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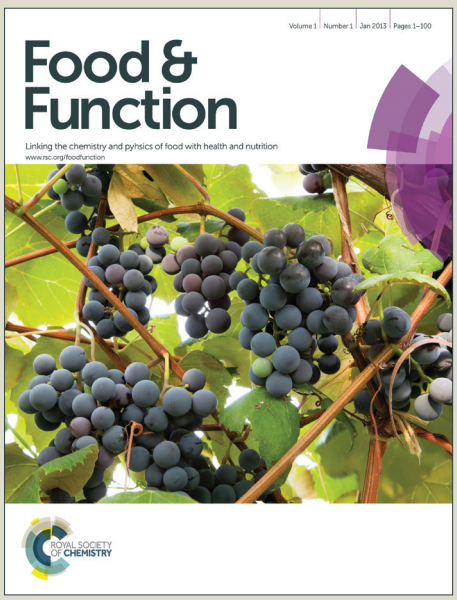

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Original research paper

Protective effects of *Drynaria fortunei* against 6-hydroxydopamine-induced oxidative damage in B35 cells via PI3K/AKT pathway

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Abstract

In this study, we demonstrated the antioxidant and protective properties of aqueous extract of two commercial Polydiaceae plants - *Drynaria fortunei* (DF) and *Pseudodrynaria coronans* (PC) against 6-hydroxydopamine (6-OHDA)-induced oxidative damage in B35 neuroblastoma cells. The contents of their phytochemical profiles were determined by spectrophotometric methods and high performance liquid chromatography using a photodiode array detector. DF extract has better effects than PC extract in scavenging ROS and inhibiting 6-OHDA autoxidation. Following exposure of B35 cells to 6-OHDA, there was a marked decrease in cell survival and the activation of intracellular antioxidant enzymes and PI3K/AKT pathway, and then the level of lipid peroxidation was increased. Pretreatment with DF extract blocked these 6-OHDA-induced cellular events. Naringin and epicatechin are major components of DF extract. These results show that DF extract exerts the protective effects against 6-OHDA toxicity *via* radical scavenging activity and the increase in the activation of PI3K/AKT pathway to elevate the levels of intracellular antioxidant enzymes including HO-1, NQO-1 and glutathione-related enzymes.

Keywords: *Drynaria fortunei*; naringin; antioxidant activity; 6-hydroxydopamine; PI3K/AKT; HO-1

Introduction

The rhizome of *Drynaria fortunei* (Kunze) J. Smith (Polydiaceae) (DF) is a major resource for the traditional Chinese medicine "Gu-Sui-Bu" in China, which is used to prevent osteoporosis and aging-associated symptoms. Accumulating evidence shows that DF has osteoprotective effects through its osteogenic differentiating and proliferating activities in cell culture and animal studies.[1-4] Flavonoids and phenylpropanoids, such as epicatechin, naringin and neoeriocitrin, are the active constituents of DF extract for its osteoprotective activities.[1-4] Other pharmacological studies indicate that DF has antioxidant and anti-apoptotic activities.[5, 6] The above phytoconstituents also have neuroprotective and anti-apoptotic activities through their antioxidant mechanisms *in vitro* and *in vivo*.[7-10] The rhizome of *Pseudodrynaria coronans* (Wall.) Ching (Polydiaceae) (PC) is an alternative medicinal resource for "Gu-Sui-Bu" in Taiwan. There are no phytochemical and pharmacological reports on PC. Therefore, this investigation is first that is aimed at comparing the phytoconstituents and reactive oxygen species (ROS) scavenging activities of two commercial "Gu-Sui-Bu" resource plants, DF and PC, because ROS are major intermediate neurotoxins and play an important role in intracellular oxidative damage due to aging-associated disorders.

Natural antioxidants, including flavonoids and phenylpropanoids, scavenge free radicals, which initiate and propagate oxidative chain reactions, thus, prevent intracellular oxidative damage.[11] Intracellular oxidative stress cascades, including biomolecules, act through ROS and deficient intracellular antioxidant defenses, which might induce the aging process and aging-associated neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).[12, 13] One of major pathological mechanisms in PD is autoxidation and enzymatic oxidation of dopamine

in the substantia nigra, which causes selective apoptosis and a loss of dopaminergic neurons.[14] 6-hydroxydopamine (6-OHDA) is a toxic oxidative dopamine metabolite that is rapidly and non-enzymatically oxidized by molecular oxygen to form *p*-quinone and ROS, such as superoxide anion and hydrogen peroxide, under physiological conditions.[15] Thus, 6-OHDA is a widely used compound for investigating pathogenesis and progression of as well as drug development for PD. Therefore, we further attempted to demonstrate the role of intracellular antioxidants and protective enzymes on the protective effects of DF extract against 6-OHDA-induced neuronal damage in B35 neuroblastoma cells because neuronal cells have several antioxidants and protective enzymes to prevent ROS formation or detoxify ROS.[16]

Materials and methods

Preparation of the herb extracts

Two Polydiaceae plants (DF and PC) were identified and provided by Hung-Chi Chang. DF or PC was extracted with distilled water, and the resulting extract was concentrated under reduced pressure to obtain DF or PC extract.[6] To assess phytoconstituents and ROS-scavenging activities, the DF or PC extract was dissolved in distilled water. To clarify the protective effects from and mechanism for 6-OHDA-induced neuronal damage, the DF or PC extract stock solutions were prepared using sterile distilled water, filtered using a 0.22 μM sterile filter and then diluted with DMEM without phenol red.

Chemicals

2-deoxyribose, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid (ferrozine), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB), 6-hydroxy-2,5,7,8-tetramethy-chroman-2-carboxylic acid (trolox), 6-OHDA, ascorbic acid, (+)-catechin, cyanidin, epicatechin, ferrous sulfate heptahydrate, Folin-Ciocalteu's reagent, reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), homovanillic acid (HVA), horseradish peroxidase (HRPase), malodialdehyde (MDA), naringin, nitroblue tetrazolium chloride (NBT), quercetin, sodium carbonate, sodium molybdate, sodium nitrate, superoxide dismutase (SOD), thiobarbituric acid (TBA), trichloroacetic acid (TCA), verbascoside, xanthine and xanthine oxidase were purchased from Sigma-Aldrich Chem. Corp. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) and all HPLC-grade solvents were purchased from Merck (Darmstadt, Germany).

