis (Scheller, Wilczok, & Imielski, 1990). Nieva Moreno, Isla, Sampietro, and Vattuone (2000) and Isla, Nieva Moreno, Sampietro, and Vattuone (2001) investigated the antioxidant activity of Argentine propolis, and reported that the correlation between flavonoid contents and antioxidant activity is significant, but other factors would be involved. Yamauchi et al. (1992) isolated benzyl caffeate as one of the antioxidants from Chinese propolis, and described that constituents other than flavonoids also contributed to the antioxidant activity of propolis.

3.3. Effect of various propolis samples on DPPH free radical

The DPPH free radical scavenging activity of various EEP samples is shown in Fig. 2. The model system of scavenging DPPH free radical is a simple method for evaluating the antioxidant activity of compounds. It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability (Chen & Hu, 1995; Tang, Kerry, Sheehan, & Buckley, 2002). We evaluated various EEP and the reference samples (VE and BHT) at the final concentration of 20 μg/ml.

As shown in Fig. 2, EEP samples from Australia (**b**), China (**f**, **g**, and **h**), Hungary (**i**), and New Zealand (**j**) had strong DPPH free radical scavenging activities of over 60%. These EEP samples had high $E^{1\%}_{1\,cm}$ values, and total polyphenol and flavonoid contents (Table 1). EEP samples from China (**f**, **g**, and **h**) and Hungary (**i**) showed strong antioxidant activities, also, in the assay system using the discoloration of β-carotene (Fig. 1). EEP samples from South Africa (**k**) and Uzbekistan (**p**), which had weak antioxidant activities in the assay system using the discoloration of β-carotene (Fig. 1), exhibited weak DPPH free radical scavenging activity. EEP from Thailand (**l**), with no antioxidant activity (Fig. 1), was observed to have extremely weak DPPH free radical scavenging activity.

In the present study, we found that Chinese propolis (**f**, **g**, and **h**) had strong DPPH free radical scavenging activity, whereas the activity of EEP from Brazil (**c**) was weak. However, several compounds with antioxidant or radical-scavenging activity have been isolated from Brazilian propolis (Basnet, Matsuno, & Neidlein, 1997; Hayashi, Komura, Isaji, Ohishi, & Yagi, 1999; Matsushige, Basnet, Kadota, & Namba, 1996). Park, Ikegaki, Alencar, and Moura (2000) reported that Brazilian propolis could be classified into 12 groups based on physicochemical characteristics, and that the biological activities of classified Brazilian propolis were also altered. The Brazilian propolis used in this study was from Minas Gerais, situated in southeastern Brazil. Thus it is considered that the antioxidant activity also differs with the region in Brazil.

The DPPH free radical scavenging activity shown in Fig. 2 seems to correlate with the antioxidant activity shown in Fig. 1. The propolis with antioxidant activity also has DPPH free radical scavenging activity. However, more detailed qualitative and quantitative analyses

