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Suppressive Effects of Ethanolic Extracts from Propolis and Its Main Botanical Origin on Dioxin Toxicity

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Suppressive effects of ethanolic extracts prepared from propolis group 12 and its main botanical origin (leaf bud of *Baccharis dracunculifolia*) on transformation of the aryl hydrocarbon receptor (AhR), the initial action of dioxin toxicity, were investigated. It was found that suppressive effects of propolis on AhR transformation were relatively higher than those of resins of its botanical origin in cell-free system and in Hepa-1c1c7 cells. When the composition of chemical ingredients was measured, propolis contained slightly higher amounts of flavonoid aglycones as compared with its botanical origin with the same characteristics. Moreover, antiradical activity, one of the typical biological activities of flavonoids, in propolis was also slightly higher than that in its botanical origin. These results indicate that not only propolis but also its botanical origin contains high amounts of flavonoid aglycones and that both of them are useful dietary sources for flavonoids with a potency to prevent dioxin toxicity.

KEYWORDS: Propolis; *Baccharis dracunculifolia*; flavonoid; dioxin; TCDD; aryl hydrocarbon receptor

INTRODUCTION

Propolis is the resinous substance collected by honeybees from various plant resins, mainly resins of leaf bud, and it has been used as a folk medicine since about 300 B.C. (*1*). Recently numerous biological activities of propolis have been reported such as antitumor, antiradical, antimicrobial, and anti-HIV activities (*2*−*5*). Previously, we classified Brazilian propolis into 12 groups on the basis of their physicochemical characteristics (*6*, *7*); of the 12 groups of propolis, group 12, which is collected from southeastern and central western Brazil, has been extensively used in foods and beverages. Moreover, it was also reported that the main botanical origin of group 12 propolis was resins of *Baccharis dracunculifolia*, and chemical constituents in resins of the leaf bud from this botanical origin were similar to those in propolis group 12 (*8*−*10*). These results strongly indicate that the biological activities of resins from leaf bud of botanical origin will be shown to be the same as those of propolis.

Recently, we have demonstrated that the ethanolic extracts of propolis suppress transformation of the aryl hydrocarbon receptor (AhR), which is a novel biological activity of propolis (*11*). AhR transformation is recognized as the initial action of dioxin toxicity. Dioxins, including the most toxic congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are mainly incorporated into the body by ingestion of contaminated foods (*12*, *13*) and bind to the cytosolic AhR; then the dioxin/AhR complex translocates to the nucleus, where it forms a heterodimer with another bHLH/PAS family protein, AhR nuclear translocator (ARNT). This heterodimer binds to the dioxin response element (DRE), which is a cis-acting element found in the 5′ regulatory regions of dioxin-responsive genes, and induces the transcription of a battery of genes and subsequent production of proteins including drug-metabolizing enzymes (*14*). Transformed AhR also alters the phosphorylation status of various proteins, including signal transduction pathways for growth factors (*15*, *16*). Thus, the suppression of AhR transformation by food components could possibly reduce the AhR-mediated biological responses caused by dioxins.

The aim of this study is to compare the suppressive effects of the ethanol extract of propolis with those of resins of leaf bud from *B. dracunculifolia* (botanical origin of propolis) on AhR transformation. The suppressive effects against TCDD-induced AhR transformation were determined in a cell-free system with the rat liver cytosolic fraction by gel retardation AhR binding (GRAB) assay and in mouse hepatoma cell linage, Hepa-1c1c7, by a newly developed assay, southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA), that is able to quantify the transformation state of AhR (*17*). Characteristics of the chemical constituents in resins of leaf bud from *B. dracunculifolia* are similar to those in propolis group 12, and both of them contain flavonoid aglycones abundantly (*9*). Certain flavonoid aglycones, such as galangin, kaempferol,

