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Identification of Metabolites in Plasma and Urine of Uruguayan Propolis-Treated Rats

SHIGENORI KUMAZAWA,*,† KAYOKO SHIMOI,‡ KATSUMI HAYASHI,† TAKESHI ISHII,† TOMOKO HAMASAKA,† AND TSUTOMU NAKAYAMA†

Laboratory of Functional Food Science and COE Program in the 21st Century, School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan, and Laboratory of Pathophysiology and Toxicology, Institute for Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

Propolis is a resinous substance collected by honeybees from various plant sources. It is extensively used in food and beverages to improve health and prevent diseases such as heart disease, diabetes, and cancer. To investigate the absorption and metabolism of the components in propolis, in the present study, we administered ethanol extracts of Uruguayan propolis (poplar type propolis) orally to rats and analyzed their plasma and urine by high-performance liquid chromatography with photodiode array and mass spectrometric detection. After deconjugation of the components by β-glucuronidase/sulfatase treatment of the specimen, pinobanksin 5-methyl ether, pinobanksin, kaempferol, chrysin, pinocembrin, and galangin were detected in plasma of rats orally administered propolis. These compounds were detected also in urine after β-glucuronidase/sulfatase treatment. Furthermore, pinobanksin 5-methyl ether, pinobanksin, chrysin, pinocembrin, and galangin were present in the urine also in free form. These results suggest that flavonoids in propolis are metabolized and circulate in the body after oral administration of propolis.

KEYWORDS: Propolis; absorption; excretion; metabolites; flavonoid

INTRODUCTION

Propolis, a natural substance collected by honeybees from buds and exudates of certain trees and plants, has been considered to be used in the beehive as a protective barrier against enemies. Propolis has been used in folk medicines in many regions of the world (*1*) and has been reported to have various biological activities such as antibacterial (*2*), antiviral (*3*), antiinflammatory (*4*), and anticancer (*5*) 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 (*6*, *7*).

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 (*8*, *9*). Because of the geographical difference, propolis from Europe, South America, and Asia has different chemical compositions (*8*−*16*). Propolis from Europe and China contains many kinds of flavonoids and phenolic acid esters (*8*−*14*). By contrast, the major components in propolis of Brazilian origin were terpenoids and prenylated derivatives of *p*-coumaric acids (*17*−*20*).

In recent years, some beverages and foodstuffs rich in polyphenols, e.g., green tea, red wine, and cocoa, have been attracting interest due to their potent antioxidant properties and possible preventive effects against cancer and coronary heart diseases (*21*−*24*). Thus, it is important to determine how polyphenol components in food are metabolized in vivo and how their metabolites function in a living system. Propolis from Europe and China contains many kinds of polyphenols such as flavonoids and phenolic acid esters as described above. Although these propolis are polyphenol-rich materials, there are few studies on the absorption and metabolism of the components in propolis.

The purpose of this study was to investigate the absorption of polyphenols in propolis, especially flavonoids, to provide pharmacokinetic profiles of the compounds in propolis and their metabolites in rats. We used Uruguayan propolis for this study because we extensively analyzed its components and clarified that it contains many kinds of flavonoids such as pinocembrin and galangin and its constituents were similar to those of propolis from Europe and China (*25*). Following the oral administration of Uruguayan propolis, metabolites in the plasma and the renal excreted urine were examined by high-performance liquid chromatography (HPLC) analysis with a PDA and MS detection.

* To whom correspondence should be addressed. Tel: +81-54-264-5525. Fax: +81-54-264-5551. E-mail: kumazawa@smail.u-shizuoka-ken.ac.jp.
† School of Food and Nutritional Sciences.
‡ Institute for Environmental Sciences.

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MATERIALS AND METHODS

Chemicals. β-Glucuronidase/sulfatase was purchased from Sigma (St. Louis, MO). Other chemicals were of analytical or HPLC grade.

Propolis Sample. Propolis collected in Uruguay was supplied from Aichi Uruguay S. A. (Montevideo, Uruguay). Crude propolis material was 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 the EEP.

