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Neuroprotection and free radical scavenging effects of *Osmanthus fragrans*

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Abstract

The ethanol extract of dried flowers *Osmanthus fragrans* (OFE) was assessed for free radical scavenging effects measured by the bleaching of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical, scavenging of the hydroxyl anion, investigation of the ferric reducing/antioxidant power (FRAP) and lipid-peroxidation inhibition in rat tissues. OFE contained a high amount of total flavonoid and polyphenol. OFE presented the effects in the metal reducing power, FRAP assay with IC_{50} values of 0.23 μg/ml, and 7.74 μg/ml, respectively. OFE presented similar activities toward the DPPH and hydroxyl anion scavenging ability with IC_{50} values of 10 μg/ml. OFE with IC_{50} values between 46 and 97 μg/ml inhibited lipid peroxidation initiated by ferrous chloride in rat brain, liver, heart and kidney mitochodrias. Moreover, the neuroprotective activity of OFE was investigated under different insults (glutamate, arachidonic acid, and 6-hydroxydopamine) in Wistar rat primary cortical neurons. OFE with EC_{50} values between 66 and 165 μg/ml attenuated the neurotoxicity on MTT and LDH assays. In addition, the AKT protein expression of excitotoxicity and oxidative stress was displayed by western blotting analysis. OFE could up-regulate the glutamate and 6-OHDA decreased AKT expression. This is the first demonstration of the neuroprotective, free radical scavenging and anti-oxidative effects of *O. fragrans*.

Introduction

Osmanthus fragrans (Oleaceae), also known as sweet olive, is a flower native to China that is valued for its delicate fruity-floral apricot aroma. It is especially valued as an additive for tea and other beverages and is native to Asia which extends from the Himalayas east through China to Japan. *O. fragrans* is used in cosmetics for the hair and skin, but is mostly used for aromatic therapy. In addition, *O. fragrans* in medicinal uses with an anti-tussive of its flowers and its essential oil used as a flavouring [1].

In the past decade, antioxidants and free radical scavengers have shown their relevance in the prevention of pathologies such as atherosclerosis, heart diseases, stroke, cancer, and arthritis, in which reactive oxygen species or free radicals are implicated [2]. A characteristic of some of the brain disorders is that oxidative stress, resulting from increased production of reactive oxygen species (ROS), increases the rate at which a brain disease progresses. Oxidative stress is thought to be a key event in the pathogenesis of cerebral ischemia. Overproduction of reactive oxygen species during ischemia/reperfusion could cause an imbalance between oxidative and anti-oxidative processes. Reactive oxygen species can damage lipids, proteins, and nucleic acids, thereby inducing apoptosis or necrosis. There is increasing

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evidence supporting the hypothesis that plant polyphenols can provide protection against neurodegenerative changes associated with cerebral ischemia [3]. Thus, protecting brain cells from free radical damage could provide therapy for these and other degenerative brain diseases .[4]. For instance, in Parkinson's disease (PD), the deposition of 6-hydroxydopamine has been suggested as the central disease causing and promoting event and has been found to be mediated by oxidative stress. In addition, AKT may promote neuron survival after ischemia [5]. Moreover, protection by some agents and in transgenic and knock-out models involves maintained AKT activity after stroke [6, 7]. The extract of *O. fragrans* only exhibited a very limited of pharmacological effects. In this study we determined the *O. fragrans* ethanolic extract (OFE) on its free radical scavenging, anti-oxidative abilities. We also measured if OFE offered protective effects in primary cultured neurons derived from rat embryonic cortical brain cells against glutamate, arachidonic acid, and 6-hydroxydopamine-induced toxicity. The possible regulated survival mechanism potentially useful for stimulating an antioxidant defense that would prevent neuronal damage resulting from a variety of oxidative stress events was also investigated.

Experimental

Chemicals

Bovine serum albumin, anti-AKT antibody, anti-GADPH antibody, 2, 4, 6-tris(2-pyridyl)-*s*-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 6-hydroxydopamine, arachidonic acid, glutamate, ferrous chloride, γ-aminobutyric acid, hydrochloric acid, dimethyl sulfoxide, ethanol, EDTA, MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide), trypan blue, gallic acid, quercetin, rutin, and the chemicals were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Basal Eagle's Medium (BME), Fetal Bovine Serum (FBS), antibiotics, glutamine and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). All reagents and chemicals were analytical grade.

Plant material

The dried flowers of *O. fragrans* were purchased from a traditional crude drug market in Taipei. A voucher specimen (OF 001) was deposited in the Department of Pharmacognosy, Taipei Medical University, Taipei, Taiwan.

