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ORIGINAL PAPER

Antioxidant Activity and Protective Effects of *Tripterygium regelii* Extract on Hydrogen Peroxide-Induced Injury in Human Dopaminergic Cells, SH-SY5Y

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Abstract The present work was conducted to investigate the antioxidant activity and neuroprotective effects of *Tripterygium regelii* extract (TRE) on H_2O_2-induced apoptosis in human dopaminergic cells, SH-SY5Y. TRE possessed considerable amounts of phenolics (282.73 mg tannic acid equivalents/g of extract) and flavonoids (101.43 mg naringin equivalents/g of extract). IC_{50} values for reducing power and DPPH radical scavenging activity were 52.51 and 47.83 μg, respectively. The H_2O_2 scavenging capacity of TRE was found to be 57.68 μM × μg^{-1} min^{-1}. By examining the effects of TRE on SH-SY5Y cells injured by H_2O_2, we found that after incubation of cells with TRE prior to H_2O_2 exposure, the H_2O_2 induced cytotoxicity was significantly reversed and the apoptotic features such as change in cellular morphology, nuclear condensation and DNA fragmentation was inhibited. Moreover, TRE was very effective attenuating the disruption of mitochondrial membrane potential and apoptotic cell death induced by H_2O_2. TRE extract effectively suppressed the up-regulation of Bax, Caspase-3 and -9, and down-regulation of Bcl-2. Moreover, TRE pretreatment evidently increased the tyrosine hydroxylase (TH) and brain-derived neurotrophic factor (BDNF) in SH-SY5Y cells. These findings demonstrate that TRE protects SH-SY5Y cells against H_2O_2-induced injury and antioxidant properties may account for its neuroprotective actions and suggest that TRE might potentially serve as an agent for prevention of neurodegenerative disease associated with oxidative stress.

Keywords Parkinson disease · *Tripterygium regelii* · Dopaminergic cell · Hydrogen peroxide · Apoptosis

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting over six million people worldwide [1]. Pathologically, PD is characterized by the marked degeneration of dopaminergic (DArgic) neurons in the substantia nigra pars compacta. Although the mechanism underlying selective degeneration of DArgic neurons is not known completely, the data indicate that oxidative stress has been reported to play important roles [2, 3]. Accumulating evidences show that oxidative stress is one of the important pathways leading to neuronal cell death in PD. Although the source of increased oxidative stress is not completely known, environmental factor, excitotoxin, dopamine homeostasis and others have gained more attention [4]. Oxidative stress may induce mitochondrial dysfunction, genetic mutation and protein aggregation, and ultimately cause cell death [5]. Oxidative damage mediated by reactive oxygen species (ROS) which can be generated following cell lysis, oxidative burst, or the

presence of an excess of free transition metals, can attack protein, deoxyneuclic acid, and lipid membranes, thereby disrupting cellular function and integrity [6, 7]. Among a great variety of ROS, hydrogen peroxide (H_2O_2) is known to play a pivotal role because it is generated from all sources of oxidative stress and can diffuse freely in and out of cells and tissues [8]. In the brain several antioxidant molecules such as ascorbate, superoxide dismutase, and glutathione peroxidase can remove ROS and protect against oxidative stress. Therefore, therapeutic strategies aimed at preventing or delaying ROS induced apoptosis might be reasonable choice for treatment of neurological diseases.

Recently, attention has been focused on searching for natural substances with neuroprotective potential that can scavenge free radicals and protect cell from oxidative damage. *Tripterygium regelii* (*T. regelii*) Sprague et Takeda is one of the most common traditional medicinal herb, native to Korea and Japan. The herb *T. regelii* has been used in traditional Korean medicine for centuries [9] and has been reported to have various pharmacological properties including anti-cancer and anti-inflammatory effects [10–12]. Diterpene-quinononones and triterpenoids were reported from *T. regelii* and have been shown that triptoquinone A and B inhibited growth of P-3888 leukemia cells in vitro [13, 14]. Over 300 compounds have been identified in the genus *Tripterygium* and many of them have been evaluated for biological activity [15]. *Tripterygium willfordii* Hook.F. is among the most studied plant of the genus *Tripterygium*. Nevertheless, no information is available regarding the effect of *T. regelii* extract against the pathogenesis of PD. Therefore, this study was designed to explore the antioxidant activity of *T. regelii* extract and evaluate its neuroprotective effects against H_2O_2-induced DArgic neuronal damage.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium:Nutrients Mix F-12 (1:1, DMEM/F-12), Fetal Bovine Serum (FBS), penicillin and streptomycin were obtained from Gibco BRL (Gaithersburg MD, USA). 1, 1–diphenyl-2-picryl hydrazyl (DPPH), butylated hydroxytoluene (BHT) and ascorbic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), DMSO, 4′, 6-diamidino-2-phenylindole (DAPI), Propidium iodide (PI) and Rhodamine 123 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Moloney-murine leukemia virus ribonuclease (M-MLV), oligo dT (deoxythymidine) primer, dNTPs (deoxynucleic acid triphosphate), Taq polymerase, specific primers (for TH, BDNF, and Actin) and 100 bp DNA ladder were purchased from the BioNEER Co. (Korea). Anti-TH antibody was purchased from Affinity BioReagents, Inc. (Golden, CO, USA). Anti-BDNF, Caspase-3, Caspase-9, Bcl-2, and Bax, antibodies were obtained from SANTA CRUZ (Santa cruz, CA, USA). Anti-actin antibody was purchased from Biomeda crop (Foster City, CA, USA). WEST-ZOL plus was obtained from INTRON biotech (Seongnam, Korea). Bicinchoninic acid protein assay kit was obtained from Pierce (Rockford, IL, USA). Protein inhibitor cocktail was obtained from Calbiochem (Darmstadt, Germany). Cytotoxicity Detection Kit (LDH assay) was purchased from Roche Applied Science (Rotkreuz, Switzerland). DeadEnd™ Fluorometric TUNEL System was purchased from Promega coporation (Madison, WI, USA).

Preparation of *Tripterygium regelii* Extract

T. regelii was collected from the Jiri-mountain, Jeollanam-do, Republic of Korea in the month of April and was authenticated by Professor Myung-Kon Kim, Department of Bio-food technology, Chonbuk National University, South Korea. A voucher specimen was deposited at Chonbuk National University. Two hundred gram of fresh *T. regelii* leaves were extracted with 2l of methanol at room temperature for 8 week. The extract was filtered through Whatman No. 1 filter paper, and concentrated using a rotary vacuum evaporator. The concentrate was freeze-dried and its yield was 1.4%. The final extract was a dark brown powder. This powder was then dissolved in phosphate buffered saline (PBS) and filtered through 0.2 μm membrane filter (Millipore, Bedford, MA, USA) and stored at 4°C. It was designated as TRE.