Determination of phytoconstituents using a spectrophotometric reader

The levels of all phytochemicals, including total phenol, phenylpropanoid, flavonols and anthocyanidins, were assayed using a 96-well microtiter spectrophotometric method. The method used to determine the total phenolic levels is based on forming blue-colored products through a redox reaction with Folin-Ciocalteu's reagent and measuring its absorbance at 725 nm. The total phenolic concentration of the samples was expressed as mg of catechin equivalents per gram of sample [17]. The method used to determine total phenylpropanoid levels is based on forming colored products using phenylpropanoid with the Arnow reagent (containing 5% (w/v) sodium nitrate and 5% sodium molybdate) and measuring its absorbance at 525 nm. The total phenylpropanoid concentration of the samples was expressed as mg

of verbascoside equivalents per g of sample.[17] The method used to determine flavonols and anthocyanidin levels is based on switching the absorbance wavelength through different hydrogen chloride concentration and measuring its absorbance at 360 and 520 nm.[18]

Determination of phytoconstituents using high performance liquid chromatography (HPLC)

Aqueous DF or PC extract was dissolved in distilled water and then filtered using a 0.22 μm filter. Stock solutions of the standards were prepared in methanol to the final concentration 10 mg/mL. All standard and sample solutions were injected into 10 μL in triplicate. The Shimadzu VP series HPLC and Shimadzu Class-VP™ chromatography data systems were used. All chromatographic operations were performed at 25 °C. The epicatechin and naringin chromatographic peaks were confirmed by comparing their retention times and UV spectra. A LiChrospher® RP-18e (250 × 4 mm, 5 μm) column (Merck KGaA, Darmstadt, Germany) was used. Certain separating conditions including the mobile phases and gradient program conditions, followed the description by Liu et al.[19]

Determination of ROS using scavenging activity assay *in vitro*

The superoxide anion, H_2O_2 and hydroxyl radical scavenging activities were determined as described previously.[17] The superoxide anion scavenging activity is based on the reaction between NBT and the superoxide anion produced from xanthine and xanthine oxidase and was determined at 560 nm for 5-min kinetics using a microplate reader (PowerWave$_X$, Bio-Tek instruments, Inc., Winooski, VT, USA). The results were expressed as the U of SOD equivalents per milligram of sample. The

H_2O_2 scavenging activity is based on HVA dimer formation through the reaction between H_2O_2 and HVA catalyzed by HRPase, which was measured by the fluorescence intensity at the excitation 315 nm and emission 425 nm using a fluorescence microplate reader (FLX800, Bio-Tek instruments, Inc., Winooski, VT, USA). The H_2O_2 scavenging activity results were expressed as μmol of trolox equivalents per gram of sample. The hydroxyl radical scavenging activity was monitored at 532 nm using the 2-deoxyribose-TBARS method. The hydroxyl radical scavenging activity results were expressed as mg of quercetin equivalents per gram of sample.

***In vitro* lipid peroxidation inhibition assay**

The whole rat brain was homogenized (100 mg/mL) in ice-cold 0.1 M phosphate buffer (pH 7.4), and then brain homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C. The reaction mixture consisted of brain homogenate, 1 mM ferrous sulfate, 5 mM ascorbic acid and sample solution. The reaction solution was incubated at 37 °C for 30 min, and the thiobarbituric acid reactive substance (TBARS) test was performed by rapidly adding 1.2% (w/v) TBA and 10% TCA. The TBARS test tubes were incubated at 90 °C for 60 min, cooled, and centrifuged at 3,000 rpm for 10 min; the absorbance of the supernatant was then determined at 532 nm.[17]

***In vitro* ferric ion reducing antioxidant power (FRAP) assay**

Briefly, 25 μL of sample solution or trolox standards were mixed with 25 μL of freshly prepared FRAP reagent, which consisted of 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl, 20 mM $FeCl_3$, and 50 mM acetate buffer (pH 3.6). The reaction mixture absorption was measured at 595 nm.

171 The results were calculated based on a standard curve obtained using trolox and
172 expressed as the relative trolox equivalent per gram of sample.[17]
173
174 ***In vitro* iron-chelating activity (ICA) assay**
175 This method is based on ferrozine-Fe^{+2} colored complex formation, and the
176 absorbance is measured at 562 nm. The percentage of inhibition of ferronize-Fe^{+2}
177 complex formation was calculated as we previously reported.[17]
178
179 **Inhibition of 6-OHDA autoxidation *in vitro***
180 Autoxidation of 6-OHDA was followed spectrophotometrically by monitoring
181 *p*-quinone formation at 490 nm.[20] The assay was conducted in a cell free system under
182 conditions that correspond to cellular 6-OHDA treatments. Stock solutions of
183 6-OHDA (100 mM) were prepared in phosphate-buffered saline solution. The
184 experiment was initiated by adding 6-OHDA to yield the final concentration 50 μM.
185 The absorbance at 490 nm was monitored for 3 min at 30-sec intervals at 37℃
186
187 **The protective effects of 6-OHDA-induced neuronal damage in B35 cells**
188 Rat B35 neuroblastoma cells were cultured in DMEM supplemented with 10%
189 fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin in a
190 water-saturated atmosphere with 5% CO_2 at 37 °C. The experiments were performed
191 24 h after the cells were seeded in 96-well sterile clear-bottom plates or a 90-mm dish.
192 The cells were plated at an appropriate density according to the scale of each
193 experiment. The DF or PC extract was treated 1 h before 6-OHDA (50 μM) was
194 added. 6-OHDA (50 μM) was used after a 24-h exposure, as described below. B35
195 neuroblastoma cells were seeded in a 90-mm dish and incubated overnight in a

