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Neuroprotective effects of Brazilian green propolis on oxytosis/ferroptosis in mouse hippocampal HT22 cells

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ABSTRACT

Propolis is a sticky dark-colored substance produced by honey bees and comprises resin, balsam, wax, essential and aromatic oils, pollen, and several other substances; it is used in food and beverages to improve health and prevent diseases. We studied the neuroprotective effects of extracts of Brazilian green propolis in the mouse hippocampal cell line HT22. Ethanol extracts of Brazilian green propolis had a more potent preventive effect on oxidative stress-induced cell death, oxytosis/ferroptosis, in HT22 cells than water extracts of Brazilian green propolis, whereas it did not protect against anticancer drug-induced apoptotic cell death. Among the primary constituents of ethanol extracts of Brazilian green propolis, only artepillin C, kaempferide, and kaempferol demonstrated neuroprotective effects against oxytosis/ferroptosis. The flavonoid derivatives kaempferide and kaempferol are antioxidants with radical-scavenging abilities that additionally induce antioxidant response element-mediated transcriptional activity, suggesting that upregulation of endogenous antioxidant defense protects against oxidative stress. In contrast, artepillin C attenuated reactive oxygen species production; however, it did not induce antioxidant response element activation. These findings indicate that the ethanol extracts of Brazilian green propolis help to prevent oxidative stress-related neuronal cell death that is involved in the pathogenesis of several neurodegenerative diseases.

1. Introduction

Propolis is a sticky dark-colored substance produced by honey bees from the sap, bark and leaf buds of various plants. Honey bees use propolis to create a sterile coating for the hive walls, repel invasion by bacteria and fungi, and strengthen thin borders. Samples of propolis have distinct colors and contain various chemical components, including phenolics such as cinnamic acid derivatives and flavonoid derivatives, fatty acids, hydrocarbons, other organic compounds, and mineral elements. The composition of propolis depends on several factors, including the collection time, geographical origin, local flora at the collection site, bee species and extraction methods. Unique types of propolis have been found in several places worldwide (Bankova et al., 1995; Bankova, 2000; Burdock, 1998).

Baccharis dracunculifolia DC (Asteraceae), a plant that is native to Brazil, is the most important source of southeastern Brazilian propolis, known as green propolis, which has various beneficial constituents (Kumazawa et al., 2003; Midorikawa et al., 2001; Park et al., 2002; Teixeira et al., 2005). The extract of Brazilian green propolis is reported to have a wide variety of biological properties including antibacterial (Bankova et al., 1996), anti-inflammatory (Machado et al., 2012), antihypertensive (Maruyama et al., 2009; Mishima et al., 2005), antihyperlipidemic (Koya-Miyata et al., 2009), antioxidative (Nakajima et al., 2009b), and antitumor (Ishiai et al., 2014) activities. Water extracts of Brazilian green propolis have been shown to be rich in chlorogenic acid, *p*-coumaric acid, and caffeoylquinic acid derivatives, whereas ethanol extracts contain artepillin C, *p*-coumaric acid, and caffeoylquinic acid derivatives (Mishima et al., 2005).

Reactive oxygen species (ROS) are generated by mitochondria under physiological conditions; however, excessive ROS production causes oxidative damage to macromolecules, including lipids, proteins, and nucleic acids. This is recognized as oxidative stress, which has been

Abbreviations: : ARE, antioxidant response element; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EEP, ethanol extracts of Brazilian green propolis; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; GSH, reduced glutathione; HO-1, heme oxygenase-1; LDH, lactate dehydrogenase; Nrf2, the nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SOD, superoxide dismutase; WEP, water extracts of Brazilian green propolis

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implicated in the progression of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (Gilgun-Sherki et al., 2001). Cellular models of endogenous oxidative stress such as glutamate-induced oxytosis in mouse hippocampal HT22 cells and erastin-induced ferroptosis in some cancer cells are an iron-dependent form of non-apoptotic cell death (Dixon et al., 2012; Maher and Davis, 1996; Tan et al., 2001). It has been suggested that oxytosis and ferroptosis are highly similar if not identical (Lewerenz et al., 2018). In HT22 cells, high concentrations of extracellular glutamate or erastin block the cystine/glutamate antiporter or system Xc^-, resulting in decreased intracellular glutathione (GSH) levels and increased ROS production that causes endogenous oxidative stress. HT22 cells lack functional ionotropic glutamate receptors; therefore, excitotoxicity can be excluded as a cause of glutamate receptor-mediated cell death triggered by Ca^{2+} influx (Maher and Davis, 1996). Previous research suggests that extracts of Brazilian green propolis and its components have antioxidant activities (Kumazawa et al., 2004). Kaempferide, isosakuranetin, aromadendrine-4′-methyl-ether, and 3-prenyl-*p*-coumaric acid have been shown to inhibit superoxide anion and total ROS production by neutrophils stimulated by opsonized zymosan (Simoes et al., 2004). Caffeoylquinic acid derivatives and artepillin C exhibited 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Izuta et al., 2009). These low molecular weight antioxidants play an extremely important role in preventing oxidative stress.

In this study, we used an *in vitro* HT22 neuronal cell model to evaluate the effect of propolis on endogenous oxidative stress. Propolis has been used as a traditional folk medicine for thousands of years in various regions worldwide; however, limited scientific research has been conducted regarding the neuroprotective effect of propolis on endogenous oxidative stress. We studied the effects of water and ethanol extracts of Brazilian green propolis on glutamate- and erastin-induced endogenous oxidative stress in which ROS has a key role in neuronal cell death. Furthermore, we identified the primary constituents in ethanol extracts of Brazilian green propolis that contribute to the neuroprotective effects and studied the potential mechanisms underlying the protective effects of these components.

2. Materials and methods

2.1. Materials

In this study, we used Brazilian green propolis (Minas Gerais State, Brazil). The propolis was extracted using water at 50 °C (water extract of propolis [WEP]) or 95% ethanol at room temperature (ethanol extract of propolis [EEP]), and its primary constituents are shown in Table 1 and Fig. 1A and B. We used the following compounds: artepillin C and drupanin, which were isolated from EEP and purified by API Co., Ltd (Gifu, Japan); baccharin (Konan Chemical Industry, Osaka, Japan); *p*-coumaric acid, kaempferide, and kaempferol (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); and isosakuranetin (Extrasynthese, Cedex, France). Extracts of propolis and its components were dissolved in dimethyl sulfoxide (DMSO) or water and stored in the dark at −20 °C. The final concentration of DMSO in the culture medium is 0.1%.

Table 1
Main constituents of ethanol extract of Brazilian green propolis

Main constituents	Amounts in EEP (mg/g)	Concentration in 25 μg/mL EEP (μM)
Artepillin C	162.0	13.5
Kaempferide	30.7	2.6
Baccharin	55.7	2.5
p-Coumaric acid	15.7	2.4
Drupanin	12.3	1.3
Dihydrokaempferide	16.1	1.3
Betuletol	16.6	1.3
Isosakuranetin	4.3	0.4
Kaempferol	1.0	0.1

2.2. Cell culture

HT22 cells (RRID:CVCL_0321) were kindly provided by Dr. David Schubert (The Salk Institute for Biological Studies, La Jolla, CA, USA). The HT22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILIM Wako Pure Chemical, Osaka, Japan, Cat# 041–29775) supplemented with 5% fetal bovine serum (HyClone Laboratories, Logan, UT, USA, Cat# SH30406.02) at 37 °C in a CO_2 incubator.

