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# Protective Effects of Chinese Propolis and Its Component, Chrysin, against Neuronal Cell Death via Inhibition of Mitochondrial Apoptosis Pathway in SH-SY5Y Cells

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Endoplasmic reticulum (ER) stress has been implicated in the pathogenesis of neurodegenerative and ischemic disorders. The purpose of this study was to evaluate the effects of Chinese propolis and its constituents [chrysin, galangin, pinocembrin, caffeic acid, and caffeic acid phenethyl ester (CAPE)] against tunicamycin-induced neuronal cell death in SH-SY5Y cells. Both Chinese propolis and chrysin concentration-dependently inhibited such cell death, the tunicamycin-induced activation of caspase-3, and the effects of tunicamycin on mitochondria [release of cytochrome *c* into the cytosol and disruption of the mitochondrial membrane potential ($\Delta\Psi_m$)]. Furthermore, Chinese propolis and chrysin each inhibited staurosporine-induced cell death. These findings indicate that the inhibitory effects of Chinese propolis against neuronal cell death induced by ER stress or staurosporine may be exerted primarily by chrysin. Moreover, the mechanism underlying the protective effects may, at least partly, involve inhibitions of caspase-3 activity and the mitochondrial apoptotic pathway.

**KEYWORDS: Chrysin; endoplasmic reticulum stress; neuroprotection; propolis; SH-SY5Y**

## INTRODUCTION

The endoplasmic reticulum (ER) regulates protein synthesis, protein folding, and intracellular $Ca^{2+}$ levels (*1*). Conditions that impair the function of the ER, collectively designated “ER stress”, can lead to an accumulation of unfolded proteins in the ER lumen (*2*). In response to ER stress, cells have developed a self-protective signal transduction pathway termed the unfolded protein response (UPR), which includes increased expression of the molecular chaperone [immunoglobulin heavy-chain binding protein (BiP)] in the ER, translational attenuation, and ER-associated degradation (*3*). However, if the damage is too severe to repair, the UPR ultimately activates an apoptotic pathway (*4*) involving a C/EBP homologous protein (CHOP) (*5*). Recent indications suggest that ER stress also activates the mitochondrial apoptotic pathway (*6, 7*).

The mitochondrial apoptotic pathway integrates a broad spectrum of extracellular and intracellular stresses. The intracellular stimuli include oxidative stress, DNA damage, and protein misfolding (*8*). Mitochondria, along with the ER, play pivotal roles in regulating the intracellular $Ca^{2+}$ content. During cellular $Ca^{2+}$ overload, mitochondria take up cytosolic $Ca^{2+}$, an event that in turn induces opening of permeability transition pores, disruption of the mitochondrial membrane potential ($\Delta\Psi_m$), release of cytochrome *c*, activation of caspases, and cell death. Reimertz et al. have reported that in SH-SY5Y cells, ER stress leads to up-regulation of the gene for a Bcl-2 homology domain 3-only protein [namely, Bcl-2 binding component 3/p53-up-regulated modulator of apoptosis (*Bbc3/PUMA*)] and to activation of the mitochondrial apoptotic pathway that is also activated by tunicamycin (*7*). A more recent study indicates that tunicamycin and thapsigargin each disrupt mitochondrial permeability and $\Delta\Psi_m$ in human cervical and colon carcinoma cell lines (*6*). These results indicate that the mitochondrial apoptotic pathway may form a pivotal part of the induction of apoptosis by ER stress.

Chinese propolis is a resinous mixture collected from plants by the honeybee, *Apis mellifera*, which uses it as a building and insulating material in the beehive, and has been used as a folk medicine since ancient times. It has important pharmacological properties, and indeed it is used for a wide variety of purposes because of its antibacterial (*9*), anti-inflammatory (*10*), antioxidative (*11, 12*), and hepatoprotective (*13*) effects and/or because of its tumoricidal activities (*14*). Moreover, Chinese propolis contains a lot of flavonoids, such as acacetin, chrysin, galangin, naringenin, and pinocembrin (*15, 16*). Flavonoids have the important effect of scavenging oxygen-derived free radicals, and use of in vitro experimental systems has shown that they

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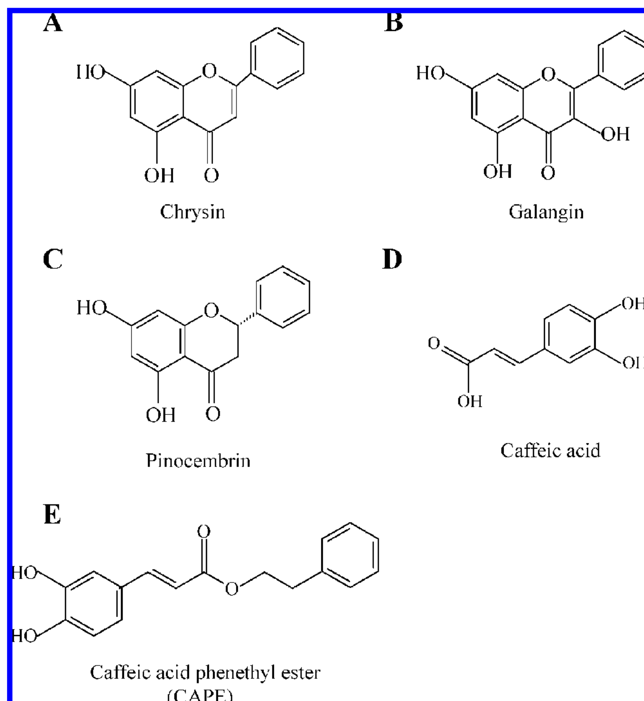


**Figure 1.** Chemical structures of (**A**) chrysin (5,7-dihydroxyflavone), (**B**) galangin (3,5,7-trihydroxyflavone), (**C**) pinocembrin (5,7-dihydroxyflavanone), (**D**) caffeic acid (3,4-dihydroxycinnamic acid *trans*-caffeate), and (**E**) caffeic acid phenethyl ester (CAPE).

also possess anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties (*17*). However, whether Chinese propolis protects against ER stress-induced neuronal cell death remains unclear.

In the present study, we aimed to evaluate the protective effects of Chinese propolis and some of its constituents (the structures of these constituents are shown in **Figure 1**) against the neuronal cell death induced by tunicamycin (an ER stress inducer) or staurosporine in SH-SY5Y cells.

## MATERIALS AND METHODS

**Materials.** Ethanol extracts of Chinese propolis and its constituents [chrysin, pinocembrin, galangin, caffeic acid, and caffeic acid phenethyl ester (CAPE)] (**Figure 1**) and Brazilian propolis were gifted by API Co. Ltd. (Gifu, Japan). The drugs used and their sources were as follows: *N*-acetylcysteine (NAC; Wako Pure Chemical Industries, Osaka, Japan), sodium hydrogen phosphate 12-water (Nacalai tesque, Kyoto, Japan), sodium dihydrogen phosphate dihydrate (Nacalai tesque), potassium chloride (Wako), paraformaldehyde (Wako), sodium chloride (Kishida Chemical, Osaka, Japan), Trolox (Sigma-Aldrich, St. Louis, MO), Alexa 546-conjugated second antibody [goat anti-mouse IgG (Molecular Probes, Eugene, OR)], mouse anticleaved caspase-3 antibody (Asp175) (Cell Signaling Technology, Danvers, MA), mouse anti-BiP/GRP78 antibody (BD Transduction Laboratories, Lexington, KY), mouse anti-$\beta$-actin antibody (Sigma), mouse anti-cytochrome *c* antibody (Santa Cruz Biotechnology, Santa Cruz, CA), dimethyl sulfoxide (DMSO; Koso Chemical, Tokyo, Japan), Dulbecco's modified Eagle's medium (DMEM; Sigma), fetal bovine serum (HyClone Laboratories, South Logan, UT), penicillin and streptomycin (Meiji Seika, Tokyo, Japan), tunicamycin (Wako), staurosporine (Cayman Chemical Co., Ann Arbor, MI), Hoechst 33342 (Invitrogen, Carlsbad, CA), and YO-PRO-1 (Molecular Probes).

