JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Ethanol Extract of Brazilian Red Propolis Induces Apoptosis in Human Breast Cancer MCF-7 Cells through Endoplasmic Reticulum Stress

Tetsuro Kamiya,* Hiroko Nishihara, Hirokazu Hara, and Tetsuo Adachi

Laboratory of Clinical Pharmaceutics, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan

ABSTRACT: Propolis, a natural product collected from plants by honey bees, is commonly used in folk medicines. Endoplasmic reticulum (ER) stress is known to induce apoptosis through the induction of CCAAT/enhancer-binding protein homologous protein (CHOP). Here, we investigated whether ethanol extracts of propolis and caffeic acid phenethyl ester (CAPE) induce apoptosis, mitochondrial dysfunction, and ER stress in human breast cancer MCF-7 cells and human fibroblasts. Among several ethanol extracts of propolis and CAPE, Brazilian red propolis (BRP) significantly reduced MCF-7 cell viability through the induction of mitochondrial dysfunction, caspase-3 activity, and DNA fragmentation but did not affect those of fibroblasts. Moreover, treatment with BRP significantly induced CHOP expression in MCF-7 cells compared to fibroblasts. Further, pretreatment with a chemical chaperone, 4-phenylbutyric acid, suppressed BRP-triggered MCF-7 cell death. Overall, we revealed that an ethanol extract of BRP induces MCF-7 cell apoptosis through, at least in part, ER stress-related signaling.

KEYWORDS: *propolis, apoptosis, mitochondrial dysfunction, endoplasmic reticulum stress*

INTRODUCTION

Propolis is a natural product made by honey bees from the gum of various plants. Accumulated evidence using high-performance liquid chromatography (HPLC) and gas chromatography−mass spectrometry has demonstrated that more than 300 compounds, including flavonoids, amino acids, and fatty acids, are present in propolis.[1,2] Propolis has been used in folk medicines and has been reported to have various biological properties, such as anticancer, antiviral, antioxidative, and antibacterial.[3−6] It is well-recognized that the main components of propolis are due to the origin of plants. Indeed, it has been reported that Chinese propolis (CP) contains pinocembrin, chrysin, and caffeic acid phenethyl ester (CAPE) at high concentrations, but Brazilian propolis (BP) contains artepillin C and baccharin, instead of the above compounds.[7−10] Recently, red propolis samples were collected in northern regions of Brazil and classified as a new type of BP, and it has been reported that Brazilian red propolis (BRP) contains daizein, biochanin A, quercetin, and naringenin.[11] Therefore, propolis-derived pharmacological activities vary according to the geographical and botanical origin. Recently, it has been reported that the ethanol extract of BRP promotes adipocyte differentiation through peroxisome proliferator-activated receptor-γ activation.[8]

Among these several propolis compounds, artepillin C and CAPE are known to show antiproliferative and anticancer properties and to induce apoptosis.[6,12] It is well-recognized that apoptosis is characterized by the activation of caspase signaling following mitochondrial dysfunction, such as increased pro-apoptotic protein Bax as well as decreased anti-apoptotic proteins Bcl-xL and Bcl-2.[13−15] Moreover, anticancer properties are related to the activation of p53 and p38 mitogen-activated protein kinase and cell cycle arrest.[16] Recently, it has been recognized that endoplasmic reticulum (ER) stress is positively related to the exacerbation of several kinds of diseases, such as type 2 diabetes, retinopathy, and metabolic disorders, through the promotion of pro-apoptotic processes.[17,18] ER stress-derived apoptosis is considered to be induced by the enhancement of CCAAT/enhancer-binding protein homologous protein (CHOP). Although propolis extracts and CAPE, a main component of CP, are known to induce apoptosis in human breast cancer MCF-7 cells through the inhibition of nuclear factor-κB (NF-κB),[6] the involvement of mitochondrial dysfunction and ER stress-related signaling in the BRP-induced apoptosis remains unknown.