2.3. Determination of cell death or viability

Cell death was determined by measuring the lactate dehydrogenase (LDH), which is a soluble cytosolic enzyme present in most eukaryotic cells, released into the culture medium upon cell death due to loss of plasma membrane integrity. The measurement was conducted using a LDH Cytotoxicity Detection Kit according to the manufacturer's protocol (Roche Diagnostics Japan, Tokyo, Japan). HT22 cells (2×10^4 cells/well) were seeded on 48-well plates (Nunc, Roskilde, Denmark) and cultured for 1 day. After the cells were treated under different experimental conditions using propolis or its constituents for 24 h in 400 μL of medium, LDH activity in the culture medium (10 μL) was assessed. The percentage cell death was calculated as 100 × (experimental release − blank)/(total release − blank), with the total release being the activity in cells lysed with 1% Triton X-100. Raw data of LDH activity in the culture medium was separately shown in Fig. S1.

Cell viability was measured using crystal violet dye which binds to proteins and DNA of viable cells. HT22 cells were seeded, cultured, and treated, as described above. Cells were fixed with methanol and stained with 0.5% crystal violet (KISHIDA CHEMICAL Co., Ltd., Osaka, Japan, #000–18972) in 10% ethanol for 15 min at room temperature. The cells were lysed in a 1% sodium dodecyl sulfate solution. The absorbance of the solution was measured photometrically at 580 nm (Saotome et al., 1989).

2.4. GSH determination

HT22 cells (2.4×10^5 cells/well) were grown on 6-well plates (Nunc, Roskilde, Denmark) for 1 day. Following treatment with the test compounds for 8 h in 3 mL of medium, cells were collected using ice-cold phosphate-buffered saline. The cell pellet was resuspended in 120 μL of 0.1 M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and it was deproteinized by adding four volumes of 25% (w/v) metaphosphoric acid. The sample was centrifuged for 10 min at 15,000 × *g*. The resultant supernatant was used for a GSH assay, according to the method provided in the study by Hissin and Hilf (1976), with minor modifications. In brief, 5 μL of the supernatant, 185 μL of 0.1 M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and 10 μL of *o*-phthaldialdehyde solution (1 mg/mL in methanol) were added to a 96-well black microplate and incubated at 25 °C for 15 min. A microplate reader (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA) was used to measure fluorescence intensity at 420 nm, with excitation at 350 nm. The cell pellet was solubilized in 75 μL of 0.2 M NaOH and used for a protein assay. GSH was normalized to cellular protein measured by the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA, Cat# 5000111JA), using γ-globulins as a standard.

2.5. Determination of ROS production, Ca^{2+} influx, and intracellular Fe^{2+} content

HT22 cells (7×10^4 cells/well) were cultured on Nunc™ Cell-

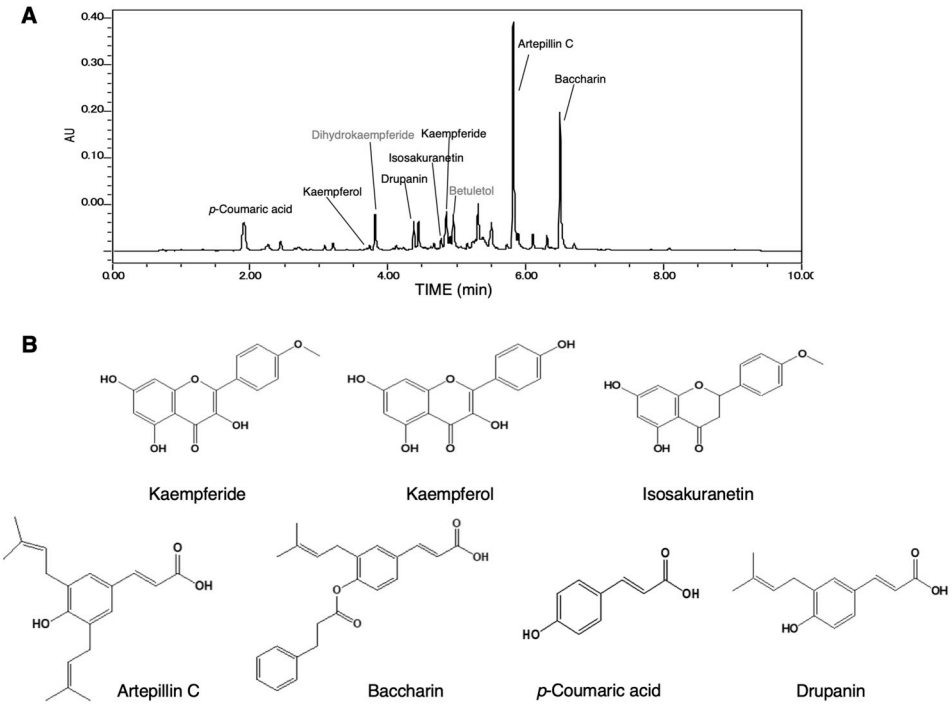

Fig. 1. (A) UPLC chromatograms of ethanol extract of Brazilian green propolis (EEP). An ultraperformance liquid chromatography (UPLC) method was used for the quantification of the constituents of EEP. EEP was filtered through a 0.2-μm filter. A 2-μL aliquot of the filtrate was injected in the Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA), which was equipped with a photodiode array detector (PDA, 210–400 nm) and a Waters Acquity BEH C18 column (100 × 2.1 mm). Eluents A and B were water and acetonitrile, respectively, both containing 0.1% (v/v) trifluoroacetic acid. After a 1 min isocratic step at 20% eluent B, the samples were eluted with a linear gradient to 80% eluent B in 8 min at a flow rate of 300 μL/min. (B) Chemical structures of the tested compounds.

Culture Treated Multidishes (12 wells, *Cat#* 150628) for 1 day, and they were treated with glutamate in the presence of test compounds in 1 mL of medium. Intracellular ROS was measured by labeling the cells with 5 μM CellROX™ Deep Red reagent (Thermo Fisher Scientific, Cat# C10422) for 30 min at 37 °C in a CO_2 incubator. Mitochondrial O_2^- was analyzed by labeling the cells with 5 μM MitoSOX (Thermo Fisher Scientific, Cat# M36008) for 15 min at 37 °C in a CO_2 incubator. To determine Ca^{2+} influx, cell cultures were loaded with 2.5 μM Fluo4-AM and 0.04% Pluronic™ F-127 (Thermo Fisher Scientific, Cat# P3000MP) for 15 min at 37 °C in a CO_2 incubator. To determine intracellular Fe^{2+}, HT22 cells (1×10^5 cells/well) were cultured on Nunc™ Cell-Culture Treated Multidishes (12 wells) for 1 day, and they were treated with test compounds in 1 mL of medium. For imaging intracellular Fe^{2+}, HT22 cells were stained with serum-free phenol red-free DMEM (Thermo Fisher Scientific, Cat# 21063029) containing 1 μM FerroOrange (Goryo Chemical Inc., Sapporo, Japan, Cat# GC904-01) for 30 min at 37 °C in a CO_2 incubator. The medium was replaced with serum-free phenol red-free DMEM after the fluorescent probes were labeled. Fluorescence measurement was conducted using fluorescence digital microscopy (BZ-X700; Keyence, Osaka, Japan). Fluorescence intensity was quantified using a Keyence image measurement and analyzing software (VH–H1A5; Keyence).

