Garcinia xanthochymus extract protects PC12 cells from H₂O₂-induced apoptosis through modulation of PI3K/AKT and NRF2/HO-1 pathways

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[ABSTRACT] The aim of the present study was to investigate the protective effects and underlying mechanisms of Garcinia xanthochymus, a perennial medicinal plant native to Yunnan, China, against H₂O₂-induced oxidative damage in rat pheochromocytoma PC12 cells. Preincubation of PC12 cells with fruit EtOAc fraction (fruit-EFr., 12.5–50 µmol·L⁻¹) of G. xanthochymus for 24 h prior to H₂O₂ exposure markedly improved cell viability and increased the activities of antioxidant enzymes (superoxide dismutase, catalase, and heme oxygenase-1 [HO-1]), prevented lactate dehydrogenase release and lipid peroxidation malondialdehyde production, attenuated the decrease of matrix metalloproteinases (MMP), and scavenged reactive oxygen species (ROS). Fruit-EFr. also reduced BAX and cytochrome C expression and improved BCL-2 expression, thereby decreasing the ratio of BAX to BCL-2. Fruit-EFr. activated the nuclear translocation of NRF2 to increase HO-1 and induced the phosphorylation of AKT. Its cytoprotective effect was abolished by LY294002, a specific inhibitor of PI3K. Taken together, the above findings suggested that fruit-EFr. of G. xanthochymus could enhance cellular antioxidant defense capacity, at least in part, through upregulating HO-1 expression and activating the PI3K/AKT pathway and that it could suppress H₂O₂-induced oxidative damage via PI3K/AKT and NRF2/HO-1 signaling pathways.

[KEY WORDS] Garcinia xanthochymus; Oxidative stress; PC12; PI3K/Akt pathway; Nrf2/HO-1 signaling pathways

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Introduction

Clinical studies have shown that there is a dramatic increase in reactive oxygen species (ROS) in specific regions in the brain tissue from patients with neurodegenerative diseases, including Parkinson’s disease and Alzheimer’s disease (AD) [1-2]. Overproduction of ROS can result in an oxidative stress situation, damaging cellular structures including proteins, lipids, and DNA, which contributes to the pathophysiology of many diseases such as neurodegenerative disorders [3]. ROS are derived from molecular oxygen, but exhibit an increased reactivity. ROS include hydrogen peroxide, superoxide anions, hydroxyl radicals, and peroxyl radicals [4].

The rat adrenal pheochromocytoma cell line (PC12) has been used as a model neuronal-like cell line for studying neurodegenerative diseases [5-7]. Exogenous H₂O₂ leads to oxidative stress and induces apoptosis of PC12 cells [8-9]. Oxidative stress is involved in neurotoxicity, neuroprotection, and neuronal repair [10]. Therefore, the regulation of ROS and apoptosis has become a research target for the prevention and treatment of neurodegenerative diseases.

Recently, an antioxidant defense mechanism involving NFE2 p45-related factor 2 (NRF2) signaling to induce en-
zymes against ROS has been reported as a new strategy for neuroprotection [11]. Previous studies have indicated that several signaling kinases such as extracellular signal-regulated kinase, mitogen activated protein kinase, and phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB, AKT), contribute to the nuclear translocation and transcriptional activation ability of NRF2 [12]. Among these pathways, the PI3K/AKT signaling pathways play a key role in the expression of NRF2-mediated downstream genes [13]. Thus, compounds that are capable of manipulating these pathways may provide protection against oxidative damage to the PC12 cells (14-16). However, the precise protective mechanism against oxidative stress is unclear.

A previous study has shown that flavonoids have the ability to stimulate the differentiation of PC12 cells [9]. Recently, it is shown that flavonoids activate the NRF2/HO-1 pathways and attenuate the production of ROS in RAW 264.7 cells [17]. Identification of phytochemicals with neurotrophic activities is a promising alternative for the treatment of neurodegenerative diseases [10].