Determination of Total Flavonoid Content

The total flavonoid content was determined according to the method of Moreno et al., [16] with some modifications. Briefly, 100 μl of TRE was diluted with 80% aqueous ethanol (900 μl). An aliquot of 100 μl was added to eppendorf tubes containing 20 μl of 10% aluminum nitrate, 20 μl of 1 M potassium acetate and 860 μl of 80% ethanol. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoid concentration was calculated using naringin as standard.

Determination of Total Phenolic Content

The total phenolic content of each extract was estimated by a colorimetric assay based on procedures described by Singleton and Rossi [17] with some modifications. Briefly, a stock solution of extract was prepared by dissolving 1 mg

of TRE in 1 ml of pure water. 25 µl of sample from stock solution was mixed with 500 µl Folin-Ciocalteu reagent. After 5 min, 500 µl of Na_2CO_3 (7.5% w/v) solution was added and the mixture was allowed to stand for 90 min with intermittent shaking. After, 90 min, the absorbance was measured at 725 nm. The final results were expressed as mg of tannic acid equivalents (TAE) per *g* of samples [18].

DPPH Radical Scavenging Activity

Free radical-scavenging activity of the extracts was estimated according to the method reported by Blois [19] with some modifications. In brief, samples were dissolved in absolute methanol and then centrifuged to remove insoluble materials. Ascorbic acid, quercetin and BHT were used as standards. 90 µl of 0.3 mM DPPH in methanol was mixed with 10 µl of TRE solutions with different concentrations (10, 50, 100, 300, and 500 µg/ml). The 96-well plate was allowed to stand at room temperature for 30 min. A control was prepared as described above without TRE, or standards. Methanol was used for baseline correction. The changes in the absorbance of all the samples and standards were measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Radical scavenging activity was calculated using the following formula:

$$\text{Radical scavenging activity}\,(\%) = (OD_{control} - OD_{samples})/OD_{control} \times 100$$

Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide (H_2O_2) scavenging activity was carried out according to the method of Aebi [20] with some modifications. Briefly, 990 µl of 75 mM phosphate buffer (pH 7.0) containing 25 mM H_2O_2 and 10 µl of 0.2 mg/ml TRE were mixed together. The mixture was incubated at 37°C for 2 min, and the absorbance was measured at 240 nm. Specific absorption coefficient of H_2O_2 $-0.03408\ cm^{-1}mM^{-1}$ was used for calculation. Results were determined using the following equation:

$$H_2O_2 \text{ scavenging activity} = \text{decreasing } H_2O_2\ (\mu M)/\{\text{sample weight (mg)} \times \text{reaction time (min)}\}$$

Reducing Power

The reducing power of extracts was determined according to the method of Oyaizu [21] with some modification. Briefly, 200 µl of TRE at various concentrations (10, 50, 100, 300, 500 µg/ml) were mixed with 0.2 M phosphate buffer solution (200 µl, pH 6.6) and 1% potassium ferricyanide (200 µl). The mixture was incubated at 50°C for 20 min. A portion (200 µl) of 10% trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 12,000×*g* for 10 min. The upper layer of solution (500 µl) was mixed with deionized water (500 µl) and 0.1% of ferric chloride (100 µl) in eppendorf tube, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid and quercetin were used as standard.

Cell Culture and Treatments

The human DA neuronal cell line, SH-SY5Y was obtained from ATTC (Rockville MD). Cells were cultured in DMEM/F12 medium (GIBCO, Gaithersburg) supplemented with 10% FBS and penicillin (100 units/ml)-streptomycin (100 µg/ml) at 37°C in 5% CO_2. Media were changed every 2 days. To examine possible toxic effects, SH-SY5Y cells were treated with the TRE extract in a concentration ranging from 1.25 to 20 µg/ml for 24 h. Similarly, cells were treated with H_2O_2 at concentrations ranging from 25 to 400 µM for 24 h. 10 µg/ml of TRE extract, which was non toxic and 100 µM H_2O_2 was chosen to evaluate the neuroprotective effects by examining cell viability. TRE extract was added 30 min prior to treatment with H_2O_2. In a single experiment each treatment was performed in triplicate.

Analysis of Cell Viability

Cell viability was determined by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. SH-SY5Y cells were seeded in 96-well plates at a density of 1×10^4 cell/well and incubated for 24 h prior to experimental treatments. The cells were then subjected to the treatments of interest. After 24 h incubation, MTT (0.5 mg/ml) was added to each wall. Following an additional 3 h incubation at 37°C, 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 540 nm using a VERSAmax micro plate reader (Molecular Devices, CA, USA). Wells without cells were used as blanks and were subtracted as background from each sample. Results were expressed as a percentage of control.

Lactate Dehydrogenase Release Assay

Cells dying by apoptosis or necrosis released Lactate Dehydrogenase (LDH) into the supernatant. The amount of LDH in the supernatant was measured with a cytotoxicity detection kit (Roche). Briefly, the cells (1×10^4 cell/well) were seeded in 96 well plates and then treated with H_2O_2 for 24 h after pre-treated with or without TRE extract for 30 min. For analysis, 100 µl supernatant was extracted from each well and was placed in separate wells of a new

96-well plate, and 100 μl catalyst solutions was added to each well and incubated at 37°C for 30 min. Absorbance was measured at 490 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Total cellular LDH was determined by lysing the cells with 2% Triton X-100 (high control); the assay medium served as a low control and was subtracted from all absorbance measurements; Cytotoxicity (%) = (exp.value − low control)/(high control − low control) × 100

Observations of Morphological Changes

Cells were seeded in 8-well chamber slide and then treated with H_2O_2 for 24 h after pre-treated with or without TRE extract for 30 min. The cells were washed twice with PBS and then fixed in 1% paraformaldehyde for 15 min. After rinses with PBS, cellular morphology was observed using a phase contrast microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed.

Nuclear Staining for Assessment of Apoptosis

Nuclear morphology was assessed by staining with 4′, 6-diamidino-2-phenylindole (DAPI).