**Propolis Extracts.** Chinese and Brazilian propolis materials were extracted using 95% ethanol with overnight filtration and concentrated under reduced pressure. The main constituents of Chinese propolis were analyzed by high-performance liquid chromatography (HPLC), the samples being injected into an HPLC system (Waters, Washington, NJ) fitted with a Shim-pack CLC-ODS (Shimazu, Kyoto, Japan) C18 column (∅ 6.0 × 150 mm). The mobile phase consisted of 1% acetic acid in 55% methanol. All constituents were measured at a wavelength of 290 nm.

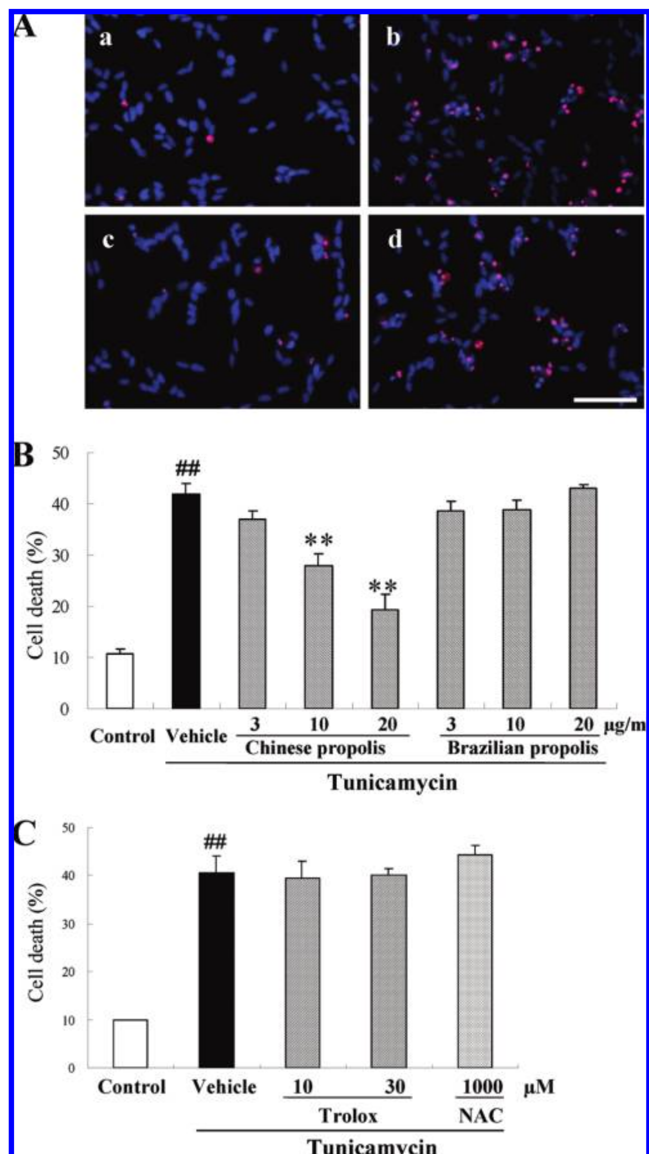


**Figure 2.** Effects of Chinese propolis against ER stress-induced cell death in SH-SY5Y cells. SH-SY5Y cells were pretreated with vehicle or with either Chinese propolis at 3–20 $\mu$g/mL or Brazilian propolis at 3–20 $\mu$g/mL for 1 h, followed by 24 h of additional incubation with 2.0 $\mu$g/mL tunicamycin. (**A**) Fluorescence micrographs of Hoechst 33342 and YO-PRO-1 staining are shown: (a) control, (b–d) tunicamycin stimulation for 24 h either alone (b) or together with (c) pretreatment with 20 $\mu$g/mL Chinese propolis or (d) pretreatment with 20 $\mu$g/mL Brazilian propolis. Scale bar represents 100 $\mu$m. (**B**) The number of dead cells was increased at 24 h after tunicamycin treatment. Pretreatment of cells with Chinese propolis at 10 or 20 $\mu$g/mL, but not with Brazilian propolis at 3–20 $\mu$g/mL, significantly reduced the tunicamycin-induced cell death (vs vehicle). (**C**) Pretreatment of cells with Trolox at 10 or 30 $\mu$M or with *N*-acetylcystein at 1 mM did not affect tunicamycin-induced cell death (vs vehicle). Data represent means and SE ($n = 6$). ##, $p < 0.01$, vs control; **, $p < 0.01$, vs vehicle (Dunnett's test).

**Cell Culture.** Human neuroblastoma (SH-SY5Y) cells were purchased from the European Collection of Cell Cultures (Wiltshire, U.K.) and maintained in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin in a humidified atmosphere containing 5% $CO_2$ at 37 °C. Cells were passaged by trypsinization

**Table 1.** Components of the Ethanol Extracts of Chinese and Brazilian Propolis

| component | Chinese propolis content[a] (%) | Chinese propolis concn[b] (μM) | Brazilian propolis content[a] (%) | Brazilian propolis concn[b] (μM) |
|---|---|---|---|---|
| caffeic acid | 1.3 | 0.74 | 0.6 | 0.31 |
| *p*-coumaric acid | 4.0 | 2.42 | 3.7 | 2.24 |
| chrysin | 3.1 | 1.22 | ND[c] | ND |
| galangin | 2.4 | 0.88 | ND | ND |
| pinocembrin | 4.9 | 1.91 | ND | ND |
| CAPE[d] | 1.7 | 0.60 | ND | ND |
| artepillin C | ND | ND | 14.0 | 4.91 |
| baccharin | ND | ND | 6.8 | 1.91 |
| drupanin | ND | ND | 1.8 | 0.78 |

[a] Values show percentage content of each constituent in Chinese or Brazilian propolis. [b] Values show concentration of each constituent in a 10 μg/mL extract of Chinese or Brazilian propolis. [c] ND, not detected. [d] CAPE, caffeic acid phenethyl ester.

every 3−5 days. SH-SY5Y cells were seeded at 1 × $10^5$ cells per well into a 12-well plate and then incubated for 24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$.

**Cell Death Assay.** SH-SY5Y cells were seeded at 1 × $10^4$ cells per well into a 96-well plate and then incubated for 24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$. The entire medium was then replaced with fresh medium containing 1% FBS, and one of the compounds was pretreated for 1 h, followed by the addition of 2.0 μg/mL tunicamycin or 30 nM staurosporine. Nuclear staining assays were carried out after a further 24 h of incubation. Cell death was assessed on the basis of combination staining with Hoechst 33342 and YO-PRO-1. Hoechst 33342 ($\lambda_{ex}$ = 360 nm, $\lambda_{em}$ > 490 nm) freely enters living cells and then stains the nuclei of viable cells, as well as those that have suffered apoptosis or necrosis. Apoptotic cells can be distinguished from viable and necrotic cells on the basis of nuclear condensation and fragmentation. YO-PRO-1 ($\lambda_{ex}$ = 491 nm, $\lambda_{em}$ > 509 nm) is a membrane-impermeable dye that is generally excluded from viable cells, whereas early-stage apoptotic and necrotic cells are YO-PRO-1-positive. At the end of the culture period, Hoechst 33342 and YO-PRO-1 dyes were added to the culture medium (at 8 and 0.2 μM,