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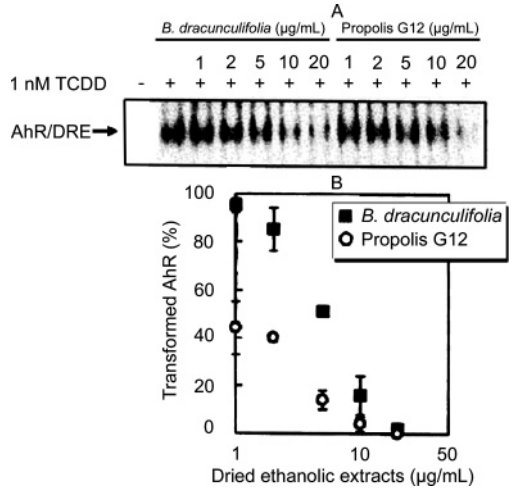

Figure 1. Suppressive effect of ethanolic extracts of propolis G12 and leaf bud of *B. dracunculifolia* on TCDD-induced AhR transformation. Suppressive effects were examined in cell-free system using rat hepatic cytosol: (**A**) typical representative result of GRAB assay; (**B**) percent of transformed AhR from the quantified density of AhR/DRE complex.

and apigenin, strongly suppress AhR transformation (*18*). However, the characteristics of the chemical constituents of propolis vary by collected areas and years. Therefore, we also determined the amounts of flavonoids in the used propolis and resins of leaf bud from *B. dracunculifolia* and measured their antiradical activity, one of the typical biological activities of flavonoids.

MATERIALS AND METHODS

Propolis and Its Botanical Origin. Brazilian propolis has been classified into 12 groups by physicochemical characteristics (*8*). Among these 12 groups of propolis, group 12 and resins of leaf bud of *B. dracunculifolia*, which is the main botanical origin of the propolis, were collected in the state of Minas Gerais, Brazil, in 2003 and used in this study.

Preparation of Ethanolic Extracts of Propolis and Resins of Leaf Bud. The ethanolic extracts of propolis and resins of leaf bud were prepared as previously described (*9*). The propolis (50 g) was extracted with 600 mL of 80% (v/v) ethanol at 60 °C for 30 min. After extraction, the mixture was centrifuged to give the supernatant, and the extracts were evaporated to dryness at 40 °C. The leaf bud was removed with a knife without breaking them into pieces, and immediately 30 g of the samples was rinsed with 300 mL of 80% ethanol at 60 °C for 1 h to remove superficial resins and then centrifuged to separate the supernatant; the extracts were then evaporated to dryness at 40 °C. The dried extracts of propolis and the resins of leaf buds from botanical origin of propolis were resolved in ethanol and used for measurement of antiradical activity and suppressive effects on AhR transformation.

Measurement of AhR Transformation in Cell-free System by GRAB Assay. Preparation of the rat liver cytosolic fraction was prepared as previously described (*18*) according to the *Guidelines for the care and use of experimental animals of Rokkodai Campus, Kobe University*, and the obtained cytosol was used as a source of AhR to determine the suppressive effects of propolis extracts by gel retardation assay (*18, 19*). Briefly, the cytosol (4 mg of protein/mL) was incubated with 1 nM TCDD in dimethyl sulfoxide in 25 mM HEPES of pH 7.4 containing 1.5 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol at 20 °C for 2 h in the dark. A control sample was incubated with dimethyl sulfoxide (10 μL/mL) alone as a vehicle. Ethanolic extracts of propolis and its botanical origin at the concentrations indicated in **Figure 1** were added to the cytosol 10 min before the addition of 1 nM TCDD. AhR transformation was measured by GRAB assay as previously described (*18, 19*).

Measurement of AhR Transformation in Hepa-1c1c7 Cells by SW-ELISA. Mouse hepatoma Hepa-lclc7 cells were grown and maintained as previously described (*20*). Briefly, the cells were seeded on 60-mm plastic dishes at the concentration of 2.5×10^5 cells/mL and incubated for 48 h. To estimate the antagonistic effect, ethanolic extracts of propolis and its botanical origin were treated with the cells for 10 min prior to the addition of 1 nM TCDD. The nuclear extract was prepared from these cells and used for measurement of AhR transformation as described previously (*17*).