The cells (1×10^3 cells/well) were seeded in 8-well chamber slide and then treated with H_2O_2 for 24 h after pre-treated with or without TRE extract for 30 min. The cells were washed twice with PBS and then fixed in 1% paraformaldehyde for 15 min. After two rinses with PBS, the cells were stained with DAPI (0.3 μM) for 10 min at 37°C in dark. Slides were washed twice with PBS and examined under fluorescent microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed.

TUNEL Assay

For in situ detection of fragmented DNA, TUNEL assay was performed using DeadEnd™ Fluorometric TUNEL System (Promega coporation, USA). Cells were cultured on 8-well chamber slide at a density of 1×10^3 cells/chamber. After treatment with H_2O_2 and TRE for 24 h, cells were washed with PBS and fixed by incubation in 4% paraformaldehyde for 20 min at 4°C. The fixed cells were then washed and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After rinses with PBS, the cells were incubated with terminal deoxynucleotidyl transferase recombinant (rTdT)-catalyzed reaction and nucleotide mixture for 60 min at 37°C in dark and then immersed in stop/wash buffer for 15 min at room temperature. The cells were then washed with PBS to remove unincorporated fluorescein-12-dUTP. After washing, cells were incubated in 1 μg/ml propidium iodide (PI) solution for 15 min in dark. The cells were observed with fluorescent microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined using the fluorescent dye Rhodamine 123. Briefly, the cells were treated with H_2O_2 for 24 h after pre-treated with or without TRE for 30 min. Cells were washed with PBS and fixed by incubation in 4% paraformaldehyde for 15 min at room temperature. After rinses with PBS, the fixed cells were incubated with 10 μg/ml Rhodamine 123 for 60 min at 37°C. The cells were washed and monitored by fluorescent microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed. The fluorescence intensity was determined using a Spectra Max Gemini EM fluorometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm excitation and 515 nm emission.

RNA Isolation and Reverse Transcription

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, USA) following the protocol provided by the company. Two micrograms of total RNA samples were reverse transcribed for each sample to be analyzed by incubation with a reverse transcription mixture containing the following constituents: 10 pmol oligo (dT) primer, 1× PCR buffer, 0.1 M DTT, 10 mM dNTPs, 20 units of RNase inhibitor, and 200 units of M-MLV. The reaction mixture was incubated for 60 min at 42°C, followed by 5 min at 70°C to inactivate the Reverse transcription (RT) enzyme. The quality of cDNA was verified by PCR amplification of β-actin.

Polymerase Chain Reaction (PCR) and the Analysis of PCR Products

The cDNA in the RT product was amplified using Taq DNA polymerase. A PCR reaction was performed in 20 μl of the total volume using 10 pmol of the following primers-TH, F: 5′- GAG GAG AAG GAG GGG AAG-3′ R: 5′- TCC AAG TCC AGG TCA GGG TC-3′; BDNF, F: 5′- GAT GAC CAT CCT TTT CCT TAC TAT GG-3′ R: 5′- CTA TCT TCC CCT TTT AAT GGT CAA T-3′; β- actin, F: 5′- CCT CTA TGC CAA CAC AGT-3′ R:5′- AGC CAC CAA TCC ACA CAG-3′. The cDNA was amplified under the following reaction conditions: denaturation at 94°C for 30 s, annealing at 57°C for 45 s for TH, at 55.8°C for BDNF and at 56°C for β-actin, polymerization at 72°C for 30 s. The cyclic process was performed 35 times for TH, BDNF and 30 times for β-actin. The PCR products were analyzed on 1.2% agarose gel and visualized by EtBr. The stained intensity of individual

bands was evaluated by Gel Quant software (DNR Bio-Imaging Systems Ltd.).

Immunoblotting

After treatment, cells were washed once with PBS and then lysed using ice-cold RIPA buffer with protease inhibitor cocktail. Cell lysates were centrifuged at 12,000×*g* for 25 min, and the protein concentrations were determined by the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as standard. The proteins were separated by 10.5% SDS–PAGE and transferred to polyvinylidine difluoride (PVDF) membrane. The Membrane was blocked with 5% (v/v) nonfat dry milk in Tris-buffered saline with Tween 20 (TBS-T) (10 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) and incubated with primary antibody for Bcl-2 (1:2000 dilution), Bax (1:1000 dilution), Caspase 3 (1:1000 dilution), Caspase 9 (1:2000 dilution), TH and BDNF (1:1000 dilution), or Actin (1:4000 dilution) for overnight at 4°C. The membrane was washed in TBS-T and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody. To reveal the reaction bands, the membrane was reacted with WEST-ZOL (plus) Western blot detection system (Intron Biotechnology, Inc., Korea) and exposed on X-ray film (BioMax MS-1, Eastman Kodak, USA).

Statistical Analysis

The data were expressed as the means ± SD. Statistical significance was assessed with one-way analysis of variance followed by a post- hoc (Bonferroni) test for multiple group comparison. Differences with *P* value less than 0.05 were considered statistically significant.

Results

Total Phenolic and Flavonoid Content

As one of the most important antioxidant plant components, phenolic compounds are widely investigated in many medicinal plant and vegetables [22]. Although most antioxidant activities from plant sources are derived from phenolic-type compounds [23], these effects do not always correlate with the presence of large quantities of phenolics. Therefore, both sets of data need to be examined together. For this, the extracts were analyzed for total phenolic and flavonoid contents. The amounts of total phenolic and flavonoid contents are shown in Table 1. The concentration of total phenolics in extracts was estimated by the Folin-Ciocalteu procedure which is considered as the best method for total phenolics determination [24]. The total phenolic content in the TRE was 282.73 ± 10.58 mg tannic acid equivalents/g of extract. The total flavonoid content was estimated by the aluminum nitrate colorimetric method and was found to be 101.43 ± 2.60 mg naringin equivalents/g of extract.

Antioxidant Activity

The antioxidant activity of TRE was evaluated by DPPH radical scavenging activity, H_2O_2 scavenging activity, and its reducing power. DPPH is a stable free radical donor, which has been widely used to test the free radical scavenging effect of natural antioxidants [25]. As shown in Fig. 1a, 52.05 ± 3.61% of DPPH radicals were scavenged when the concentration of TRE was 50 μg/ml, which is, however, higher than BHT (27.48 ± 3.98%) and lower than that of ascorbic acid (92.67 ± 2.40%), a potent antioxidant to scavenge radicals and quercetin (69.30 ± 0.91), a known flavonoid. In addition, the scavenging activity of these extracts increased with the increase in their concentrations from 10 to 500 μg/ml. The average IC_{50} concentrations for the TRE, quercetin and vitamin C were found to be 47.83 ± 0.26 μg, 41.83 ± 8.94 μg and 3.24 ± 0.15 μg, respectively (Table 1). These results clearly indicated that TRE must be a potent source of compounds that can donate hydrogen atoms to act as an antioxidant.