Animals and Diets. Male SD rats, 7−9 weeks old (SLC, Hamamatsu, Japan), weighing 180−200 g, were housed in an air-conditioned room under 12 h dark/12 h light cycles, with free access to tap water and CE-2 commercial food pellets (Crea Japan, Tokyo, Japan) at first; then, this diet was changed to a synthetic basal diet, which consists of 38% corn starch, 25% casein, 10% α-starch, 8% cellulose powder, 6% minerals, 5% sugar, 2% vitamins, and 6% lard (Oriental Yeast, Tokyo, Japan) 1 week before the experiments. Animals were maintained and handled according to Guidelines for the Regulation of Animal Experimentation Committee of the University of Shizuoka.

Sample Preparation of Blood and Urine. EEP was dissolved in propyleneglycol before administering to rats to avoid the toxicity of ethanol. Rats (n = 4/group) fasted overnight were anesthetized with ether at 1.0, 2.0, 4.0, 8.0, and 24.0 h following administration of EEP (15 mg/kg in propyleneglycol) by gastric intubation, and blood was withdrawn from the abdominal aorta into heparinized tubes. Nontreated specimens were used as a control or 0 h plasma. In addition, the urine was collected for 24 h following administration of EEP (15 mg/kg in propyleneglycol) using a metabolic cage (n = 4/group). Plasma and urine samples were analyzed by HPLC with PDA and MS detection.

HPLC−PDA Analysis of Metabolites in Plasma and Urine. The samples (0.5 mL) of plasma and urine were acidified with the same volume of 0.01 M oxalic acid. This solution was applied to a Sep-Pak C_{18} cartridge. After the cartridge was washed with 0.01 M oxalic acid and distilled water, the methanol eluate was obtained. The eluate was evaporated to dryness, and the residue was dissolved in 100 μL of methanol. After centrifugation for 2 min at 0 °C and 15 000 rpm, the supernatants were used for HPLC analysis. The metabolites of propolis were analyzed chromatographically with a Jasco HPLC system (Tokyo, Japan) using a 250 mm × 4.6 mm i.d. Capcell Pak C18 UG120 column (Shiseido, Tokyo, Japan). The mobile phase consisted of 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient was 20−80% B in 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.

For detection of the conjugates, each sample was acidified with 1 M acetate buffer (pH 4.5) and was preincubated for 2 min at 37 °C. Solutions were treated with 2.8×10^3 units/mL of β-glucuronidase and 2.5×10^2 units/mL of sulfatase for 20 min at 37 °C, and then, the same volume of 0.01 M oxalic acid was added. The mixtures were centrifuged for 5 min at 8000 rpm. Supernatants were prepared and analyzed in the same way as described above.

LC-MS Analysis. LC-MS (ESI method) analysis was performed in a Thermo Electron (San Jose, CA) LCQ mass spectrometer with a Shiseido HPLC SI-1 apparatus using a 250 mm × 2.0 mm i.d. Capcell Pak C18 UG120 column (Shiseido) and UV detection (280 nm). The gradient conditions of the mobile phase were the same as described above.

The operating parameters of MS 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 mode.

RESULTS

Identification of Propolis Metabolites in Rat Plasma. **Figure 1** shows the HPLC chromatograms of the ethanol extracts of Uruguayan propolis and those of the plasma extracts from rats analyzed with or without β-glucuronidase/sulfatase treatment for samples obtained 1 h after administration of propolis. Three metabolite peaks (A, 17.1 min; B, 21.3 min; C, 23.1 min) were observed in the chromatogram of the plasma extracts with β-glucuronidase/sulfatase treatment (**Figure 1c**). These peaks were not observed before the treatment with β-glucuronidase/sulfatase (**Figure 1b**). These results demonstrated that peaks A−C were glucuronide and/or sulfate conjugates.