Reversed-Phase High-Performance Liquid Chromatography (RPHPLC). The analysis of chemical ingredients from propolis and leaf bud extracts was performed by RPHPLC with a liquid chromatograph equipped with a YCM Pack ODS-A column and a photodiode array (SPD-M10-A, Shimadzu Co., Kyoto, Japan) (*8, 9*).

Determination of Antiradical Activity. The free radical scavenging efficiency of the ethanolic extracts of propolis and leaf bud was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method as previously described (*21, 22*). Respective samples (20 μL each) were mixed with 980 μL of methanol, and the mixture was added to 2 mL of DPPH solution (10 mg/L in 80% methanol). The absorbance was measured for 150 s at 517 nm. Because a loss of absorbance means the reduction of DPPH by an antioxidant, the degree of discoloration of the solution indicates the scavenging activity of the added sample. The antiradical activity was calculated as a percentage of DPPH discoloration.

RESULTS AND DISCUSSION

Suppressive Effects of Propolis and Its Botanical Origin on AhR Transformation in a Cell-free System. Brazilian propolis has been classified into 12 groups by physicochemical characteristics (*6, 7*). Previously, it was reported that propolis group 12 showed stronger suppressive effects on dioxin-mediated AhR transformation as compared with vegetable extracts (*11*). In this study, propolis group 12 and its main botanical origin were compared for suppressive effects on AhR transformation as a marker for dioxin toxicity.

Suppressive effects of ethanolic extracts of propolis G12 and its main botanical origin were measured by gel retardation assay in a cell-free system as described under Materials and Methods. The results are shown in **Figure 1**, and the upper panel (**A**) shows the typical representative result of the GRAB assay, whereas the bottom one (**B**) shows percent of transformed AhR from the quantified density of the AhR/DRE complex in panel **A**. Data are represented as the mean ± SD from triplicate experiments. It was confirmed that propolis group 12 suppressed AhR transformation dose-dependently in the cell-free system, the same as our previous study (*11*). Resins of *B. dracunculifolia* also suppressed AhR transformation in a dose-dependent manner. When the 50% inhibitory concentration (IC_{50}) was determined, the IC_{50} values of propolis G12 and resins of *B. dracunculifolia* were 0.74 and 4.6 μg/mL, respectively. These data indicate that the ethanolic extract of resins of *B. dracunculifolia* contains the antagonistic ingredients for AhR, although its suppressive effect was lower than that of propolis.

Suppressive Effects of Propolis and Its Botanical Origin on AhR Transformation in Hepa-lclc7 Cells. We also attempted to examine whether these two samples of extracts suppress AhR transformation in mouse hepatocytes by SW-ELISA, which is a more quantitative method for the evaluation of AhR transformation than the GRAB assay (*17*). In this experiment, mouse hepatoma Hepa-lclc7 cells were used because this cell linage is sensitive to TCDD. As shown in **Figure 2**, both extracts of propolis G12 and its botanical origin at 10 μg/mL significantly suppressed AhR transformation induced by l nM TCDD. When extracts were used at 25 μg/mL, propolis G12 and botanical origin suppressed 60 and 43% of 1 nM TCDD-induced AhR transformation, respectively, and the suppressive effects were stronger than that of respective

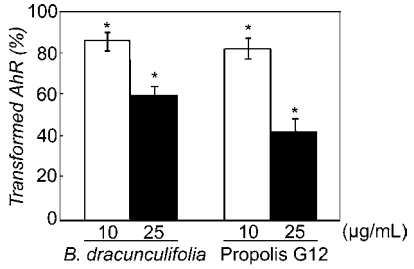

Figure 2. Suppressive effects of ethanolic extracts of propolis G12 and leaf bud of *B. dracunculifolia* on TCDD-induced AhR transformation in mouse hepatoma Hepa-1c1c7 cells.