Fe(III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties [26]. Figure 1b shows the reducing powers of the TRE. Like the DPPH radical

Table 1 Phenolic contents and antioxidant activities of TRE

Sample	Flavonoid compound (mg NE/g)	Phenolic compound (mg TAE/g)	DPPH IC50 (μg)	Reducing power lC50 (μg)	Hydrogen peroxide scavenging activity (μM × μg^{-1} min^{-1})
TRE	101.43 ± 2.60	282.73 ± 10.58	47.83 ± 0.26	52.51 ± 0.21	57.68 ± 3.61
Vit C	–	–	3.24 ± 0.15	24.00 ± 0.32	+
Quecctin	–	–	41.83 ± 8.94	43.21 ± 0.96	+

Each value represents the mean ± SD of three determinations

NE naringin equivalents; *TAE* Tannic acid equivalents

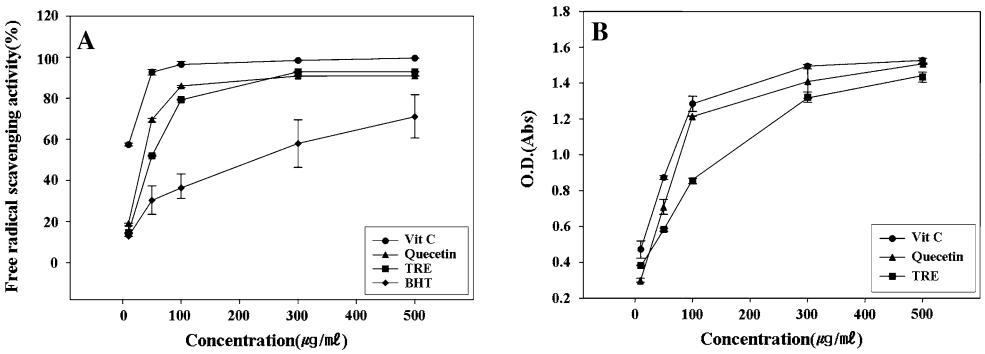

Fig. 1 **a** Scavenging effects of TRE, BHT, ascorbic acid (Vit C) and quercetin on DPPH radical, and **b** reducing power of TRE, Vit C, and quercetin. Each value represents the mean ± SD of three determinations

scavenging activity, the reducing power of TRE was concentration dependent. The reducing power of TRE was 0.854 ± 0.015 at 100 μg/ml and 1.322 ± 0.028 at 300 μg/ml. However, ascorbic acid showed slightly higher activity with a reducing power of 1.299 ± 0.028 and 1.477 ± 0.026 at 100 and 300 mg/ml, respectively. The IC_{50} of TRE was 52.51 ± 0.21 μg while that for quercetin and vitamin C were 43.21 ± 0.96 μg, 24.00 ± 0.32 μg, respectively (Table 1). These results demonstrate the electron donor properties of TRE for neutralizing free radicals by forming stable products.

H_2O_2 is one of the reactive oxygen species. The H_2O_2 scavenging activity was determined as a measure of the antioxidant activity of TRE. As can be seen from Table 1, TRE had an effective H_2O_2 scavenging capacity. Scavenging activity of TRE was found to be 57.68 ± 3.61 $\mu M \times \mu g^{-1}\ min^{-1}$.

These data showed that TRE could be a source of compounds with potent antioxidant activity.

Effects of TRE Against H_2O_2-Induced Cytotoxicity

Initial experiments were first performed to determine whether H_2O_2 alone or TRE alone was toxic to human dopaminergic cells, SH-SY5Y. Cells were exposed to various concentrations of H_2O_2 (25, 50, 100, 200, and 400 μM) for 24 h and cell survival was assessed by MTT assay. As shown in Fig. 2a, H_2O_2 induced a dose dependent cytotoxicity in SH-SY5Y cells. In the presence of 100 μM H_2O_2, there is only 58.98% of viable cells as compared to control cells. To evaluate whether TRE influences neuronal cytotoxicity, SH-SY5Y cells were treated with various concentrations of TRE (1.25, 2.5, 5, 10, and 20 μg/ml) for 24 h. These concentrations of TRE did not show any cytotoxicity in SH-SY5Y cells. Consequently, the treatment of 100 μM H_2O_2 and 10 μg/ml of TRE for 24 h were chosen in the subsequent experiments.

Next, we attempted to determine the effects of TRE on neuronal protection via on MTT reduction assay. To determine the protective effects of TRE against H_2O_2-induced loss of cell viability, SH-SY5Y cells were pre-treated with 10 μg/ml TRE extract for 30 min, followed by 100 μM H_2O_2 for 24 h. As shown in Fig. 2b, H_2O_2 induced loss of cell viability was significantly attenuated by TRE treatment. The effects of TRE could also be confirmed by the morphological observation (Fig. 2c). Morphological changes were observed in SH-SY5Y cells when treated with 100 μM H_2O_2 for 24 h (Fig. 2c, b). In this case, the survival cells showed a reduction of cytoplasm and diminution of tack on the plate, in contrast to the typical morphology of cells presented by the untreated control group (Fig. 2c, a). This effect was remarkably reverted when the cells were exposed to H_2O_2 in the presence of TRE (Fig. 2c, d), indicating that TRE offered protection to the H_2O_2 induced damage in SH-SY5Y cells.

To further investigate the protective effect of TRE, the release of LDH was measured (Fig. 2d). LDH release is increased as the number of dead cells increases. As shown in Fig. 2d, release of LDH was increased significantly after exposure to 100 μM H_2O_2, indicating that H_2O_2 caused cytotoxicity in SH-SY5Y cells. In contrast, TRE-treated cells showed decreased release of LDH compared with H_2O_2-exposed cell group. The protective effect of TRE on H_2O_2-induced cytotoxicity determined by LDH assay was similar to that determined by MTT assay. TRE rescued the viability of cells against the neurotoxicity induced by H_2O_2, suggesting the protective effect of TRE.