196 water-saturated atmosphere with 5% CO_2 at 37°C. The cell morphology was observed
197 24 h after 6-OHDA exposure using a phase-contrast microscope (Nikon, Tokyo,
198 Japan).
199 The MTT assay is based on the ability of living cells to reduce MTT to insoluble
200 formazan, which is measured at 570 nm to study cell survival as we previously
201 reported.[21] Briefly, 24 h after 6-OHDA exposure, the medium was replaced and MTT
202 was added to each well. After incubating for 2 h at 37°C, the cells were washed with
203 PBS, and DMSO was added. The experiments were performed in triplicate over four
204 independent experiments. Cell viability was expressed as the percentage relative to
205 untreated cells, which served as the control group (designated 100% viable).
206
207 **Intracellular antioxidant enzyme and GSH level measurements**
208 Following incubation for 24 h with 6-OHDA, B35 neuroblastoma cells were
209 collected from culture dishes and sonicated on ice. The solution was centrifuged for
210 15 min at 4°C to eliminate cell debris, and the supernatant was used in enzyme
211 activity assays. The antioxidant enzyme activities, including for GPx and GR, were
212 measured as we previously reported.[21] The GPx and GR activities were expressed as
213 mU/mg of protein. The GSH levels were determined described previously.[21] Briefly,
214 the lysates (20 μg/50 μL) or GSH standard was pipetted into each well of a 96-well
215 plate. The reaction solution, included DTNB, NADPH and GR, was added to each
216 well and was recorded at 405 nm for 5 min in a microplate reader. The GSH levels
217 were expressed as pmol/mg of protein.
218
219 **The lipid peroxidation assay in B35 cells**

Lipid peroxidation was measured using the TBARS assay in cell cultures. Briefly the lysates (200 μg/100 μL) or MDA standard was pipetted into 1.5 mL tubes, and a TBA test was performed. Next, the supernatant absorbance at 532 nm was determined.[21] The experiments were performed in triplicate over three independent trials. The MDA levels were expressed as nmol/mg of protein.

Western blot analysis

Twenty-four hours after 6-OHDA exposure, the cells were subjected to western blot analyses to determinate of the levels of phosphoinositide 3-kinase (PI3K)/AKT pathway, heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase (NQO-1). Briefly, the protein samples were extracted from the cell and quantified using a Bradford protein assay kit (Bio-Rad), followed by electrophoretic separation through SDS-PAGE. After transferring the protein samples to PVDF membranes, the samples were incubated with primary antibodies against PI3K, AKT, phospho-AKT (serine 473) (*p*-AKT (ser)), phospho-AKT (threonine 308) (*p*-AKT (thr)), HO-1 or NQO-1, overnight at 4 °C and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG. The images were scanned using an LAS-4000 mini imaging system (Fujifilm, Kanagawa, Japan), and the optical density data were analyzed using MultiGauge v3.0 software (Fujifilm, Kanagawa, Japan). For the western blot analyses, β-actin served as an internal control.

Statistical Analyses

All results were expressed as the mean ± standard deviation (SD). The significant differences were calculated using SPSS software with a one-way ANOVA followed by Scheffe's test, and P values < 0.05 were considered significant.

Results

DF or PC extract phytoconstituents

The levels of total phenols, flavonoids, phenylpropanoids and anthocyanidins in the DF or PC extract were measured using 96-well microtiter spectrophotometric methods and are shown in **Table 1**. The levels of total phenols, flavonoids, phenylpropanoids and anthocyanidins in each gram of DF extract were equivalent to 268.41 mg catechin, 13.67 mg quercetin, 21.05 mg verbascoside and 2.07 mg cyanidin. However, the levels of total phenols, flavonoids, phenylpropanoids and anthocyanidins in each gram of PC extract were equivalent to 130.10 mg catechin, 7.42 mg quercetin, 4.50 mg verbascoside and 1.22 mg cyanidin.

The phytoconstituents of the DF or PC extract were further assayed using high performance liquid chromatography. Their chromatographs are shown in **Figure 1**; the certain phytoconstituent peak zones differ between the DF and PC extracts. Each gram of DF extract contained 26.23 mg of naringin and 1.00 mg of epicatechin. Each gram of PC extract contained 10.27 mg of naringin and 1.46 mg of epicatechin (**Table 1**).

The ROS-scavenging and lipid peroxidation inhibiting activities of DF or PC extract *in vitro*

The scavenging activity of DF or PC extract against ROS was also investigated using 96-well microtiter spectrophotometric methods. The scavenging activity of each gram of DF extract against superoxide anion, H_2O_2 and hydroxyl radical was equivalent to 11.46 U of SOD, 927.57 μmol of trolox and 14.95 mg of quercetin. The scavenging activity of each gram of PC extract was equivalent to 9.43 U of SOD,

582.60 μmol of trolox and 4.40 mg of quercetin (**Table 2**). Next, their lipid peroxidation-inhibiting effects were evaluated using the Fe^{2+}/ascorbate method, for which rat brain homogenate was used as the oxidizable biomolecule target. The IC_{50} of DF extract against lipid peroxidation is 32.43 mg/mL and the IC_{50} of PC extract is 117.99 mg/mL (**Table 2**). Furthermore, their iron-chelating and reducing power activities were further investigated using 96-well microtiter spectrophotometric methods (ICA and FRAP tests). The reducing power of each gram of DF extract is equivalent to 1034.65 μmol of trolox and better than that of PC extract (each gram is equivalent to 622.64 μmol trolox) (**Table 2**). However, the above extracts did not have iron-chelating capacities at the concentrations used in the hydroxyl radical scavenging and lipid peroxidation-inhibiting assays (data not shown).