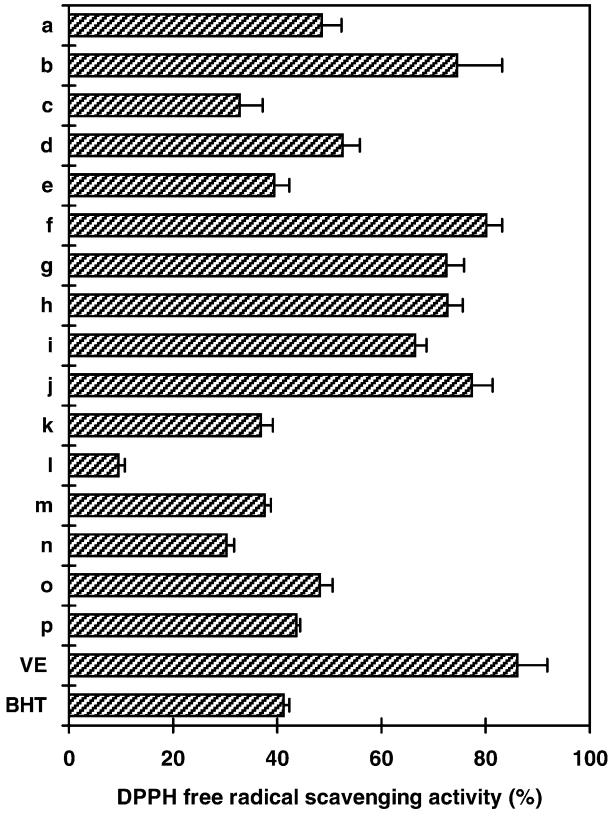


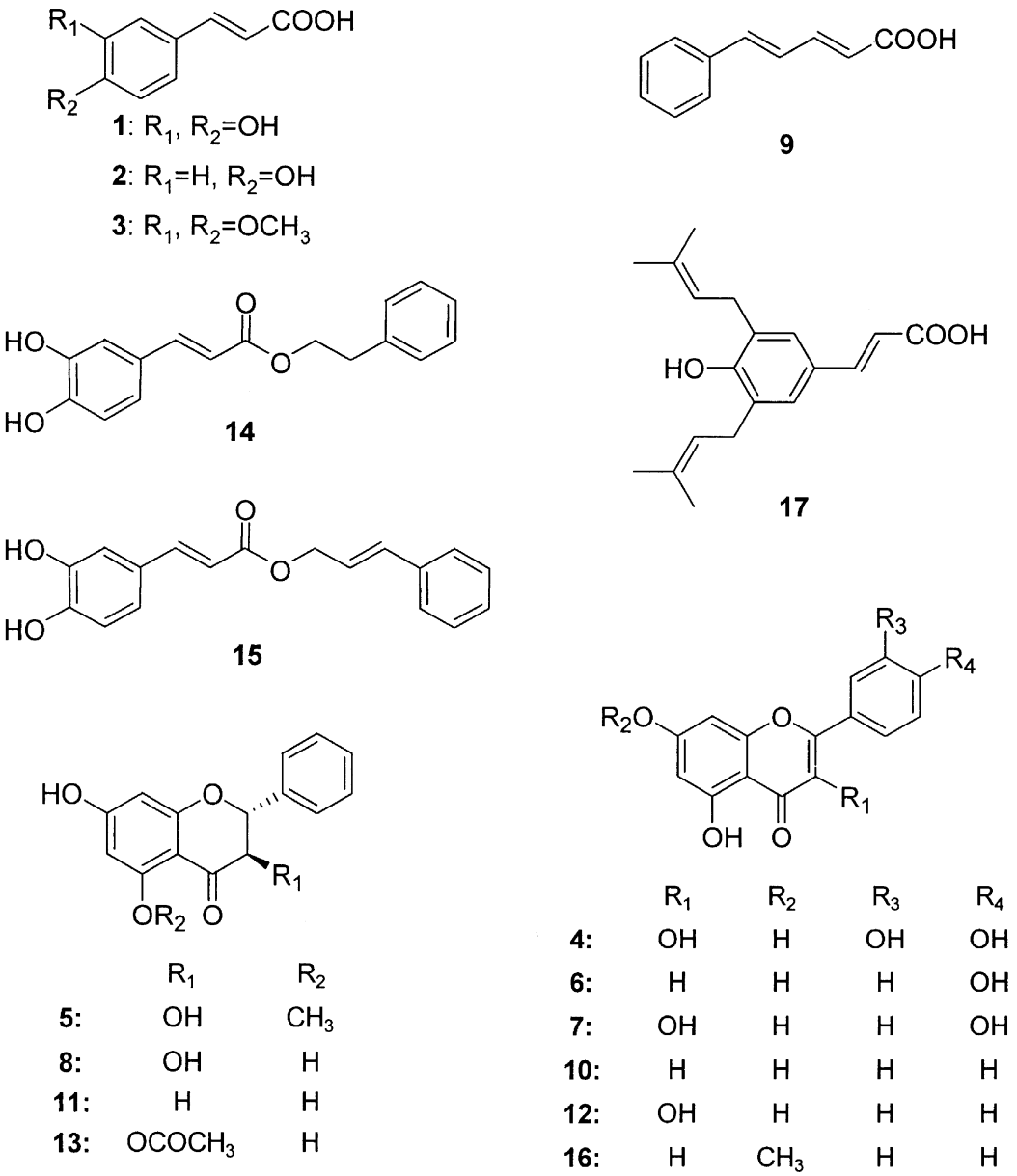
Fig. 2. DPPH radical scavenging activity of EEP (**a**–**p**) of various geographic origins. **a**, Argentina; **b**, Australia; **c**, Brazil; **d**, Bulgaria; **e**, Chile; **f**, China (Hebei); **g**, China (Hubei); **h**, China (Zhejiang); **i**, Hungary; **j**, New Zealand; **k**, South Africa; **l**, Thailand; **m**, Ukraine; **n**, Uruguay; **o**, United States; **p**, Uzbekistan; VE, α-tocopherol; BHT, butylated hydroxytoluene. EEP and the reference samples (VE and BHT) were used for the assay at the final concentration of 20 μg/ml. Measurements were carried out in triplicate. Means and standard deviations are indicated.

