Oral administration of Brazilian propolis exerts estrogenic effect in ovariectomized rats

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ABSTRACT — Propolis, a natural product derived from plants by honeybees, is a mixture of several hundred chemicals, including flavonoids, coumaric acids, and caffeic acids, some of which show estrogen-like activity. In this study, the estrogenic activity of crude ethanolic extract of Brazilian propolis was determined using several in vitro and in vivo assays. Propolis was found to bind to human estrogen receptors (ERs). Furthermore, propolis induced the expression of estrogen-responsive genes in ER-positive MCF-7 and Ishikawa cells. These in vitro assays suggest that propolis exerts estrogenic activity; therefore, in vivo experiments were conducted using ovariectomized rats. Oral administration of propolis (55 or 550 mg/kg/day for 3 days) significantly increased uterine wet weight and luminal epithelium thickness in comparison with the corresponding values in the corn oil-treated control group. Moreover, propolis induced ductal cell proliferation in the mammary glands. These effects were completely inhibited by full ER antagonist ICI 182,780, confirming that the effects of propolis are mediated by the ER. Our data show that oral intake of propolis induces estrogenic activity in ER-expressing organs in vivo and suggest that Brazilian propolis is a useful dietary source of phytoestrogens and a promising treatment for postmenopausal symptoms.

Key words: Brazilian propolis, Phytoestrogens, Postmenopausal women, Natural products, Oral intake

INTRODUCTION

Estrogen plays a key role in a broad range of biological systems, including the reproductive system, the central nervous system, and metabolic systems. Decreased estrogen production in menopausal women leads to symptoms such as hot flush, insomnia, and osteoporosis. To relieve these menopausal symptoms, estrogen alone or in combination with progestin has been prescribed as estrogen or hormone replacement therapy (ERT or HRT) (Grodstein et al., 1997). Equine estrogens (equilin and equilenin) have also been used for postmenopausal women; however, equine and human estrogens induce several types of DNA lesions such as DNA oxidation and bulky DNA-adenine dimer formation (Okamoto et al., 2008a, 2010; Okahashi et al., 2010). Accordingly, long-term treatment with estrogens has been reported to increase the incidence of breast cancer (Grodstein et al., 1997; Chlebowski et al., 2010; Beral et al., 2011). To improve quality of life for postmenopausal women, it is important that clinicians and researchers develop alternative ERT strategies that do not confer a significant risk of cancer.

Some natural products such as flavonoids have been reported to show estrogen receptor (ER)-mediated biological effects in several assays (Kuiper et al., 1998; Ito et al., 2006; Okamoto et al., 2006). Dietary intake of equol, a metabolite of the soybean isoflavone daidzein, prevented typical postmenopausal symptoms such as bone loss and fat accumulation in Japanese women because of its estrogenic potency (Ishimi, 2010). Genistein, a potent estrogenic isoflavone, has been reported to reduce postmenopausal symptoms in women (Taylor et al., 2009). In addition, daidzein and its active metabolite equol have an ability to suppress the proliferation of human breast cancer xenografts implanted in athymic nude mice (Liu et al., 2012). These reports suggest that phytoestrogens can be used for treatment of postmenopausal symptoms accompanied by cancer chemoprevention.
Propolis is a resinous product that is derived from plants by honeybees for the purpose of sealing small gaps in the hive structure, and is distinct from beeswax. It has a long history of use as a folk medicine, and several biological effects of propolis such as anti-inflammatory, antimicrobial, anti-viral, and anti-oxidative activities have been reported (Burdock, 1998). Propolis contains several hundred chemicals, including flavonoids, coumaric acids, and caffeic acids, some of which are known to include estrogenic effects (Kuiper et al., 1998; van der Woude et al., 2005). Song et al. (2002) reported that subcutaneous administration of propolis showed estrogenic activity in immature rat. However, xenobiotics injected via subcutaneous route avoid first-pass metabolism as well as gastrointestinal absorption steps. For considering clinical use, propolis must be confirmed the estrogenic effectiveness by oral administration.

In the present study, the estrogenic activity of crude ethanolic extract of Brazilian propolis (EEP) was determined using several in vitro assays based on competitive binding to the ER and expression of estrogen inducible genes progesterone receptor (PR) and trefoil factor 1 (TFF-1; formerly pS2). We also determined the in vivo estrogenic activity of EEP via oral administration in ovariectomized (OVX) rats.

