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

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In this study, we demonstrate the antioxidant and protective properties of the 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 showed better effects than PC extract in scavenging ROS and inhibiting 6-OHDA autoxidation. Following exposure to 6-OHDA, B35 cells showed a marked decrease in cell survival and the activation of intracellular antioxidant enzymes and the PI3K/AKT pathway, and then an increased level of lipid peroxidation. 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 protective effects against 6-OHDA toxicity *via* radical scavenging activity and an increase in the activation of the PI3K/AKT pathway to elevate the levels of intracellular antioxidant enzymes including HO-1, NQO-1 and glutathione-related enzymes.

**Introduction**

The rhizome of *Drynaria fortunei* (Kunze) J. Smith (Polydiaceae) (DF) is a major source 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. Flavonoids and phenylpropanoids, such as epicatechin, naringin and neoeriocitrin, are the active constituents of DF extract due to their osteoprotective activities. Other pharmacological studies indicate that DF has antioxidant and anti-apoptotic activities. The above phytoconstituents also have neuroprotective and anti-apoptotic activities through their antioxidant mechanisms *in vitro* and *in vivo*. The rhizome of *Pseudodrynaria coronans* (Wall.) Ching (Polydiaceae) (PC) is an alternative medicinal source for “Gu-Sui-Bu” in Taiwan. There are no phytochemical and pharmacological reports on PC. Therefore, this investigation is the first one to compare the phytoconstituents and reactive oxygen species (ROS) scavenging activities of two commercial plant sources of “Gu-Sui-Bu”, DF and PC, because ROS are the 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 preventing intracellular oxidative damage. 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).

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 anions and hydrogen peroxide, under physiological conditions. 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 in 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 to detoxify ROS.\textsuperscript{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.\textsuperscript{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 \( \mu \)m 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'-dithiobis(2-nitrobenzoic acid) (DTNB), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 6-OHDA, ascorbic acid, (+)-catechin, cyanidin, epicatechin, ferrous sulfate heptahydrate, Folin–Ciocalteu’s reagent, reduced glutathione (GSH), glutathione peroxidase (GPx), 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\(_2\)O\(_2\)) 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 the formation of 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.\textsuperscript{17} The method used to determine total phenylpropanoid levels is based on the formation of colored products from the reaction of 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.\textsuperscript{17} The method used to determine flavonols and anthocyanidin levels is based on switching the absorbance wavelength through different hydrochloride concentrations and measuring its absorbance at 360 and 520 nm.\textsuperscript{18}

**Determination of phytoconstituents using high performance liquid chromatography (HPLC)**

The aqueous extract of DF or PC was dissolved in distilled water and then filtered using a 0.22 \( \mu \)m filter. Stock solutions of the standards were prepared in methanol to the final concentration 10 mg mL\(^{-1}\). All standard and sample solutions were injected at 10 \( \mu \)L volumes in triplicate. The Shimadzu VP series HPLC and Shimadzu Class-VPTM 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 \( \mu \)m) column (Merck KGaA, Darmstadt, Germany) was used. Certain separating conditions including the mobile phases and gradient program conditions followed the description by Liu \textit{et al.}\textsuperscript{19}

**Determination of ROS using scavenging activity assay in vitro**

The superoxide anion, H\(_2\)O\(_2\) and hydroxyl radical scavenging activities were determined as described previously.\textsuperscript{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 from 5 min kinetics using a microplate reader (PowerWaveX, Bio-Tek instruments, Inc., Winooski, VT, USA). The results were expressed as the U of SOD equivalents per milligram of sample. The H\(_2\)O\(_2\) scavenging activity is based on HVA dimer formation through the reaction between H\(_2\)O\(_2\) and HVA catalyzed by HRPase, which was measured by the fluorescence intensity at 315 nm excitation and 425 nm emission using a fluorescence microplate reader (FLX800, Bio-Tek instruments, Inc., Winooski, VT, USA). The H\(_2\)O\(_2\) scavenging activity results were expressed as \( \mu \)mol 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\(^{-1}\)) in ice-cold 0.1 M phosphate buffer (pH 7.4), and then the 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 the 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 3000 rpm for 10 min; the absorbance of the supernatant was then determined at 532 nm.\textsuperscript{17}

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

Briefly, 25 \( \mu \)L of sample solution or trolox standards were mixed with 25 \( \mu \)L 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. The results were calculated based on a standard curve obtained using trolox and expressed as the relative trolox equivalent per gram of sample.17

**In vitro iron-chelating activity (ICA) assay**

This method is based on ferrozine–Fe$^{2+}$ colored complex formation, and the absorbance is measured at 562 nm. The percentage of inhibition of ferrozine–Fe$^{2+}$ complex formation was calculated as we previously reported.17

**Inhibition of 6-OHDA autoxidation in vitro**

Autoxidation of 6-OHDA was followed spectrophotometrically by monitoring p-quinone formation at 490 nm.28 The assay was conducted in a cell-free system under conditions that correspond to cellular 6-OHDA treatments. Stock solutions of 6-OHDA (100 mM) were prepared in phosphate-buffered saline solution. The experiment was initiated by adding 6-OHDA to yield the final concentration 50 μM. The absorbance at 490 nm was monitored for 3 min at 30 s intervals at 37 °C.