of the compounds with antioxidant activity will be necessary to elucidate the antioxidant activity of propolis.

3.4. HPLC analysis of various propolis samples

Major components in EEP samples were identified by HPLC analysis with PDA and MS detection. The chemical structures of the compounds identified are shown in Fig. 3. Previously, we isolated and identified 33 compounds, which are 18 flavonoids, 4 aromatic carboxylic acids, and 11 phenolic acid esters from Uruguayan propolis (Kumazawa, Hayashi et al, 2002). Compounds that could not be obtained from commercial sources were isolated from Uruguayan proplis, as authentic compounds to identify each component.

Fig. 4 shows the HPLC chromatograms of EEP samples **a**–**p**. The identified peaks, by the HPLC analysis with PDA and MS, are indicated by the numbers **1**–**17**. In order to identify each peak, UV spectra and the selected ion monitoring (SIM) of MS spectra of all peaks were compared with those of authentic samples. Several common compounds were observed in EEP samples, other than those from Brazil (**c**) and Thailand (**l**). However, the HPLC chromatogram pattern of EEP from South Africa (**k**) was slightly different from those of EEP samples other than from Brazil (**c**) and Thailand



	R_1	R_2
5:	OH	CH_3
8:	OH	H
11:	H	H
13:	$OCOCH_3$	H

	R_1	R_2	R_3	R_4
4:	OH	H	OH	OH
6:	H	H	H	OH
7:	OH	H	H	OH
10:	H	H	H	H
12:	OH	H	H	H
16:	H	CH_3	H	H

Fig. 3. Structures of the constituents identified from propolis. **1**, Caffeic acid; **2**, *p*-coumaric acid; **3**, 3,4-dimethoxycinnamic acid; **4**, quercetin; **5**, pinobanksin 5-methyl ether; **6**, apigenin; **7**, kaempferol; **8**, pinobanksin; **9**, cinnamylideneacetic acid; **10**, chrysin; **11**, pinocembrin; **12**, galangin; **13**, pinobanksin 3-acetate; **14**, phenethyl caffeate; **15**, cinnamyl caffeate; **16**, tectochrysin; **17**, artepillin C.

(**l**). Brazilian propolis has been reported to have a characteristic chemical composition, different from those of propolis from Europe, Asia, and North America (Fujimoto, Nakamura, & Matsuka, 2001; Marcucci & Bankova, 1999; Tazawa, Warashina, & Noro, 2000). The main compounds found in Brazilian propolis are prenylated derivatives of *p*-coumaric acid (Tazawa et al., 2000), as described above.

Each component was quantitatively analysed from the calibration curve of the HPLC chromatogram using authentic compounds. However, peaks **13** (pinobanksin 3-acetate) and **14** (phenethyl caffeate) overlapped on the HPLC chromatogram. We attempted to separate these peaks, using various HPLC conditions, but could not separate them completely. Thus, concerning the quantitative analysis of peaks **13** and **14**, we used the softwear "Xcalibur" (ThermoElectron, CA, USA) for quantitative determination, based on the ion intensity of MS spectrum. The SIM chromatograms and mass spectra of peaks **13** and **14** of EEP from Argentina (**a**) are shown in Fig. 5 as an example. The pseudomolecular ions $(M-H)^-$ of **13** (m/z 313) and **14** (m/z 283) were detected in the negative ESI mode, and each peak could be determined. It was reported previously that LC/MS