MATERIALS AND METHODS

Ethanol Extracts of Propolis. Ethanol extracts of CP [identification number (IN) 060501], BRP (IN 20100630), and BP (IN 100819), provided by Api Co., Ltd. (Gifu, Japan), were obtained by the addition of 3.5 volumes of 95% ethanol to 50 g of propolis at room temperature for 24 h and filtration. There major constituents and amounts were previously determined by HPLC and shown in Table 1.[8−11,19]

Cell Culture. Human breast cancer MCF-7 cells and human fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Human prostate cancer LNCaP cells and human breast cancer ZR-75-1 cells were grown in RPMI1640 supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were kept in an atmosphere of 5% CO_2/95% air at 37 °C. The cells were grown to confluence in a 96-well microplate (seeded at a density of 3×10^4 cells/well) for measurement of cell viability or a 6 cm culture dish (seeded at a density of 1×10^5 cells/mL) for others and treated with the test reagents, including propolis ethanol extracts (up to 20 μg/

Received: June 21, 2012
Revised: October 10, 2012
Accepted: October 15, 2012
Published: October 15, 2012

Table 1. Major Constituents in the Ethanol Extract of CP, BRP, and BP

CP	%[a]	BRP	%[b]	BP	%[a]
pinocembrin	8.4	formononetin	1.1	artepillin C	14.0
chrysin	5.0	quercetin	0.2	baccharin	6.8
CAPE	1.7	biochanin A	0.1	di-*O*-caffeoylquinonic acids	6.4
caffeic acid	1.3	daidzein	0.04	*p*-coumaric acid	2.5

[a]Quantity of constituents of propolis used in this study. [b]Average quantity of constituents of propolis described in ref 19.

Table 2. Primer Sequences and PCR Conditions Used in This Study

gene	sequences	T_m (°C)	cycle	size (bp)
Bax	S; 5′-CAGCTCTGAGCAGATCATGA-3′ AS; 5′-GCCTTGAGCACCAGTTTGCT-3′	60	25	330
Bcl-xL	S; 5′-AGGCAGGCGACGAGTTTGAAC-3′ AS; 5′-CGGCTCTCGGCTGCTGCATT-3′	60	25	337
Bcl-2	S; 5′-GATGTCCAGCCAGCTGCACCTG-3′ AS; 5′-CACAAAGGCATCCCAGCCTCC-3′	60	27	256
CHOP	S; 5′-CCTTCCAGTGTGTGGGACTT-3′ AS; 5′-GTGTGTTTTCCTTTTGCCGT-3′	60	27	259
β-actin	S; 5′-CAAGAGATGGCCACGGCTGCT-3′ AS; 5′-TCCTTCTGCATCCTGTCGGCA-3′	60	20	275

mL), CAPE (Api Co., Ltd., Gifu, Japan; up to 2 μM), daidzein (Fujicco Co., Ltd., Kobe, Japan; up to 10 μg/mL), and biochanin A (Sigma-Aldrich Co., St. Louis, MO; up to 10 μg/mL). Pretreatment with 4-phenylbutyric acid (PBA, LKT Lab., St. Paul, MN; up to 2 mM) and Z-VAD-FMK (Sigma-Aldrich Co., St. Louis, MO; 10 μM) was added 1 h prior to the addition of the ethanol extract of propolis. The cells were washed with ice-cold phosphate-buffered saline (PBS) and then used for measurements of cell viability, determination of apoptosis, reverse transcriptional-polymerase chain reaction (RT-PCR) analysis, and western blotting.

Measurement of Cell Viability. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to estimate the cytotoxicity of ethanol extracts or constituents of propolis. After treatment of these cells with propolis, the culture medium was aspirated and the cells were added to 110 μL of 10% FCS−DMEM containing MTT (CHEMICON Int., Inc., Temecula, CA) and were then incubated for 3 h at 37 °C in a humidified atmosphere of 5% CO_2/95% air. After incubation, the cells were added to 100 μL isopropanol containing 0.04 N HCl and were then mixed thoroughly to dissolve the MTT formazan. Finally, the MTT formazen was measured at 595 nm with a reference wavelength of 655 nm.