2.6. Determination of superoxide anion scavenging activity in vitro

Superoxide anions were measured using a SOD Assay Kit -WST according to the manufacturer's recommended protocol (Dojindo Laboratories, Cat# S311). The reduction of WST-1 to WST-1 formazan by the superoxide anion was measured spectrophotometrically at 450 nm.

2.7. Stable antioxidant response element-driven reporter system

HT22 cells that stably expressed antioxidant response element (ARE)-luciferase reporter plasmids were generated according to the procedure described in a previous study (Hirata et al., 2018). HT22-ARE cells (2×10^4 cells/well) were grown on 48-well plates (Nunc, Roskilde, Denmark) and cultured for 1 day. After the cells were treated under different experimental conditions of propolis or its components for 8 or 16 h in 400 μL of medium, the culture medium was removed, and the cells were lysed with 50 μL of 1 × passive lysis buffer (Promega Corporation, Madison, WI, USA, Cat# E1941). Luciferase activity was measured using the Luciferase Assay System (Promega Corporation, Cat# E1500).

2.8. Western blotting

Western blotting was performed according to the procedure described in a previous study (Shibata et al., 2017). The membranes were probed with the following primary antibodies at the indicated dilutions: anti-heme oxygenase 1 (HO-1; 1:2000; mouse monoclonal, Enzo Life Science, Inc., Farmingdale, NY, USA, RRID:AB_10617276), anti-Mn superoxide dismutase (SOD; 1: 5000; rabbit polyclonal, Enzo Life Science, Inc., RRID:AB_2039585), anti-Cu/Zn SOD (1: 3000; rabbit polyclonal, Enzo Life Science, Inc., RRID:AB_2039583), anti-γ-glutamyl cysteine-synthase catalytic subunit (γ-GCSc; 1:1000; mouse monoclonal, Santa Cruz Biotechnology, Inc., Dallas, TX, USA, sc-390811), anti-γ-glutamyl cysteine-synthase regulatory subunit (γ-GCSm; 1:1000; mouse monoclonal, Santa Cruz Biotechnology, Inc., sc-55586), anti-glutathione reductase (1:1000; mouse monoclonal, Santa Cruz Biotechnology, Inc., sc-133245, RRID:AB_2295121), anti-glutathione peroxidase 1 (GPx1; 1:2000; rabbit polyclonal, GeneTex, Inc., Irvine, CA, USA, GTX116040, RRID:AB_2037097), anti-glutathione peroxidase 4 (GPx4; 1:1000; rabbit monoclonal, Abcam, Cambridge, UK, abcam125066, RRID: AB_10973901), anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH; 1:2000; mouse monoclonal, Acris Antibodies, Inc., San Diego, CA, USA, RRID:AB_1616730), anti-NAD(P)H:quinone oxidoreductase 1 (NQO1; 1:1000; rabbit monoclonal, Abcam, abcam80588, RRID:AB_1603750), and anti-thioredoxin (1:1000; rabbit polyclonal, Proteintech, Rosemont, IL, USA, 14999-1-AP, RRID:AB_2272597). Blots were washed and incubated with secondary antibodies conjugated to horseradish peroxidase (1: 2000; Cell Signaling Technology, Beverly, MA, USA, Cat#7074, #7076). Immunoreactive bands were visualized using enhanced chemiluminescence (GE Healthcare UK Ltd., Cat# RPN2106) or SuperSignal™ West Dura Extended Substrate (Thermo Fisher Scientific, Cat# 34075).