**Protective effects against 6-OHDA-induced neuronal damage in B35 cells**

Rat B35 neuroblastoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units per mL penicillin and 100 μg mL$^{-1}$ streptomycin in a water-saturated atmosphere with 5% CO$_2$ at 37 °C. The experiments were performed 24 h after the cells were seeded in 96-well sterile clear-bottom plates or a 90 mm dish. The cells were plated at an appropriate density according to the scale of each experiment. The DF or PC extract was treated 1 h before 6-OHDA (50 μM) was added. 6-OHDA (50 μM) was used after a 24 h exposure, as described below. B35 neuroblastoma cells were seeded in a 90 mm dish and incubated overnight in a water-saturated atmosphere with 5% CO$_2$ at 37 °C. The cell morphology was observed 24 h after 6-OHDA exposure using a phase-contrast microscope (Nikon, Tokyo, Japan).

The MTT assay is based on the ability of living cells to reduce MTT to insoluble formazan, which was measured at 570 nm to study cell survival as we previously reported.21 Briefly, 24 h after 6-OHDA exposure, the medium was replaced and MTT was added to each well. After incubating for 2 h at 37 °C, the cells were washed with PBS, and then DMSO was added. The experiments were performed in triplicate over four independent experiments. Cell viability was expressed as the percentage relative to untreated cells, which served as the control group (designated 100% viable).

**Intracellular antioxidant enzyme and GSH level measurements**

Following incubation for 24 h with 6-OHDA, B35 neuroblastoma cells were collected from culture dishes and sonicated on ice. The solution was centrifuged for 15 min at 4 °C to eliminate the cell debris, and the supernatant was used in enzyme activity assays. The antioxidant enzyme activities, including for GPx and GR, were measured as we previously reported.21 The GPx and GR activities were expressed as mU mg$^{-1}$ of protein. The GSH levels were determined as described previously.21 Briefly, the lysates (20 μg/50 μL) or GSH standard was pipetted into each well of a 96-well plate. The reaction solution, including DTNB, NADPH and GR, was added to each well and was recorded at 405 nm for 5 min using a microplate reader. The GSH levels were expressed as pmol mg$^{-1}$ of protein.

**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$^{-1}$ of protein.

**Western blot analysis**

Twenty-four hours after 6-OHDA exposure, the cells were subjected to western blot analyses to determine the levels of the phosphoinositide 3-kinase (PI3K)/AKT pathway, heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase (NQO-1). Briefly, protein samples were extracted from the cells 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 Fig. 1; 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).

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 anions, $\text{H}_2\text{O}_2$ and hydroxyl radicals 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$^{3+}$/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$^{-1}$ and the IC$_{50}$ of PC extract is 117.99 mg mL$^{-1}$ (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, which is better than that of the PC extract (each gram is equivalent to 622.64 µmol trolox) (Table 2). However, the above extracts did not show iron-chelating capacities at the concentrations used in the hydroxyl radical scavenging and lipid peroxidation-inhibiting assays (data not shown).

![Fig. 1](image) HPLC chromatograms of *Drynia fortunei* (DF) or *Pseudodrynaria coronans* (PC) extract at 280 nm. Traces: (A) standard, (B) DF at 5 mg mL$^{-1}$, and (C) PC at 10 mg mL$^{-1}$.

### Table 1  The phytoconstituents of aqueous extracts of Polydiaceae plants$^a$

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total phenolics (mg of catechin per g)</th>
<th>Flavonols (mg of quercetin per g)</th>
<th>Phenylpropanoids (mg of verbascoside per g)</th>
<th>Anthocyanin (mg of cyanidin per g)</th>
<th>Epicatechin (mg g$^{-1}$)</th>
<th>Naringin (mg g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Drynia fortunei</em></td>
<td>268.41 ± 4.41</td>
<td>13.67 ± 0.26</td>
<td>21.05 ± 1.04</td>
<td>2.07 ± 0.04</td>
<td>1.00 ± 0.02</td>
<td>26.23 ± 0.34</td>
</tr>
<tr>
<td><em>Pseudodrynaria coronans</em></td>
<td>130.10 ± 0.25</td>
<td>7.41 ± 0.10</td>
<td>4.50 ± 0.12</td>
<td>1.22 ± 0.04</td>
<td>1.46 ± 0.08</td>
<td>10.27 ± 0.88</td>
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</table>