Garcinia xanthochymus, a tree that can grow up to 10–20 m, belongs to the Garcobe family. It is distributed widely in Yunnan, Guangxi, Guangdong, Hainan, Taiwan, and subtropical coastal areas of China. It is used as an insecticide, cathartic, for detoxification and for other purposes as a traditional medicinal plant [18]. Previous phytochemical investigations have revealed that the fruit, leaves, pulp juice, stem bark, and seeds of this tree contain a number of flavonoids, triterpenoids, and xanthones [19-22]. Pharmacology experiments have indicated that extracts from G. xanthochymus, especially its fruit, have potent antioxidant properties [20,21,22]. However, the precise protective mechanism of the fruit EtOAc fraction (fruit-EFr.) of G. xanthochymus against oxidative stress is unclear. Therefore, we hypothesized that the protective function of G. xanthochymus may involve mechanisms other than radical scavenging and antioxidant activities. Herein, we evaluated the cytoprotective effect of fruit-EFr. against neurotoxic H2O2-induced oxidative stress, and assessed whether the PI3K/AKT and NRF2/HO-1 pathways contributed to the protective effect of G. xanthochymus in PC12 cells.

Materials and Methods

Materials

The cell line PC12 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 culture medium, horse serum, and fetal bovine serum were from Hyclone (Thermo Scientific, Logan, UT, USA). Phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, H2O2, penicillin, streptomycin, Hochest 33258, 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA), and MTT were from Sigma Chemical Co (St. Louis, MO, USA). JC-1, radioimmunoprecipitation assay lysis buffer, and BCA protein assay kit were from Beyotime (Haimen, China). Lipid peroxidation malondialdehyde (MDA), LDH (lactic dehydrogenase), CAT (catalase), and total superoxide dismutase (T-SOD) assay kits were from Nanjing Jiancheng (Nanjing, China). Antibodies against BAX, BCL-2, NRF2, phospho-AKT, and AKT were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against cytochrome C and HO-1 were from Epitomics (Burlingame, CA, USA). Antibodies against β-actin and the ECL chemiluminescent substrate were from Wuhan Boster Biological Co., Ltd. (Wuhan, China). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA, USA).

Preparation of the extracts of G. xanthochymus leaves and fruits

The leaves and fruits of G. xanthochymus were obtained from Xishuangbanna Prefecture, Yunnan Province, China, and were identified by Prof. ZHAO Yin-Hong from the Xishuangbanna Ethnic Medicine Research Institute (Yunnan, China). The dried leaves and fruits of G. xanthochymus were powdered and extracted three times with 95% ethanol at room temperature. The ethanol extracts from the leaves and fruits were then partitioned with petroleum ether, EtOAc, and n-BuOH through liquid-liquid extraction. Each fraction, including petroleum ether (PFr), ethyl acetate (EFr), n-butanol (BFr), and the water fraction (WFr), were stored in a dry cabinet. The four fractions from each part of G. xanthochymus were then subjected to antioxidant analysis. Each extract was dissolved in DMSO (Biosharp, Hefei, China) as a stock solution and further diluted to its final concentrations when needed. The final concentration of DMSO added to every well culture was less than 0.5%.

Total phenolic content

Aliquots of the extracts were used for testing the total phenolic content present in the samples. A sample of 100 µL was added to 1 mL of 10% (F/P) Folin-Ciocalteu, incubated for 5 min, to which 1 mL of 10% Na2CO3 was then added. The mixture was incubated at 37 °C for 60 min before reading the absorbance at 760 nm using a microplate reader (Biochrom Asys UVM 340 Microplate, Cambridge, UK). All the aliquots of samples and Gallic acid standards were analyzed in triplicate. Gallic acid equivalents were established in milligrams per 100 mg of dry fraction.