Animals

The Laboratory Animal Ethics Committee of the Taipei Medical University approved the study protocol. Wistar rats were purchased from the Center of Experimental Animals, National Taiwan University, Taiwan. Rats were housed in plastic cages with controlled temperature and humidity and bred at the experimental animal center in Taipei Medical University. All experiments were performed in accordance with the guidelines for animal experiments of Taipei Medical University and the guiding principles for the care and use of laboratory animals approved by the Chinese Society of Laboratory Animal Sciences, Taiwan. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Extraction and sample preparation

The dried flower of *O. fragrans* was refluxed and extracted twice with ten volumes of ethanol. The combined filtration was evaporated and concentrated under vacuum at 40 °C to about 1/10 volume and freeze dried to obtain the ethanolic extract of *O. fragrans* (OFE). Then it was dissolved in DMSO/PBS solution (final DMSO is under 0.1%) for analysis and evaluation of the following biological assays (free radical scavenging activity, antioxidant capacity and neuroprotecive evaluation).

Free radical scavenging and anti-oxidative ability measurement

Quenching of DPPH by O. fragrans

The free radical scavenging capacities of OFE was tested by their ability to bleach the stable 1,1-diphenyl-2 picryl-hydrazyl radical (DPPH). DPPH radical scavenging activity was investigated by the modified method of Blois [8]. Briefly, 0.4 ml of an ethanolic solution of 500 μM DPPH was mixed

with 0.5 ml of 100 mM Tris–HCl buffer, pH 7.4, and 0.1 ml of Tris–HCl buffer without (% scavenging tube) or with different concentrations of OFE (from 1 to 100 μg/ml). The tubes were incubated in the dark at room temperature for 20 min, and the absorbance was recorded at 528 nm by Elisa spectrophotometer (μQuant, Bio-Tek Instruments, INC). The antioxidant capacity is given as percent (%) DPPH scavenging as follows:

$$(\%\text{ of decoloration}) = \frac{1 - \text{absorbance of compound/extract}}{\text{absorbance of blank}} \times 100$$

The degree of decoloration indicates the free radical scavenging efficiency of the substances. For extracts, values are reported as mean ± SD of four determinations, the half-maximal inhibitory concentration (IC_{50}) was calculated by linear regression analysis and expressed as mean of three determinations. Gallic acid was used as a free radical scavenger reference compound.

Metal chelating activity of O. fragrans

Ferrous ions Fe (II) chelation of each extract was estimated [9]. Briefly, 50 μl of 2 mM $FeCl_2$ was added to the OFE (1 ml). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured by Elisa spectrophotometer (μQuant, Bio-Tek Instruments, INC) at 562 nm. EDTA was used as a positive control.

Hydroxyl radical (^{-}OH) inhibitory activity of O. fragrans

Hydroxyl radical scavenging activity was determined by assessing the ability of deoxyribose to compete with OFE for hydroxyl radicals. OFE was dissolved in 10% ethanol (final concentration: 0.4%). In the assay for the hydroxyl radical scavenging, the reaction mixture (1.0 ml), comprising 30 mM KH_2PO_4–KOH buffer (pH 7.4), 2 mM 2-deoxyribose, 0.1 mM $FeCl_3 \cdot 6H_2O$, 104 μM EDTA, OFE (1–400 μg/ml in above buffer), 1.0 mM H_2O_2, and 0.1 mM L-ascorbic acid, was incubated at 37 °C for 1 h. The thiobarbituric acid reactive substance level was determined after terminating the reaction by the addition of TBA reagent. Quercetin was used as a positive control [10].

Ferric reducing ability assay (FRAP) of O. fragrans

The ferric reducing ability of each standard solution was measured according to a modified protocol developed [11]. To prepare the FRAP reagent, a mixture of 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM ferric chloride (10:1:1, v/v/v) was made. 0.1 ml of OFE was added to 1.9 ml of reagent. Readings at the absorption maximum (593 nm) were taken every 15 second using an Elisa spectrophotometer (μQuant, Bio-Tek Instruments, INC), and the reaction was monitored for up to 10 min. Trolox solution was used to perform the calibration curves. The data was also expressed as trolox equivalent antioxidant capacity (TEAC) milligrams per gram of dry weight.

Estimation of the total phenolic contents

The method [12] downscaled to 1 ml final volume was followed to determine the amounts of total phenolics in OFE (100 μl) was mixed with 500 μl of 1:10 Folin-Ciocalteau's reagent followed by the addition of Na_2CO_3 (400 μl, 7.5%). After incubating the reaction mixture at 50 °C for 30 min, the absorbance at 600 nm was recorded by an Elisa spectrophotometer (μQuant, Bio-Tek Instruments, INC). Gallic acid monohydrate was used as the reference standard. The total phenolic content of OFE is expressed as gallic acid equivalents (μg of GAE)/mg dry weight of the samples.

Estimation of the total flavonoids

About 1 ml of OFE was placed in a 10 ml volumetric flask. Distilled water was added to make 5 ml, and 0.3 ml $NaNO_2$ (1:20) was added. 3 ml $AlCl_3$ (1:10) was added 5 min later. After 6 min, 2 ml 1 M NaOH was added and the total was made up to 10 ml with distilled water. The solution was mixed well again and the absorbance was measured against a blank at 510 nm by an Elisa spectrophotometer (μQuant, Bio-Tek Instruments, INC). Rutin was used as the standard for a calibration curve [13].