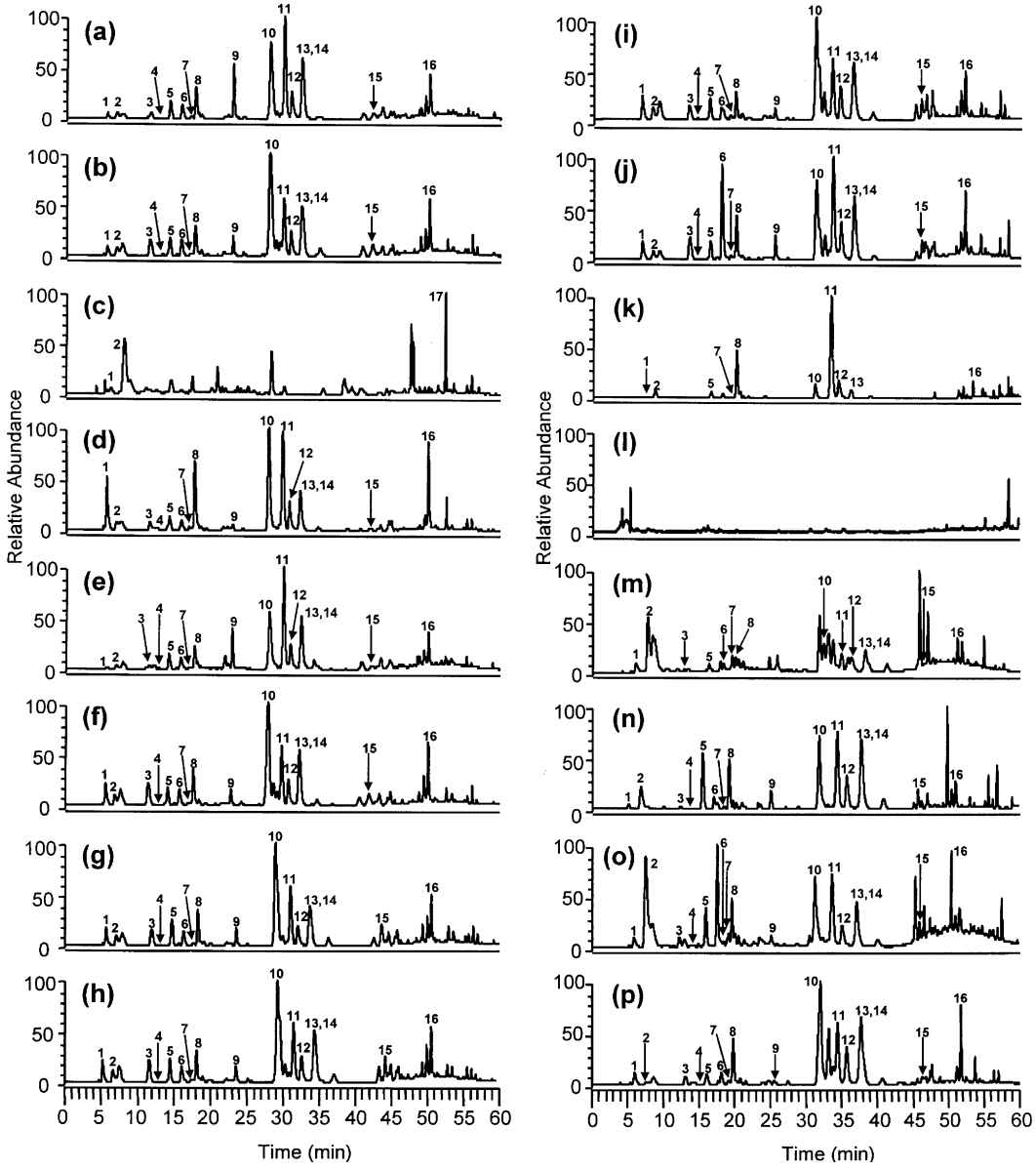


Fig. 4. HPLC chromatograms of EEP of various geographic origins. **(a)** Argentina; **(b)** Australia; **(c)** Brazil; **(d)** Bulgaria; **(e)** Chile; **(f)** China (Hebei); **(g)** China (Hubei); **(h)** China (Zhejiang); **(i)** Hungary; **(j)** New Zealand; **(k)** South Africa; **(l)** Thailand; **(m)** Ukraine; **(n)** Uruguay; **(o)** United States; **(p)** Uzbekistan. In each line the numbers of peaks represent the same compounds. **1**, Caffeic acid; **2**, *p*-coumaric acid; **3**, 3,4-dimethoxycinnamic acid; **4**, quercetin; **5**, pinobanksin 5-methyl ether; **6**, apigenin; **7**, kaempferol; **8**, pinobanksin; **9**, cinnamylideneacetic acid; **10**, chrysin; **11**, pinocembrin; **12**, galangin; **13**, pinobanksin 3-acetate; **14**, phenethyl caffeate; **15**, cinnamyl caffeate; **16**, tectochrysin; **17**, artepillin C.