DF or PC extract inhibits 6-OHDA autoxidation *in vitro*

The DF or PC extract inhibiting activity against *p*-quinone production from 6-OHDA autoxidation under cell-free physiological conditions was also investigated through 96-well microtiter spectrophotometric methods. The reaction mixture (only 6-OHDA) absorbance at 490 nm was considered 100% of *p*-quinone produced from 6-OHDA. The DF extract at 25 - 250 μg/mL inhibited absorbance at 490 nm in a concentration-dependent manner ($P < 0.05$, $P < 0.001$). The PC extract at only 50 - 250 μg/mL inhibited absorbance at 490 nm ($P < 0.05$, $P < 0.01$) (**Figure 2**)

The protective effects of the DF extract against 6-OHDA-induced neuronal damage in B35 neuroblastoma cells

We further evaluated the protective effects of DF extract at 10 - 250 μg/mL against 6-OHDA-induced neuronal damage in B35 neuroblastoma cells using the MTT assay.

Incubation with 50 μM 6-OHDA to B35 neuroblastoma cells for 24 h, cell viability was decreased to 46.3% compared with the control ($P < 0.001$) (**Fig. 3(A)**). The DF extract at 50 - 250 μg/mL increased the cell viability against 6-OHDA in a concentration-dependent manner ($P < 0.01$, $P < 0.001$) (**Fig. 3(A)**). Furthermore, we observed morphological alterations of B35 neuroblastoma cells through phase-contrast microscopy. Incubation with 50 μM 6-OHDA for 24 h decreased the cell number and cell shrinkage (**Figure 3(B)-a and 3(B)-b**). The DF extract (50 - 100 μg/mL) attenuated the morphological changes (**Figure 3(B)-c and 3(B)-d**).

GSH cycle involvement in the protective effects of DF extract

To clarify the protective mechanism of the DF extract against 6-OHDA-induced neuronal damage in B35 neuroblastoma cells, we measured the intracellular antioxidant defenses including the levels of GSH and related antioxidant enzymes activities. The intracellular GSH levels as well as GPx and GR activities in B35 neuroblastoma cells treated with 50 μM 6-OHDA for 24 h were lower ($P < 0.01$) (**Table 3**). The level of MDA in B35 neuroblastoma cells treated with 50 μM 6-OHDA for 24 h was greater ($P < 0.01$) (**Table 3**). The DF extract at 100 μg/mL partially restored the intracellular GSH levels, and partially reversed the decrease in activities of GPx and GR by 50 μM 6-OHDA in B35 neuroblastoma cells ($P < 0.05$) (**Table 3**). The DF extract at 100 μg/mL also decreased the level of MDA, which was increased by 6-OHDA ($P < 0.05$) (**Table 3**).

Reversal of PI3K/AKT-, NQO-1- and HO-1-mediated damage through the protective effects of DF extract

Because neuronal damage due to 6-OHDA mainly occurs through the PI3K/AKT pathway and a decrease in detoxifying enzymes such as HO-1 and NQO-1,[32, 33] we assayed the levels of PI3K/AKT pathway proteins, HO-1 and NQO-1 in B35 neuroblastoma cells treated with 6-OHDA and DF extract. The protein immunoblot assay is shown in **Figure 4(A)**. 6-OHDA decreased the levels of PI3K ($P < 0.01$) but did not alter the level of AKT ($P > 0.05$) (**Figure 4(B)** and **Figure 4(C)**). However, 6-OHDA decreased the ratio of *p*-AKT (thr) to AKT and *p*-AKT (ser) to AKT ($P < 0.001$) (**Figure 4(D), Figure 4(E)** and **Figure 4(F)**). 6-OHDA further decreased the levels of HO-1 and NQO-1 in B35 neuroblastoma cells ($P < 0.01$ for HO-1, $P < 0.05$ for NQO-1) (**Figure 4(G)** and **Figure 4(H)**). The DF extract at 50 ~ 100 μg/mL restored the levels of PI3K and NQO-1 as well as the ratio of *p*-AKT to AKT (especially *p*-AKT (ser) vs AKT) ($P < 0.01$, $P < 0.001$), but only the DF extract at 100 μg/mL could restore the levels of HO-1 and ratio of *p*-AKT (thr) to AKT ($P < 0.05$, $P < 0.01$) (**Figure 4(B-H)**).

Discussion

Gu-Sui-Bu is a common traditional Chinese medicine used to prevent aging-associated disorders, including PD, for centuries. DF is a major resource for Gu-Sui-Bu, and PC is an alternative resource in Taiwan. According to phytochemical reports on DF,[3, 4, 19] flavonoids and phenylpropanoids are major phytoconstituents of DF. Our data indicate that the DF extract had higher levels of total phenolics and flavonoids (approximately two times) compared with the PC extract. Next, we further compared the DF and PC extract chromatograms and matched them with other reports.[19] The DF extract might contain phenolic acids, dihydroxychromone, epicatechin and naringin. The PC extract might contain epicatechin and naringin.

Epicatechin and naringin are co-existing phytoconstituents in the DF and PC extracts; however, the certain phytoconstituent peak zones differ between the DF and PC extracts. The DF extract contained higher levels of naringin (approximately two times) than the PC extract. Hence, we suggest that the DF extract has higher phenolic levels, especially for naringin.

These phenolic compounds have been shown to correlate with the plant antioxidant activities.[17, 22] Moreover, in the pathogenesis of PD, both neurotoxic ROS and highly reactive, redox-cycling DA-derived quinones are formed due to enzymatic breakdown by monoamine oxidase (MAO) or autoxidation of excess cytosolic dopamine.[15] ROS mainly include superoxide anion, H_2O_2 and hydroxyl radical. Superoxide anion is produced in a cell's mitochondrion and has been implicated in the pathophysiology of certain diseases such as PD. H_2O_2 yields the highly reactive hydroxyl radical, which is the most reactive and severely damages adjacent biomolecules, such as polyunsaturated fatty acids.[11] Our results also indicate that the DF extract had higher ROS scavenging and lipid peroxidation-inhibiting activities as well as reducing power capacity (approximately two times) compared with the PC extract. Moreover, at the concentrations used in the hydroxyl radical scavenging and lipid peroxidation-inhibiting assays, neither the DF nor the PC extract had the capacity to chelate iron. From the above results, we suggested that the DF or PC extract inhibited lipid peroxidation in brain homogenate systems mainly by terminating oxidative chain reactions through its radical scavenging capacity and reducing power; however, certain reports indicate that the activity of certain antioxidant compounds may correlate with iron-chelation.[11] Furthermore, at the concentrations used in the ROS-scavenging activity assays, the DF extract also inhibited *p*-quinone production from 6-OHDA autoxidation in a concentration-dependent manner; this effect was