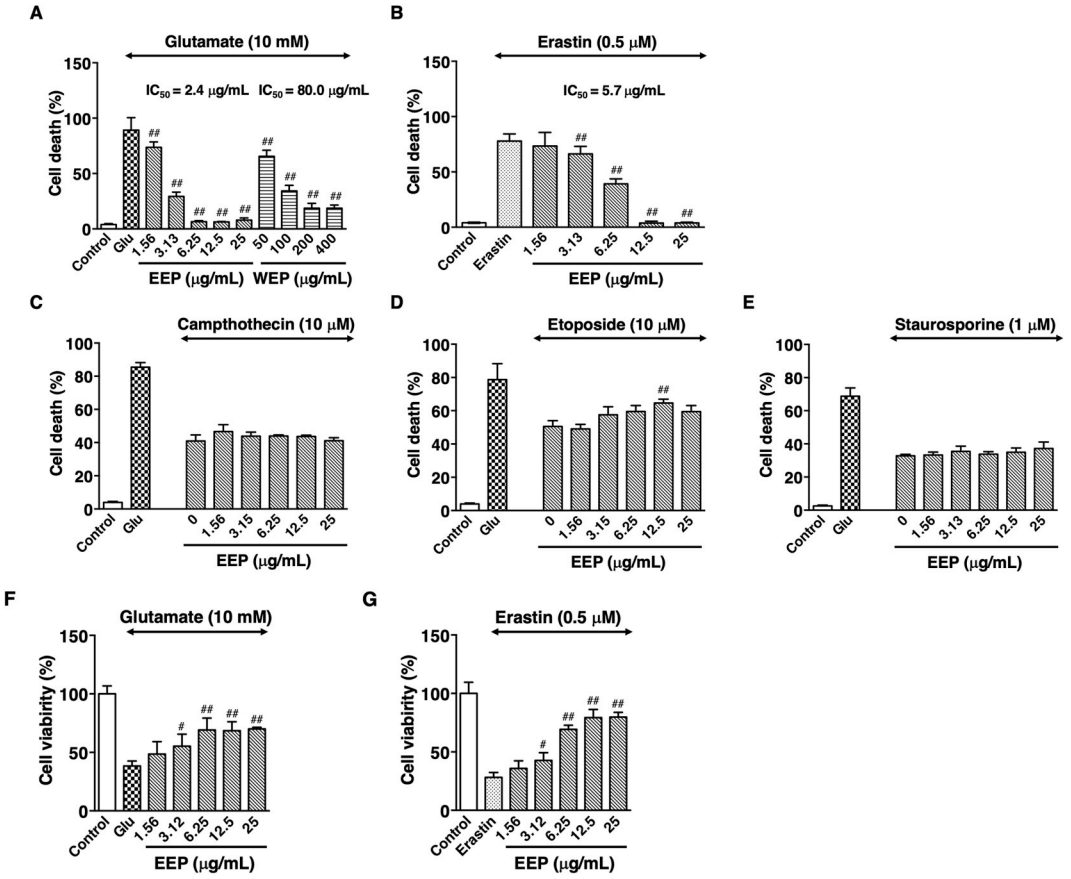

Fig. 2. (A)(B) Effects of ethanol and water extracts of Brazilian green propolis (EEP and WEP, respectively) on oxidative stress-induced cell death. HT22 cells were incubated with 10 mM glutamate or 0.5 μM erastin and the indicated concentrations of EEP and WEP for 24 h; cell death was determined by measuring LDH activity. Data represent the means ± SD of at least four independent cultures. $^{\#\#}P < 0.01$, difference from glutamate or erastin alone.(C)(D)(E) Effects of EEP on apoptosis-induced cell death. HT22 cells were incubated with 10 μM camptothecin, 10 μM etoposide, or 1 μM staurosporine and indicated concentrations of EEP for 24 h; cell death was determined by measuring LDH activity. Data represent the means ± SD of at least four independent cultures. $^{\#\#}P < 0.01$, difference from etoposide alone. (F)(G) Effects of EEP on glutamate- and erastin-induced cytotoxicity. HT22 cells were incubated with 10 mM glutamate or 0.5 μM erastin and the indicated concentrations of EEP for 24 h; cell viability was determined by cresyl violet assay. Data represent the means ± SD of at least four independent cultures. $^{\#\#}P < 0.01$, difference from glutamate or erastin alone.

2.9. Statistical analysis

Statistical analysis of the numerical data was performed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). We conducted one-way analysis of variance followed by Dunnett's multiple comparison test to measure significant differences between a control column and the multiple columns.

3. Results

3.1. Brazilian green propolis specifically protects HT22 cells from oxidative stress-induced cell death

First, we studied the effect of extracts of Brazilian green propolis on endogenous oxidative stress in mouse hippocampal HT22 cells. Glutamate or erastin was used to induce endogenous oxidative stress. High concentrations of extracellular glutamate or erastin prevent cystine import, resulting in decreased GSH levels and increased ROS and lipid peroxide production in the cells (Dixon et al., 2012; Tan et al., 2001). Treatment of HT22 cells with glutamate resulted in > 80% cell death, as determined by LDH activity (Fig. 2A). EEP and WEP prevented glutamate-induced cell death at 1.56–25 μg/mL and 50–400 μg/mL, respectively, in a concentration-dependent manner (Fig. 2A, Fig. S1A). The IC_{50} values (the concentration causing 50% inhibition) of EEP and WEP were 2.4 μg/mL and 80.0 μg/mL, respectively. EEP and WEP were not cytotoxic at concentrations up to 25 μg/mL and 400 μg/mL, respectively (data not shown). Furthermore, EEP protected HT22 cells from erastin-induced cell death; the IC_{50} value of EEP influencing erastin-induced cell death was 5.7 μg/mL (Fig. 2B, Fig. S1B). WEP showed a protective effect against erastin-induced cell death; however, the effect was less than that observed with EEP (data not shown). The effects of EEP on glutamate- and erastin-induced cytotoxicity were confirmed by cell viability assay using crystal violet (Fig. 2F and G). From these findings, we further evaluated the neuroprotective effect of EEP against oxidative stress in HT22 cells.

We studied the effect of EEP on apoptotic cell death induced by camptothecin and etoposide (topoisomerase inhibitors) or staurosporine (a broad-spectrum inhibitor of protein kinases) to determine whether the effect of EEP is specific to oxidative stress, which is distinct from apoptosis, necrosis, and autophagy (Dixon et al., 2012; Lewerenz et al., 2018; Tan et al., 2001). Significant cell death was observed following HT22 cell treatment with camptothecin, etoposide, and staurosporine alone for 24 h; however, the extent of cell death was less than that observed following treatment with glutamate and erastin (Fig. 2C–E). This was likely because these agents induce apoptosis, resulting in the loss of membrane integrity in the late phase of cell death. In contrast to the potent protective effect of EEP against glutamate- and erastin-induced cell death, EEP did not prevent camptothecin and staurosporine-induced cell death to any extent, and it had little effect on etoposide-induced cell death. These findings indicate that EEP