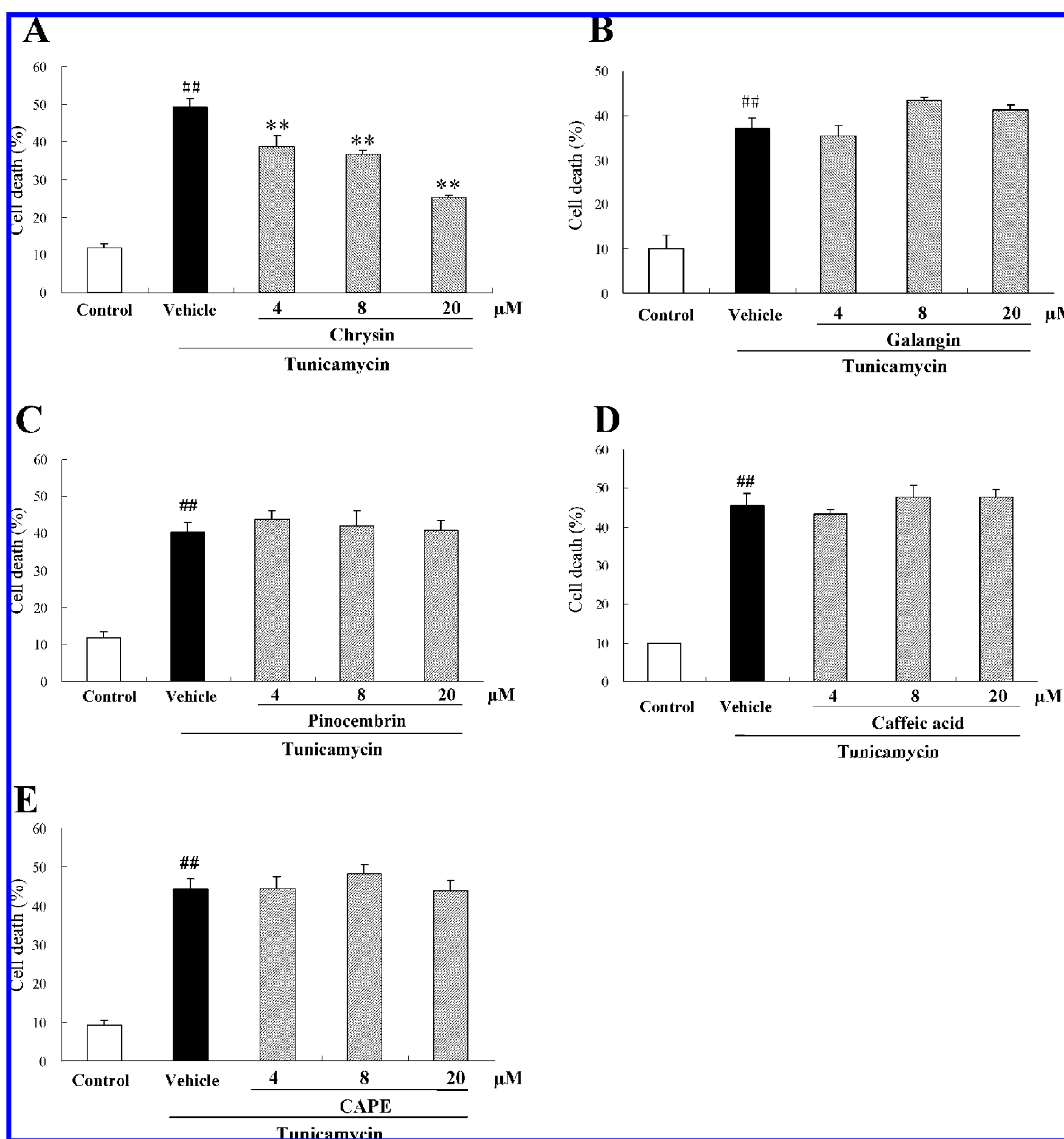


**Figure 3.** Neuroprotective effects of various constituents of Chinese propolis against ER stress-induced cell death in SH-SY5Y cells. SH-SY5Y cells were pretreated for 1 h with vehicle or with one of the propolis constituents [(**A**) chrysin, (**B**) galangin, (**C**) pinocembrin, (**D**) caffeic acid, or (**E**) CAPE], followed by 24 h of additional incubation with 2.0 μg/mL tunicamycin. Data represent means and SE ($n$ = 6). ##, $p$ < 0.01, vs control; **, $p$ < 0.01, vs vehicle (Dunnett's test).

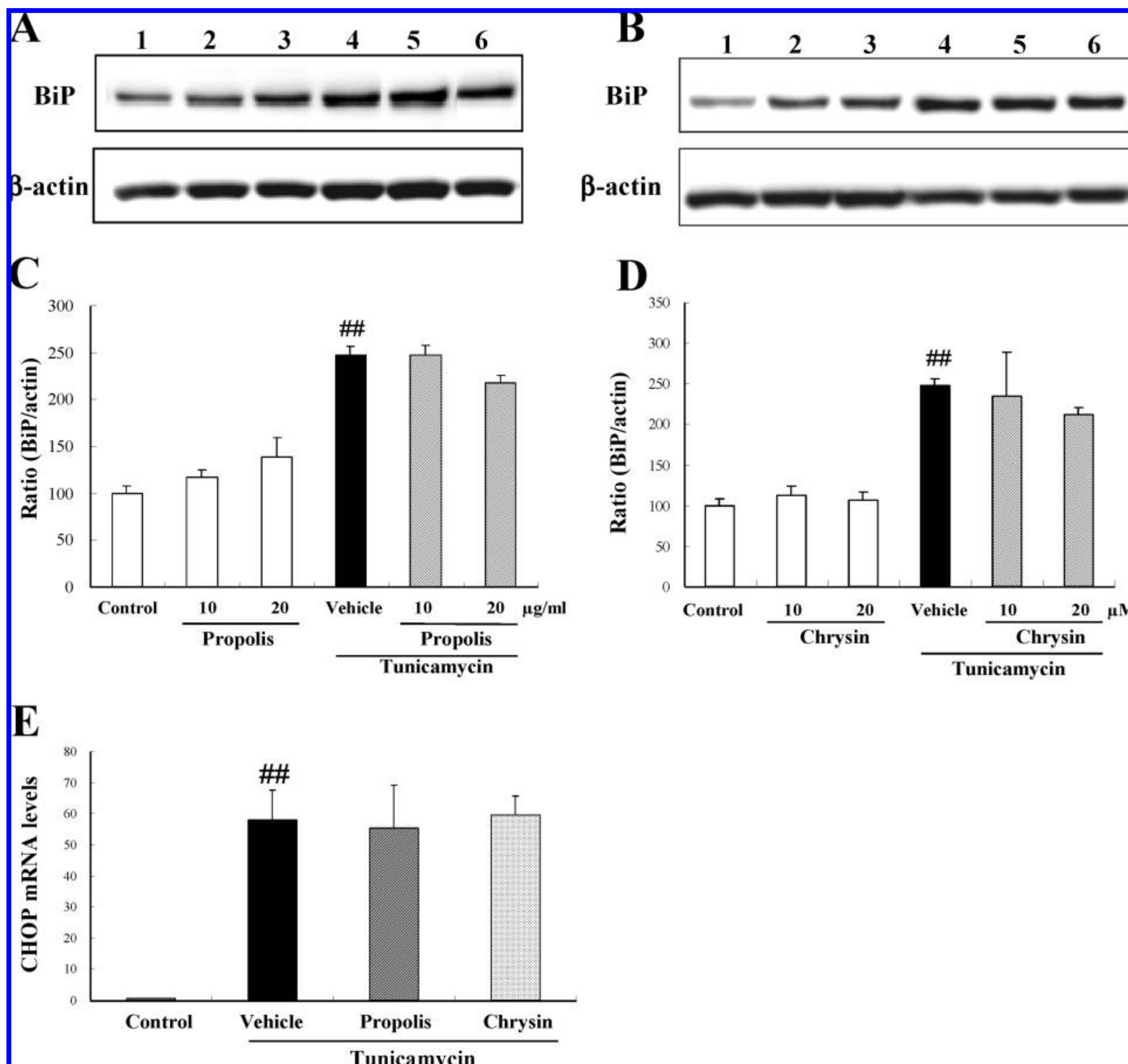


**Figure 4.** Effects of Chinese propolis and chrysin on BiP and CHOP expressions in SH-SY5Y cells. (**A**, **B**) BiP expression was detected using Western blotting. (**A**) Pretreatment with vehicle (lanes 1 and 4) or with Chinese propolis at either 10 $\mu$g/mL (lans 2 and 5) or 20 $\mu$g/mL (lanes 3 and 6) was followed by 24 h of additional incubation with 2.0 $\mu$g/mL tunicamycin. Subsequent analysis was by Western blot (anti-BiP/GRP78 antibody or anti-$\beta$-actin antibody). (**B**) Pretreatment with vehicle (lanes 1 and 4) or with chrysin at either 10 $\mu$M (lanes 2 and 5) or 20 $\mu$M (lanes 3 and 6) was followed by 24 h of additional incubation with 2.0 $\mu$g/mL tunicamycin. Western blot analysis was as in (**A**). (**C**, **D**) BiP expression was quantified by densitometry and corrected by reference to $\beta$-actin. (**E**) Measurements of CHOP mRNA levels were performed after pretreatment with Chinese propolis at 10 $\mu$g/mL or with chrysin at 20 $\mu$M followed by 6 h of additional incubation with 2.0 $\mu$g/mL tunicamycin. Data represent means and SE ($n$ = 6). ##, $p < 0.01$, vs control (Dunnett's test).

respectively) for 15 min. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). A total of at least 300 cells per condition were counted in a blind manner by a single observer (H.I.).