369 greater than for the PC extract. Based on the above phytochemical and antioxidant
370 results, we suggest that ROS-scavenging activities of the DF or PC extract positively
371 correlate with the levels of all phytochemicals; this relationship is consistent with
372 earlier reports.[17, 22] The enhanced ROS-scavenging activity of the DF extract
373 compared with the PC extract may be due to the DF extract enrichment phenolic
374 compounds, such as naringin, because their antioxidant activities are consistent with
375 the pharmacological activities of naringin and epicatechin.[10, 23]

376 Similar to PD pathogenesis, 6-OHDA is a selective dopaminergic neurotoxin that
377 induces PD-like cell or animal models via excessive ROS and *p*-quinone generation
378 during 6-OHDA autoxidation.[15] We further found that, at 50 - 250 μg/mL, DF extract
379 increased cell viability against 6-OHDA in a concentration-dependent manner and
380 reversed the 6-OHDA-induced morphological changes in B35 neuroblastoma cells.
381 Naringin is an active DF compound identified in our HPLC results and other reports
382 that also protect against rotenone-induced neuronal damage in SH-SY5Y cells.[9]
383 Therefore, we suggest that the DF extract protect against 6-OHDA-induced neuronal
384 damage in B35 neuroblastoma cells partially via scavenging ROS and inhibiting
385 *p*-quinone. However, intracellular redox imbalance is mainly due to an imbalance
386 between generating and eliminating the free radicals, specifically caused by lower
387 intracellular antioxidant defenses.[11, 13] Neuronal intracellular antioxidant defenses
388 mainly include several antioxidants and protective enzymes that prevent ROS
389 formation or detoxify ROS.[16] GSH recycling includes GSH and related enzymes. such
390 as glutathione peroxidase (GPx) and GR, and is a major intracellular antioxidant
391 defense. In PD patients, the degree of in symptom severity correlates with
392 intracellular GSH loss in substantia nigra.[24] Many oxidants, such as 6-OHDA and
393 H_2O_2, could deplete the intracellular GSH levels and decrease GPx and GR activation

394 in cell culture and animals.[21, 25] Our results are consistent with the above reports;[21, 25]
395 incubation with 50 μM 6-OHDA in B35 neuroblastoma cells for 24 h decreased the
396 GSH cycle activities and increased the oxidative damage. The DF extract at 100
397 μg/mL reversed the GSH cycle activities that were decreased by 50 μM 6-OHDA in
398 B35 neuroblastoma cells, which in turn, decreased the oxidative damage. Hence, we
399 suggest that the DF extract attenuated the 6-OHDA-induced neuronal damage by
400 upregulating the antioxidant status via intracellular GSH regeneration and its radical
401 scavenging activity. In fact, naringin which is a major DF extract constituent also
402 protected against the neuronal damage caused by 3-nitropropionic acid or kainic acid
403 via antioxidant activity and intracellular GSH regeneration.[7, 8] Certain reports further
404 indicated that naringin and epicatechin have neuroprotective activities against
405 rotenone, aluminum and amyloid β peptide *in vitro* and *in vivo*. [7-10, 26] Based on the
406 above results, we suggest that DF is a potential medicinal plant that protects against
407 PD in a comparison between two commercial Polydiaceae plants.
408 AKT plays a pivotal role in fundamental cellular functions, such as cell
409 proliferation and survival, by phosphorylating a variety of enzymes, including
410 pro-apoptotic regulators, detoxifying and antioxidant proteins, and transcription
411 factors.[27] AKT is mainly activated through phosphorylation of T308 (AKT (thr)) and
412 S473 (AKT (ser)) by receptor tyrosine kinases, G-protein-coupled receptors, mTOR
413 complex 2 (mTORC2), DNA-dependent protein kinase (DNA-PK), and other stimuli
414 that induce phosphatidylinositol 3,4,5 triphosphates (PtdIns(3,4,5)P3) production
415 through PI3K.[28] Hence, certain reports indicate that AKT is an important therapeutic
416 target for the treating diabetes, stroke, and neurodegenerative disease.[27, 28] In this
417 study, we found that treating with 6-OHDA for 24 h decreased the levels of PI3K and
418 AKT phosphorylation, especially at S473, in B35 neuroblastoma cells. Hence,

6-OHDA mainly inhibited full activation of AKT via obstructing AKT phosphorylation at S473, which causes neuronal damage. The DF extract increased the PI3K levels and restored AKT phosphorylation. Among the various cytoprotective enzymes modulated by the AKT cascade, HO-1 and NQO-1 play an important role in neuroprotective functions.[29] Recent, mounting evidence indicates that a pharmacological inducer of HO-1 expression may maximize the intrinsic antioxidant potential of cells.[30] NQO-1 is a key enzyme that detoxifies reactive quinones produced from autoxidation and enzymatic oxidation of dopamine.[31] Our data also indicate that 6-OHDA decreased the HO-1 and NQO-1 levels in B35 neuroblastoma cells. Hence, our results are consistent with other reports that 6-OHDA decreases the HO-1 and NQO-1 expression via inhibiting phosphorylation of the PI3K/AKT pathway, causing neuronal damage.[32, 33] DF also reversed the HO-1 and NQO-1 levels that were decreased by 6-OHDA. Therefore, we suggest that the DF extract induced AKT phosphorylation and further activated HO-1 and NQO-1 expression to counteract the neurotoxicity and decrease the neuronal damage caused by 6-OHDA in B35 cells.