Effects of TRE Against H_2O_2-Induced Apoptosis

Cell body shrinkage, nuclear condensation, and DNA fragmentation are hallmarks of apoptosis. We investigated whether TRE extract prevents apoptosis induced by H_2O_2 in SH-SY5Y cells. DAPI staining revealed that the control SH-SY5Y cells exhibited normal regular and oval shaped nuclei (Fig. 3a). The nuclear morphology of cells exposed to TRE alone was intact and similar to that of untreated control cells (Fig. 3c). However, H_2O_2-treated cells (Fig. 3b) exhibited highly condensed and fragmented nuclei morphologies, characteristics of apoptosis. In contrast, TRE pre-treatment inhibited these apoptotic features (Fig. 3d). Further, H_2O_2 was shown to induce apoptosis by

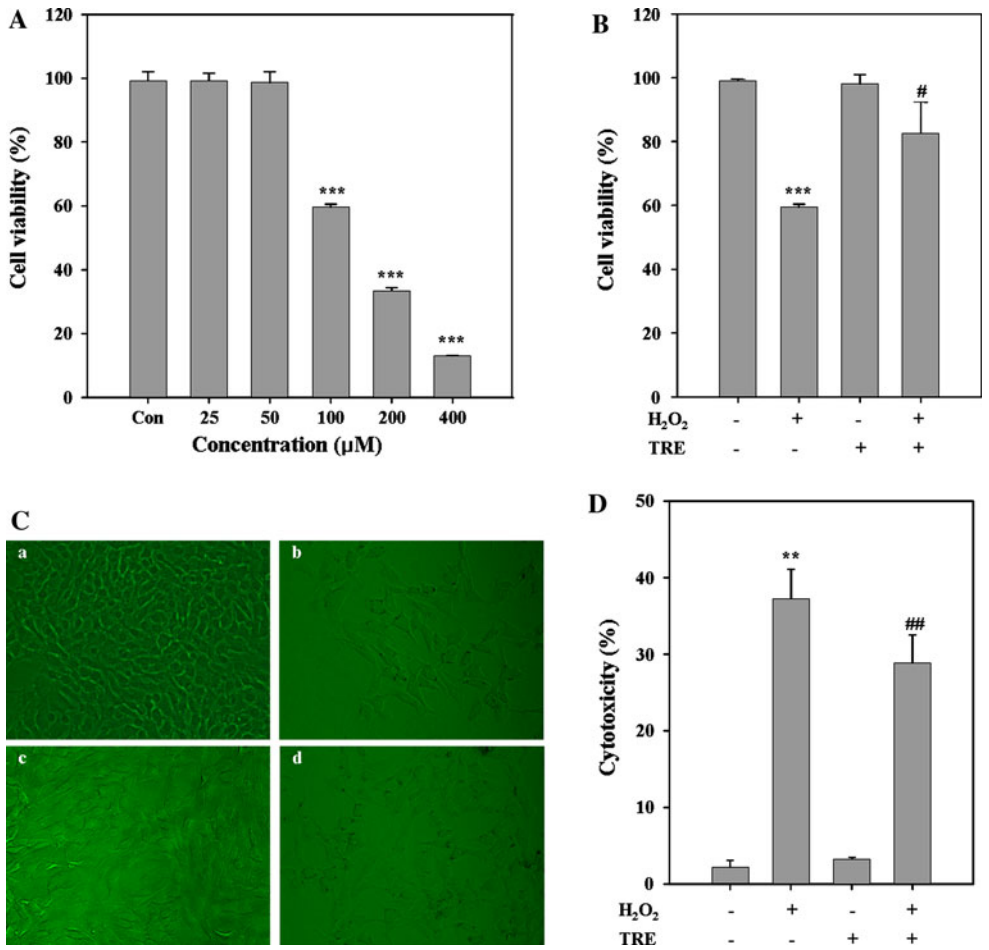

Fig. 2 Neuroprotective effects of TRE on H_2O_2 induced cytotoxicity in SH-SY5Y cells. **a** Dose-dependent effects of H_2O_2 on SH-SY5Y cell viability. SH-SY5Y cells were exposed to different concentration of $H_2O_2$for 24 h. Cell viability was assessed using MTT assay. **b** Protective effect of TRE on H_2O_2-induced cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were pre-treated with 10 μg/ml TRE for 30 min then treated with H_2O_2 (100 μM) for 24 h and Cell viability was measured using the MTT assay. **c** Effects of TRE on H_2O_2 induced morphological alterations in SH-SY5Y cells. Morphological studies were conducted by phase-contrast microscopy. Control cells without any treatment (a), 100 μM H_2O_2 (b), 10 μg/ml TRE (c), and cells pretreatment with 10 μg/ml TRE for 30 min then treatment with H_2O_2 (100 μM) (d). (D) SH-SY5Y cells were pre-treated with 10 μg/ml TRE for 30 min then treated with H_2O_2 (100 μM) for 24 h and Cell toxicity was measured by LDH assay. The data are represented as means ± SD of three independent experiments. Con; untreated control. ** $P < 0.01$, *** $P < 0.001$ versus control group; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ versus H_2O_2 treated group

causing DNA strand breaks, as determined by TUNEL assay and PI staining (Fig. 3f, J). Pretreatment of TRE significantly inhibited the H_2O_2-induced apoptosis (Fig. 3h, L). These results demonstrate that TRE decreased level of cell death, nuclear condensation and DNA fragmentation and indicates that TRE has an anti-apoptotic effect in SH-SY5Y cells.

Effects of TRE on the H_2O_2-Induced Reduction of the Mitochondrial Membrane Potential

The diffusion and accumulation of Rodamine- 123 in mitochondria is proportional to the degree of Mitochondrial Membrane Potential (MMP) [27–29]. A collapse of the mitochondrial trans-membrane potential has been linked to several models for apoptosis [30]. To examine if H_2O_2 induced apoptosis and its rescue by TRE involve on MMP pathway in SH-SY5Y cells, measurement of MMP was carried out using Rodamine 123. The corresponding changes of fluorescence intensity of Rodamine-123 were measured and presented in Fig. 4b. As shown in Fig. 4, when SH-SY5Y cells were exposed to 100 μM H_2O_2 for 24 h, the mitochondrial membrane potential was significantly decreased, indicating the release of Rodamine-123 from mitochondria into the cytosol. However, the cells pre-incubated with TRE prior to the addition of H_2O_2, showed a markedly increased in the mitochondrial membrane potential as compared with H_2O_2-treated cells. These results showed that TRE extract suppressed the H_2O_2-induced decrease of mitochondrial membrane potential.