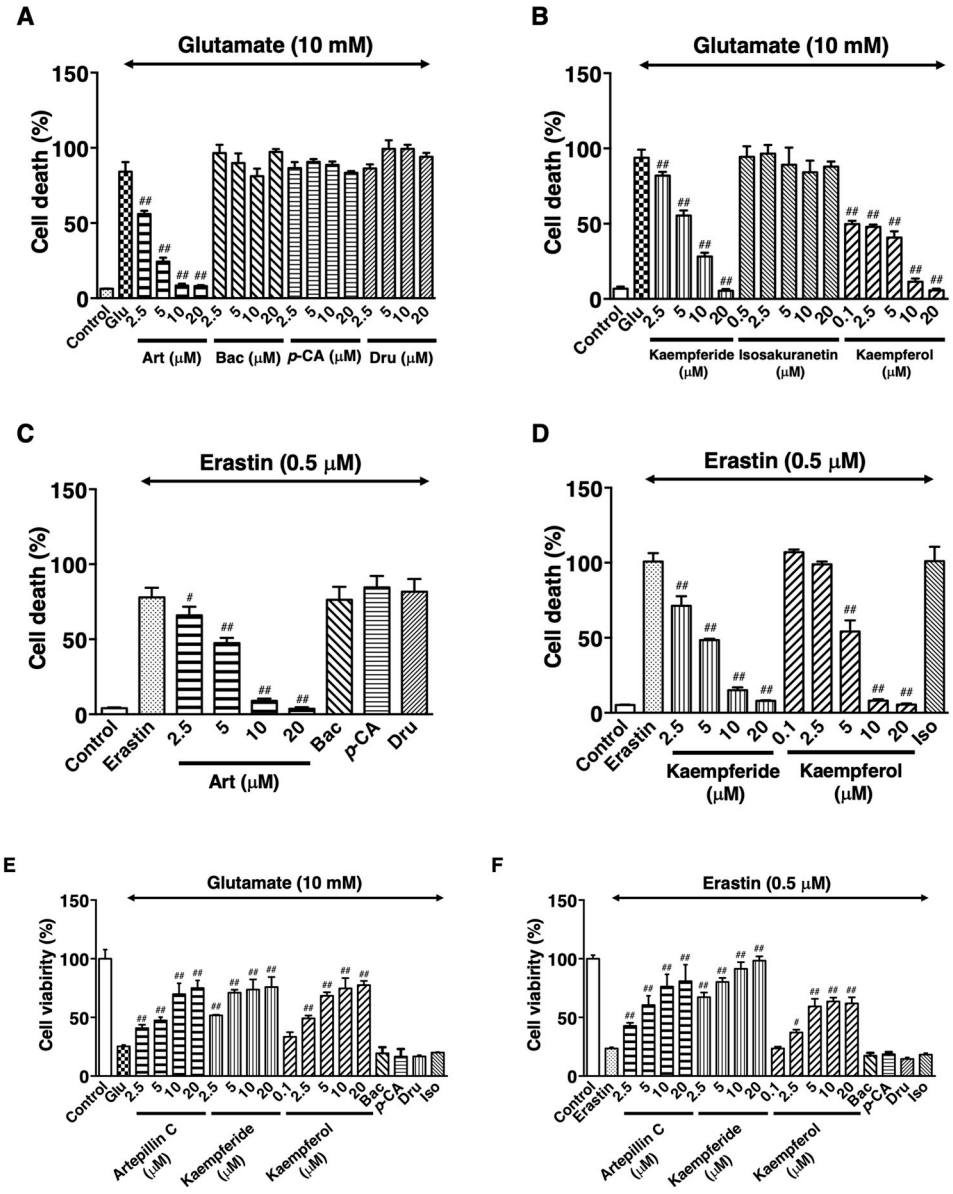

Fig. 3. Effects of artepillin C, kaempferide, baccharin, *p*-coumaric acid, drupanin, isosakuranetin, and kaempferol on glutamate- and erastin-induced cytotoxicity. (A)(B)(E) HT22 cells were incubated with 10 mM glutamate and indicated concentrations of artepillin C (Art), kaempferide, baccharin (Bac), *p*-coumaric acid (*p*-CA), dorupanin (Dru), isosakuranetin, and kaempferol for 24 h. (C)(D)(F) HT22 cells were incubated with 0.5 μM erastin and indicated concentrations of artepillin C (Art), kaempferide, and kaempferol or 20 μM baccharin (Bac), 20 μM *p*-coumaric acid (*p*-CA), 20 μM dorupanin (Dru), and 20 μM isosakuranetin for 24 h. Data represent the means ± SD of at least four independent cultures. $^{\#\#}P < 0.01$, difference from glutamate or erastin alone.

selectively prevents oxidative stress-induced cell death; however, it does not protect against apoptotic cell death.

3.2. Artepillin C, kaempferide, and kaempferol showed a protective effect against oxidative stress-induced cell death

To identify the components of EEP involved in inhibiting oxidative stress-induced cell death, we studied the impact of artepillin C, kaempferide, baccharin, *p*-coumaric acid, drupanin, isosakuranetin, and kaempferol, which are the primary components of EEP (Table 1, Fig. 1A and B) and are commercially available, on glutamate- and erastin-induced cytotoxicity. Artepillin C prevented both glutamate- and erastin-induced cell death at IC_{50} values of 3.1 μM and 5.3 μM, respectively (Fig. 3A, C, E, F). Although kaempferide, kaempferol, and isosakuranectin are structurally similar, only kaempferide and kaempferol inhibited glutamate- and erastin-induced cell death in a concentration-dependent manner (Fig. 3B, D, E, F). Based on the component analysis shown in Table 1, the concentrations of artepillin C and kaempferide in 25 μg/mL EEP are estimated to be 13.5 μM and 2.6 μM, respectively, indicating that both artepillin C and kaempferide contribute to neuroprotective activities in the EEP. Despite the kaempferol concentration being low, its potent neuroprotective activity could enable it to contribute to the biological activity of the EEP. In contrast, baccharin, *p*-coumaric acid, drupanin, and isosakuranetin had no effect on oxidative stress–induced cell death (Fig. 3A–D).

3.2.1. EEP, artepillin C, kaempferide, and kaempferol did not affect the decrease in GSH by glutamate

Exposure of HT22 cells to a high glutamate concentration results in GSH depletion because of intercellular cystine depletion. Because GSH is a major intracellular antioxidant, decreased intracellular GSH levels cause increased intracellular ROS production. Further, we studied the effects of EEP and components in EEP, such as artepillin C, kaempferide, and kaempferol, which influence oxidative stress, on glutamate-induced GSH depletion. As shown in Fig. 4A–D, in glutamate-treated HT22 cells, GSH decreased significantly compared with the control. EEP, artepillin C, kaempferide, and kaempferol did not prevent GSH depletion, suggesting that the recovery of GSH levels does not contribute to the neuroprotective effect of EEP and components in EEP against glutamate-induced oxidative stress.

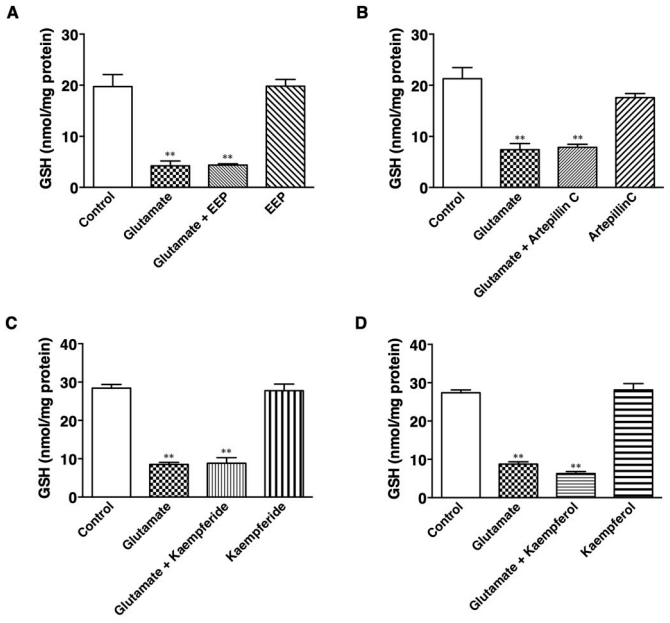

Fig. 4. (A–D) Effects of ethanol extracts of Brazilian green propolis (EEP), artepillin C, kaempferide, and kaempferol on glutamate-induced GSH depletion. HT22 cells were incubated with 10 mM glutamate and 25 μg/mL EEP, 20 μM artepillin C, 20 μM kaempferide or 20 μM kaempferol for 12 h. Data represent the means ± SD of at least four independent cultures. $^{**}P < 0.01$, difference from the control.

3.2.2. EEP, artepillin C, kaempferide, and kaempferol suppressed intracellular ROS and mitochondrial superoxide anion production and increased intracellular Ca^{2+} concentration

Next, we studied the effect of EEP, artepillin C, kaempferide, and kaempferol on intracellular ROS production. The HT22 cells were treated with glutamate or erastin in the presence of EEP, artepillin C, kaempferide, and kaempferol for 12 h or 8 h and were then labeled using the fluorescent probes Deep Red and MitoSOX. We observed intense red fluorescence of each reagent in the glutamate- and erastin-treated cells compared with the control; the fluorescence was suppressed in the presence of EEP or artepillin C, kaempferide and kaempferol (Fig. 5A, B, D, E; Figs. S2A and B). The effect of kaempferide on mitochondrial superoxide anion production could not be ascertained because it increased the fluorescence of MitoSOX. The fluorescence of Fluo-4 that was enhanced by glutamate and erastin was greatly decreased by EEP, artepillin C, kaempferide, and kaempferol (Fig. 5C, F; Fig. S2C), indicating their protective effect against oxidative stress-induced cell death by inhibiting intracellular ROS production and Ca^{2+} influx. Furthermore, these compounds exhibited superoxide anion scavenging ability *in vitro*. In the experiment using a SOD assay kit, EEP and kaempferide exhibited stronger superoxide anion scavenging activity than artepillin C and kaempferol (Fig. 5G).