Conclusion

In conclusion, DF extract had the higher phenolic levels, especially for phenylpropanoids, and exhibited the higher radical scavenging potency compared to PC extract. Its antioxidant activity can be correlated with its reducing power, which is not due to iron chelation, and this antioxidant activity depends on the phenolic antioxidants such as naringin and epicatechin.[19] The DF extract has a protective effect against 6-OHDA-induced neuronal damage *in vitro*. Naringin and epicatechin are its major active compounds because naringin and epicatechin can protect against

444 neuronal damage caused by rotenone and amyloid β peptide.[9, 26] This protective
445 mechanism might be related to its radical scavenging capacity and its ability to
446 activate intracellular antioxidant defenses, including GSH recycling as well as HO-1
447 and NQO-1 via the phosphorylation in the PI3K/AKT pathway (**Figure 5**). Hence, we
448 suggest that DF extract has potential therapeutic benefits for treating aging-associated
449 symptoms and neurodegenerative disorders. However, the expressions of detoxifying
450 and antioxidant enzymes such as GSH-related enzymes, HO-1 and NQO-1 are
451 coordinated and induced via a nuclear factor-E2-related factor 2 (Nrf2)-dependent and
452 antioxidant response element (ARE)-mediated mechanism.[34] Recent studies have
453 reported that Nrf2 is a master redox regulator that upregulates HO-1 to protect
454 dopaminergic neurons against 6-OHDA-induced neurotoxicity.[35, 36] Studies have also
455 suggested that Nrf2 nuclear translocation requires the activation of several signal
456 transduction pathways, such as PI3K/AKT or the mitogen-activated protein kinase
457 (MAPK) pathways.[32, 34, 36] In addition, naringin is a major active compound in DF
458 extract and has also been shown to protect against 3-nitropropionic acid-induced
459 apoptosis via downregulating the pro-apoptotic gene Bax and the upregulating the
460 anti-apoptotic genes Bcl-2 and Bcl-X(L).[7] The expression of pro-apoptotic genes is
461 also modulated by AKT.[27, 28] Therefore, the anti-apoptotic effects from DF extract and
462 the role of pro-apoptotic genes and Nrf2 translocation mediated by PI3K/AKT in the
463 neuroprotective effects from DF extract must be further investigated.

464

465 ## Acknowledgments

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468 101-2120-M-009-008 and NSC102-2320-B-039-037.

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533

Table 1. The phytoconstituents of aqueous extracts of Polydiaceae plants.

Samples	Total phenolics (mg of catechin / g)	Flavonols (mg of quercetin / g)	Phenylpropanoids (mg of verbascoside / g)	Anthocyanidin (mg of cyanidin / g)	Epicatechin (mg / g)	Naringin (mg / g)
Drynaria fortunei	268.41 ± 4.41	13.67 ± 0.26	21.05 ± 1.04	2.07 ± 0.04	1.00 ± 0.02	26.23 ± 0.34
Pseudodrynaria coronans	130.10 ± 0.25	7.41 ± 0.10	4.50 ± 0.12	1.22 ± 0.04	1.46 ± 0.08	10.27 ± 0.88

Data were expressed as mean ± SD (*n*=3).

Table 2. ROS scavenging activities of aqueous extracts of Polydiaceae plants.

Samples	$O_2^{\cdot}$ scavenging (U of SOD / mg)	H_2O_2 scavenging (μmol of trolox / g)	$OH^{\cdot}$ scavenging (mg of quercetin / g)	IC_{50} of lipid peroxidation (mg / mL)	Reducing power (μmol of trolox / g)
Drynaria fortunei	11.46 ± 0.31	927.57 ± 7.61	14.95 ± 0.31	32.43 ± 0.84	1034.65 ± 21.44
Pseudodrynaria coronans	9.43 ± 0.81	582.60 ± 9.49	4.40 ± 1.46	117.99 ± 10.38	622.64 ± 14.81

Data were expressed as mean ± SD (n=3).

Table 3. Effects of aqueous extract of *Drynaria fortunei* (DF) on antioxidant makers and MDA levels in B35 Cells exposed to 50 μM 6-OHDA

Samples	GSH (pmol/mg of protein)	GR (mU/mg of protein)	GPx (mU/mg of protein)	MDA (nmol/mg of protein)
Control	6.77 ± 0.21**	15.31 ± 0.65**	143.52 ± 8.27**	7.67 ± 0.33**
6-OHDA	3.08 ± 0.19	9.83 ± 0.38	76.24 ± 7.92	16.23 ± 0.97
DF 10 μg/mL + 6-OHDA	3.15 ± 0.13	10.37 ± 0.52	81.46 ± 6.12	14.76 ± 1.08
DF 50 μg/mL + 6-OHDA	3.21 ± 0.17	10.85 ± 0.71	87.37 ± 9.16	14.12 ± 0.84
DF 100 μg/mL + 6-OHDA	4.47 ± 0.23*	13.74 ± 0.47*	121.45 ± 7.53*	9.69 ± 0.43*

B35 cells were treated with the above samples plus 50 μM 6-OHDA. Data are expressed as mean ± SEM ($n = 4$). * $p < 0.05$, ** $p < 0.01$ as compared to the 6-OHDA group.