**Western Blot Analysis.** Cells were washed with PBS, harvested, and lysed in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma) and phosphates inhibitor cocktails 1 and 2 (Sigma). Lysates were centrifuged at 12000*g* for 15 min at 4 °C. Supernatants were collected and boiled for 5 min in SDS sample buffer (Wako). Equal amounts of protein were subjected to 5−20% SDS-PAGE gradient gels and then transferred to poly(vinylidene difluoride) membranes. After blocking with Block Ace (Snow Brand Milk Products Co. Ltd., Tokyo, Japan) for 30 min, the membranes were incubated with the primary antibody (anti-BiP/GRP78 antibody, anticleaved caspase-3 antibody, or anti-$\beta$-actin antibody). Subsequently, the membrane was incubated with the secondary antibody [HRP-conjugated goat anti-mouse IgG (Pierce Biotechnology, Rockford, IL)]. The immunoreactive bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and then measured using GelPro (Media Cybernetics, Silver Spring, MD).

**Total RNA Isolation and Reverse Transcription of RNA.** Cells were collected by trypsinization. Total RNA was isolated using an RNAiso Kit (Takara, Siga, Japan). Reverse transcription was performed using a PrimeScript RT reagent kit (Takara) according to the manufacturer's instructions.

**Real-Time Quantitative RT-PCR Analysis.** Real-time PCR was performed using a Thermal Cycler Dice Real Time System TP800 with SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions. The primer pair for CHOP was designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi). The primer sequences for CHOP were sense 5′-GACCTGCAAGAGGTCCTGTC-3′ and antisense 5′-TGTGACCTCTGCTGGTTCTG-3′, and those for $\beta$-actin were sense 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and antisense 5′-ATGGAGCCACCGATCCACA-3′. Real-

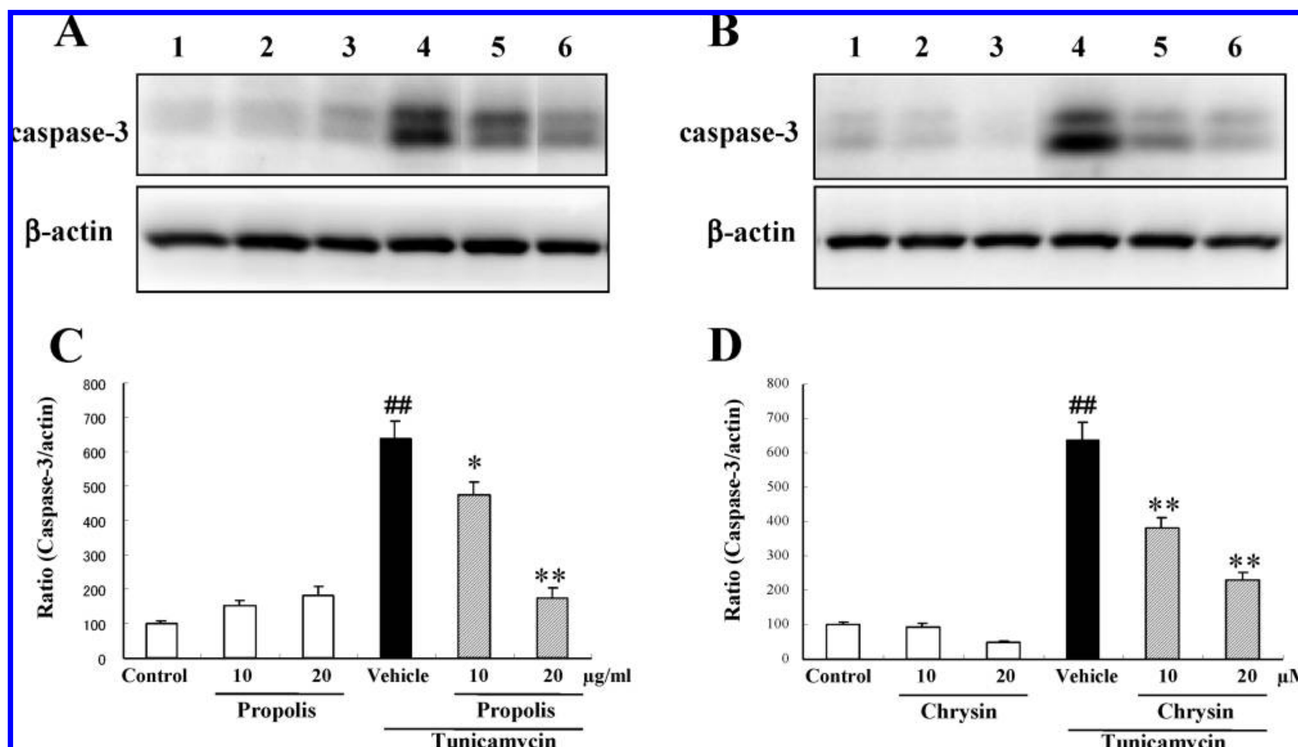


**Figure 5.** Effects of Chinese propolis and chrysin on caspase-3 activity (as assessed using Western blot) in SH-SY5Y cells. (**A**) Cleaved caspase-3 expression in SH-SY5Y cells was assessed after 1 h of pretreatment with vehicle (lanes 1 and 4) or with Chinese propolis at either 10 $\mu$g/mL (lanes 2 and 5) or 20 $\mu$g/mL (lanes 3 and 6) followed by 24 h of additional incubation with 2.0 $\mu$g/mL tunicamycin (lanes 4−6). Subsequent analysis was by Western blot (anticleaved caspase-3 antibody or anti-$\beta$-actin antibody). (**B**) Cleaved caspase-3 expression in SH-SY5Y cells was assessed after 1 h of pretreatment with vehicle (lanes 1 and 4) or with chrysin at either 10 $\mu$M (lanes 2 and 5) or 20 $\mu$M (lanes 3 and 6) followed by 24 h of additional incubation with 2.0 $\mu$g/mL tunicamycin (lanes 4−6). Western blot analysis was as in (**A**). (**C**, **D**) Cleaved caspase-3 expression was quantified by densitometry and corrected by reference to $\beta$-actin. Data represent means and SE ($n = 6$). ##, $p < 0.01$, vs control; $*$, $p < 0.05$, and $**$, $p < 0.01$, vs vehicle (Student's $t$ test).

time PCR conditions were as follows: initial denaturation at 95 °C for 10 s, annealing and extension step at 60 °C for 30 s. For analysis of the dissociation curve, samples were subjected to 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s.

**Immunofluorescence.** After treatment with tunicamycin in the presence or absence of Chinese propolis at 10 $\mu$g/mL or chrysin at 20 $\mu$M for 24 h, cells were washed twice in PBS and then fixed with 4% paraformaldehyde at 25 °C for 15 min. After being blocked for 30 min with PBS containing 3% normal goat serum, the cells were incubated for 1 h with mouse anti-cytochrome *c* antibody. After washing, Alexa 546-conjugated second antibody (goat anti-mouse IgG) was added for 1 h. After the cells had then been washed three times with PBS, Hoechst 33324 (10 $\mu$g/mL) was added and incubated for 5 min. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus). Experiments were repeated a total of three times.

**Mitochondrial Membrane Potential ($\Delta\Psi_m$).** To measure $\Delta\Psi_m$, the fluorescent dye JC-1 (Cell Technology, Mountain View, CA) was used. JC-1 exists as a monomer at low $\Delta\Psi_m$ and emits green fluorescence, but it forms aggregates and emits red fluorescence at higher $\Delta\Psi_m$ levels. After treatment of cells with tunicamycin in the presence or absence of Chinese propolis at 10 $\mu$g/mL or chrysin at 20 $\mu$M for 24 h, cells were collected by trypsinization and incubated for 15 min at 37 °C with JC-1. Thereafter, cells were washed with loading buffer and measured using an Olympus IX70 inverted epifluorescence microscope (olympus) or Varioskan Flash (Thermo Electron Corp., Vantaa, Finland) with respective excitation and emission wavelengths of 485 and 535 nm for the monomer and 550 and 600 nm for the aggregates.