Cell culture and treatment

The PC12 cells were cultured in RPMI 1640 medium containing 5% horse serum, 10% fetal bovine serum, 100 U·mL−1 of penicillin, and 100 µg·mL−1 of streptomycin in a humidified incubator at 5% CO2, and the culture medium was changed every 2 or 3 days. In all experiments, the cells were pretreated for 30 min with or without different concentrations of extracts (final concentration, 12.5, 25, or 50 µg·mL−1) or quercetin (25 µmol·L−1), which served as a positive control, before the addition of H2O2 (final concentration, 400 µmol·L−1) for an additional 4-h incubation.

Measurements of cell viability and LDH

The cells were seeded in 96-well plates at a density of 2 × 104 cells/well. After 24 h of subculture, the cells were
exposed to extracts or quercetin for 30 min; after 400 µmol·L⁻¹ H₂O₂ was added and the incubation was continued for 4 h. Extracts and quercetin were applied at concentrations demonstrated to be non-toxic to the cells. After the treatment, the culture medium was aspirated and retained for LDH determination. The rate of absorbance was read at 490 nm on a microtiter plate reader. The data were expressed as the mean percent of LDH release from the maximum control. The viability of cells was assessed using MTT assay (final concentration, 0.5 mg·mL⁻¹) [24], and the value for each treatment group was converted to a percentage of the control.

**Assays for antioxidant enzymes and lipid peroxidation**

The PC12 cells (2 × 10⁵ cells/mL) were plated in culture plates and cultured for 24 h. After experimental treatments, the cells were lysed on ice and centrifuged (12 000 r·min⁻¹, 10 min) at 4 °C. The supernatants were collected for enzyme activity assays. The CAT, superoxide dismutase (SOD), and MDA activities were determined using kits according to the manufacturer’s suggested protocols.

**Determination of mitochondrial transmembrane potential (△Ψm)**

JC-1 was used for detecting mitochondrial transmembrane potential (MMP). The PC12 cells were treated with 400 µmol·L⁻¹ of H₂O₂ for 4 h after extracts or quercetin pretreatment. Then, they were incubated with 10 µmol·L⁻¹ of JC-1 at 37 °C in dark, followed by washing with JC-1 buffer solution (1 ×) twice. Flow cytometry was used to analyze the change of △Ψm. The results of flow cytometry were analyzed using Flowjo software (Tree Star, San Carlos, CA, USA) and were expressed as % relative green fluorescence as compared with that of the total cells.

**Determination of intracellular ROS**

After the cells were cultured with or without pretreatments, they were collected, re-suspended in phosphate-buffered saline, and incubated with DCFH-DA at a final concentration of 10 µmol·L⁻¹ for 30 min at 37 °C. Then, the cells were washed with phosphate-buffered saline and harvested for fluorescence-activated cell sorting analysis. These cells were excited with a 488-nm argon ion laser in a flow cytometer (BD FACSCalibur™Flow Cytometry, BD Bioscience, San Jose, CA, USA). The data were collected with at least 10 000 events.

**Western blotting analysis**

After incubation, the cells were dissolved in radio-immunoprecipitation assay lysis buffer and boiled at 100 °C for 5 min with 5 × protein loading dye. The samples were then subjected to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins in the gel were transferred onto an immobilon-polyvinylidene difluoride membrane and the membrane was incubated with the primary antibody overnight (HO-1 1 : 1 000; NRF2 1 : 500) at 4 °C. Then, the membranes were washed with TBST (20 mmol·L⁻¹ of Tris-HCl, pH 7.4, 137 mmol·L⁻¹ of NaCl, and 0.05% Tween-20) three times at room temperature for 10 min each. Horse radish peroxidase conjugated anti-rabbit or anti- mouse IgG was used as the secondary antibody, and the membrane was incubated at room temperature for 2 h. The proteins were detected with BeyoECL Plus gel imaging system (Bio-Rad, Hercules, CA, USA). β-actin was used as an internal loading control to normalize the amount of protein analyzed.