MATERIALS AND METHODS

Chemicals

Ethanolic extract of Brazilian propolis (EEP, 55% (w/v)) was a gift from Yamada Bee Farm Corp. (Okayama, Japan). 17β-Estradiol (E2), corn oil, and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries (Osaka, Japan). [2,4,6,7,16,17-3H]17β-Estradiol (H-E2, 161 Ci/mmol) was purchased from GE Health Care (Buckinghamshire, UK). ICI 182,780 was obtained from Toecris Bioscience (Ellisville, MO, USA).

Estrogen receptor binding assay

To determine the ER binding potency of EEP, the ERα and ERβ binding affinity of EEP was tested using a spin column assay with commercially available full-length hERα or hERβ (Life Technologies, Carlsbad, CA, USA) according to a previously reported method (Okamoto et al., 2008b). Briefly, various concentrations of EEP (5.5 × 10^{-4} to 55 μg/mL) were incubated at room temperature for 1 hr in binding buffer containing hERα or hERβ (1.5 nM) and 3H-labeled E2 (1 nM). After incubation, the mixture was applied onto a macro spin column containing G-25 Sephadex (Harvard Apparatus, Holliston, MA, USA) according to the manufacturer’s instructions. ER-bound 3H-E2 was separated from free 3H-E2 by gel filtration. The ER-bound 3H-E2 was dissolved in a scintillation cocktail (Nacalai, Kyoto, Japan) and radioactivity was counted using a scintillation counter (Hitachi Aloka Medical, Ltd., Tokyo, Japan). A binding curve was generated and IC50 value (the concentration that produced 50% inhibition of ER-3H-E2 binding) was calculated using a single binding site competition model and Prism 5 statistical analysis software (Graphpad Software, San Diego, CA, USA).

Cell culture

MCF-7 (breast cancer) and Ishikawa (endometrial cancer) ER-positive cancer cell lines were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), 1% penicillin/streptomycin (Wako Pure Chemical Industries), and 2 mM L-glutamine (Life Technologies), and cultured at 37°C under a humidified 5% CO2 atmosphere. Ishikawa cells were maintained in DMEM supplemented with 5% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine under the same atmospheric conditions as MCF-7 cells. Cells were plated onto a 24-well culture plate at 5 × 10^4 cells/well and allowed to attach overnight. The next day, the medium was replaced with phenol red-free DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% charcoal-stripped FBS (Equitech Bio, Kerrville, TX, USA), 1% penicillin/streptomycin, and 2 mM L-glutamine, and incubated for a day. Test chemicals were dissolved in DMSO and applied to cells at final DMSO concentrations of less than 0.1%. After 24 hr of chemical treatment, cells were collected and used immediately in downstream assays or stored at -80°C until use.

Real-time quantitative PCR analysis

Total RNA was isolated using a spin column (High Pure RNA Purification Kit, Roche Diagnostics, Indianapolis, IN, USA), and 500 ng of total RNA was used as a template for first-strand cDNA synthesis using a kit (PrimeScript RT-PCR kit, Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. Real-time RT-PCR analysis was performed using SYBR green reagent (LightCycler 480 SYBR green I master, Roche Diagnostics). Primer pairs used for quantification of PR, TFF-1, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, used as an internal standard) were as follows: PR forward, 5′-TCTGAAGGGCAATAGGAAGG-3′; PR reverse, 5′-CGGATTTTATCAACGATGCAG-3′; TFF-1 for-
Antigen retrieval was carried out in an autoclave at 121°C and were sliced using a microtome (thickness, 5 μm). Glands were stained with hematoxylin, cleaned up under xylene, and mounted. Images were taken under a stereomicroscope (Stemi SV11, Carl Zeiss, Jena, Germany) for at least 3 days in each solvent. After rehydration, the glands were defatted in ethanol, acetone, chloroform, and ethanol again and processed as a whole mount. The glands were dehydrated in ethanol, acetone, chloroform, and ethanol again and mounted on glass slides. Images were captured under a digital microscope (Keyence, Osaka, Japan) and mounted on glass slides. Images were captured using a digital microscope (Keyence) and the thickness of the uterine luminal epithelium was measured using analytical software (Keyence). Pathological evaluation was carried out by 2 independent investigators.

Fixed uterine tissues were embedded in paraffin. The sections were prepared at a thickness of 5 μm, stained with H&E, and mounted on glass slides. Images were captured under a digital microscope (Keyence) and the thickness of the uterine luminal epithelium was measured using analytical software (Keyence).