**Statistical Analysis.** Data are presented as the means ± SE. Statistical comparisons were made using a one-way ANOVA followed by Dunnett's test or Student's *t* test [using STAT VIEW version 5.0 (SAS Institute, Cary, NC)]. $P < 0.05$ was considered to indicate statistical significance.

## RESULTS

**Effects of Chinese Propolis against Tunicamycin-Induced Neuronal Cell Death.** We first examined whether ethanol extracts of Chinese and Brazilian propolis might protect against the neuronal cell death induced by tunicamycin in SH-SY5Y cells. We identified living and dead cells by means of Hoechst 33324 and YO-PRO-1 staining, respectively. By comparison with control, tunicamycin significantly increased the number of dead cells (stained with YO-PRO-1) (**Figure 2A**). Preteatment with Chinese propolis at 3−20 $\mu$g/mL protected against this cell death in a concentration-dependent manner, the effect being significant at 10 and 20 $\mu$g/mL. In contrast, Brazilian propolis at 3−20 $\mu$g/mL did not affect the cell death (**Figure 2B**). For an indication of whether the protective effects of Chinese propolis might be caused by its antioxidant activity, we evaluated the effects of the vitamin E analogue Trolox and *N*-acetylcysteine (NAC). However, neither Trolox at 10 and 30 $\mu$M nor NAC at 1000 $\mu$M had any effect on tunicamycin-induced cell death (**Figure 2C**).

**Effects of Constituents of Chinese Propolis against Tunicamycin-Induced Neuronal Cell Death.** Because Chinese propolis exhibited the above neuroprotective effect, we analyzed the constituents of Chinese and Brazilian propolis using HPLC (**Table 1**). Chinese propolis contained abundant flavonoids (chrysin, 3.1%; galangin, 2.4%; pinocembrin, 4.9%) and other distinctive constituents (caffeic acid, 1.3%;

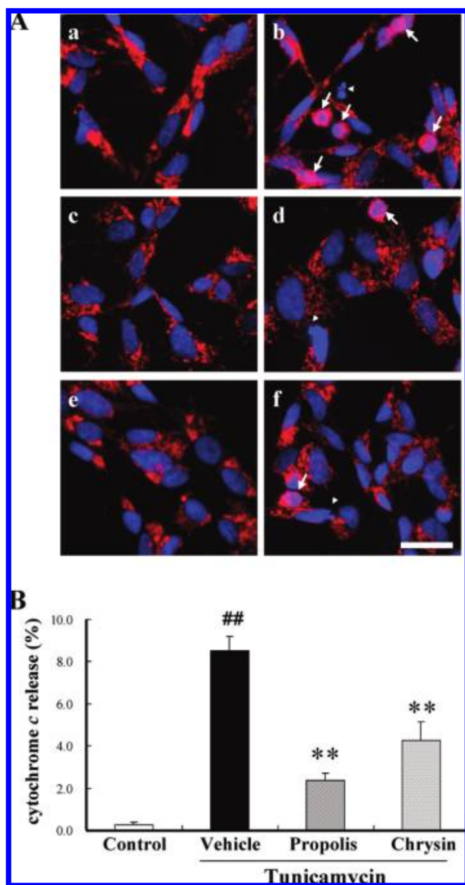


**Figure 6.** Effects of Chinese propolis and chrysin on release of cytochrome *c*. (**A**) Immunofluorescence staining with anticytochrome *c* (red) and Hoechst 33324 (blue) is shown. The pictures show pretreatment with vehicle (a, b) or with Chinese propolis at 10 $\mu$g/mL (c, d) or with chrysin at 20 $\mu$M (e, f) followed by 24 h of additional incubation with 2.0 $\mu$g/mL tunicamycin (b, d, f). Arrows and triangles indicate cytochrome *c* released from mitochondria. Scale bar represents 25 $\mu$m. (**B**) Cytochrome *c* release from mitochondria is quantified. The rate of cytochrome *c* release is expressed as the percentage of cytochrome *c* released from mitochondria. Data represent means and SE ($n = 6$). ##, $p < 0.01$, vs control; **, $p < 0.01$, vs vehicle (Student's *t* test).

CAPE, 1.7%) (the structures of these constituents are shown in **Figure 1**). On the other hand, Brazilian propolis contained abundant cinnamic acid analogues (artepilin C, 14.0%; baccharin, 6.8%; drupanin, 1.8%). It seemed likely that some constituents present in only Chinese propolis might be responsible for the protective effects described above, because Brazilian propolis had no such effects. We therefore examined various constituents of Chinese propolis (chrysin, galangin, pinocembrin, caffeic acid, and CAPE) to see if they would protect against the cell death induced by tunicamycin. Of these, only chrysin (concentration-dependently, at 4−20 $\mu$M) protected against this cell death (**Figure 3**).

**Effects of Chinese Propolis and Chrysin against Expressions of ER Stress-Related Proteins.** To examine whether Chinese propolis and/or chrysin might affect the ER stress pathway, we first detected BiP expression using Western blot (**Figure 4A**,**B**). Tunicamycin significantly up-regulated BiP expression (versus control), and neither Chinese propolis at 10 and 20 $\mu$g/mL nor chrysin at 10 and 20 $\mu$M affected the level of BiP expression induced by tunicamycin (**Figure 4C**,**D**). Next, we measured the CHOP mRNA level using real-time quantitative RT-PCR. Tunicamycin up-regulated the CHOP mRNA level over 50-fold (versus control), and neither Chinese propolis at 10 $\mu$g/mL nor chrysin at 20 $\mu$M altered this elevated level (**Figure 4E**).

**Effects of Chinese Propolis and Chrysin against Tunicamycin-Induced Caspase-3 Activation.** To examine the effects of Chinese propolis and chrysin against the tunicamycin-induced activation of caspase-3, we detected the cleaved (active) form of caspase-3 using Western blot (**Figure 5A**,**B**). Tunicamycin increased the band intensity of cleaved caspase-3 (versus control), and Chinese propolis at 10 and 20 $\mu$g/mL and chrysin at 10 and 20 $\mu$M strongly decreased this intensity (**Figure 5C**,**D**).

**Effects of Chinese Propolis and Chrysin against Release of Cytochrome *c* from Mitochondria.** To clarify the mechanism underlying the above protective effects, we focused on the mitochondrial apoptotic pathway. Reimertz et al. reported that in SH-SY5Y cells, the ER stressor tunicamycin activates the mitochondrial apoptotic pathway, leading to a release of cytochrome *c* from mitochondria into the cytosol (*7*). In normal cells, cytochrome *c* is localized within mitochondria, whereas after an increase in mitochondrial permeability the localization shifts to the cytosol. We evaluated the effects of Chinese propolis and chrysin against the tunicamycin-induced release of cytochrome *c*. In control cells, cytochrome *c* was localized almost exclusively to the mitochondria. However, after tunicamycin treatment, cytochrome *c* was released from mitochondria into the cytosol, although Chinese propolis at 10 $\mu$g/mL and chrysin at 20 $\mu$M decreased this release (**Figure 6A**). As shown in **Figure 6B**, tunicamycin significantly up-regulated the rate of release of cytochrome *c* (versus control), and Chinese propolis at 10 $\mu$g/mL and chrysin at 20 $\mu$M significantly reduced this elevated rate.