Assay of Caspase-3 Activity. After treatment of MCF-7 cells and fibroblasts with ethanol extracts of propolis and CAPE, the cells were washed twice with ice-cold PBS and collected by centrifugation at 550*g* for 10 min. The pellets were lysed on ice for 10 min in lysis buffer. Caspase-3 activity was then assayed using an Apopcyto caspase-3 colorimetric assay kit according to the protocol of the manufacturer (MBL, Nagoya, Japan).

Determination of DNA Fragmentation. After treatment of MCF-7 cells and fibroblasts with ethanol extracts of propolis and CAPE, the cells were washed twice with ice-cold PBS and lysed on ice for 20 min in lysis buffer [10 mM Tris-HCl at pH 8.0 containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X-100]. The lysates were centrifuged at 13000*g* for 10 min. The supernatants were deprotenized by digestion with 200 μg/mL proteinase K at 50 °C for 30 min, extracted once with an equal volume of phenol/chloroform/isoamylalcohol mixture (25:24:1), and then precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. After centrifugation at 17700*g* for 10 min, the pellets were washed with 70% ethanol and resuspended in 20 μL of 10 mM Tris-HCl at pH 8.0 containing 1 mM EDTA and 10 μg/mL RNase. The DNA solution was incubated at 37 °C for 1 h, and 5 μg DNA was separated on 1.5% (w/v) agarose gel, stained with ethidium bromide, and photographed.

RT-PCR Analysis. The cDNA was prepared, and RT-PCR was performed by the methods described in our previous report.[20] The primer sequences and PCR conditions used in this study were shown in Table 2. These PCR products were loaded onto a 2% (w/v) agarose gel for electrophoresis, and densitometric analysis of the PCR products was performed with Multi Gauge, version 3.0 (Fuji Film, Tokyo, Japan).

Western Blotting. Whole cell extracts were prepared in lysis buffer as described previously.[21] Extracts containing 20 μg of protein were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 12% (w/v) polyacrylamide gels followed by transferring electrophoretically onto polyvinylidene difluoride (PVDF) membranes. Subsequently, the membranes were incubated with the respective specific primary antibodies (CHOP, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and actin, Millipore Co., Billerica, MA; 1:1000). The blots were incubated with biotin-conjugated goat anti-rabbit or anti-mouse antibody (Zymed Lab., San Francisco, CA; 1:1000), followed by incubation with ABC reagents (Vector Laboratories, Inc., Burlingame, CA; 1:5000). Finally, the bands were detected using SuperSignal West Pico (Thermo Scientific, Rockford, IL) and imaged using a LAS-3000 UV mini (Fuji Film).

Statistical Analysis. Data are expressed as the means ± standard deviation (SD) of three independent experiments. Statistical evaluation of the data was performed using analysis of variation (ANOVA), followed by post-hoc Bonferroni tests. A p value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Propolis, a natural product made by honey bees, is known to have several chemical components and to show biological properties, such as antiviral, antibacterial, antioxidative, and anticancer. We previously reported that ethanol extracts of CP, BRP, and BP show antioxidative property and suppress reactive-oxygen-species-derived cell death.[22] On the other hand, it has been reported that CAPE has anticancer properties by inhibiting NF-κB and activating caspase and Fas signaling in