**Statistical analyses**

The data were presented as means ± SD of three independent experiments and were analyzed with Prism 5.0 software (Graph Pad Software, San Diego, CA, USA). One-way analysis variance (ANOVA) was applied to determine the significance of differences among different groups. The differences were considered statistically significant when P < 0.05 and highly significant when P < 0.01.

**Results**

**Contents of total phenols**

The total phenolic contents of fractions from G. xanthochymus are shown in Table 1. The phenolic contents of different parts from G. xanthochymus decreased in the following order: leaf-EFr. > leaf-BFr. > leaf-PFr. > leaf-WFr.; fruit-EFr. > fruit-PFr. > fruit-BFr. > fruit-WFr. The ethyl acetate fraction from leaves had the highest content of total phenols (32.22% ± 1.05%). In the present study, ethyl acetate seemed to be the best solvent that concentrated the most phenolic substances of intermediate polarity [25].

<table>
<thead>
<tr>
<th>Plant part</th>
<th>Phenols/(mg GA/100 mg)a</th>
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<tbody>
<tr>
<td>Fruit</td>
<td></td>
</tr>
<tr>
<td>PFr.</td>
<td>8.23 ± 0.37</td>
</tr>
<tr>
<td>EFr.</td>
<td>10.35 ± 0.79</td>
</tr>
<tr>
<td>BFr.</td>
<td>2.10 ± 0.12</td>
</tr>
<tr>
<td>WFr.</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td>Leaf</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.80 ± 0.24</td>
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<tr>
<td></td>
<td>32.22 ± 1.05</td>
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<td></td>
<td>14.90 ± 0.60</td>
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<td>1.05 ± 0.07</td>
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*a mg gallic acid equivalent per 100 mg of the fraction.

**Fruit-EFr. protective effects against H₂O₂-induced cytotoxicity**

To investigate the potential protective effects of the extracts against H₂O₂-induced cytotoxicity, the injury effects of H₂O₂ at different concentrations on the PC12 cells were measured using an MTT assay. In preliminary studies, H₂O₂ was applied to cells at various concentrations (50, 100, 200, 400, and 800 µmol·L⁻¹) and cell viability was assessed at various times (4, 8, 12, and 24 h). H₂O₂ could gradually decrease the cell viability as the concentration increased (data not shown). However, there was no difference in treatment with H₂O₂ concentrations greater than 400 µmol·L⁻¹ for different times. A 4-h treatment of 400 µmol·L⁻¹ H₂O₂ decreased the cell viability to 51.20%, compared with the vehicle group. Therefore, we used a moderate concentration of H₂O₂ (400 µmol·L⁻¹) for a 4-h exposure to assess extract-mediated protection.
As shown in Fig. 1, pretreatment with fruit-PFr., fruit-EFr., and leaf-BFr. and quercetin (at 25 µmol·L\(^{-1}\) as the control) significantly resulted in a dose-dependent protection against \(\text{H}_2\text{O}_2\)-induced cytotoxic effects (\(P < 0.001\)); fruit-PFr., fruit-EFr., and leaf-BFr at concentrations of 50 µmol·L\(^{-1}\) for 4 h before the co-culture with \(\text{H}_2\text{O}_2\), restoring the cell viability to 96.14% ± 2.6%, 93.57% ± 3.1%, and 69.99% ± 2.2%, respectively. Treatment with extracts significantly decreased the LDH release (Fig. 3). Our results showed that fruit-PFr. and fruit-EFr. extracts of \(G. \text{xanthochymus}\) could suppress \(\text{H}_2\text{O}_2\)-induced cytotoxicity in PC12 cells at a concentration of 50 µg·mL\(^{-1}\). The results indicated that the fruit EtOAc fraction (fruit-EFr.) might be the most active part of \(G. \text{xanthochymus}\) in decreasing the release of LDH.