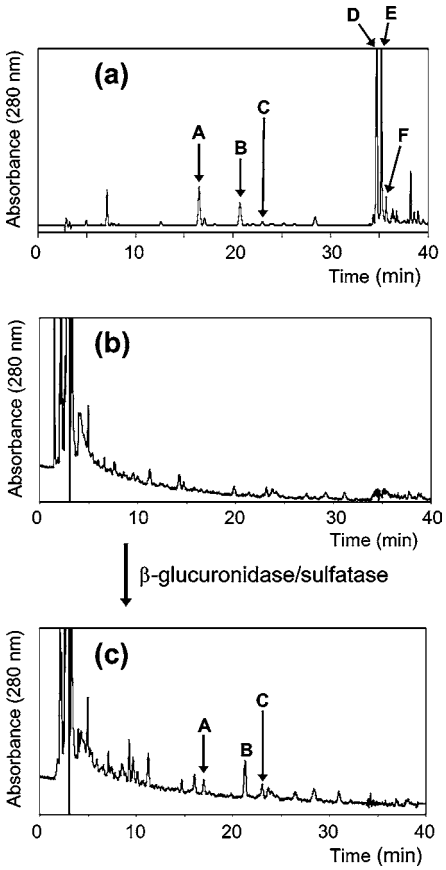

Figure 1. HPLC chromatograms of (**a**) the ethanol extracts of Uruguayan propolis and the plasma extracts from rats 1 h after administration of propolis (**b**) without and (**c**) with β-glucuronidase/sulfatase treatment. (A) Pinobanksin 5-methyl ether, (B) pinobanksin, (C) kaempferol, (D) chrysin, (E) pinocembrin, and (F) galangin.

The plasma extract from propolis administered rats was analyzed by HPLC with PDA and MS detection. **Figure 2** shows the ESI mass spectra of peaks A−C. The negative ESI-MS of peaks A−C showed molecular ion peaks at m/z 285.2, 271.2, and 285.1 $(M - H)^-$, respectively. Furthermore, their retention times and UV absorption spectra obtained by PDA were compared with those of authentic compounds isolated from Uruguayan propolis. On the basis of the mass spectra, retention times, and UV spectra, peaks A, B, and C were identified as pinobanksin 5-methyl ether, pinobanksin, and kaempferol, respectively. **Figure 3** shows the HPLC chromatograms of the plasma extracts from rats analyzed with β-glucuronidase/sulfatase treatment for samples obtained 2, 4, 8, and 24 h after administration of Uruguayan propolis. Peaks D−F (D, 34.8 min; E, 35.3 min; F, 36.9 min) were detected in the samples 4 h after administration of propolis by SIM scanning of the negative ESI-MS (**Figure 3b**) although these peaks were not detected in the samples 1 and 2 h after administration of propolis (**Figure 1**). The presence of peaks D−F was confirmed also in the plasma samples of 8 and 24 h after administration of propolis by SIM scanning (**Figure 3c**,**d**). Peaks D−F were identified as chrysin, pinocembrin, and galangin, respectively, as described below (**Figure 6**), but their amounts in the plasma samples were very low.