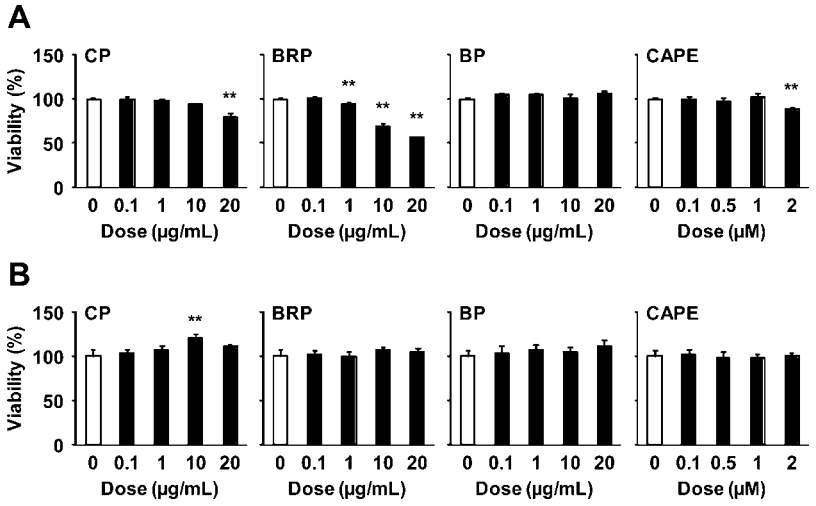

Figure 1. Effect of ethanol extracts of CP, BRP, and BP and CAPE on cell viability in (A) MCF-7 cells and (B) fibroblasts. MCF-7 cells and fibroblasts were treated with the indicated concentrations of ethanol extracts of propolis and CAPE for 24 h. After treatment, cell viability was measured. (**, $p < 0.01$ versus vehicle).

human breast cancer MCF-7 cells;[6] however, whether an ethanol extract of BRP has anticancer properties remains unclear. Accordingly, we first investigated the effect of ethanol extracts of CP, BRP, and BP and CAPE on the cell viability of human breast cancer MCF-7 cells and human fibroblasts. As shown in Figure 1A, ethanol extracts of CP and BRP and CAPE significantly decreased MCF-7 cell viability in a dose-dependent manner but an ethanol extract of BP did not affect MCF-7 cell viability. On the other hand, these extracts did not induce cell death in fibroblasts (Figure 1B), suggesting that ethanol extracts of propolis and CAPE induce cell death only in MCF-7 cells. Because it is well-recognized that the main components of propolis are due to the origin of plants,[23,24] differences in the contained components were considered to be associated with differences in the induction of MCF-7 cell death.

The activation of caspase-signaling pathways, such as caspase-3, caspase-6, caspase-8, and caspase-9, is known to be closely related to apoptotic processes.[25] Indeed, it has been reported that propolis-induced apoptosis in MCF-7 cells and human osteogenic carcinoma SAOS-2 cells is mediated by the induction of caspase signaling.[26,27] In this study, treatment with ethanol extracts of CP and BRP significantly induced caspase-3 activities in MCF-7 cells compared to fibroblasts (Figure 2A). Moreover, especially in BRP-treated MCF-7 cells, DNA fragmentation was observed (Figure 2B). On the other hand, ethanol extracts of propolis and CAPE did not induce caspase-3 activities and DNA fragmentation in fibroblasts. We next investigated the involvement of caspase signaling in ethanol extracts on BRP-induced apoptosis using Z-VAD-FMK, a pan-caspase inhibitor. Pretreatment with Z-VAD-FMK significantly suppressed cell death and DNA fragmentation induced by an ethanol extract of BRP (panels C and D of Figure 2), suggesting that caspase signaling plays a pivotal role in apoptosis induced by an ethanol extract of BRP.

Mitochondrial dysfunction, such as increased pro-apoptotic protein Bax as well as decreased anti-apoptotic proteins Bcl-xL and Bcl-2, is considered to be closely associated with the induction of apoptosis.[13−15] We therefore investigated whether an ethanol extract of BRP led to changes in the expression of pro- and anti-apoptotic proteins in MCF-7 cells and fibroblasts.