**Effects of fruit-EFr. on antioxidant activities and lipid peroxidation caused by \(\text{H}_2\text{O}_2\)**

Lipid peroxidation is one of the earliest recognized and most extensively studied manifestations of oxygen toxicity in biology. In the present study, the MDA level was quantified by the thiobarbituric acid reactive substances assay. A significant increase by 116.1% ± 4.9% of the MDA level compared with the control group was observed in PC12 cells exposed to 400 µmol·L\(^{-1}\) \(\text{H}_2\text{O}_2\) for 4 h (\(P < 0.01\)). However, when the cells were pretreated with 12.5–50 µg·mL\(^{-1}\) fruit-EFr. or 25 µmol·L\(^{-1}\) quercetin for 24 h, the MDA levels were reduced by 32.2% ± 2.4%, 52.1% ± 1.3%, 60.6% ± 0.8%, and 49.6% ± 2.5% respectively, as compared with the PC12 cells treated with \(\text{H}_2\text{O}_2\) (\(P < 0.05\); Fig. 4A).

Antioxidant enzymes, such as SOD and CAT, are the primary defense substances to protect living systems from oxidative damage. Exposure of the PC12 cells to \(\text{H}_2\text{O}_2\) (400 µmol·L\(^{-1}\)) for 4 h significantly reduced the amount of CAT (Fig. 4C) and the activity of SOD (Fig. 4B) to 8.2% ± 0.9% and 31.2% ± 1.6%, respectively, as compared with the control group (\(P < 0.001\)). Pretreatment of the PC12 cells with fruit-EFr. or quercetin for 24 h significantly attenuated the decrease in CAT and SOD activity induced by \(\text{H}_2\text{O}_2\) (\(P < 0.01\)).

**Effects of fruit-EFr. on intracellular ROS caused by \(\text{H}_2\text{O}_2\)**

The effects of fruit-EFr. or quercetin on the intracellular ROS levels of the PC12 cells were examined by flow cytometric DCFH-DA assays to measure DCF fluorescence. Exposure of the cells to 400 µmol·L\(^{-1}\) \(\text{H}_2\text{O}_2\) for 4 h significantly increased the intracellular ROS level to 237% ± 5.9% of the control (Fig. 5). Such intracellular ROS accumulation was significantly eliminated by pretreatment (24 h) with fruit-EFr. or quercetin (\(P < 0.05\)). Fruit-EFr. reduced the level of ROS by 59% ± 2.4%, while quercetin (25 µmol·L\(^{-1}\)) reduced the level of ROS by 56% ± 3.2% in \(\text{H}_2\text{O}_2\)-treated PC12 cells.

**Effects of fruit-EFr. on the ratio of BAX/BCL-2 and the release of cytochrome C**

The BCL-2 family members are important mediators of apoptosis. To investigate the effects of fruit-EFr. on BCL-2, BAX, and cytochrome C activity after \(\text{H}_2\text{O}_2\) exposure, the PC12 cells were treated with 400 µmol·L\(^{-1}\) of \(\text{H}_2\text{O}_2\) for 12 h. The BAX/BCL-2 ratio was increased significantly to 6.2 ± 0.4 compared with the control group (\(P < 0.01\)), and the release of cytochrome C from the mitochondria to the cytosol was increased significantly (\(P < 0.01\)) (Fig. 6). However, the BAX/BCL-2 ratio in the PC12 cells pre-incubated with 50 µg·mL\(^{-1}\) fruit-EFr. almost returned to the normal levels and so did the amount of released cytochrome C.
Fig. 2 Effects of fruit-EFr. on the MMP level in PC12 cells treated with H$_2$O$_2$. Cells were pretreated with 12.5–50 μg·mL$^{-1}$ fruit-EFr. or 25 μmol·L$^{-1}$ quercetin for 30 min and then incubated in the presence of 400 μmol·L$^{-1}$ H$_2$O$_2$ for 4 h. MMP was measured using a JC-1 assay kit. Results were expressed as the ratio of red and green fluorescence and are shown as means ± SD ($n = 3$). Data were compared with control or H$_2$O$_2$ groups by one-way ANOVA tests. Significant differences ($P < 0.05$) were observed in the H$_2$O$_2$-induced cytotoxicity.