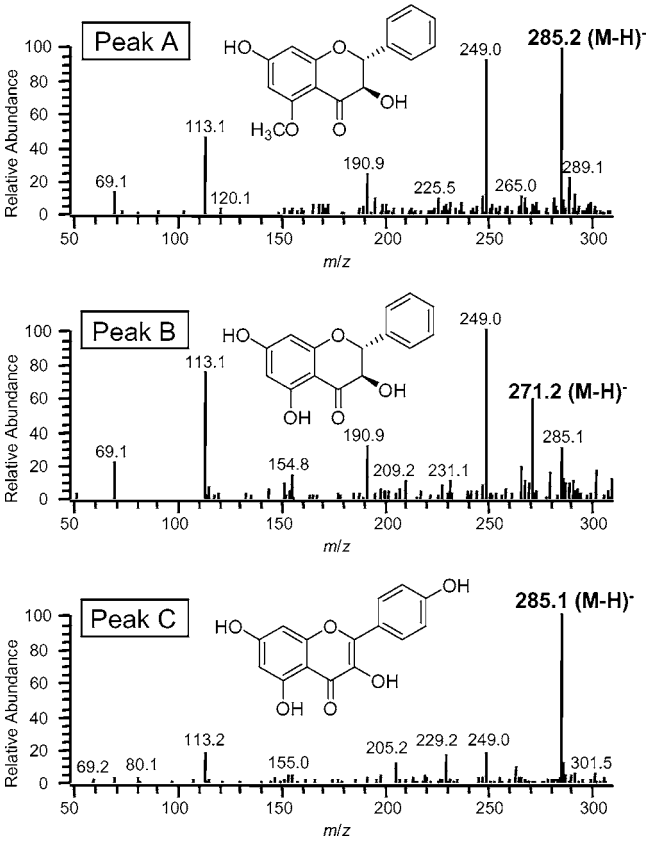

Figure 2. Mass spectra and structures of peaks **A**, **B**, and **C** after β-glucuronidase/sulfatase treatment of the plasma extracts from propolis-administered rats. (A) Pinobanksin 5-methyl ether, (B) pinobanksin, and (C) kaempferol.

The time courses of the concentration of the conjugates of pinobanksin 5-methyl ether, pinobanksin, and kaempferol (peaks A−C) in rat plasma after oral administration of propolis are shown in **Figure 4**. Quantification of peaks D−F was not established due to the small amounts and overlapping of each peak. The amounts of the conjugates of peaks A−C were determined as those of the free form after treatment with β-glucuronidase/sulfatase. The concentrations of the conjugates of pinobanksin 5-methyl ether and pinobanksin in rat plasma increased to the highest level 1 h after administration of propolis in propyleneglycol and decreased gradually. On the other hand, the kaempferol conjugate reached its maximum concentration within the first 2 h after administration.

Identification of Propolis Metabolites in Rat Urine. Figure 5 shows the HPLC chromatograms before and after β-glucuronidase/sulfatase treatment of the 24 h urine extracts from propolis administered rats. Peaks A−F were detected also in the samples after β-glucuronidase/sulfatase treatment of the 24 h urine extracts from propolis administered rats. Furthermore, peaks A, B, D, E, and F were observed before the treatment with β-glucuronidase/sulfatase. Thus, these compounds were excreted in a free form. **Figure 6** showed the negative ESI-MS and chemical structures of peaks D, E, and F. These peaks gave more definitive molecular ion peaks than those observed in the plasma samples.

Table 1 shows the contents of peaks A−F in rat urine 24 h following oral administration of propolis. The contents of these peaks increased by the treatment with β-glucuronidase/sulfatase, indicating that these peaks are excreted even in a conjugated form. The content of each peak in EEP is also shown in **Table 1**. Pinobanksin was excreted most efficiently on a basis of the content in EEP.

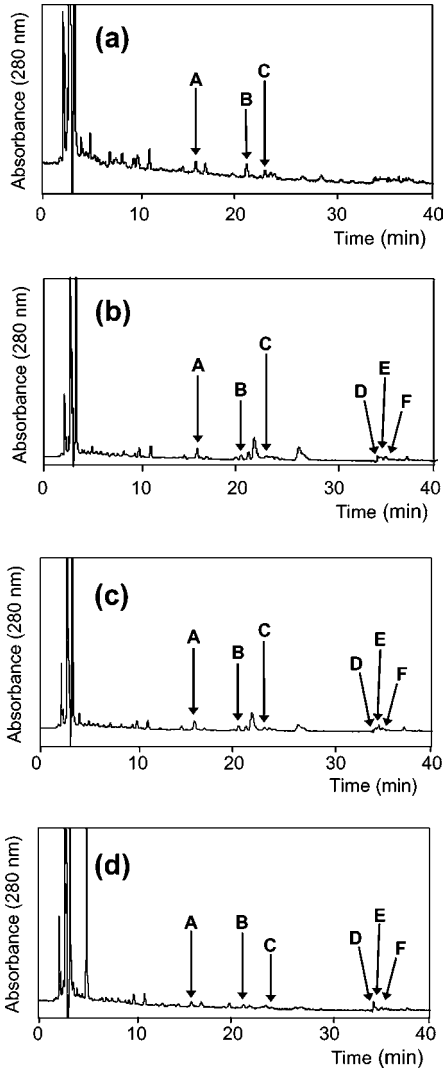

Figure 3. HPLC chromatograms of the plasma extracts from rats (**a**) 2, (**b**) 4, (**c**) 8, and (**d**) 24 h after administration of propolis with β-glucuronidase/sulfatase treatment.