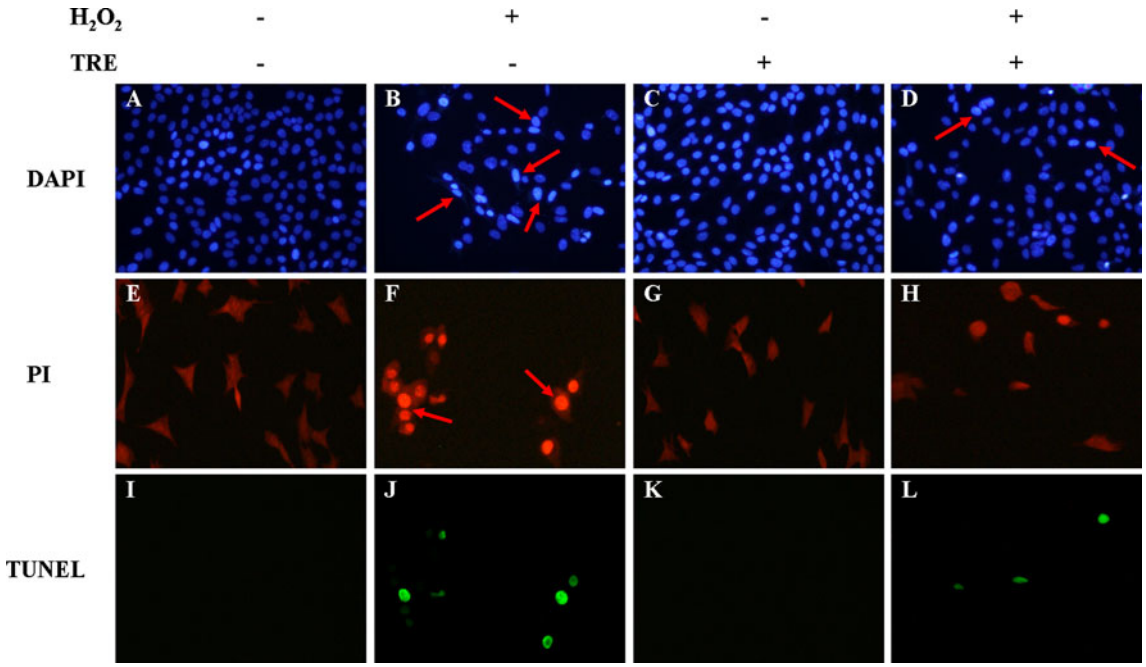

Fig. 3 Inhibition of H_2O_2 induced apoptosis in SH-SY5Y cells by TRE. Cells were pre-treated with TRE (10 μg/ml, 30 min) then treated with vehicle or H_2O_2 (100 μM 24 h) Morphological apoptosis was determined by staining with DAPI (A-D), TUNEL (I-L), and PI (E–H). *Arrows* indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic cells. TRE suppresses these apoptotic features. Each image is representative of three experiments. Pictures were taken using a fluorescent microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed at 100× magnification

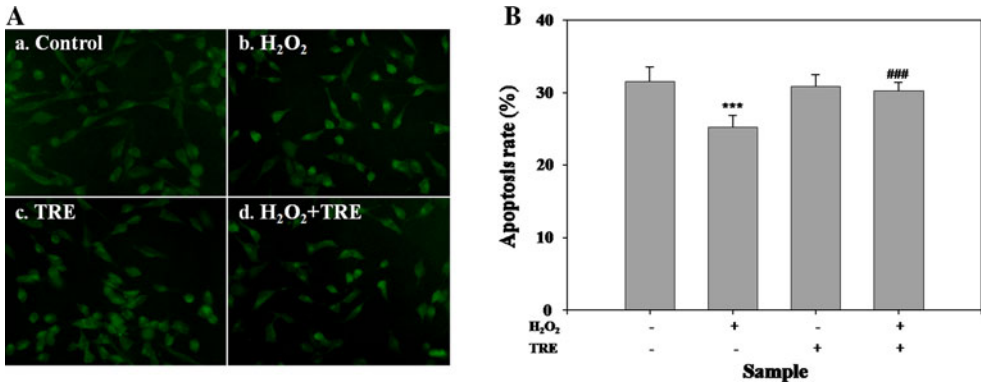

Fig. 4 Effect of TRE on H_2O_2-induced decrease of mitochondrial membrane potential. SH-SY5Y cells were pretreated with TRE (10 μg/ml) for 30 min followed by 100 μM $H_2O_2$for 24 h. Cells were incubated with Rhodamine 123 and the membrane potential was monitored by fluorescent microscope (Nikon, Eclipse TE 2000-U, Japan) (**a**). Control cells without any treatment (*a*), 100 μM H_2O_2 (*b*), 10 μg/ml TRE (*c*), and cells pretreatment with 10 μg/ml TRE for 30 min then treatment with H_2O_2 (100 μM) (*d*). The fluorescence intensity was determined using a Spectra Max Gemini EM fluorometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm excitation and 515 nm emission (**b**). The reduced fluorescence of Rhodamine 123 was determined as the reduced mitochondrial membrane potential. Results are expressed as mean ± SD of three independent experiments. *** $P < 0.001$ versus control group; ### $P < 0.001$ versus H_2O_2 -treated group

Effects of TRE on Bcl-2 and Bax Protein Expression in H_2O_2 Treated SH-SY5Y Cells

The Bax to Bcl-2 expression ratio can be used to determine whether a cell has undergone apoptosis. In H_2O_2-treated cells, the expression of Bcl-2 protein was downregulated whereas the expression of Bax protein was up-regulated (Fig. 5a, c), which resulted in a high Bax to Bcl-2 ratio, indicating that the H_2O_2-induced apoptosis in SH-SY5Y cells is probably mediated by the mitochondrial pathway. However, pretreatment with TRE attenuated the change in Bax and Bcl-2 that was induced by H_2O_2, resulting in a