Fig. 3 After pretreatment with extracts of G. xanthochymus, LDH activity in culture supernatants was measured with a colorimetric LDH assay kit. The results were expressed as the values of absorbance at 570 nm and the percentage (%) of LDH leakage to the external medium, respectively. Results shown are means ± SD ($n = 3$). Data were compared with the control or H$_2$O$_2$ groups by one-way ANOVA tests. Significant differences ($P < 0.05$) were observed in the H$_2$O$_2$-induced cytotoxicity. *$P < 0.01$ vs control; **$P < 0.01$ vs H$_2$O$_2$

Protective ability of fruit-EFr. via modulation of the PI3K/AKT pathway

The PI3K/AKT pathway plays an important role in the regulation of many important cell functions, such as maintaining the cell’s viability and the antioxidant system [26]. To further investigate whether extracts of G. xanthochymus prevented H$_2$O$_2$-induced injury by activating the PI3K/AKT pathway, we evaluated intracellular PI3K/AKT pathway activation by detecting phosphorylated AKT (pAKT) expression. We used PI3K inhibitor treatments for further verification. We found that fruit-EFr. significantly up-regulated pAKT expression, similar to quercetin pretreatment (Fig. 7A). Then, we used a specific PI3K inhibitor, LY294002, for further confirmation (Fig. 7A). After pretreatment with (10 μmol·L$^{-1}$) for 0.5 h, AKT phosphorylation or activation by fruit-EFr. was completely blocked. This suggested that the PI3K/AKT pathway might play a key role in the protective ability of fruit-EFr.

Effects of Fruit-EFr. on HO-1 expression and the nuclear accumulation of NRF2

HO-1 is considered an important enzyme for cellular defense against oxidative stress. Thus, we used Western blotting analysis to evaluate whether fruit-EFr. could induce HO-1 expression in the PC12 cells. The exposure of the PC12 cells to fruit-EFr. for 24 h upregulated the expression of HO-1 in a concentration-dependent manner. NRF2 is a transcription factor that can bind the ARE sequence of promoters, and it is responsible for regulating HO-1 gene expression. Therefore, we explored whether fruit-EFr. could activate NRF2 and be involved in the induction of HO-1 expression. As shown in Fig. 7B, Western blotting analysis indicated that fruit-EFr. obviously enhanced HO-1 expression and NRF2 accumulation in the nucleus. In other words, it might significantly increase NRF2-mediated transcriptional activation. These findings suggested that the induction of HO-1 and NRF2-mediated ARE activation by fruit-EFr. might be linked to its antioxidative properties.
Fig. 4  Effects of fruit-EFr. on the MDA, T-SOD, and CAT levels in PC12 cells treated with H\textsubscript{2}O\textsubscript{2}. Cells were pretreated with 12.5–50 μg·mL\textsuperscript{−1} fruit-EFr. or 25 μmol·L\textsuperscript{−1} quercetin for 24 h and then incubated in the presence of 400 μmol·L\textsuperscript{−1} H\textsubscript{2}O\textsubscript{2} for 4 h. Activities of MDA (A), T-SOD (B), and CAT (C) were measured using an acolorimetric assay kit. Results shown are means ± SD (n = 3). Data were compared with the control or H\textsubscript{2}O\textsubscript{2} groups by one-way ANOVA tests. Significant differences (P < 0.05) were observed on the H\textsubscript{2}O\textsubscript{2}-induced cytotoxicity. *P < 0.01 vs control; *P < 0.05 vs H\textsubscript{2}O\textsubscript{2}; **P < 0.01 vs H\textsubscript{2}O\textsubscript{2}.