**Effects of Chinese Propolis and Chrysin against Tunicamycin-Induced $\Delta\Psi_m$ Disruption.** It is known that an increase in mitochondrial permeability leads to a disruption of $\Delta\Psi_m$. To examine whether Chinese propolis and/or chrysin might prevent the disruption of $\Delta\Psi_m$ induced by tunicamycin, we used the lipophilic cation JC-1 to evaluate $\Delta\Psi_m$. The red fluorescence emitted by the aggregate form of JC-1 was observed in the polarized mitochondrial membrane, but when the mitochondrial membrane was depolarized, the green fluorescence emitted by the monomeric form of JC-1 was observed in the cytosol (using fluorescence microscopy; **Figure 7A**). By comparison with control, tunicamycin increased the emission of green fluorescence and decreased that of red fluorescence. Pretreatment with Chinese propolis at 10 $\mu$g/mL or with chrysin at 20 $\mu$M altered both the green and red fluorescences, and we obtained $\Delta\Psi_m$ values from the ratio of red/green fluorescence intensities (using a multiplate reader). Tunicamycin significantly decreased $\Delta\Psi_m$ (versus control), and Chinese propolis or chrysin partially prevented this effect of tunicamycin (**Figure 7B**). These results indicate that both Chinese propolis and chrysin inhibit the mitochondrial apoptotic pathway at least partly via inhibitions of two effects of tunicamycin (namely, depolarization of the mitochondrial membrane potential and cytochrome *c* release from mitochondria).

**Effects of Chinese Propolis and Chrysin against Staurosporine-Induced Neuronal Cell Death.** Because mitochondrial permeabilization plays a pivotal causal role in the cell death induced by staurosporine (*18*), we examined whether Chinese propolis and/or chrysin might also protect SH-SY5Y cells against the neuronal cell death induced by a broad kinase inhibitor, staurosporine. Staurosporine significantly induced the cell death (versus control), and Chinese propolis at 10−20 $\mu$g/

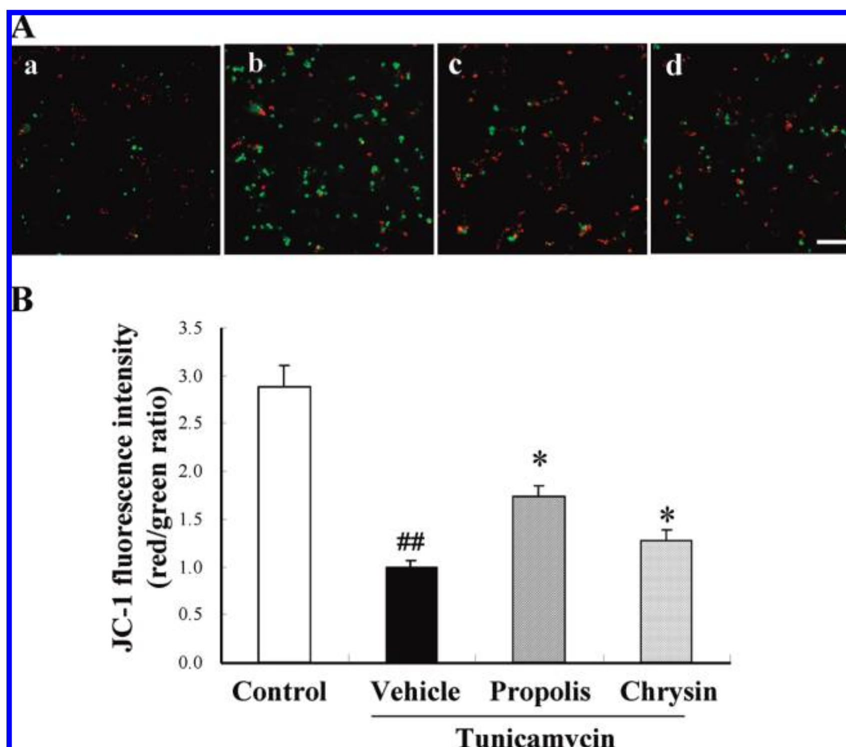


**Figure 7.** Effects of Chinese propolis and chrysin on depolarization of mitochondrial membrane potential in SH-SY5Y cells. (**A**) Representative fluorescence microscopy of JC-1 staining at 24 h after addition of 2 $\mu$g/mL tunicamycin as shown by nontreated cells (a), tunicamycin treatment (b), pretreatment with 10 $\mu$g/mL Chinese propolis at 1 h before tunicamycin (c), and pretreatment with 20 $\mu$M chrysin at 1 h before tunicamycin (d). (**B**) $\Delta\Psi_m$ (based on JC-1 staining) is quantified. Cells with polarized mitochondrial membranes display red fluorescence within mitochondria. When the mitochondrial membrane is depolarized, JC-1 is released from mitochondria and is then expressed as green fluorescence in the cytosol. From the red/green fluorescence ratio, we obtained the $\Delta\Psi_m$ value. Chinese propolis and chrysin each improved $\Delta\Psi_m$ (vs tunicamycin alone). Scale bar represents 50 $\mu$m. Data represent means and SE ($n = 6$). ##, $p < 0.01$, vs control; $*$, $p < 0.05$, vs vehicle (Student's $t$ test).

mL and chrysin at 4 and 8 $\mu$M significantly inhibited this effect, but Brazilian propolis at 3−20 $\mu$g/mL did not (**Figure 8A**,**B**). Finally, we established that neither Trolox at 10−30 $\mu$M nor NAC at 1000 $\mu$M had any effect on the cell death induced by staurosporine (**Figure 8C**).

## DISCUSSION

In the present study, we evaluated the effects of Chinese propolis and several of its constituents (chrysin, pinocembrin, galangin, caffeic acid, and CAPE) and also the effects of Brazilian propolis against ER stress-induced cell death in SH-SY5Y cells. Ethanol extracts of Chinese propolis (10−20 $\mu$g/mL) and chrysin (4−20 $\mu$M; a major flavonoid present in Chinese propolis) partially suppressed the inhibition of neuronal cell death by tunicamycin. However, the other constituents (pinocembrin, galangin, caffeic acid, and CAPE) and Brazilian propolis had no such effects (**Figures 2** and **3**). Additionally, we found that Chinese propolis and chrysin inhibited three individual effects of tunicamycin: namely, activation of caspase-3, depolarization of the mitochondria membrane potential, and mitochondrial cytochrome *c* release (**Figures 5** and **6**). Furthermore, both Chinese propolis (10−20 $\mu$g/mL) and chrysin (4 and 8 $\mu$M) partially suppressed the neuronal cell death induced by staurosporine.

In this study, we used two different drugs, tunicamycin and staurosporine, to induce neuronal cell death in SH-SY5Y cells. The glucosamine-containing nucleoside antibiotic, tunicamycin, produced by the genus *Streptomyces*, inhibits both N-linked glycosylation and the formation of N-glycosidic protein−carbohydrate linkages (*19*) and induces ER stress. Staurosporine, on the other hand, is a nonspecific protein kinase inhibitor that is widely used to initiate cell death in mammalian nucleated cells and has effects such as cell shrinkage, phosphatidylserine exposure, loss of plasma membrane integrity, $\Delta\Psi_m$ reduction, cytochrome *c* release, nuclear chromatin condensation and fragmentation, and DNA degradation (*18*). Because propolis has antioxidant activity [in particular, constituents such as dicaffeoylquinic acid and some hydroxyflavones have strong antioxidant activities (*20, 21*)], the effects of two antioxidants, Trolox and NAC, against the cell death induced by tunicamycin and staurosporine in SH-SY5Y cells were also examined. However, neither Trolox at 10−30 $\mu$M nor NAC at 1 mM had any effect on such cell death (**Figures 2B** and **8C**). These results suggest that the protective effects of Chinese propolis against tunicamycin- and staurosporine-induced cell death are independent of its antioxidant activity.