Estrogenic activity of Brazilian propolis

Non-target analysis was carried out to estimate the structure of estrogenic compounds in Brazilian propolis. High-resolution mass spectra were acquired using a quadrupole time-of-flight (QTOF) mass spectrometer (Impact HD, Bruker Daltonics Inc., Billerica, MA, USA) equipped with an HPLC system (Nexera, Shimadzu, Kyoto, Japan). Mass spectral conditions were as follows: ion source, electro-chemical ionization; nebulizer gas, 2.0 bar; dry gas, 8.0 L/min; dry temperature, 200°C; and ion polarity, positive mode. HPLC separation was carried out using the following conditions: column, ODS (2.0 i.d. x 150 mm, 3 μm); column oven, 40°C; mobile phase A/B, 0.1% formic acid/methanol; A/B gradient program, linear from 100/0 to 0/100 over 60 min; flow rate, 0.2 mL/min, and injection volume, 1 μL. EEP was diluted 100-fold with methanol before it was applied to the high-resolution LC-QTOF system. Data analysis was performed on TargetAnalysis software (Bruker Daltonics Inc.).

Statistical analysis

Each experiment was repeated at least three times. The values are expressed as the mean value ± S.D. The
significance of the differences between mean values were assessed using Student’s t-test. All computations were calculated using the Microsoft Excel program.

RESULTS

ER-binding potency

Because ligand binding to ER is the primary mediator of the action of estrogens, a competitive ER binding assay using ³H-labeled E2 was carried out to determine the ER-binding potency of EEP. As shown in Fig. 1, EEP competitively bound to both ERs. The IC₅₀ value of EEP for hERα was 96.8 μg/mL, and the IC₅₀ value of EEP for hERβ was 15.3 μg/mL. This assay showed that EEP was about 6 times as potent at the hERβ in comparison with its potency at the hERα.

Estrogen-inducible gene expression

After ligand binding, ER agonists promote the expression of estrogen-inducible genes such as PR and TFF-1. Using ER-positive human breast cancer MCF-7 cells and endometrial cancer Ishikawa cells, PR and TFF-1 expression was determined using real-time quantitative PCR analysis (Fig. 2). In MCF-7 and Ishikawa cells, EEP induced the expression of estrogen-inducible genes at the highest concentration used in this study (5.5 μg/mL).

Estrogenic effect on mammary gland in OVX rats

In order to confirm the estrogenic effect of EEP in vivo, OVX rats were treated with 55 or 550 mg/kg EEP by gavage for 3 days. E2 (0.3 μg/kg, s.c.) was administered as a positive control. Whole-mount analysis clearly showed the development of mammary lobuli and ducts in the E2-treated rats (Fig. 3c). However, little or no difference was seen in the EEP (550 mg/kg)-treated rats in comparison with the control group (Fig. 3a and 3b). To evaluate EEP-induced cell proliferation, paraffin-fixed mammary gland sections were prepared and the expression of cell proliferation marker Ki67 antigen in the mammary epithelium was determined by immunostaining analysis (Fig. 3d). In the E2-treated (0.3 μg/kg) group, 79.5% of the mammary epithelial cells were Ki67-positive, whereas in the control group, 8.8% of the mammary epithelial cells were Ki67-positive. Oral EEP treatment increased the percentage of Ki67-positive cells in a dose-dependent manner (15.1% by treatment with 55 mg/kg and 33.2% by treatment with 550 mg/kg). In addition, pretreatment with ICI 182,780 (1 mg/kg) diminished the increase in Ki67-positive cells observed in the group treated with a high dose (550 mg/kg) of EEP.

Estrogenic effect of EEP in the OVX rat uterus

The estrogenic effect of EEP in the uterus was determined based on EEP-induced increases in uterine wet weight and epithelial thickness. In comparison with the control treatment, E2 (0.3 μg/kg) treatment increased uterine wet weight and epithelial thickness by 3.98- and 3.34-fold (Fig. 4). In the EEP-treated rats, uterine wet weight and epithelial thickness significantly increased in a dose-dependent manner. In comparison with the control treatment, EEP at a dose of 55 and 550 mg/kg increased uterine weight 1.40- and 1.81-fold, respectively, and increased uterine epithelial thickness 1.27- and 1.34-fold, respectively. The effects of EEP on uterine wet weight and epithelial thickness were completely suppressed by pretreatment with ICI 182,780.