Fig. 5  Fruit-EFr. effects on H\textsubscript{2}O\textsubscript{2}-induced ROS production in PC12 cells. Cells were pretreated with 12.5–50 μg·mL\textsuperscript{−1} fruit-EFr. or 25 μmol·L\textsuperscript{−1} quercetin for 24 h and then incubated in the presence of 400 μmol·L\textsuperscript{−1} H\textsubscript{2}O\textsubscript{2} for 4 h. Intracellular levels of ROS were measured with DCFH-DA. Results shown are means ± SD (n = 3). Data were compared with the control or H\textsubscript{2}O\textsubscript{2} groups by one-way ANOVA tests. Significant differences (P < 0.05) were observed in the H\textsubscript{2}O\textsubscript{2}-induced cytotoxicity. *P < 0.05 vs control; *P < 0.01 vs H\textsubscript{2}O\textsubscript{2}; **P < 0.01 vs H\textsubscript{2}O\textsubscript{2}. 
Fig. 6  Fruit-EFr. effects on the ratio of BAX/BCL-2 and the release of cytochrome C. (A) BAX, BCL-2, and cytochrome C (B) protein were detected by Western blotting. Results shown are means ± SD (n = 3). Data were compared with the control or H_2O_2 groups by one-way ANOVA tests. Significant differences (P < 0.05) were observed on the H_2O_2-induced cytotoxicity. **P < 0.01 vs control; * P < 0.05 vs H_2O_2; ** P < 0.01 vs H_2O_2.

Fig. 7  Effects of Fruit-EFr. on HO-1 expression and the nuclear accumulation of NRF2. (A)pAKT, AKT, HO-1, and NRF2. (B) protein were detected by Western blotting. Results shown are means ± SD (n = 3). Data were compared with the control or H_2O_2 groups by one-way ANOVA tests. Significant differences (P < 0.05) were observed in the H_2O_2-induced cytotoxicity. **P < 0.01 vs control; * P < 0.05 vs H_2O_2; ** P < 0.01 vs H_2O_2.

Discussion

Increasing evidence supports the idea that oxidative stress is implicated in the mechanism of cellular injuries in a variety of human diseases, including neurodegenerative disorders [27]. Many researchers have focused on non-vitamin antioxidants, such as polyphenols, that can scavenge free radicals and protect cells from oxidative damage with few side effects [28]. Intake of antioxidants is one feasible way to prevent oxidative stress-mediated cellular injuries to augment the oxidative defense capacity [29]. Our previous work [20-21, 23] has identified antioxidant activity in extracts of *G. xanthochymus*. These properties, along with the multiple aforementioned bioactivities of polyphenols, make *G. xanthochymus* interesting plants in the search for new natural products to treat neurodegenerative diseases.

H_2O_2 is one of the major ROS and is widely used as an inducer of oxidative stress in vitro [30]. PC12 cells derived from a rat pheochromocytoma have been widely used for neurological studies [31-32]. In the present study, the H_2O_2-induced PC12 cells are used as a model for neurological study. Significant increases in ROS accumulation and lipid peroxidation were observed in H_2O_2-treated PC12 cells. H_2O_2-induced ROS accumulation was efficiently removed by
fruit-EFr. or quercetin. This finding was consistent with earlier reports that H$_2$O$_2$ induces lipid peroxidation in rat neurons [31]. Lipid peroxidation is essential for assessing the role of oxidative injury in pathophysiological disorders [34-35]. Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of saturated or unsaturated lipids [36].

Our results from the present study showed that the fruit-EFr. of _G. xanthochymus_ decreased the MDA content and the production of LDH in the PC12 cells, improved their SOD activity, and protected the PC12 cells against injuries caused by exposure to H$_2$O$_2$. Moreover, treatment with extracts from _G. xanthochymus_ showed a decrease in ROS generation and lipid peroxidation and an increase in cell viability after H$_2$O$_2$ treatment.