analysis is an excellent technique for identifying the constituents in propolis (Kumazawa, Tazawa, Noro, & Nakayama, 2000). Midorikawa et al. (2001) also applied the LC/MS technique for analyzing propolis samples from Brazil, Peru, China, and The Netherlands. On the other hand, Tazawa et al. (2000) reported that 3D-HPLC analysis (PDA analysis) is an effective method for chemical evaluation of propolis. Further the combination of HPLC–PDA and MS has been reported to be a powerful approach for the rapid identification of phytochemical constituents in botanical extracts (He, 2000). The current study also shows that HPLC, with PDA and MS detection, can provide information on each peak in propolis for identification, based on comparison with standard compounds.

The results of the quantitative analysis of all EEP samples are shown in Table 2. Values are expressed as means of triplicated analyses for each sample. Caffeic acid (**1**) and *p*-coumaric acid (**2**) were detected in EEP samples, except for EEP from Thailand (**l**). Particularly, EEP from the United States (**o**) contained the largest amount of **2** (19.4 mg/g of EEP) in all EEP samples. 3,4-Dimethoxycinnamic acid (**3**) was detected in all EEP samples, other than from Brazil (**c**), South Africa (**k**), and Thailand (**l**). All EEP samples from China (**f**, **g**, and **h**) contained **3** over 7 mg/g of EEP. Quercetin (**4**) was present in propolis other than from Brazil (**c**), South Africa (**k**), Thailand (**l**), and Ukraine (**m**). Uruguayan propolis (**n**) especially contained a large amount of pinobanksin 5-methyl ether (**5**) (51.0 mg/g of EEP). Apigenin (**6**) was detected in the highest amount in EEP from New Zealand (**j**) (78.3 mg/g of EEP), but propolis from South Africa (**k**) did not contain **6**. Kaempferol (**7**) was detected in EEP samples other than from Brazil (**c**) and Thailand (**l**). Propolis from Ukraine (**m**) and the United States (**o**) contained **7** over 10 mg/g of EEP. Pinobanksin (**8**) was detected in almost all EEP samples and its amount was high. Propolis from Bulgaria (**d**) had the highest amount of **8** (84.8 mg/g of EEP) in all EEP samples. Although EEP from Brazil (**c**), South Africa (**k**), Thailand (**l**), and Ukraine (**m**) did not contain cinnamylidenacetic acid (**9**), EEP from Argentina (**a**) and Chile (**e**) contained much **9** (ca. 30 mg/g of EEP). Chrysin (**10**) is one of the representive flavonoids of propolis (Marcucci & Bankova, 1999) but it was not detected in propolis from Brazil (**c**) and Thailand (**l**). Propolis from China (**f**, **g**, and **h**), Oceania (**b** and **j**), and Bulgaria (**d**) contained a large amount of **10**, over 100 mg/g of EEP. Flavonoids such as pinocembrin (**11**), galangin (**12**), pinobanksin 3-acetate (**13**) and tectochysin (**16**), were abundantly detected in EEP samples other than from Brazil (**c**) and Thailand (**l**). Phenethyl caffeate (**14**) and cinnamyl caffeate (**15**) were seen in most EEP samples but not in propolis from Brazil (**c**), South Africa (**k**), and Thailand (**l**). Artepillin C (**17**) was detected only in Brazilian propolis (**c**).