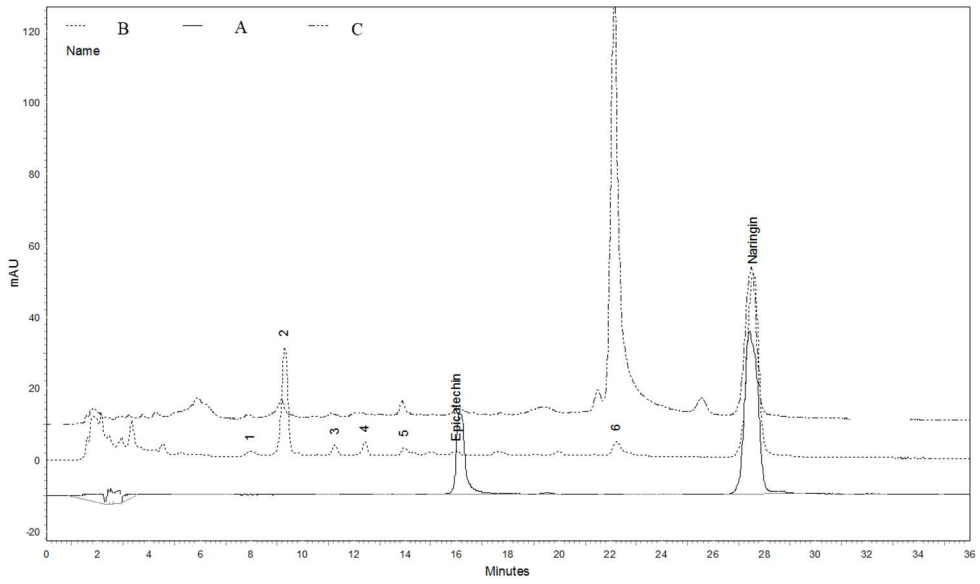

Figure 1. HPLC chromatograms of *Drynaria fortunei* (DF) or *Pseudodrynaria coronans* (PC) extract at 280 nm. Trace: (A) Standard, (B) DF at 5 mg/mL, (C) PC at 10 mg/mL.
240x141mm (150 x 150 DPI)

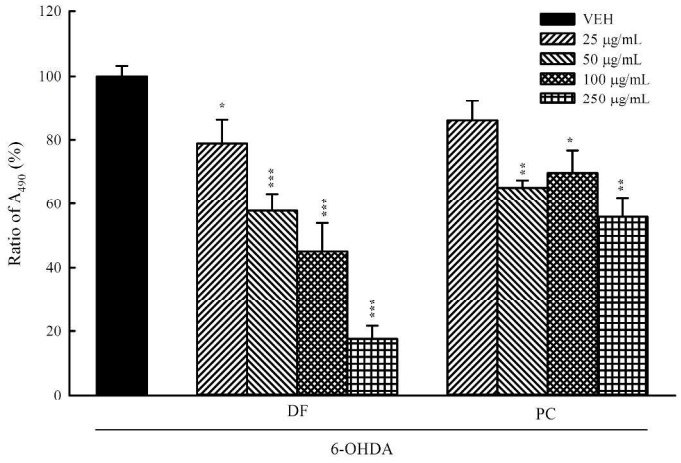

Figure 2. Effect of *Drynaria fortunei* (DF) or *Pseudodrynaria coronans* (PC) extract on p-quinolone production from 6-hydroxydopamine (6-OHDA) autoxidation. Data are expressed as mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with VEH group.
435x305mm (300 x 300 DPI)

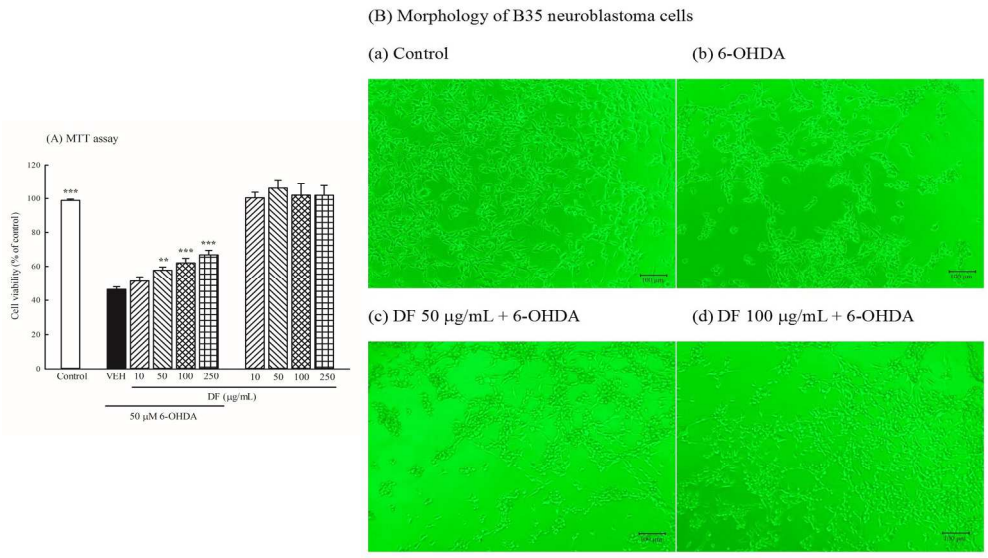

Figure 3. Effect of *Drynaria fortunei* (DF) extract on 6-hydroxydopamine (6-OHDA)-induced toxicology in B35 cells. Data are expressed as mean ± SEM (n = 4). (A) Cell viability was measured by MTT assay. (B) Cell morphology was visualized by a phase-contrast microscope (100×). (B)-a, control; (B)-b, B35 cells exposed to 50 μM 6-OHDA; (B)-c, B35 cells treated with 100 μg/mL of DF extract plus 50 μM 6-OHDA; (B)-d, B35 cells treated with 100 μg/mL of PC extract plus 50 μM 6-OHDA. ** $p < 0.01$, *** $p < 0.001$, compared with 6-OHDA/VEH group.
442x249mm (150 x 150 DPI)