DISCUSSION

Recently, the bioavailability, pharmacokinetics, and metabolism of polyphenols, including flavonoids, have been focused on the chemoprevention of oxidative stress by active oxygen species and free radicals in aging and disease (*26*). There have been many studies showing that flavonoids in a free form or a glycosylated form are absorbed from the intestinal tract and are metabolized to glucuronide or sulfate conjugates (*27*−*32*). These metabolites circulate in the blood and are excreted into urine and bile.

In the present study, we investigated the absorption of polyphenols in Uruguayan propolis in rats. We previously isolated many kinds of flavonoids and phenolic acid esters from this propolis (*25*). In the propolis, those flavonoid compounds were present in the aglycone form but not as glycosides. Most metabolites from flavonoids found in rat plasma were in the form of glucuronide or sulfate conjugates (*29, 30*). Thus, we analyzed the metabolites of the administered propolis after the treatment with β-glucuronidase/sulfatase. We reported previously that LC-MS analysis is an excellent technique for

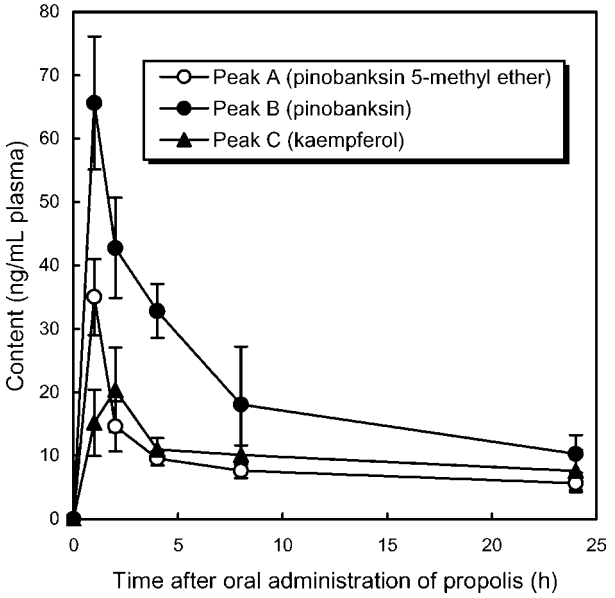

Figure 4. Contents of the conjugates of peaks A, B, and C in plasma of rats after oral administration of propolis. The amount of the conjugate of each component was determined as that of the free form analyzed after treatment with β-glucuronidase/sulfatase. Each value represents the mean ± SD ($n = 4$).

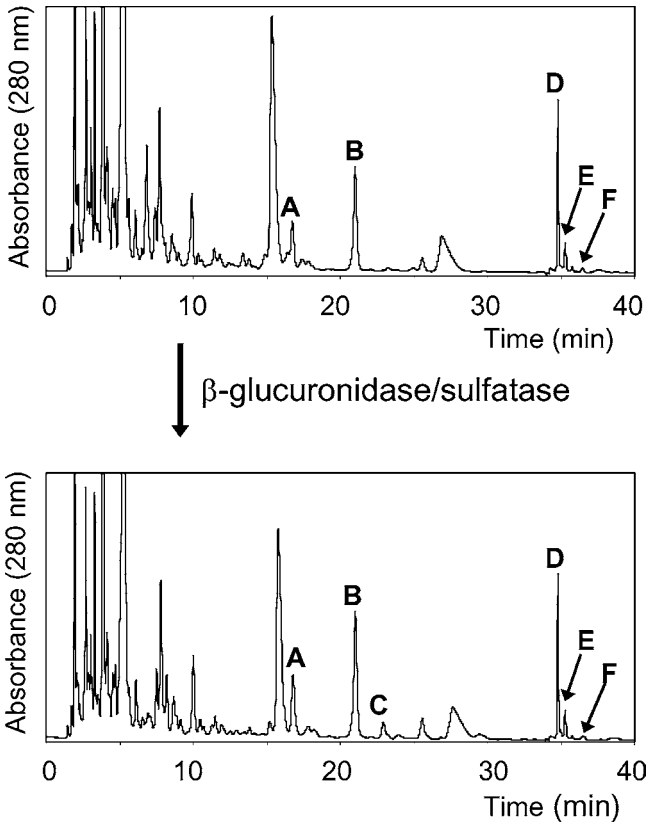

Figure 5. HPLC chromatograms before and after β-glucuronidase/sulfatase treatment of the urine extracts from propolis-administered rats.

identifying the constituents in propolis (*9*, *33*). Here, not only MS but also PDA detection was applied for the identification of each peak in propolis.