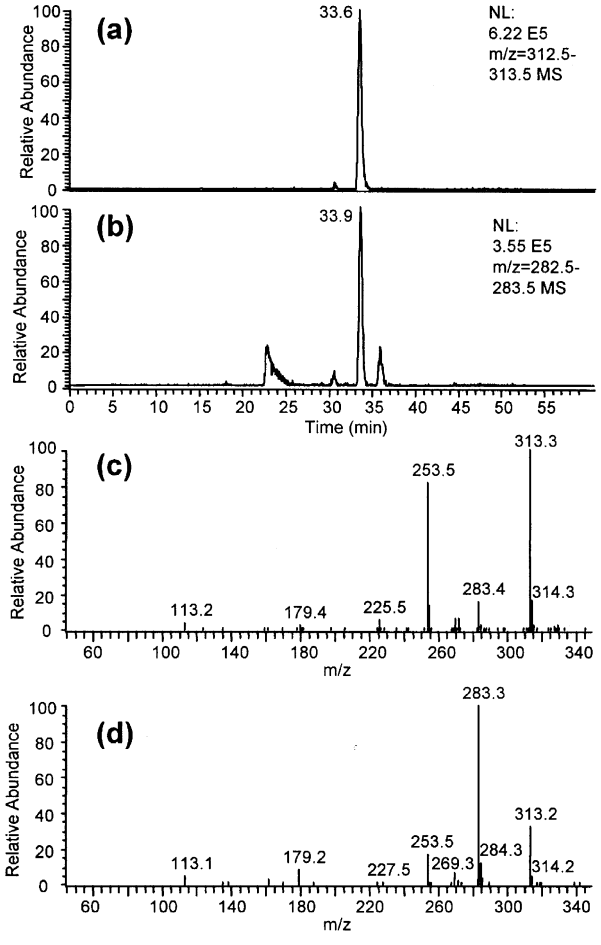


Fig. 5. Selective ion chromatograms (SIM) and mass spectra of EEP from Argentina (sample **a** in Fig. 6). **(a)** Ion chromatogram of the ion *m*/*z* 313. **(b)** Ion chromatogram of the ion *m*/*z* 283. **(c)** Mass spectrum of the compound eluting at t_R 33.6 min. Identified as **13** (pinobanksin 3-acetate). **(d)** Mass spectrum of the compound eluting at t_R 33.9 min. Identified as **14** (phenethyl caffeate).

Seventeen compounds were identified from 16 kinds of propolis and the quantitative values of each compound were determined in the present study. Nagai, Sakai, Inoue, Inoue, and Suzuki (2001) reported that propolis may contain compounds such as quercetin, flavones, isoflavones, flavonones, anthocyanins, and catechins. However, quercetin is a minor component of propolis, and is not found in some propolis samples (Table 2). Further isoflavones, anthocyanins, and catechins were not detected in the propolis samples used in this study (data not shown). Bankova et al. (2000) reported that the characteristic compounds of the propolis from Europe, Asia, and North America are pinocembrin, pinobanksin, pinobanksin 3-acetate, chrysin, galangin, caffeate (benzyl, phenylethyl, prenyl), and that the source plant is *Populus* spp. (poplar). We detected these compounds in the EEP samples other than those from Brazil, South Africa, and Thailand. Thus, the source plant of the propolis, other than that from Brazil, South Africa, and Thailand, is assumed to be the poplar