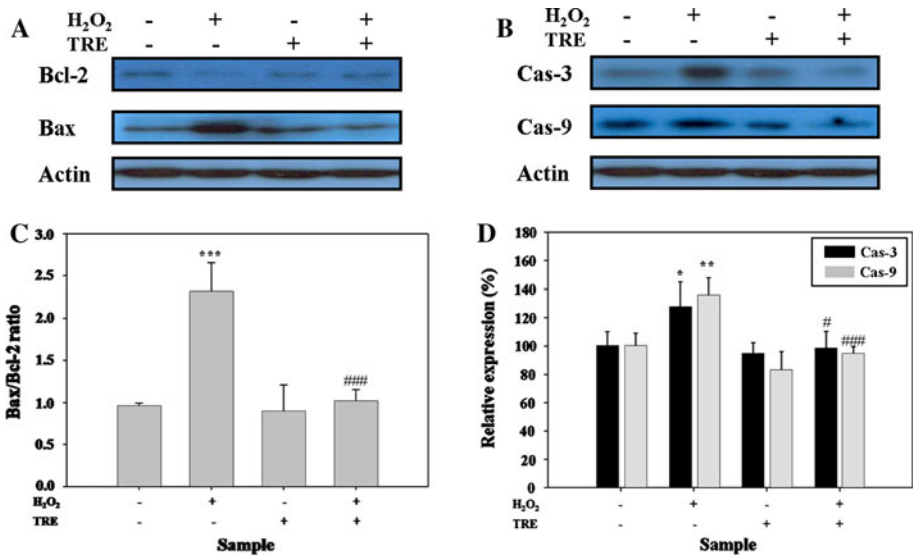

Fig. 5 TRE prevents H_2O_2 induced changes in the expression levels of apoptotic proteins. Effects of TRE on Bcl-2 and Bax expression (**a**, **c**). Effects of TRE on cleaved caspase-3 and cleaved caspase-9 expression (**b**, **d**). SH-SY5Y cells were pretreated with TRE (10 μg/ml) for 30 min followed by 100 μM H_2O_2 for 24 h. Expression of Bcl-2, Bax, caspase-3, and caspase-9 were assessed by immunoblots and intensity of each band was estimated by densitometric analysis. Actin was used as an internal loading control. Results are expressed as mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control; # $P < 0.05$, ### $P < 0.001$ versus H_2O_2 treated group

decrease in the Bax to Bcl-2 ratio. TRE extract treatment alone maintained the Bax to Bcl-2 ratio compared with control.

Effects of TRE on the H_2O_2 -Induced Caspase-3, and Caspase-9 Activation

We further investigated the effect of TRE on caspase signaling. Caspase-3 and caspase-9 play an important role in apoptosis, their expression levels were examined by Western blot. As shown in Fig. 5b and d, expression of Caspase-3 and Caspase-9 were markedly increased with the treatment of H_2O_2. In contrast, TRE pretreatment significantly attenuated the Caspase-3 and Caspase-9 expression in cells treated with H_2O_2. These results suggest that TRE inhibited downstream apoptotic signaling including the Caspase-3, and Caspase-9.

Effects of TRE on TH and BDNF Expression

As TH plays a key role as a rate-limiting enzyme in the dopamine biosynthesis pathway and BDNF regulates the proliferation, differentiation and survival of dopaminergic neurons, we examined the effect of TRE on TH and BDNF expression. As illustrated in Fig. 6a and b, the mRNA and protein levels of TH and BDNF were dramatically decreased by H_2O_2-treatment in SH-SY5Y cells. However, treatment with TRE notably induced the expression of these enzymes and maintained the TH and BDNF levels significantly even after H_2O_2-treatment.

Discussion

Progresses in understanding the pathogenesis of neurodegenerative disorders open new avenues for the development of potential neuroprotective therapeutic strategies. Many studies have shown that the oxidative stress is an important mediator of cellular damage in various neurological disorders including PD. Oxidative stress induces ROS such as H_2O_2 and superoxide anion which results in mitochondrial dysfunction, protein misfolding, genetic mutation and finally cell death [31]. Suppression of ROS by antioxidants might be an effective strategy in inhibiting oxidative stress-induced cell death. Therefore, the use of anti-oxidant agents as a way of neuroprotection could be a potential therapy to slow or ameliorate the progression of neurodegenerative diseases [32]. In this study, we found that TRE exhibited potent DPPH radical scavenging activity, H_2O_2 scavenging activity and strong reducing power. These results suggest that TRE could be a prominent natural source of strong antioxidant compounds.

Although the composition of TRE extract remains to be elucidated, previously a number of triterpenes, diterpenes, sesquiterpenes and alkaloids as a major compound have been isolated from this plant and their biological activities have been documented [15, 33–39]. Several studies have shown that celastrol, a quinone methide triterpenoid isolated from *T. regelii*, possesses various biological properties including chemopreventive, antioxidant, neuroprotective and anti-inflammatory actions [33, 37, 38]. Recently, Kim et al. [38] showed that celastrol effectively suppresses the

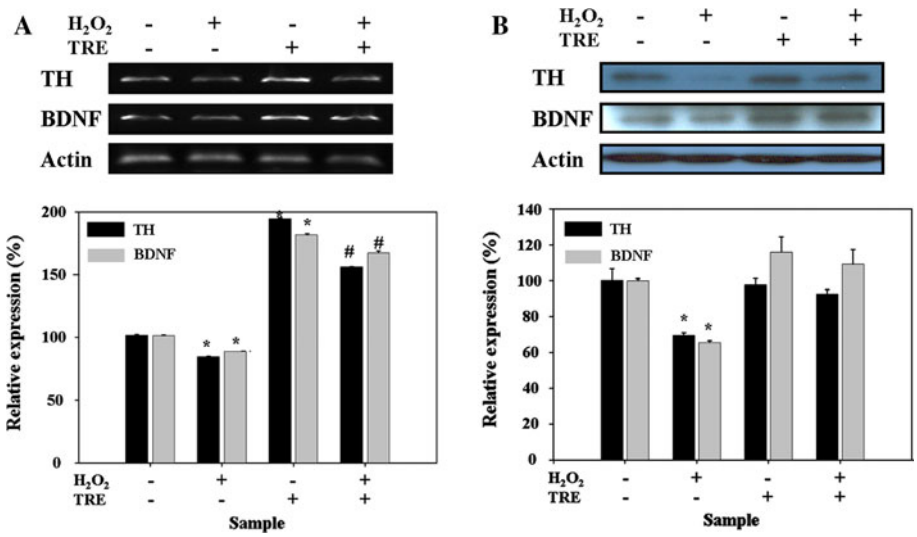

Fig. 6 Effects of TRE on TH and BDNF expression. SH-SY5Y cells were pretreated with TRE (10 μg/ml) for 30 min followed by 100 μM H_2O_2 for 24 h. Expression of the TH and BDNF were detected by PCR (**a**), and by immunoblot assay (**b**). Actin was used as an internal loading control. Results are expressed as mean ± SD of three independent experiments. * $P < 0.05$, compared with untreated control; # $P < 0.05$, versus H_2O_2 treated group