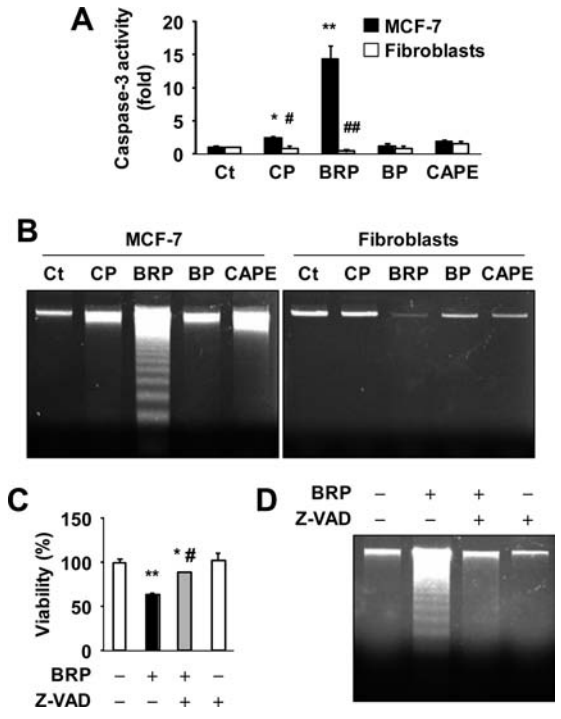

Figure 2. Ethanol extract of BRP induces apoptosis in MCF-7 cells but not in fibroblasts. MCF-7 cells and fibroblasts were treated with 20 μg/mL CP, BRP, and BP or 2 μM CAPE for 24 h. After treatment, (A) caspase-3 activities were measured and (B) DNA fragmentation was determined. (*, $p < 0.05$ versus vehicle; **, $p < 0.01$ versus vehicle; #, $p < 0.05$ versus MCF-7 cells; and ##, $p < 0.01$ versus MCF-7 cells). MCF-7 cells were pretreated with (+) or without (−) 10 μM Z-VAD-FMK (Z-VAD) for 1 h and then treated with (+) or without (−) 20 μg/mL BRP for 24 h. After treatment, (C) cell viability was measured and (D) DNA fragmentation was determined. (*, $p < 0.05$ versus vehicle; **, $p < 0.01$ versus vehicle; and #, $p < 0.05$ versus BRP-treated cells).

As shown in Figure 3, the expression of Bax mRNA in MCF-7 cells was bell-shaped, whereas Bcl-xL and Bcl-2 mRNA in MCF-7 cells were decreased. Moreover, the reduction of Bcl-2 by 20 μg/mL BRP was determined as early as 12 h, reaching 21% by 24 h (data not shown); however, in fibroblasts,

dx.doi.org/10.1021/jf303004n | J. Agric. Food Chem. 2012, 60, 11065−11070

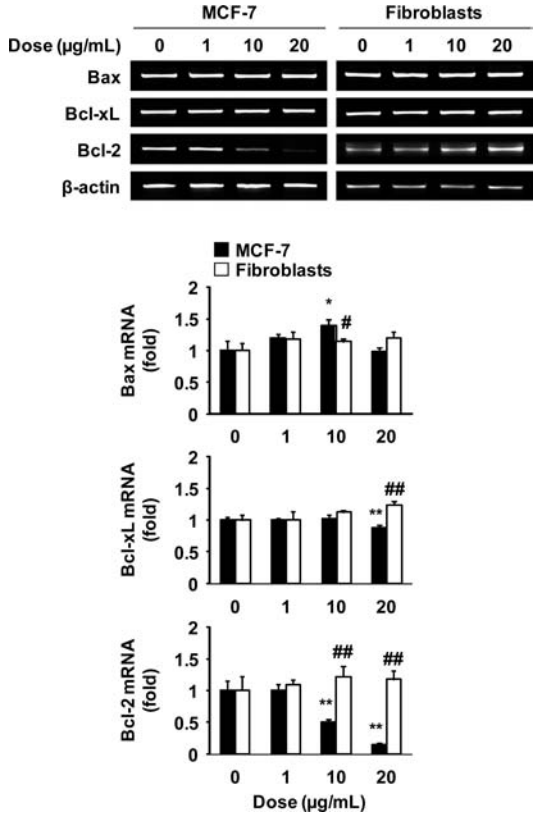

Figure 3. Ethanol extract of BRP induces mitochondrial dysfunction in MCF-7 cells but not in fibroblasts. MCF-7 cells and fibroblasts were treated with the indicated concentrations of an ethanol extract of BRP for 24 h. After treatment, Bax, Bcl-xL, and Bcl-2 mRNA were measured. RT-PCR data were normalized using β-actin levels. (*, $p < 0.05$ versus vehicle; **, $p < 0.01$ versus vehicle; #, $p < 0.05$ versus MCF-7 cells; and ##, $p < 0.01$ versus MCF-7 cells).