3.2.3. EEP, artepillin C, kaempferide, and kaempferol suppressed intracellular ROS and mitochondrial superoxide anion production and increased intracellular Ca^{2+} concentration

Next, we studied the effect of EEP, artepillin C, kaempferide, and kaempferol on intracellular ROS production. The HT22 cells were treated with glutamate or erastin in the presence of EEP, artepillin C, kaempferide, and kaempferol for 12 h or 8 h and were then labeled using the fluorescent probes Deep Red and MitoSOX. We observed intense red fluorescence of each reagent in the glutamate- and erastin-treated cells compared with the control; the fluorescence was suppressed in the presence of EEP or artepillin C, kaempferide and kaempferol (Fig. 5A, B, D, E; Figs. S2A and B). The effect of kaempferide on mitochondrial superoxide anion production could not be ascertained because it increased the fluorescence of MitoSOX. The fluorescence of Fluo-4 that was enhanced by glutamate and erastin was greatly decreased by EEP, artepillin C, kaempferide, and kaempferol (Fig. 5C, F; Fig. S2C), indicating their protective effect against oxidative stress-induced cell death by inhibiting intracellular ROS production and Ca^{2+} influx. Furthermore, these compounds exhibited superoxide anion scavenging ability *in vitro*. In the experiment using a SOD assay kit, EEP and kaempferide exhibited stronger superoxide anion scavenging activity than artepillin C and kaempferol (Fig. 5G).

3.3. EEP, kaempferide, and kaempferol, but not artepillin C, activated ARE

Propolis is known to exhibit a strong antioxidant effect, and artepillin C, kaempferide, and kaempferol are known to scavenge ROS directly (Izuta et al., 2009; Kumazawa et al., 2004; Nakajima et al., 2009b; Simoes et al., 2004; Saw et al., 2014). To examine the indirect mechanism of ROS-scavenging effect of EEP, artepillin C, kaempferide, and kaempferol, we investigated whether these substances activate the nuclear factor erythroid 2-related factor 2 (Nrf2)-ARE pathway, which is crucial in upregulating cytoprotective genes and enzymes in response to oxidative stress. ARE, a cis-acting regulatory enhancer, is contained in the 5′ flanking region of various phase II detoxification enzymes and antioxidant proteins, including HO-1, NQO1, γ-glutamyl cysteine-synthase, and Cu/Zn SOD (Wasserman and Fahl, 1997). We measured ARE activity using HT22 cells that stably expressed the ARE reporter plasmid, which contains the ARE core sequence of the rat NQO gene (HT22-ARE cells). EEP strongly enhanced ARE activation in a concentration-dependent manner; however, artepillin C, baccharin, *p*-coumaric acid, drupanin, and isosakuranetin showed little effect on ARE activation (Fig. 6A and B). Kaempferide increased ARE activation in a concentration-dependent manner (Fig. 6B). Because the concentration of kaempferide in 25 μg/mL EEP is estimated to 2.6 μM, as described above, kaempferide in EEP may contribute to ARE induction. Moreover, kaempferol, a demethylated product of kaempferide, is present at low concentrations in EEP; however, it strongly enhanced ARE activation in a concentration-dependent manner (Fig. 6B).

3.4. EEP increased HO-1 protein levels

Finally, we studied the effects of EEP and artepillin C on protein expression, including HO-1, NQO-1, γ-GCSc, γ-GCSm, glutathione reductase, thioredoxin, and Cu/Zn SOD, which are reported to be upregulated by the Nrf2-ARE pathway. EEP strongly induced HO-1 expression. Although artepillin C did not exhibit ARE activation, it increased the HO-1 level in a weak manner; however, it was not statistically significant. EEP induced HO-1 protein more strongly in the presence of glutamate and erastin (Fig. 7A and B). EEP and artepillin C had no effect on NQO1, γ- GCSc, γ-GCSm, glutathione reductase, thioredoxin, and Cu/Zn SOD expression (Fig. 7A, B, Fig. S3). Furthermore, they had no effect on an intracellular antioxidant enzyme, such as Mn-SOD, GPx1, and GPx4, an essential regulator of ferroptosis (Yang et al., 2014). These findings indicate that the upregulation of HO-1 may contribute to the protective effect of EEP against oxidative stress-induced ROS production.

4. Discussion

In this study, we showed that extracts of propolis, a traditional folk medicine, inhibit endogenous oxidative stress, oxytosis/ferroptosis, in mouse hippocampal HT22 cells. The neuroprotective activity of EEP was much greater than that of WEP. Table 1 lists the constituents of EEP, including artepillin C, baccharin, kaempferide, kaempferol, dihydrokaemferide, betuletol, isosakuranetin, *p*-coumaric acid, and drupanin. Artepillin C, kaempferide, and kaempferol, but not baccharin, isosakuranetin, *p*-coumaric acid, and drupanin, inhibited oxytosis/ferroptosis strongly. Artepillin C and kaempferol, but not baccharin, *p*-