$^a$ Data are expressed as mean ± SD ($n$ = 3).
DF or PC extract inhibits 6-OHDA autoxidation in vitro

The inhibiting activity of the DF or PC extract against p-quinone production from 6-OHDA autoxidation under cell-free physiological conditions was also investigated by 96-well microtiter spectrophotometric methods. The reaction mixture (only 6-OHDA) absorbance at 490 nm was considered to be 100% of p-quinone produced from 6-OHDA. The DF extract at 25–250 μg mL⁻¹ inhibited the absorbance at 490 nm in a concentration-dependent manner (*p < 0.05, **p < 0.001). The PC extract at only 50–250 μg mL⁻¹ inhibited the absorbance at 490 nm (*p < 0.05, *p < 0.01) (Fig. 2).

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⁻¹ against 6-OHDA-induced neuronal damage in B35 neuroblastoma cells using the MTT assay. After incubating B35 neuroblastoma cells with 50 μM 6-OHDA for 24 h, the cell viability decreased to 46.3% compared with the control (P < 0.001) (Fig. 3(A)). The DF extract at 50–250 μg mL⁻¹ 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 using phase-contrast microscopy. Incubation with 50 μM 6-OHDA for 24 h decreased the cell number and cell shrinkage (Fig. 3(B)-a and 3(B)-b). The DF extract (50–100 μg mL⁻¹) attenuated the morphological changes (Fig. 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 enzyme 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 higher (*p < 0.01) (Table 3). The DF extract at 100 μg mL⁻¹ 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⁻¹ also decreased the level of MDA, which was increased by 6-OHDA (*p < 0.05) (Table 3).

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>O₂⁻ scavenging (U of SOD per mg)</th>
<th>H₂O₂ scavenging (μmol of trolox per g)</th>
<th>OH⁻ scavenging (mg of quercetin per g)</th>
<th>IC₅₀ of lipid peroxidation (mg mL⁻¹)</th>
<th>Reducing power (μmol of trolox per g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drynaria fortunei</td>
<td>11.46 ± 0.31</td>
<td>927.57 ± 7.61</td>
<td>14.95 ± 0.31</td>
<td>32.43 ± 0.84</td>
<td>1034.65 ± 21.44</td>
</tr>
<tr>
<td>Pseudodrynaria coronans</td>
<td>9.43 ± 0.81</td>
<td>582.60 ± 9.49</td>
<td>4.40 ± 1.46</td>
<td>117.99 ± 10.38</td>
<td>622.64 ± 14.81</td>
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</table>

* Data are expressed as mean ± SD (n = 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 Fig. 4(A). 6-OHDA decreased the levels of PI3K (P < 0.01) but did not alter the level of AKT (P > 0.05) (Fig. 4(B) and (C)). However, 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) (Fig. 4(G) and (H)). The DF extract at 50–100 μg mL⁻¹ 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⁻¹ could restore the levels of HO-1 and the ratio of p-AKT (thr) to AKT (P < 0.05, P < 0.01) (Fig. 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 source for Gu-Sui-Bu, and PC is an alternative source in Taiwan. According to phytochemical reports on DF,3,4,19 flavonoids and phenylpropanoids are the 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. 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, 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. 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. ROS mainly include superoxide anion, H$_2$O$_2$ and hydroxyl radical. The superoxide anion is produced in a cell’s mitochondrion and has been implicated in the pathophysiology of certain diseases such as PD. H$_2$O$_2$ yields the highly reactive hydroxyl radical, which is the most reactive and severely damages adjacent biomolecules, such as polyunsaturated fatty acids. 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 suggest that the DF or PC extract inhibits 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. 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 greater than for the PC extract. Based on the above phytochemical and antioxidant results, we suggest that ROS-scavenging activities of the DF or PC extract positively correlate with the levels of all phytochemicals; this relationship is consistent with earlier reports. The enhanced ROS-scavenging activity of the DF extract compared with the PC extract may be due to the DF extract enrichment with phenolic compounds, such as naringin, because their antioxidant activities are consistent with the pharmacological activities of naringin and epicatechin.