Table 2
Content 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**
Caffeic acid (**1**)	0.7	1.7	1.6	7.2	0.4	3.3	2.8	2.4	3.1	2.8	0.2	–	0.8	0.7	0.8	1.5
p-Coumaric acid (**2**)	1.8	3.6	27.4	3.5	1.9	4.0	4.0	3	3.7	3.1	1.5	–	8.9	8.4	19.4	0.9
3,4-Dimethoxycinnamic acid (**3**)	2.2	8.6	–	4.0	1.8	10.1	7.4	7.9	5.2	9.2	–	–	0.8	1.1	2.0	2.6
Quercetin (**4**)	2.2	4.8	–	4.7	1.5	4.4	3.8	0.8	4.4	1.2	–	–	–	2.5	3.8	0.8
Pinobanksin 5-methyl ether (**5**)	15.0	23.8	–	19.7	18.8	19.8	26.2	21.1	21.8	20.0	5.9	–	7.5	51.0	23.8	10.8
Apigenin (**6**)	12.0	18.4	–	13.4	14.2	17.1	17.1	14.3	9.0	78.3	–	–	3.9	14.8	0.6	8.1
Kaempferol (**7**)	2.3	3.9	–	5.0	1.4	2.1	2.6	2.5	4.8	3.7	1.0	–	10.9	2.5	10.3	3.8
Pinobanksin (**8**)	22.5	32.1	–	84.8	21.4	36.1	35.0	22.5	21.3	36.3	31.4	–	6.6	36.52	23.2	29.4
Cinnamylideneacetic acid (**9**)	30.4	14.6	–	6.3	31.2	11.2	12.7	10.5	7.8	18.1	–	–	–	13.7	5.2	2.9
Chrysin (**10**)	68.5	138.6	–	120.4	66.3	127.3	137.9	137.9	82.9	101.9	11.2	–	12.9	77.3	39.4	87.0
Pinocembrin (**11**)	68.7	58.7	–	94.4	86.2	54.8	61.5	46.9	51.2	99.7	69.8	–	9.2	75.0	46.7	44.5
Galangin (**12**)	32.5	42.5	–	45.6	37.7	39.6	33.5	32.6	44.2	58.2	18.9	–	13.4	48.8	21.5	41.4
Pinobanksin 3-acetate (**13**)	56.3	79.7	–	41.2	63.4	52.5	64.2	51.2	59.9	66.2	7.7	–	14.7	80.0	27.6	58.4
Phenethyl caffeate (**14**)	8.6	10.4	–	5.6	7.4	29.2	24.5	19.3	15.4	12.0	–	–	2.6	12.4	7.2	18.6
Cinnamyl caffeate (**15**)	6.6	16.6	–	0.7	6.1	16.3	20.3	14.4	13.8	12.7	–	–	2.1	8.9	7.5	2.2
Tectochrysin (**16**)	31.4	58.2	–	96.9	33.1	62.0	45.4	35.5	39.0	62.2	7.7	–	12.4	23.8	36.1	7.3
Artepillin C (**17**)	–	–	43.9	–	–	–	–	–	–	–	–	–	–	–	–	–

–: not detected.
[a] Values are expressed as mean of triplicate analyses for each sample.

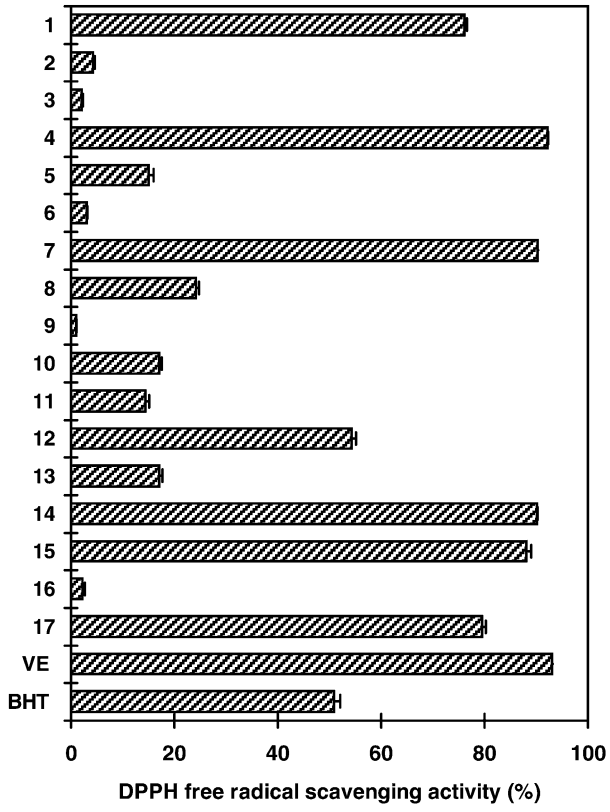


Fig. 6. DPPH free radical scavenging activity of the constituents identified in propolis. **1**, caffeic acid; **2**, p-coumaric acid; **3**, 3,4-dimethoxycinnamic acid; **4**, quercetin; **5**, pinobanksin 5-methyl ether; **6**, apigenin; **7**, kaempferol; **8**, pinobanksin; **9**, cinnamylideneacetic acid; **10**, chrysin; **11**, pinocembrin; **12**, galangin; **13**, pinobanksin 3-acetate; **14**, phenethyl caffeate; **15**, cinnamyl caffeate; **16**, tectochrysin; **17**, artepillin C; VE, α-tocopherol; BHT, butylated hydroxytoluene. Each sample was used for the assay at the final concentration of 20 μg/ml.