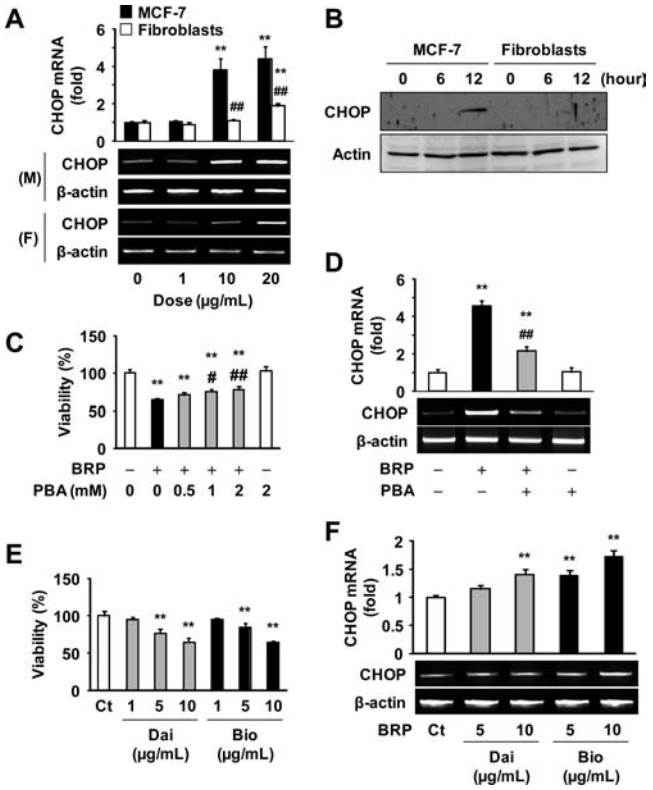

Figure 4. Involvement of ER stress in the ethanol extract of BRP-induced apoptosis in MCF-7 cells. MCF-7 cells (M) and fibroblasts (F) were treated with the (A) indicated concentrations of an ethanol extract of BRP for 24 h or (B) 20 μg/mL BRP for the indicated hours. After treatment, (A) CHOP mRNA and (B) protein levels were measured. RT-PCR data were normalized using β-actin levels (**, $p < 0.01$ versus vehicle; ##, $p < 0.01$ versus MCF-7 cells). MCF-7 cells were pretreated with the (C) indicated concentrations of PBA or (D) with (+) or without (−) 2 mM PBA for 1 h and then treated with (+) or without (−) 20 μg/mL BRP for 24 h. After treatment, (C) cell viability and (D) CHOP mRNA were measured. RT-PCR data were normalized using β-actin levels (**, $p < 0.01$ versus vehicle; #, $p < 0.05$ versus BRP-treated cells; and ##, $p < 0.01$ versus BRP-treated cells). MCF-7 cells were treated with the indicated concentrations of daidzein (Dai) or biochanin A (Bio) for 24 h. After treatment, (E) cell viability and (F) CHOP mRNA were measured. RT-PCR data were normalized using β-actin levels (**, $p < 0.01$ versus vehicle).

treatment with BRP did not affect these expressions, suggesting that the significant induction of Bax and reduction of Bcl-xL and Bcl-2 might be involved in BRP-induced apoptosis.

It is well-known that ER stress contributes to the exacerbation of several diseases through the induction of apoptosis.[17,18] Under ER stress conditions, cells trigger a set of pathways known as the unfolded protein response, which is mediated by the upregulation of chaperones, such as glucose-regulated protein 78 kDa (GRP78), GRP94, and oxygen-regulated protein 150 kDa.[28,29] On the other hand, pro-apoptotic pathways are also activated and mediated by the induction of CHOP.[30,31] It has been reported that propolis suppressed ER stress-induced cell death in neuronal cells,[32] suggesting the utility of propolis against ER stress-triggered disturbances of tissue and cell homeostasis; however, in this study, we observed that treatment with an ethanol extract of BRP significantly induced the expression of CHOP in MCF-7 cells compared to that in fibroblasts (panels A and B of Figure 4), suggesting that ER stress may be involved in BRP-induced apoptosis in MCF-7 cells. Moreover, pretreatment with 4-phenylbutyric acid (PBA), an ER stress inhibitor,[33,34] partially but significantly suppressed the ethanol extract of BRP-triggered cell death (Figure 4C) and induction of CHOP in MCF-7 cells (Figure 4D). These results indicate that ER stress plays a major role in the ethanol extract of BRP-triggered apoptosis.