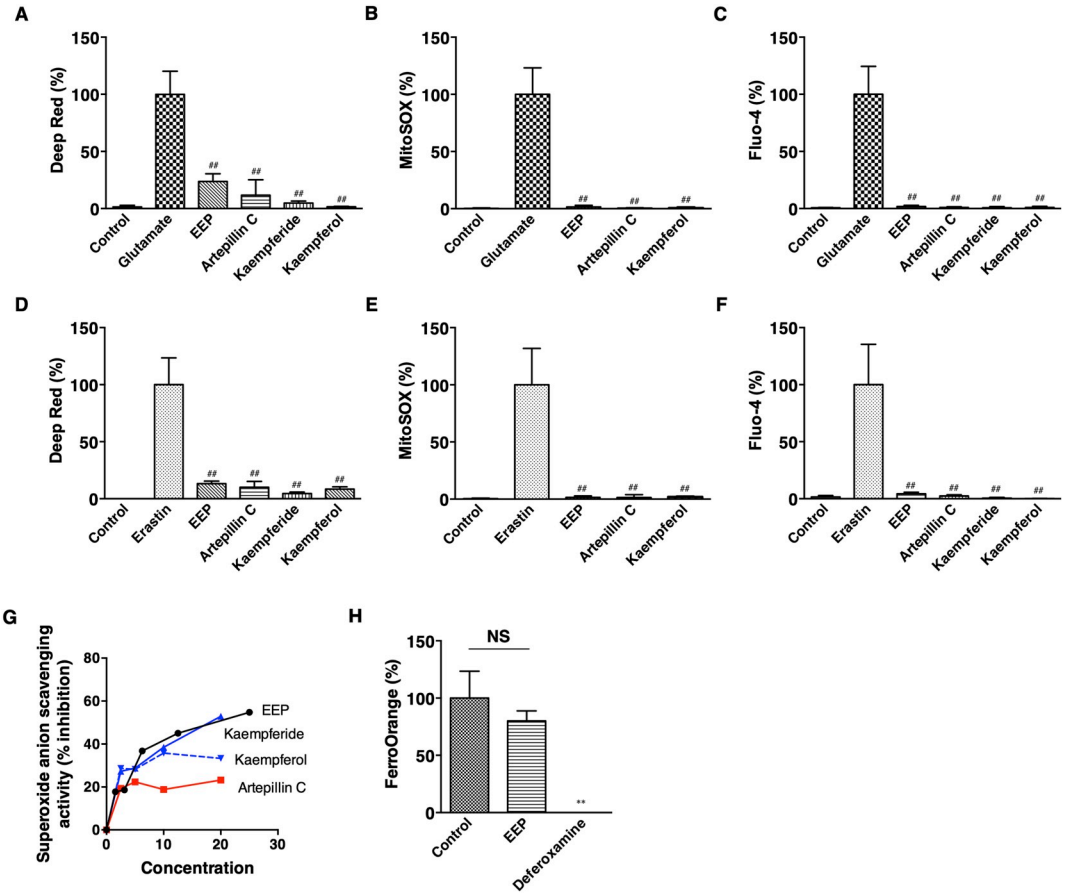

Fig. 5. Effects of ethanol extracts of Brazilian green propolis (EEP), artepillin C, kaempferide, and kaempferol on glutamate- and erastin-induced reactive oxygen species (ROS) production and Ca^{2+} influx in HT22 cells, superoxide anion scavenging ability *in vitro* and intracellular Fe^{2+} in HT22 cells. (A)(B)(D)(E) HT22 cells were treated with 10 mM glutamate or 0.5 μM erastin and 25 μg/mL EEP, 20 μM artepillin C, or 20 μM kaempferol for 12 h or 8 h. ROS production was determined as described in the Materials and methods section. (C)(F) HT22 cells were treated with 10 mM glutamate or 0.5 μM erastin and 25 μg/mL EEP, 20 μM artepillin C, 20 μM kaempferide, or 20 μM kaempferol for 12 h or 8 h Ca^{2+} influx was determined as described in the Materials and methods section. (G) Effects of EEP, artepillin C, kaempferide, and kaempferol on superoxide anion scavenging ability *in vitro*. Superoxide anions were measured using a SOD Assay Kit-WST-1 as described in the Materials and methods section. The data shown are from a representative experiment performed in duplicate and independently repeated at least twice with comparable results. % Inhibition: Percentage of inhibition in relation to control (DMSO). (H) HT22 cells were treated with 25 μg/mL EEP for 16 h. Deferoxamine (50 μM) was used as a positive control of the Fe^{2+} chelator. Intracellular Fe^{2+} was determined as described in the Materials and methods section. Data represent the means ± SD of at least four independent cultures. $^{**}P < 0.01$, difference from the control; $^{\#\#}P < 0.01$, difference from glutamate or erastin alone; NS, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

coumaric acid, and drupanin, have been reported to show strong DPPH radical scavenging activity (Izuta et al., 2009; Kumazawa et al., 2004; Saw et al., 2014), and kaempferide has been reported to have a potent inhibitory effect on ROS production by rabbit neutrophils stimulated by serum-opsonized zymosan particles (Simoes et al., 2004). Consistent with these findings, EEP, artepillin C, kaempferide, and kaempferol inhibited glutamate- and erastin-induced ROS production (Fig. 5A, B, D, E). These compounds also exhibited superoxide anion scavenging ability *in vitro* (Fig. 5G). However, they had no effect on glutamate-induced GSH depletion (Fig. 4A–D), suggesting that the protective effect of propolis against oxidative stress in the cells occurs after GSH depletion and that among the components of EEP, artepillin C, kaempferide, and kaempferol are involved in preventing oxidative stress by direct ROS scavenging. Various neuroprotective antioxidants have been identified from natural sources. 17-*β* Estradiol, vitamin E, polyphenolic bioflavonoids, and melatonin can function as antioxidants and prevent oxidative cell death in HT22 cells (Behl, 2000; Herrera et al., 2007; Ishige et al., 2001). Overall, ROS-scavenging substances are effective in alleviating oxytosis/ferroptosis.

The Nrf2-ARE pathway is a well-known to be one of the most important cellular endogenous defense mechanisms against oxidative stress (Itoh et al., 2004). In this study, we assessed the effect of EEP and its components on ARE activation (Fig. 6A and B). EEP remarkably enhanced ARE activation at concentrations similar to those that prevent oxidative stress-induced cell death. Ethanol extracts of *Baccharis* propolis that primarily comprise chlorogenic acid, kaempferide, and artepillin C have been reported to promote Nrf2 nuclear translocation and induce HO-1, thioredoxin reductase 1, and γ-GCSm expression in RAW 264.7 cells (Zhang et al., 2016). Interestingly, artepillin C, the most abundant anti-oxidative stress component in EEP, showed little effect on ARE activation, indicating that the protective efficacy of artepillin C is not dependent on the upregulation of endogenous antioxidant defenses. In contrast, the neuroprotective effect of EEP is dependent on ROS scavenging and upregulation of endogenous antioxidant defenses, indicating that extracts of propolis attenuate endogenous oxidative stress by multiple antioxidant mechanisms. Kaempferide and kaempferol activate ARE in HT22 cells; however, their concentrations in EEP may not be sufficient for maximum ARE activation by EEP. Dihydrokaempferide and betuletol, which are structurally similar to kaempferide and kaempferol but have not been examined in this study, may induce ARE activation; furthermore, EEP may contain unidentified components that induce ARE activation.

Based on the results of the ARE reporter assay, EEP strongly induced HO-1 protein expression (Fig. 7). However, no significant change was

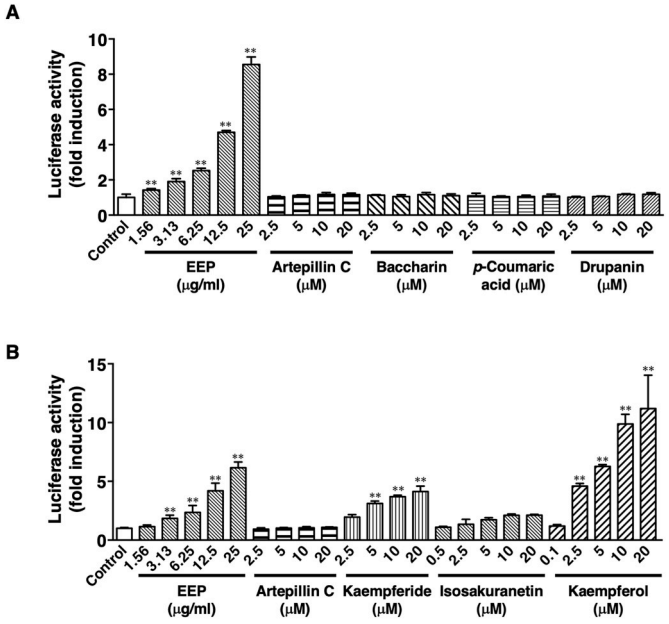

Fig. 6. Effects of ethanol extracts of Brazilian green propolis (EEP) and its primary constituents on ARE activation. HT22-ARE cells were incubated with indicated concentration of EEP and its primary constituents for 8 h (A) or 16 h (B). ARE-driven reporter activity was measured as described in the Materials and methods section. Data represent the means ± SD of at least four independent cultures. $^{**}P < 0.01$, difference from the control.

observed in the expression of other ARE-driven genes that encode phase II detoxification enzymes and antioxidant proteins such as NQO-1, γ-GCSc, γ-GCSm, glutathione reductase, thioredoxin, and Cu/Zn SOD. This observation does not mean that other ARE-regulated genes did not respond to EEP. In fact, mRNA expression of ARE-driven genes such as NQO-1 and γ-GCSc was increased in response to ARE inducers in HT22 cells (data not shown). These findings suggest that the effect of EEP on glutamate-induced ROS production is partially because of the upregulation of HO-1. Accumulating evidence has indicated that the upregulation of HO-1 is accompanied by neuroprotection against various stimuli (Nitti et al., 2018); however, direct evidence regarding the neuroprotective function of HO-1 against oxytosis/ferroptosis is unavailable and requires further study.