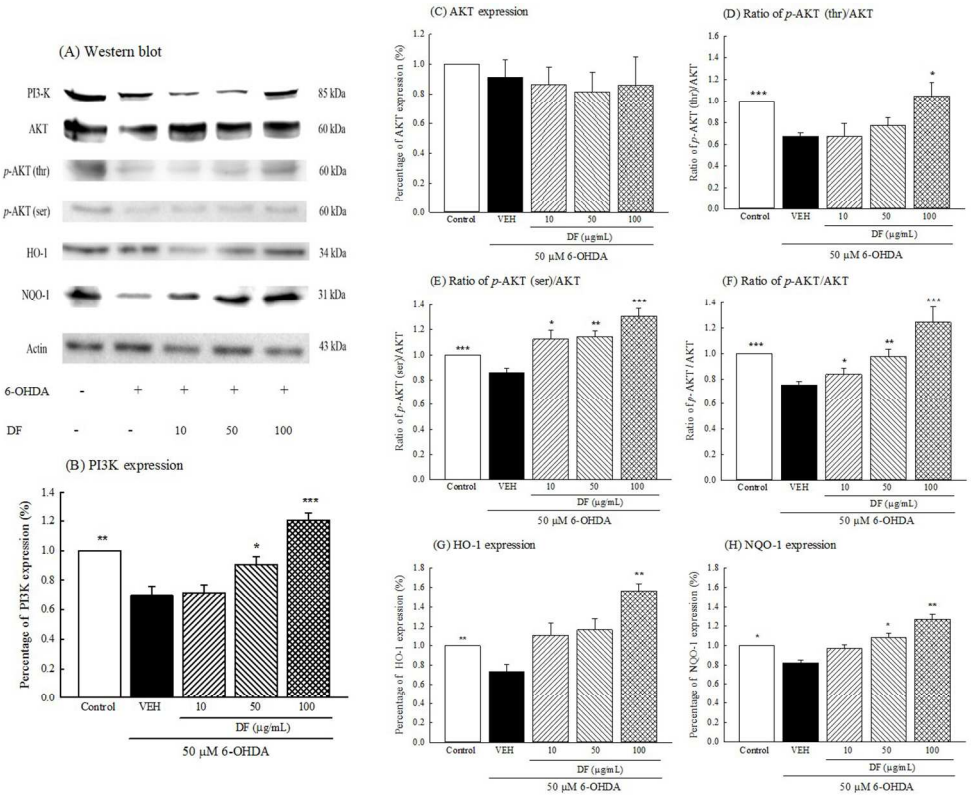

Figure 4. Effect of *Drynaria fortunei* (DF) on 6-hydroxydopamine (6-OHDA)-induced alteration of PI3-K and AKT activation in B35 cells. (A) Protein was determined by immunoblot assay, (B) Levels of PI3-K expression, (C) Levels of AKT expression, (D) Ratio of p-AKT (thr) / AKT, (E) Ratio of p-AKT (ser) / AKT, (F) Levels of HO-1 expression, (G) Levels of NQO-1 expression. Data are expressed as mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with 6-OHDA group.
233x184mm (150 x 150 DPI)

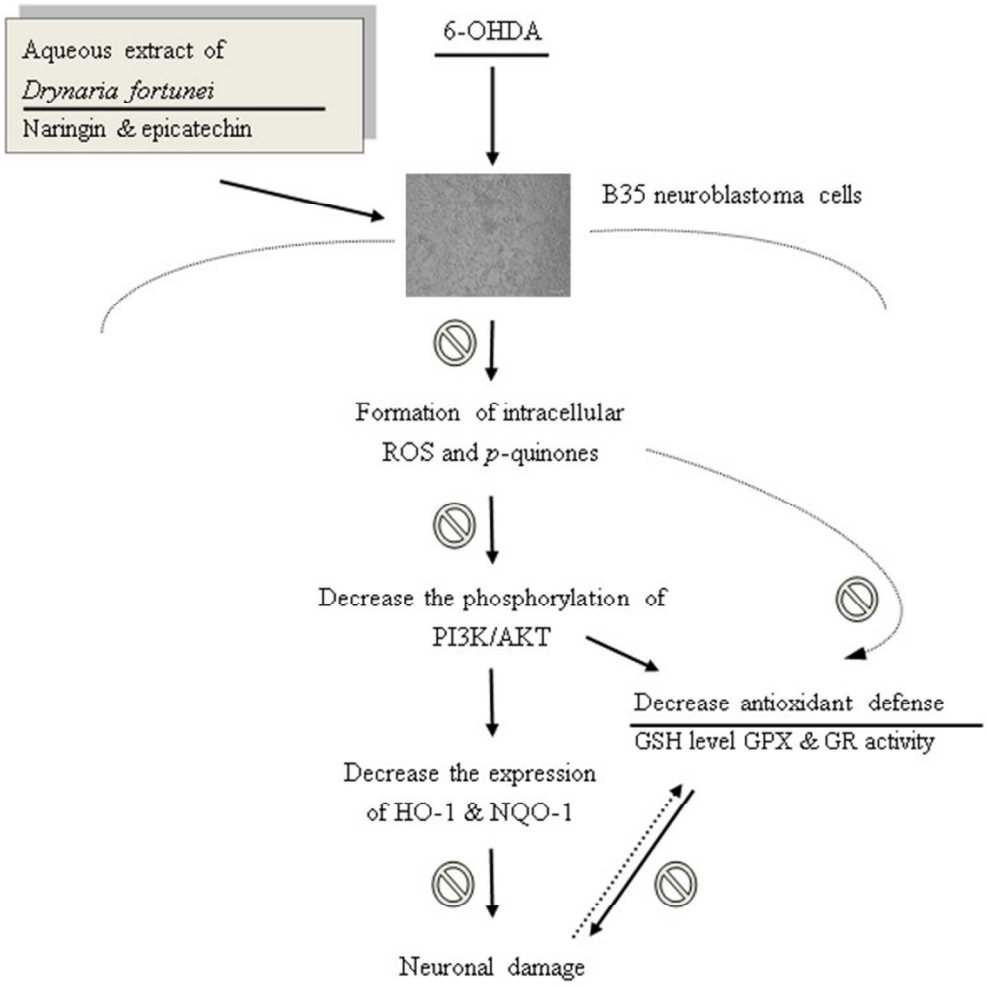

Figure 5. The biological action of *Drynaria fortunei* (DF) as a potential antioxidant and protective plant against oxidative stress caused by 6-hydroxydopamine (6-OHDA). Prohibition sign indicates that the inhibitory effect of *Drynaria fortunei*.
126x126mm (150 x 150 DPI)