inflammatory responses accompanying the direct inhibition of nitric oxide, prostaglandin E_2, cyclooxygenase-2 and inflammatory cytokines in murine macrophages. Moreover, it has been reported that some quinone-methide triterpenes such as celastrol, pristimerin, tingenone, and iguesterin including celastrol isolated from *T. regelii* exhibited inhibitory effects for SARS-CoV $3CL^{pro}$ (severe acute respiratory syndrome coronavirus (SARS- CoV) [39]. Natural triterpenoid pristimerin was found to induce mitochondrial cell death through ROS-dependent activation of both Bax and PARP-1 in human cervical cancer cells [40]. In addition, regelin and regelinol, new antitumour ursine type triterpenoids have been reported from this plant [10]. Previous studies have shown the anticancer activities of some sesquiterpenes such as triptofordin F-2, triptogelin A-1, and triptogelin C-1 from *T. regelii* [41]. Furthermore, diterpene quinoids, triptoquinones A-F isolated from *T. regelii* have been reported to have anti-inflammatory activities [34, 42]. These studies have demonstrated the potential of TRE to reduce inflammation and autoimmune responses. However, its relationship with neurodegenerative disease is unexplored. In this study we found that TRE could modulate PD-related neurotoxins-induced cell death in human DArgic neurons.

Several studies have shown that H_2O_2-induces neuronal cell death with more or less necrotic and/or apoptotic characteristics depending on concentrations and exposure time [43–45]. High concentration of H_2O_2 -induces necrotic forms of cell death [46]. However, moderate concentrations of H_2O_2 induce DNA cleavage and are associated with morphological evidence of apoptosis [6]. Our results confirmed that treatment of SH-SY5Y cells with H_2O_2 resulted in a dose dependent viability loss (Fig. 2a). However, pre-treatment with TRE (10 μg/ml) efficiently prevented the loss of cell viability (Fig. 2b, d), which is further confirmed by morphological observations (Fig. 2c). These results indicated that TRE significantly protected SH-SY5Y cells from H_2O_2-induced cytotoxicity through the inhibition of both apoptotic and necrotic process. Further, in the present study, we showed a protective effect of TRE against H_2O_2-induced cell death in human DArgic cells, SH-SY5Y. Apoptosis is the process of cell death characterized by cell shrinkage, nuclear condensation, DNA fragmentation and membrane blabbing. These apoptotic features in situ were detected by DAPI, TUNEL and PI staining (Fig. 3). Interestingly, TRE significantly attenuated these features, indicating that TRE may possess an inhibitory effect on H_2O_2 induced apoptosis.

Apoptosis is mediated through extrinsic pathway by death receptor and intrinsic pathway by mitochondria. Ultimately, these pathways activate caspases, and activated caspases induce cell death. Bcl-2 family consists of two groups; anti-apoptotic group (Bcl-2 and Bcl-xL) and pro-apoptotic group (Bak, Bax, Bid), and they play an important role in mitochondrial related apoptosis pathway. Bcl-2, one of anti-apoptotic factor, residing in the outer mitochondrial membrane inhibits Cytochrome c release [47]. The pro-apoptotic factor Bax, resides in the cytosol. Translocation of Bax to the mitochondrial membrane might lead to loss of mitochondrial membrane potential and an increase in mitochondrial permeability. Increased mitochondrial permeability results in the release of Cytochrome c from the mitochondria [48]. Released Cytochrome c triggers activation of caspase-9 which in turn activates caspase-3, and activated Caspase-3 induces cell death. In this study we examined the modulation of the gene expression of Bcl-2 and Bax after treatments with H_2O_2. Our data reveal that Bax/Bcl-2 protein ratio is increased following a treatment with H_2O_2, but it decreases with the administration of TRE prior to H_2O_2. These results suggest

that TRE can indeed diminish H_2O_2-induced apoptotic cell death. Moreover, we demonstrated that TRE prevented depolarization of mitochondrial membrane potential induced by H_2O_2 as detected by Rodamine 123 (Fig. 4). In addition, our present findings show that H_2O_2 induces an activation of caspase-3 and caspase-9, while TRE can prevent these events, supporting a strong anti-apoptotic potential for TRE.

Altogether, our results demonstrate that the H_2O_2-induced apoptosis in SH-SY5Y cells can be reverted by TRE.

TH is the major rate limiting enzyme of catecholamine biosynthesis in DA and noradrenergic neurons [49]. The expression of TH plays a critical role in survival and differentiation of DArgic neurons. Dramatically reduced numbers of TH-positive neurons was observed in brains of primate and rodent models of Parkinson disease and increased activity of TH in parkinson rats resulted in effective DA production and relief of disease symptoms [50]. It has been reported previously that BDNF is the most important neurotrophic factor for the differentiation and survival of midbrain DA neurons [51, 52]. BDNF can protect DA neurons against neurotoxins in vivo and in vitro [53, 54]. Therefore, the increase of TH and BDNF expression induced by TRE treatment may contribute to their neuroprotective effects.

Although TH and BDNF were upregulated by TRE, the extent of increase differed among them. The degrees of induction were different at the transcriptional as well as translational level. In addition, there was clearly a quantitative difference between TH and BDNF mRNA and protein levels following TRE treatment. This disparity is not surprising since it is well known that the volume of mRNA resulting from gene expression commonly does not lead to equivalent quantities of the target protein [55]. Furthermore, these discrepancies may be due to differences in time course, mode of analysis and most importantly, the experimental conditions.

In conclusion, we found that TRE has DPPH radical scavenging activity, reducing power and H_2O_2-scavenging activity and neuroprotective effects. The mechanism underlying the protective effects of TRE in H_2O_2-injured SH-SY5Y cells might be related to the inhibition of apoptotic features such as change in cellular morphology, nuclear condensation and DNA fragmentation, maintenance of MMP stability, increased Bcl-2 activity and decrease of Caspase-3, caspase-9, and Bax activation through mitochondrial-dependent pathway. Moreover, TRE increased the TH and BDNF in SH-SY5Y cells. A probable underlying mechanism of this novel action of TRE may be associated with the presence of flavonoids in the extract, which are a source of antioxidants, since oxidative process are important in the pathogenesis of several disorders including PD. The components with potential antioxidant activity are promising candidates for use as new therapeutic agents against PD. Therefore, TRE may be a highly valuable candidate for the treatment of neurodegenerative disorders associated with oxidative stress.

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