Research has shown that extracts of Brazilian green propolis have inhibited various types of stress-induced cell death in different cells. In retinal ganglion cells, water extracts of Brazilian green propolis and their constituents, including caffeoylquinic acid derivatives, artepillin C, and *p*-coumaric acid, exhibited neuroprotective effects against oxygen-glucose deprivation-induced cell damage (Nakajima et al., 2009a). Furthermore, ethanol extracts of Brazilian green propolis have been shown to inhibit hydrogen peroxide- or serum deprivation-induced cell damage in PC12 or SH-SY5Y cells (Ni et al., 2017; Shimazawa et al., 2005). Brazilian green propolis also mediated positive potentials in a mouse model of focal cerebral ischemia by permanent middle cerebral artery occlusion (Shimazawa et al., 2005). Here we demonstrated that EEP exhibited neuroprotective activity against glutamate-induced oxytosis and erastin-induced ferroptosis and that a cinnamic acid derivative, artepillin C, and the flavonoid derivatives kaempferide and kaempferol are effective components of EEP. Because EEP did not inhibit staurosporine-, etoposide-, and camptothecin-induced cell death, EEP was found to have little effect on apoptotic cell death. There is a possibility that the lack of apoptosis inhibition is due to the compounds not reaching the nucleus; etoposide and camptothecin-induced apoptosis requires caspase activation, indicating that propolis at least did not act on the apoptotic signaling pathway in the cytoplasm. This characteristic is similar to ferrostatin-1, liproxstatin-1, and GIF-0726-r and its derivatives, which are specific inhibitors of glutamate- and erastin-induced death but not apoptotic cell death induced by agents such as staurosporine and camptothecin (Dixon et al., 2012; Hirata et al., 2018). The ethanol extract of Northeast Portuguese propolis has been shown to attenuate staurosporine-induced caspase-3 activation (Cardoso et al., 2011); This reflects that the properties and chemical composition of propolis vary with geographical origin.

In conclusion, we showed that extracts of Brazilian green propolis prevented endogenous oxidative stress-induced cell death in mouse hippocampal HT22 cells. These findings indicate that propolis is a valuable home remedy for suppressing oxidative stress. Among the components of EEP, artepillin C, kaempferide, and kaempferol showed neuroprotective activity, with a distinct mechanism of action. Artepillin C, kaempferide, and kaempferol are potent ROS-scavenging antioxidants, whereas kaempferide and kaempferol, but not artepillin C, promoted ARE activation (Fig. 8). This indicates that kaempferide and kaempferol effectively protect against neuronal oxidative stress using multiple antioxidant defense mechanisms, although their

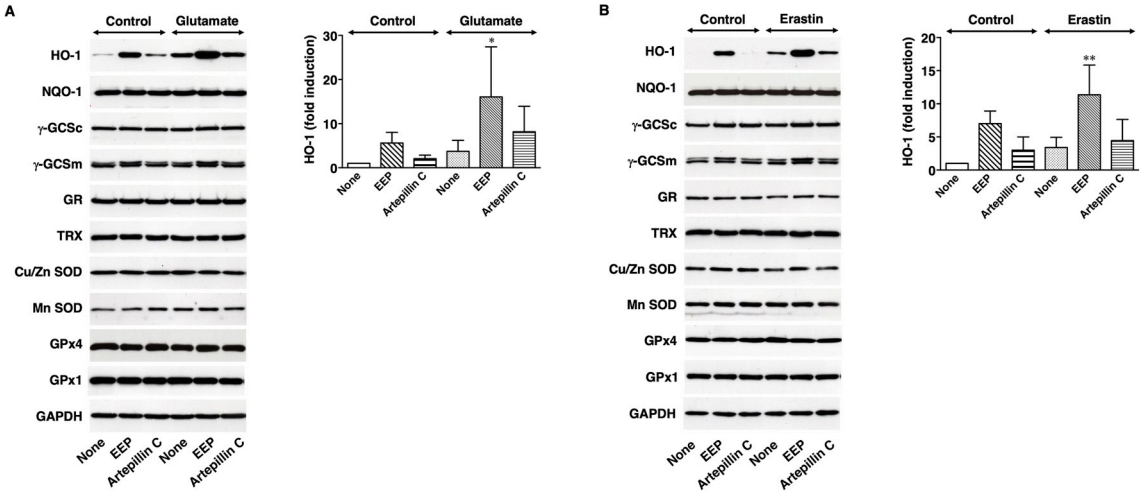

Fig. 7. Effects of ethanol extracts of Brazilian green propolis (EEP) and artepillin C on antioxidant protein expressions. HT22 cells were incubated with EEP (25 μg/mL) or artepillin C (20 μM) in the presence or absence of 10 mM glutamate (A) or 0.5 μM erastin (B) for 12 h or 8 h. Whole cell lysates (20 μg protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies, as described in the Materials and methods section. (Left) Representative immunoblots of typical data are shown. (Right) The band intensity of HO-1 was quantified using Image J. Data represent the means ± SD of four independent cultures. $^{*}P < 0.05$, $^{**}P < 0.01$; difference from the control.

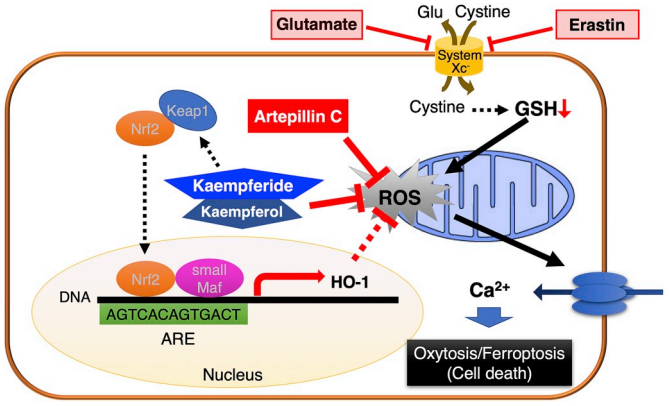

Fig. 8. Model for the mechanisms of alleviation of endogenous oxidative stress by artepillin C, kaempferide, and kaempferol.

concentrations in EEP are lower than that of artepillin C, which is generally accepted to be a primary active ingredient in EEP. Several compounds in extracts of propolis are believed to explicate various beneficial biological effects; moreover, a synthetic supplement containing artepillin C, kaempferide, and kaempferol can easily provide stable quality control and can replace natural propolis extracts to improve health and prevent neurodegenerative diseases. Such an approach will overcome not only the limited availability of propolis but also the difference in its chemical composition that occur based on the extraction method, the geographical location, botanical origin, and bee species.

Conflicts of interest

None of the authors declare any financial interest or conflict of interest related to the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110669.

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