Because both Chinese propolis and chrysin displayed neuroprotective effects against the ER stressor, tunicamycin, we thought that they might alter the expressions of ER stress-related proteins (BiP and CHOP). However, neither of them altered the expression levels of BiP and CHOP mRNA seen after tunicamycin application (**Figure 4**). These data suggest that the observed defensive effects of Chinese propolis and chrysin may be independent of alterations in the levels of protein expression induced by ER stress. On the other hand, Oda et al. have reported that in SH-SY5Y cells, caspase-4 is not expressed and Z-LEVD-FMK, a caspase-4-specific inhibitor, does not inhibit cell death induced by tunicamycin (*22*). Moreover, we tried to detect caspase-4 with Western blot, but we could not detect pro- and activated-caspase-4 using an anti-caspase-4/TX antibody (Medical and Biological Laboratories, Nagoya, Japan)

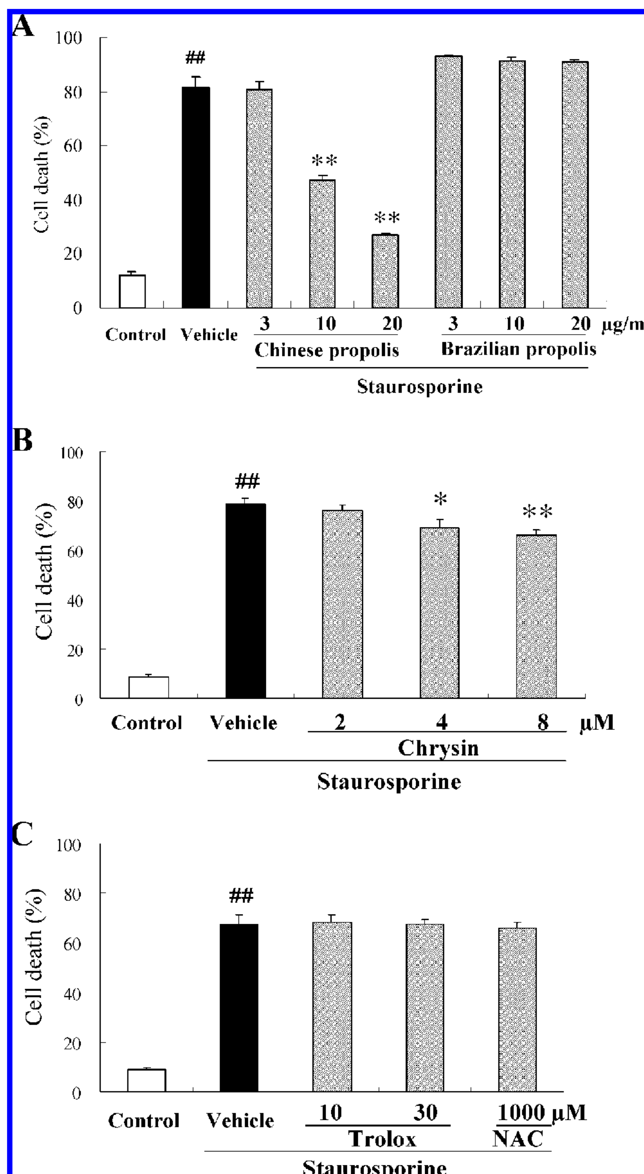


**Figure 8.** Effects of Chinese propolis against staurosporine-induced neuronal cell death in SH-SY5Y cells. Cell-death rate was measured after pretreatment with (**A**) Chinese or Brazilian propolis at 3−20 $\mu$g/mL, (**B**) chrysin at 2−20 $\mu$M, or (**C**) Trolox at 10−30 $\mu$M or NAC at 1 mM (each pretreatment being followed by 24 h of additional incubation with 30 nM staurosporine). Data represent means and SE ($n = 6$). ##, $p < 0.01$, vs control; $*$, $p < 0.05$, and $**$, $p < 0.01$, vs vehicle (Dunnett's test).

(unpublished data, Izuta et al.). These findings indicate that in the SH-SY5Y cells, caspase-4 may not play a crucial role in ER stress-induced cell death.

In the present study, we focused on the common apoptosis pathway activated by tunicamycin or staurosporine, because Chinese propolis and chrysin protected against neuronal cell death induced by tunicamycin and staursporine. Previous studies have indicated (a) that both tunicamycin and thapsgargin increase mitochondrial permeability in human cervical and colon carcinoma cell lines (*6*), whereas (b) a novel compound [2,3-dihydroxypropyl-5-bromo-*N*-(2-methyl-3-trifluoromethyl-phenyl)anthranilate] protects mouse thymocytes against thapsigargin-induced $\Delta\Psi_m$ reduction (*23*). These findings indicated that increased mitochondrial permeability may play an important role in the cell death induced by ER stress. Therefore, we evaluated the pharmacological action of these compounds against the mitochondrial apoptosis pathway. Both Chinese propolis (10 $\mu$g/mL) and chrysin (20 $\mu$M) inhibited cytochrome *c* release from mitochondria and the $\Delta\Psi_m$ depolarization seen after tunicamycin treatment (**Figures 6** and **7**). Moreover, both Chinese propolis at 10−20 $\mu$g/mL and chrysin at 4 and 8 $\mu$M partially protected against the neuronal cell death induced by staurosporine, a broad kinase inhibitor (**Figure 8**). Taken together, these findings indicate that Chinese propolis and chrysin may prevent the activation of the mitochondrial apoptotic pathway that is induced by agents such as tunicamycin, through inhibitions of $\Delta\Psi_m$ depolarization and cytochrome *c* release.

The preventive effects of Chinese propolis and chrysin against $\Delta\Psi_m$ loss induced by tunicamycin (**Figure 7**) were weaker than the effects of other experiments (**Figures 2**, **3**, **5**, and **6**). To explain the differential potency among the experiments, we have thought two hypotheses. First, the differences may depend on the sensitivity of each experiment. Cytochrome *c* release can be definitely detected, because little cytochrome *c* release is detected in normal conditions. On the other hand, measurements of mitochondrial membrane potential ($\Delta\Psi_m$) are comparative assay, and even after tunicamycin treatment, $\Delta\Psi_m$ levels do not reach zero. Therefore, it is difficult to clearly detect the effects of Chinese propolis and chrysin against $\Delta\Psi_m$ loss in this assay. Second, Chinese propolis and chrysin might have effected downstream signals of $\Delta\Psi_m$ loss in the mitochondrial apoptosis pathway. Therefore, these compounds had very weak potency against $\Delta\Psi_m$ depolarization and had strong potency against cytochrome *c* release.

The flavonoid chrysin, which is present at high levels in both honey and propolis and also in many plant extracts (*24*), exhibits anticarcinogenic (*25*), antiviral (*26*), antioxidant (*27*), and anti-inflammatory activities. The molecular mechanisms underlying its anti-inflammatory effects can be explained in part by suppressions of the promoter activities of the pro-inflammatory enzymes cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) (*28*). Furthermore, chrysin has been reported to protect mesencephalic cultures from injury by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium hydrochloride (which has been shown by DNA fragmentation studies and tyrosine hydroxylase immunocytochemistry of dopamine neurons to occur by apoptosis in vitro) (*29*). In addition, Yamazaki and co-workers demonstrated that diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), suppressed the neuronal cell death induced by two types of ER-inducing agents (thapsigargin and tunicamycin) in SH-SY5Y cells (*30*). Moreover, (a) lipopolysaccharide-induced inflammation in the lung has been shown to be attenuated in CHOP-knockout mice (*31*), and (b) studies of primary intestinal epithelial cells from inflamed interleukin 10-knockout mice and patients with inflammatory bowel disease have revealed activated ER stress responses in the intestinal epithelium (*32*). These reports indicate that inflammatory responses may induce ER stress. Therefore, it is possible that the anti-inflammatory effects of chrysin and diclofenac are in part dependent on their inhibitory effects against ER stress. Moreover, the potency of chrysin against neuronal cell death induced by tunicamycin was comparatively strong, whereas the potency against neuronal cell death induced by staurosporine was weaker than that of Chinese propolis. This result indicates that chrysin may specifically inhibit neuronal cell death induced by ER stress rather than staurosporine. In addition to that, it is possible that Chinese propolis includes

the contents that protect neuronal cell death induced by staurosporine.

In the present study, we compared two kinds of propolis collected in China and Brazil and found that the propolis collected in China and its constituent, chrysin, protected neuronal cell death induced by tunicamycin or staurosporine in SH-SY5Y cells. It is known that chrysin is included in Argentine, Italian, and Spanish samples of propolis (*15*). Therefore, a propolis including chrysin may have the protective effects against neuronal cell death.

In conclusion, both Chinese propolis and one of its constituents, chrysin, inhibit the neuronal cell death induced by tunicamycin in SH-SY5Y cells. Our results suggest that the mechanism underlying their protective effects is partly dependent on inhibitions of both caspase-3 activity and the mitochondrial apoptotic pathway.