tree. Recently *Baccharis dracunculifolia* is reported to be an important source of Brazilian propolis, and to contain altepillin C (Bankova et al., 1999; Midorikawa et al., 2001). We also detected artepilin C in Brazilian propolis. Thus the origin plant of the Brazilian propolis used for this study may be *Baccharis dracunculifolia*. However, the source of the propolis from South Africa and Thailand is unknown. Particularly, the HPLC chromatogram of EEP from Thailand (**l**) showed a characteristic pattern without phenolics. Some components of EEP from Ukraine (**m**) were the same as those of EEP from Europe and China, whose origin plant is considered to be the poplar tree, but unknown components were also present (Fig. 4). Bankova et al. (2000) reported that *Betula verrucosa* (birch) is mentioned as the source plant in North Russia. Thus the origin plant of the propolis from Ukraine could be birch.

3.5. Effect of constituents in propolis on DPPH free radical

The effects of compounds **1**–**17** on scavenging of the DPPH free radical at the final concentration of 20 μg/ml are presented in Fig. 6. Marked differences in the activity were observed. Compounds **1**, **4**, **7**, **14**, **15**, and **17** exhibited strong DPPH free radical scavenging activity, over 60%. One of the common structural features for radical scavenging activity is the *o*-dihydroxy (catechol) phenyl structure, as seen in compounds **1**, **4**, **14**, and **15**. Bors, Heller, Michel, and Saran (1990) also reported that the *o*-dihydroxy phenyl ring is an important structure for the antioxidant activity of flavonoids.

Burda and Oleszek (2001) reported that the antioxidant activity depended on the presence of a flavonol structure or free hydroxyl group at the C-4′ position. The effect of **7** on scavenging the DPPH free radical, which has no *o*-dihydroxy phenyl ring, would be attributed to the flavonol structure with a free hydroxyl group at the C-4′ position. Compound **17** also showed strong DPPH free radical scavenging activity. However, **17** does not possess the *o*-dihydroxy phenyl ring, nor is it a flavonoid. The DPPH free radical scavenging activity of **17** has already been reported by Banskota et al. (2001a).

The propolis of various geographic origins had different antioxidant activities, as shown in Figs. 1 and 2. These differences are attributable the quantities of the compounds with antioxidant activity in propolis, because EEP samples **a**, **b**, **f**, **g**, **h**, **i**, and **j** that had antioxidant activity contained antioxidative compounds **1**, **4**, **7**, **14**, and **15** (Table 2). The antioxidant activity of Brazilian propolis was weaker than those of European and Chinese propolis, as shown in Figs. 1 and 2. However, in Brazilian propolis, the water extracts have been reported to possess stronger antioxidant activity than the methanol extracts (Banskota et al., 2000). The reason that we could not observe strong antioxidant activity of Brazilian propolis in this study might be because we used ethanol extracts. Actually, several antioxidative compounds, such as propol {3 - [4 - hydroxy - 3 - (3 - oxo - but - 1 - enyl) - phenyl]-acrylic acid} (Basnet et al., 1997) and quinic acid derivatives (Matsushige et al., 1996) were isolated from the water extracts of Brazilian propolis. In addition, the in vivo antioxidative activity of Brazilian propolis has been also reported (Sun et al., 2000).

The naturally occurring polyphenols are expected to help reduce the risk of various life-threatening diseases, including cancer and cardiovascular diseases, due to their antioxidant activities. Thus propolis with antioxidant activity may protect humans from deleterious oxidative processes. Banskota et al. (2001a) also reported that the antioxidative activity of propolis is due to its phenolic constituents, which also possess antitumour and antihepatotoxic activities.

4. Conclusion

In this study, the in vitro antioxidant activity of various propolis samples was investigated. Major constituents were identified in propolis and quantitatively analysed. Differences were observed in the constituents and quantitative values in the propolis of various geographic origins. Thus, it was difficult to evaluate the quality of propolis.

The source of European propolis is considered to be poplar trees. However, European propolis that was not of poplar origin has recently been reported (Bankova, Popova, Bogdanov, & Sabatini, 2002). This finding also illustrates the diversity of propolis. There is a clear need to clarify the quality and quantity of the constituents in propolis, in order to evaluate its biological activity.

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