Peaks A (pinobanksin 5-methyl ether), B (pinobanksin), C (kaempferol), D (chrysin), E (pinocembrin), and F (galangin) were detected in the plasma of rats administered propolis. These peaks were detected only after the treatment with β-glucuronidase/sulfatase. The presence of the free form of these compounds (A−F) in rat plasma was not confirmed since the molecular ions of these peaks were not detected by SIM of MS spectra without enzyme treatment (data not shown). This suggested that peaks A−F are present in rat plasma as the conjugated form. Peaks D−F were not detected until 2 h after administration of propolis, suggesting that the compounds corresponding to these peaks are absorbed into the body gradually and slowly. The new peaks other than peaks A−F also appeared after the enzyme treatment, but we could not identify these peaks since they were present in only trace amounts. On the other hand, peaks A−F were detected also after β-glucuronidase/sulfatase treatment of the urine extracts

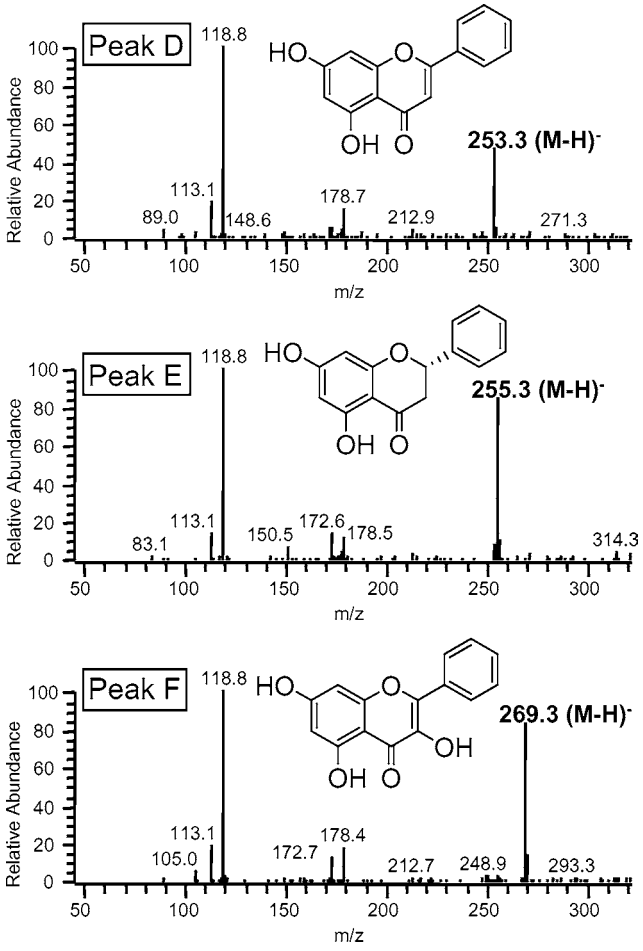

Figure 6. Mass spectra and structures of peaks **D**, **E**, and **F** after β-glucuronidase/sulfatase treatment of the urine extracts from propolis-administered rats. (D) Chrysin, (E) pinocembrin, and (F) galangin.

Table 1. Contents of Peaks A–F in EEP and in 24 h Urine of Rats after Oral Administration of Propolis[a]

	propolis (μg/mg of EEP)[b]	urine (μg/24 h urine)	
		+ enzyme	− enzyme
peak A (pinobanksin 5-methyl ether)	22.16	3.89 ± 0.35	2.38 ± 0.45
peak B (pinobanksin)	9.94	5.94 ± 0.69	4.76 ± 0.58
peak C (kaempferol)	3.41	0.18 ± 0.01	ND[c]
peak D (chrysin)	55.58	5.36 ± 0.41	4.72 ± 0.44
peak E (pinocembrin)	38.56	2.37 ± 0.06	2.12 ± 0.19
peak F (galangin)	16.98	1.49 ± 0.07	1.47 ± 0.06

[a] Values are means ± SD ($n = 4$). [b] Values are means of triplicate analyses. [c] Not detected.

obtained from propolis-administered rats. The peaks other than peak C were detected without enzyme treatment. Thus, these components (A, B, D, E, and F) proved to be excreted into the urine in a free form.