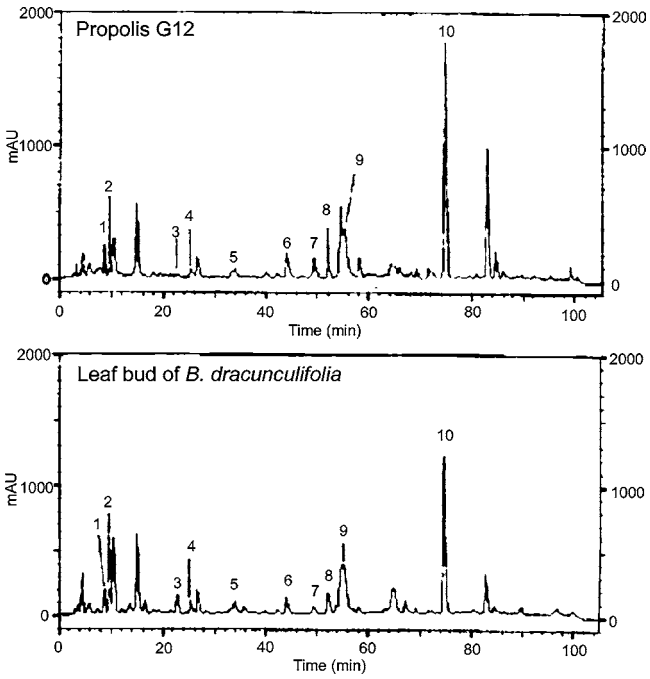

Figure 3. RPHPLC of ethanolic extracts of propolis G12 and leaf bud of *B. dracunculifolia*: 1, coumaric acid; 2, ferulic acid; 3, cinnamic acid; 4, quercetin; 5, kaempferol; 6, pinobanksin-3-acetate; 7, chrysin; 8, galangin; 9, kaempferide; 10, artepillin C (3,5-diprenyl-4-hydroxycinnamic acid).

extracts at 10 μg/mL. These results indicate that certain active components in both extracts enter hepatocytes and reveal the suppressive effects, but the effects weakened compared with those in the cell-free system.

Chemical Ingredients of Propolis and Its Botanical Origin. To confirm the chemical ingredients of propolis and its botanical origin, the contents of flavonoids and other phenolic compounds were determined, and the results were shown in **Figure 3** and **Table 1**. Both propolis and resins of leaf bud contained the same flavonoid aglycones such as quercetin, kaempferol, pinobanksin-3-acetate, chrysin, galangin, and kaempferide, but the contents of them in propolis were slightly higher than those in the resins of leaf bud. Both of them also contained coumaric acid, ferulic acid, cinnamic acid, and artepillin C. These results are almost the same as in our previous paper (*9*), although the composition of the chemical ingredients is different from that in the previous paper. This difference may be due to the different samples, that is, differences in the collection areas and years. Generally, flavonoids exist as glycosides in the plant, but the results in this study and a previous one (*9*) indicate that flavonoids exist as aglycones in leaf bud. Therefore, it is predicted that flavonoid aglycones in the ethanol extract of leaf bud show the same biological activities, including the suppressive effects on AhR transformation, as the extract of propolis does.

Table 1. Contents[a] of Flavonoids and Other Chemical Ingredients in Propolis G12 and Leaf Bud from *B. dracunculifolia* by RPHPLC

peak	retention time (min)	compound	propolis G12	leaf bud from *B. dracunculifolia*
1	8.85	coumaric acid	6.08	5.20
2	9.67	ferulic acid	2.76	7.10
3	22.77	cinnamic acid	0.71	5.39
4	26.9	quercetin	2.50	2.05
5	33.22	kaempferol	1.23	1.03
6	45.43	pinobanksin-3-acetate	27.40	17.30
7	50.33	chrysin	6.10	3.30
8	52.09	galangin	4.46	7.72
9	55.93	kaempferide	13.70	10.57
10	75.54	artepillin C	58.39	47.19

[a] Contents of ingredients are expressed as milligrams of respective compounds in 1 g of propolis and leaf bud from *B. dracunculifolia*.