Similar to PD pathogenesis, 6-OHDA is a selective dopaminergic neurotoxin that induces PD-like cell or animal models via excessive ROS and p-quinone generation during 6-OHDA autoxidation. We further found that, at 50–250 µg mL⁻¹, DF extract increased the cell viability against 6-OHDA in a concentration-dependent manner, and reversed the 6-OHDA-induced morphological changes in B35 neuroblastoma cells. Naringin is an active DF compound identified in our HPLC results and other reports that also protect against rotenone-induced neuronal damage in SH-SY5Y cells. Therefore, we suggest that the DF extract protects against 6-OHDA-induced neuronal damage in B35 neuroblastoma cells partially via scavenging ROS and inhibiting p-quinone. However, the intracellular redox imbalance is mainly due to an imbalance between generating and eliminating the free radicals, specifically caused by reduced intracellular antioxidant defenses. Neuronal intracellular antioxidant defenses mainly include several antioxidants and protective enzymes that prevent ROS formation or detoxify ROS. GSH recycling includes GSH and related enzymes, such as glutathione peroxidase (GPx) and GR, and is a major intracellular antioxidant defense. In PD patients, the degree of symptom severity correlates with intracellular GSH loss in substantia nigra. Many oxidants, such as 6-OHDA and H₂O₂, could deplete the intracellular GSH levels and decrease GPx and GR activation in cell culture and animals. Our results are consistent with the above reports; incubation of B35 neuroblastoma cells with 50 µM 6-OHDA for 24 h decreased the GSH cycle activities and increased the oxidative damage. The DF extract at 100 µg mL⁻¹ reversed the GSH cycle activities that were decreased by 50 µM 6-OHDA in B35 neuroblastoma cells, which in turn, decreased the oxidative damage. Hence, we suggest that the DF extract attenuated the 6-OHDA-induced neuronal damage by upregulating the antioxidant status via intracellular GSH regeneration and its radical scavenging activity. In fact, naringin which is a major DF extract constituent also protected against the neuronal damage caused by 3-nitropropionic acid or kainic acid via antioxidant activity and intracellular GSH regeneration. Certain reports further indicated that naringin and epicatechin have neuroprotective
activities against rotenone, aluminum and amyloid β peptide in vitro and in vivo. Based on the above results, we suggest that DF is a potential medicinal plant that protects against PD in a comparison between two commercial Polydiaceae plants.

AKT plays a pivotal role in fundamental cellular functions, such as cell proliferation and survival, by phosphorylating a variety of enzymes, including pro-apoptotic regulators, detoxifying and antioxidant proteins, and transcription factors. AKT is mainly activated through phosphorylation of T308 (AKT (thr)) and S473 (AKT (ser)) by receptor tyrosine kinases, G-protein-coupled receptors, mTOR complex 2 (mTORC2), DNA-dependent protein kinase (DNA-PK), and other stimuli that induce phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P3) production through PI3K. Hence, certain reports indicate that AKT is an important therapeutic target for treating diabetes, stroke, and neurodegenerative diseases. In this study, we found that treating with 6-OHDA for 24 h decreased the levels of PI3K and AKT phosphorylation, especially at S473, in B35 neuroblastoma cells. Hence, 6-OHDA mainly seems to inhibit 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. Recently, mounting evidence indicates that a pharmacological inducer of HO-1 expression may maximize the intrinsic antioxidant potential of cells. NQO-1 is a key enzyme that detoxifies reactive quinones produced from autoxidation and enzymatic oxidation of dopamine. 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. 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 higher phenolic levels, especially for phenylpropanoids, and exhibited 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 phenolic antioxidants, such as naringin and epicatechin. The DF extract has a protective effect against 6-OHDA-induced neuronal damage in vitro. Naringin and epicatechin are its major active compounds because both can protect against neuronal damage caused by rotenone and amyloid β peptide. This protective mechanism might be related to its radical scavenging capacity and its ability to activate intracellular antioxidant defenses, including GSH recycling as well as HO-1 and NQO-1 via phosphorylation in the PI3K/AKT pathway (Fig. 5). Hence, we suggest that DF extract has potential therapeutic benefits for treating aging-associated symptoms and neurodegenerative disorders. However, the expressions of detoxifying and antioxidant enzymes such as GSH-related enzymes, HO-1 and NQO-1 are coordinated and induced via a nuclear factor-E2-related factor 2 (Nrf2)-dependent and antioxidant response element (ARE)-mediated mechanism. Recent studies have reported that Nrf2 is a master redox regulator that upregulates HO-1 to protect dopaminergic neurons against 6-OHDA-induced neurotoxicity. Studies have also suggested that Nrf2 nuclear translocation requires activation of several signal transduction pathways, such as PI3K/AKT or mitogen-activated protein kinase (MAPK) pathways. In addition, naringin is a major active compound in DF extract and has also been shown to protect against 3-nitropropionic acid-induced apoptosis via downregulating the pro-apoptotic gene Bax and upregulating the anti-apoptotic genes Bcl-2 and Bcl-X(L). The expression of pro-apoptotic genes is also modulated by AKT. Therefore, the anti-apoptotic effects from DF extract and the role of pro-apoptotic genes and Nrf2 translocation mediated by PI3K/AKT in the neuroprotective effects from DF extract must be further investigated.

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References