## ABBREVIATIONS USED

Bbc3/PUMA, Bcl-2 binding component 3/p53-up-regulated modulator of apoptosis; BiP, immunoglobulin heavy-chain binding protein; CAPE, caffeic acid phenethyl ester; CHOP, C/EBP homologous protein; COX2, cyclooxygenase 2; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; HPLC, high-performance liquid chromatography; iNOS, inducible nitric oxide synthase; NAC, *N*-acetylcysteine; UPR, unfolded protein response; $\Delta\Psi_m$, mitochondrial membrane potential.

## LITERATURE CITED

(1) Rao, R. V.; Ellerby, H. M.; Bredesen, D. E. Coupling endoplasmic reticulum stress to the cell death program. Cell Death Differ. **2004**, *11*, 372–380.
(2) Kaufman, R. J. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. **1999**, *13*, 1211–1233.
(3) Cudna, R. E.; Dickson, A. J. Endoplasmic reticulum signaling as a determinant of recombinant protein expression. Biotechnol. Bioeng. **2003**, *81*, 56–65.
(4) Oyadomari, S.; Araki, E.; Mori, M. Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells. Apoptosis **2002**, *7*, 335–345.
(5) Wang, X. Z.; Lawson, B.; Brewer, J. W.; Zinszner, H.; Sanjay, A.; Mi, L. J.; Boorstein, R.; Kreibich, G.; Hendershot, L. M.; Ron, D. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol. Cell. Biol. **1996**, *16*, 4273–4280.
(6) Sharaf, el. dein. O.; Maillier, E.; Poncet, D.; Kroemer, G.; Lemaire, C.; Brenner, C. Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. Oncogene **2008**, *27*, 285–299.
(7) Reimertz, C.; Kögel, D.; Rami, A.; Chittenden, T.; Prehn, J. H. Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptotic pathway. J. Cell Biol. **2003**, *162*, 587–597.
(8) Crow, M. T.; Mani, K.; Nam, Y. J.; Kitsis, R. N. The mitochondrial death pathway and cardiac myocyte apoptosis. Circ. Res. **2004**, *95*, 957–970.
(9) Bankova, V.; Marcucci, M. C.; Simova, S.; Nikolova, N.; Kujumgiev, A.; Popov, S. Antibacterial diterpenic acids from Brazilian propolis. Z. Naturforsch. C **1996**, *51*, 277–280.
(10) Mirzoeva, O. K.; Calder, P. C. The effect of propolis and its components on eicosanoid production during the inflammatory response. *Prostaglandins. Leukotrienes Essent. Fatty Acids* **1996**, *55*, 441–449.
(11) Krol, W.; Czuba, Z.; Scheller, S.; Gabrys, J.; Grabiec, S.; Shani, J. Anti-oxidant property of ethanolic extract of propolis (EEP) as evaluated by inhibiting the chemiluminescence oxidation of luminol. Biochem Int. **1990**, *21*, 593–597.
(12) Scheller, S.; Wilczok, T.; Imielski, S.; Krol, W.; Gabrys, J.; Shani, J. Free radical scavenging by ethanol extract of propolis. Int. J. Radiat. Biol. **1990**, *57*, 461–465.
(13) Basnet, P.; Matsushige, K.; Hase, K.; Kadota, S.; Namba, T. Four di-*O*-caffeoyl quinic acid derivatives from propolis. Potent hepatoprotective activity in experimental liver injury models. Biol. Pharm. Bull. **1996**, *19*, 1479–1484.
(14) Chen, C. N.; Weng, M. S.; Wu, C. L.; Lin, J. K. Comparison of radical scavenging activity, cytotoxic effects and apoptosis induction in human melanoma cells by Taiwanese propolis from different sources. Evidence-Based Complement. Alternat. Med. **2004**, *1*, 175–185.
(15) Volpi, N.; Bergonzini, G. Analysis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry. J. Pharm. Biomed. Anal. **2006**, *42*, 354–361.
(16) Gardana, C.; Scaglianti, M.; Pietta, P.; Simonetti, P. Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography−tandem mass spectrometry. J. Pharm. Biomed. Anal. **2007**, *45*, 390–399.
(17) Middleton, E., Jr. Effect of plant flavonoids on immune and inflammatory cell function. Adv. Exp. Med. Biol. **1998**, *439*, 175–182.
(18) Arnoult, D.; Akarid, K.; Grodet, A.; Petit, P. X.; Estaquier, J.; Ameisen, J. C.; Arnoult, D. On the evolution of programmed cell death: apoptosis of the unicellular eukaryote *Leishmania major* involves cysteine proteinase activation and mitochondrion permeabilization. Cell Death Differ. **2002**, *9*, 65–81.
(19) Mahoney, W. C.; Duksin, D. Biological activities of the two major components of tunicamycin. J. Biol. Chem. **1979**, *254*, 6572–6576.
(20) Arora, A.; Nair, M. G.; Strasburg, G. M. Structure−activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. Free Radical Biol. Med. **1999**, *24*, 1355–1363.
(21) Nakajima, Y.; Shimazawa, M.; Mishima, S.; Hara, H. Water extract of propolis and its main constituents, caffeoylquinic acid derivatives, exert neuroprotective effects via antioxidant actions. Life Sci. **2007**, *80*, 370–377.
(22) Oda, T.; Kosuge, Y.; Arakawa, M.; Ishige, K.; Ito, Y. Distinct mechanism of cell death is responsible for tunicamycin-induced ER stress in SK-N-SH and SH-SY5Y cells. Neurosci. Res. **2008**, *60*, 29–39.
(23) Muramatsu, Y.; Maemoto, T.; Iwashita, A.; Matsuoka, N. Novel neuroprotective compound SCH-20148 rescues thymocytes and SH-SY5Y cells from thapsigargin-induced mitochondrial membrane potential reduction and cell death. Eur. J. Pharmacol. **2007**, *563*, 40–48.
(24) Williams, C. A.; Harborne, J. B.; Newman, M.; Greenham, J.; Eagles, J. Chrysin and other leaf exudate flavonoids in the genus *Pelargonium*. Phytochemistry **1997**, *46*, 1349–1353.
(25) Cárdenas, M.; Marder, M.; Blank, V. C.; Roguin, L. P. Antitumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines. Bioorg. Med. Chem. **2006**, *14*, 2966–2971.
(26) Critchfield, J. W.; Butera, S. T.; Folks, T. M. Inhibition of HIV activation in latently infected cells by flavonoid compounds. AIDS Res. Hum. Retroviruses **1996**, *12*, 39–46.
(27) Lapidot, T.; Walker, M. D.; Kanner, J. Antioxidant and prooxidant effects of phenolics on pancreatic $\beta$-cell in vitro. J. Agric. Food Chem. **2002**, *50*, 7220–7225.
(28) Cho, H.; Yun, C. W.; Park, W. K.; Kong, J. Y.; Kim, K. S.; Park, Y.; Lee, S.; Kim, B. K. Modulation of the activity of pro-inflammatory enxymes, COX-2 and iNOS, by chrysin derivatives. Pharmacol. Res. **2004**, *49*, 37–43.
(29) Mercer, L. D.; Kelly, B. L.; Horne, M. K.; Beart, P. M. Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures. Biochem. Pharmacol. **2005**, *69*, 339–345.

(30) Yamazaki, T.; Muramoto, M.; Oe, T.; Morikawa, N.; Okitsu, O.; Nagashima, T.; Nishimura, S.; Katayama, Y.; Kita, Y. Diclofenac, a non-steroidal anti-inflammatory drug, suppresses apoptosis induced by endoplasmic reticulum stresses by inhibiting caspase signaling. *Neuropharmacology* **2006**, *50*, 558–67.

(31) Endo, M.; Mori, M.; Akira, S.; Gotoh, T. C/EBP homologous protein (CHOP) is crucial for the induction of caspase-11 and the pathogenesis of lipopolysaccharide-induced inflammation. *J. Immunol.* **2006**, *176*, 6245–6253.

(32) Shkoda, A.; Ruiz, P. A.; Daniel, H.; Kim, S. C.; Rogler, G.; Sartor, R. B.; Haller, D. Interleukin-10 blocked endoplasmic reticulum stress in intestinal epithelial cells: impact on chronic inflammation. *Gastroenterology* **2007**, *132*, 190–207.

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