As a traditional medicine, _G. xanthochymus_ has long been advocated as a way to protect against cancer, oxidative stress, inflammation, and diabetes [38], but few studies have evaluated its efficacy and possible mode of action. The present study demonstrated that fruit-EFr. effectively protected the PC12 cells from H$_2$O$_2$-induced injury in a dose-related manner.

Cells have several antioxidant mechanisms that act as a detoxifying system to prevent ROS-induced damage. In this respect, SOD maintains a very low steady-state intracellular level of superoxide [37], H$_2$O$_2$ can be removed by CAT. The elimination of H$_2$O$_2$ is critical for reducing oxidative stress, and the combined action of CAT provides a repair mechanism for oxidized membrane components [38]. Our present studies showed that cells treated with H$_2$O$_2$ had a marked decrease in cell survival and an elevation of oxidative stress characterized by MDA production, LDH release, and a reduction in SOD and CAT activities. However, when the PC12 cells are pre-incubated with fruit-EFr., these H$_2$O$_2$-induced cellular events were blocked. Our results also showed that fruit-EFr. was as efficient as quercetin in protecting the PC12 cells against H$_2$O$_2$-induced oxidative damage.

Western blotting analysis revealed that BAX and cytochrome C expression were increased in the PC12 cells under H$_2$O$_2$ stress. However, they were significantly reduced by fruit-EFr. (25 or 50 µg·mL$^{-1}$) or quercetin (25 µmol·L$^{-1}$). We found that H$_2$O$_2$ induced the activation of BAX and cytochrome C in the PC12 cells. The expression of H$_2$O$_2$-activated BAX and cytochrome C were reduced by fruit-EFr. The expression of BCL-2 was increased by fruit-EFr. at the same time, which can inhibit apoptosis. In the present study, we demonstrated that fruit-EFr. could suppress H$_2$O$_2$-induced apoptosis and inhibit the release of cytochrome C from the mitochondria to the cytoplasm. It also could activate the phosphorylation of AKT and promote the expression of NRF2 and HO-1.

LY294002, a specific inhibitor of PI3K, was used to explore the linkage between the protective effects of fruit-EFr. on the PC12 cells exposed to hydrogen peroxide and the PI3K signaling cascade. When the cells were pretreated with LY294002, the phosphorylation of AKT was inhibited. Our results showed that fruit-EFr. enhanced the phosphorylation of AKT and induced the differentiation of the PC12 cells, and it might be involved in PI3K-mediated signaling pathways that are vital to cell viability. By activating PI3K/AKT signaling, the mRNA expression of HO-1 [39], GPx activity [40], and catalase activity [41] are increased. These are important mechanisms for promoting cell survival.

We also focused on the up-regulation of the NRF2/HO-1 pathway. HO-1, one of the phase II cytoprotective enzymes, is a ubiquitous and redox-sensitive inducible stress protein [42] that is transcriptionally regulated by a variety of stimuli. It has been widely accepted that increasing HO-1 expression represents an adaptive response that confers resistance to oxidative injury [43]. Our data suggested that fruit-EFr. might modulate PI3K/AKT and NRF2/HO-1 signaling to up-regulate the antioxidant system.

In conclusion, the results of the present study demonstrated that fruit-EFr. could protect PC12 cells from H$_2$O$_2$-induced damage. The protective ability may be mediated via PI3K/ AKT and NRF2/HO-1 signaling modulation. Owing to its cell-protective effects and the data from previous studies [20-21, 23] supporting the fact that the edible and medicinal fruit of _G. xanthochymus_ can clearly acts as a protective agent against oxidative stress, we suggest it has a potential use as a dietary supplement in the management of diseases where oxidative stress mechanisms are implicated; however, clinical efficacy must be demonstrated first in the future.

References


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