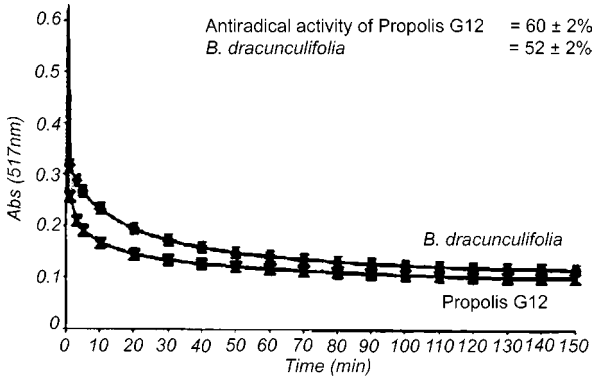

Figure 4. Antiradical activity of ethanolic extracts of propolis G12 and leaf bud of *B. dracunculifolia*.

Antioxidant Activity of Propolis and Its Botanical Origin. Finally, we measured the antiradical activity as one of the typical biological activities of propolis, and the results are shown in **Figure 4**. As described by Kumazawa et al. (*23*), the model system of scavenging DPPH free radical is a simple method for evaluating the antioxidant activity of compounds; therefore, the antiradical activity of propolis was used for the evaluation of the antioxidant activity of the propolis. As shown in **Table 1**, concentrations of quercetin, kaempferol, and artepillin C in propolis G12 are higher than in leaf bud from *B. dracunculifolia*. The antiradical activities of propolis and its botanical origin are 60 $\pm$ 2 and 52 $\pm$ 2%, respectively. The results show that the antiradical activity of propolis was slightly higher than that of its botanical origin, suggesting that differences in the activity were dependent on differences in the contents of polyphenols in both ethanol extracts. Kumazawa et al. also reported that quercetin, kaempferol, and artepillin C exhibited strong DPPH free radical scavenging activity, >60% (*23*). Therefore, it was confirmed that resins of leaf bud from *B. dracunculifolia* reveal the same biological activities as propolis group 12.

Dioxins invade the human body mainly through diet and produce various toxic effects through AhR transformation. Therefore, if some components in food suppress AhR transformation, AhR-mediated biological responses caused by dioxins can be reduced. Among food components, flavonoids have considerable possibilities to suppress dioxin toxicity because their structures match the AhR pocket (*24*), in addition to their biological activities such as antioxidative activity, anticarcinogenicity, and the inhibition of several enzymes including protein kinases and cytochrome P450 (*25*, *26*). Certain flavonoids,

especially flavones and flavonols in aglycones, inhibit AhR transformation antagonistically (*18*). Because propolis contains flavonoid aglycones abundantly, it is a good candidate as a food possessing suppressive effects on AhR transformation. Indeed, propolis groups 12 and 3 showed stronger suppressive effects on AhR transformation as compared with vegetable extracts in our previous paper (*11*). In addition to propolis, we have demonstrated that resins of leaf bud from *B. dracunculifolia*, the botanical origin of propolis G12, suppressed AhR transformation in this study. Ethanolic extract from the resins of leaf bud has a unique property in that it abundantly contains various flavonoid aglycones but not glycosides. It is apparent that flavonoid profiles in both propolis G12 and *B. dracunculifolia* mainly consisted of pinobanksin-3-acetate, kaempferide, galangin, chrysin, and kaempferol in this study. Therefore, these flavonoid aglycones will contribute suppressive effects on AhR transformation. Moreover, some active ingredients in propolis and the resins of leaf bud reveal the suppressive effects in hepatocytes. Further study is needed to clarify whether the oral intake of propolis or its botanical origin suppresses AhR transformation in the liver.

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