Determination of the estrogenic constituents of Brazilian propolis

To evaluate the active estrogenic constituents of Brazilian propolis, non-target mass spectrometry analysis was performed using a high-resolution QTOF mass spectrometer (Fig. 5). The base peak chromatogram showed that EEP contained a large number of compounds (Fig. 5a). As shown in Fig. 5b, high-resolution extracted ion chromatograms indicated the existence of compounds with molecular weights ([M+H]+ ± 0.002 mass units) equivalent to those reported for phytoestrogens for-
Fig. 2. Effect of EEP on E2-inducible gene expression in MCF-7 and Ishikawa cells. Human breast cancer MCF-7 cells (A) and endometrial Ishikawa cells (B) were exposed for 24 hr to EEP (5.5 × 10^3 to 5.5 μg/mL) in phenol red-free DMEM containing 10% charcoal-stripped FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine. PR and TFF-1 gene expression was determined by real-time quantitative PCR analysis as described in the Materials and Methods. Gene expression was normalized to GAPDH expression. Data are expressed as mean ± S.E.M. (n > 3).

Fig. 3. Effect of EEP on mammary gland histology in OVX rats. A-C, Images (30 × magnification) of whole mount preparations of mammary glands from OVX rats treated with corn oil, EEP (550 mg/kg, p.o.), or E2 (0.3 μg/mL, s.c.). D, Percentages of Ki67-positive cells in mammary epithelium. Data are expressed as mean ± S.D. (n > 3).
mononetin (m/z 269.0814), naringenin (m/z 273.0763), biochanin A (m/z 285.0763), kaempferol (m/z 287.0556), and quercetin (m/z 303.0505).

**DISCUSSION**

Propolis contains hundreds of phenolic compounds and is thus an attractive source of bioactive materials such as phytoestrogens for prevention of post-menopausal symptoms. Song *et al.* (2002) reported that Korean propolis showed estrogenic activity based on rat uterotrophic assay in which ethanolic or ether extracts of Korean propolis were subcutaneously administered to immature Sprague-Dawley rats (20-21 days old). Oral administration would be the most suitable route of propolis administration in humans, and its absorption and metabolism should be considered in the context of the gastrointestinal tract. In the present study, we demonstrated that EEP exerts estrogenic action in OVX rats (5 weeks old) at doses of 55 and 550 mg/kg/day (equivalent to 3.3 and 33 g/day, respectively, for a 60 kg person) via oral administration. This effect would be induced by compounds containing in EEP, because EEP showed estrogenic activity in several *in vitro* assays. Although immature rats are sensitive to E2 and genistein, a representative estrogenic flavonoid, in comparison with mature OVX rats (Tinwell *et al.*, 2000; Kanno *et al.*, 2003), Korean propolis needed about 10-fold higher dosage (500 mg/kg/day) than Brazilian propolis to increase uterine weight. Therefore, the estrogenic potency of Brazilian propolis would be expected to be stronger than that of Korean propolis reported by Song *et al.* (2002). It is expected that differences in the effects of Brazilian and Korean propolis would be due to differences in their constituent compounds and/or metabolic activation of constituent compounds via a first-pass metabolic effect.

Several groups have identified bioactive components of propolis (Shimizu *et al.*, 2004; Li *et al.*, 2007; Szliszka *et al.*, 2011), including phytoestrogens kaempferol, naringenin, and quercetin (Kuiper *et al.*, 1998; van der Woude *et al.*, 2005). Although the estrogenic components of the EEP used in this study have not been identified, our preliminary experiments utilizing a high-resolution QTOF-mass spectrometer indicated that EEP contained known phytoestrogens kaempferol, quercetin, naringenin, biochanin A (4′-O-methyl-genistein), and formononetin (4′-O-methyl-daidzein) (Fig. 5). Kaempferol, quercetin, and naringenin have low bioavailability because of their hydrophilicity. In contrast to kaempferol, quercetin, and naringenin, biochanin A and formononetin are relatively lipophilic and exert estrogenic activity via metabolic 4′-O-demethylation (Tolleson *et al.*, 2002). Therefore,
lipophilic phytoestrogens would contribute to the estrogenic activity of EEP in OVX rats.

Natural estrogens and equine estrogens are currently prescribed to postmenopausal women, but these potent estrogens exert severe side effects, such as increased cancer risk, and thus alternative estrogens are needed to support continued treatment of such patients. Although further experiments must be conducted, our data suggest that oral intake of propolis produces estrogenic activity in estrogen target organs in vivo. Therefore, Brazilian propolis represents a useful dietary source of phytoestrogens and a promising treatment for postmenopausal symptoms.

Conflict of interest---- The authors declare that there is no conflict of interest.

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