Next, we investigated the effect of daidzein and biochanin A on MCF-7 cell apoptosis because these compounds are reported to be main constituents of the ethanol extract of BRP. Treatment with daidzein and biochanin A significantly reduced cell viability (Figure 4E). Moreover, we also determined the induction of CHOP when MCF-7 cells were treated with daidzein and biochanin A (Figure 4F). Recently, it has been reported that propolis activates estrogen-receptor-dependent signaling.[35] Therefore, we next investigated the involvement of estrogen receptor signaling in the viability of MCF-7 cells (estrogen receptor α positive and β positive)[36] in comparison to those of human prostate cancer LNCaP cells (estrogen receptor α negative and β positive)[37] and human breast cancer ZR-75-1 cells (estrogen receptor α positive and β negative).[36] Treatment with ethanol extracts of propolis induced cell death in LNCaP cells (Figure 5A), but not in ZR-75-1 cells (Figure 5B). These results suggested the possibility that ethanol extracts of propolis-induced cell death might depend upon estrogen receptor β-derived signaling.

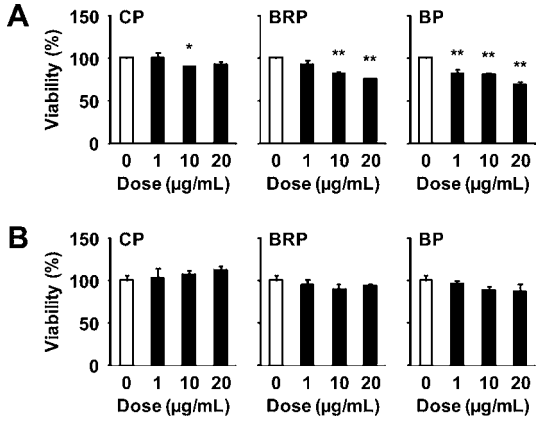

Figure 5. Effect of ethanol extracts of CP, BRP, and BP on cell viability in (A) LNCaP cells and (B) ZR-75-1 cells. These cells were treated with the indicated concentrations of ethanol extracts of propolis for 24 h. After treatment, cell viability was measured (*, $p < 0.05$ versus vehicle; **, $p < 0.01$ versus vehicle).

However, additional studies will be necessary to determine the exact molecular mechanisms governing the relationship between estrogen receptor-derived signaling and MCF-7 cell apoptosis.

In this study, we investigated whether an ethanol extract of BRP induces MCF-7 cell apoptosis and first revealed that apoptosis was, at least in part, mediated by ER stress-related signaling. Although these results were in contrast to previous reports that mentioned that propolis acts as an inhibitor of ER stress, a property that significantly induces ER stress only in cancer cells might contribute to the development of novel anticancer drugs.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +81-58-230-8100. Fax: +81-58-230-8105. E-mail: tekamiya@gifu-pu.ac.jp.

Funding

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion for Science (TK, 23790190; TA, 21590169).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The propolis ethanol extracts used in this study were provided by Api Co., Ltd. (Gifu, Japan).

ABBREVIATIONS USED

ER, endoplasmic reticulum; CHOP, CCAAT/enhancer-binding protein homologous protein; CAPE, caffeic acid phenethyl ester; BRP, Brazilian red propolis; CP, Chinese propolis; BP, Brazilian propolis; NF-κB, nuclear factor-κB; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcriptional-polymerase chain reaction; PBA, 4-phenylbutyric acid; GRP78, glucose-regulated protein 78 kDa; Dai, daidzein; Bio, biochanin A.

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