There are several studies evaluating the antioxidant activity of propolis (*34*−*37*). We reported that the in vitro antioxidant activity of propolis from various geographic origins was correlated with the contents of antioxidative compounds such as kaempferol and phenethyl caffeate (*9*, *16*). On the other hand, Sun et al. reported that the in vivo antioxidative activity of Brazilian propolis (*38*). The major components in Brazilian propolis are terpenoids and prenylated derivatives of *p*-coumaric acids, not flavonoids (*19*). Kolankaya et al. reported that Turkish propolis showed a protective effect against alcohol-induced liver damage and improved lipid profile (*39*). Because they reported that Turkish propolis contains many flavonoids such as galangin, quercetin, kaempferol, apigenin, pinobanksin, pinocembrin, and pinostrobin, it is assumed that flavonoids in propolis are absorbed into the body and cause the scavenging free radicals, thereby protecting against lipid peroxidation.

Recently, many studies on the absorption and metabolism of flavonoids have been reported (*27*−*32*). For example, quercetin, which is a flavonoid in onion, has been shown to be absorbed and to be bioavailable in humans (*27*). Furthermore, the study of the intestinal absorption of isoflavonoids has also been reported (*40*). However, there are no reports concerning the absorption and the metabolism of the characteristic flavonoids in propolis such as pinocembrin and galangin.

Table 1 shows the contents of peaks A−F in propolis and in 24 h urine following oral administration of propolis. Peak B, pinobanksin, was detected in a higher amount in the urine than other peaks with and without enzyme treatment. We previously reported that Uruguayan propolis contains many pinobanksin derivatives with various alkyl esters bonded at the 3-position (*25*). The ester bond of the 3-position of these pinobanksin derivatives is considered to be cleaved by hydrolysis after the compounds are administered. Thus, the content of pinobanksin in the urine shown in **Table 1** would include the content derived from its derivatives with ester bonds. Therefore, pinobanksin was detected in the highest amount in the urine.

We previously reported that the free metabolite of eriodictyol, a flavonoid in lemon fruit, was detected in urine but was not detected in plasma (*41*). This suggests that the conjugated metabolites were hydrolyzed to free aglycones in the kidneys and then excreted into urine (*41*, *42*). Also in the present study, the free metabolites were detected not in the plasma (**Figures 1** and **3**) but in the urine (**Figure 5**). This suggests a similar mechanism of metabolism. We searched for the methylated metabolites of peaks A−F in plasma and urine using SIM of MS spectra but could not find them. This might be because these compounds were present in only trace amounts. Methylated metabolites of flavonoids such as quercetin and eriodictyol have been detected in rat urine (*32*, *41*).

Kaempferol and its metabolite are reported to have antioxidant activity (*9*, *43*). We detected kaempferol in plasma as shown in **Figures 1** and **3**. Thus, following administration of Uruguayan propolis, plasma is expected to have antioxidant activity and thereby is resistant to lipid peroxidation. The chemical composition of propolis is extremely complex, and more than 150 constituents have been identified so far (*8*). Propolis contains many polyphenols including kaempferol (*9*). Thus, the polyphenols other than kaempferol, which have antioxidant activity, are expected to be present in plasma.

In the present study, we demonstrated for the first time the presence of several flavonoids from propolis in rat plasma and urine. This suggests that flavonoids in propolis are absorbed from intestinal cells and protect the lipid membranes from oxidative injury. However, several unknown minor peaks for metabolites of propolis extracts were detected in the rat plasma and urine after administration of propolis extracts. Further investigation of these unknown peaks is also required.

ABBREVIATIONS USED

EEP, ethanol extracts of propolis; ESI, electrospray ionization; MS, mass spectrometry; PDA, photodiode array; SIM, selected ion monitoring.

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