Preparation of primary cortical neuron cultures

Primary cultures of cortical neurons were taken from pregnant rat with starting weights of 225 ± 25 g, with free access to food and water, under a controlled temperature (21 ± 1 °C) and a 12: 12 h light: dark cycle (light on 07.00 h). Rats at 17 days of gestation were deeply anesthetized with a maintenance of halothane. Briefly, description was based on a previous study [13]. All cultured cells were initially incubated for 45 min in a humidified incubator with 5% CO_2 at 37 °C for monolayer attachment, after which the medium was replaced by serum-free Basal Medium Eagle (BME). The primary cultured cells obtained under these conditions appeared to contain mostly cortical neurons. The day of plating was counted as 0 days-*in-vitro* (DIV), and different stages of cultured neurons were used in this study.

Neuron cell viability measurement

The medium was removed from 12 DIV neuronal cells and replaced with fresh medium. The primary cultured neuron cell viability was assessed by the mitochondria reduction of MTT assay. Cells were seeding and drug treated in 24-wells as 5×10^5 cells/well and incubated for 24 h. MTT, yellowish in color, was dissolved in PBS. Mitochondrial respiration, an indicator of cell viability, was assayed by the mitochondrial-dependent reduction of MTT to formazan. Each well plate was incubated with MTT (0.25 mg/ml) for 4 h. The cells were solubilized in 0.04 N HCl in isopropanol. The extent of the reduction was measured by an Elisa spectrophotometer (μQuant, Bio-Tek Instruments, INC) at absorbance 600 nm [14].

Cell membrane leakage level by lactate dehydrogenase release (LDH assay)

Cells were grown in 24 wells as 5×10^5 cells/well, and applied with OFE and neurotoxic insults for 24 h. About 0.5 ml of culture medium was incubated with 0.1 mg of β-NADH in 1.2 ml of 0.1 M phosphate buffer for 5–15 min at room temperature. The absorbance at a wavelength of 340 nm was measured for 2 min immediately after 0.1 M sodium pyruvate was added. The unit activity of LDH was defined as the decrease of A 340 absorption in 1 min by an Elisa spectrophotometer (μQuant, Bio-Tek Instruments, INC), multiplied by 1000 in 1 ml of sample [14].

Chemicals-induced neurotoxicity and protective effects of O. fragrans

For the neurotoxicity study, various concentrations of each rice extract was added to cultured neurons to pre-treat 1 h, followed by incubation with a combined treatment of with/without 50 μM glutamate, arachidonic acid or 6-hydroxydopamine in primary cortical neuron then followed by a 24-h incubation in BME medium [14–16]. After 24 h, the cell and cultured medium were measured as described in 2.5.1 and 2.5.2, respectively.

Determination of lipid peroxidative product of O. fragrans on rat brain, liver, kidney and heart tissue

Preparation of rat brain, heart, liver, and kidney mitochondrias

Rat brain, heart, liver, and kidney were removed quickly, perfused immediately with ice-cold normal saline and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing potassium chloride (1.17%) using a Potter Elvehjem homogenizer. The homogenate was centrifuged at $800 \times g$ for 5 min at 4 °C in a refrigerated centrifuge to separate the nuclear debris. The supernatant so obtained was centrifuged at $10{,}500 \times g$ for 20 min at 4 °C to get the mitochondrias.

Protein determination

Tissue lysate (brain, liver, kidney, and heart) protein was determined by the method using bovine serum albumin as the standard. Protein was estimated by the method of Lowry et al. [17].

Thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) assay

The concentration of MDA, a compound that is produced during lipid peroxidation, was determined by using the thiobarbituric acid (TBA) method. Pre-treatment of OFE at various concentrations, then two volumes of TBA reagent (containing 0.375% thiobarbituric acid, 0.25 M HCl, and 0.1 mM ethylenediamine tetraacetic acid) were added to the cell homogenate and boiled for 40 min at 100 °C. After cooling and centrifugation at 3000 rpm/min for 10 min, the absorbances of the supernatants were measured at 532 nm. The percentage of inhibition of the formation of TBARS (% inhibition of lipid peroxidation) was calculated as follows: where A1, A2, and A_t are the absorbance values at 535 nm for the unprotected sample, the blank, and the test sample, respectively [18].

$$(\%\ \text{inhibition of lipid peroxidation}) = \frac{(A1 - At)}{(A1 - A2)} \times 100$$

O. fragrans on AKT expression by western blotting

Cortical neuron (2 ml, 1×10^6 cells/ml), grown in 6-well plates were incubated with or without OFE for 24 h. Cell pellets were lysed with lysis buffer solution containing 40 mM Tris–HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% nonide P-40, and the protease inhibitor. Cells were washed with ice-cold phosphate-buffered saline and stored at −20 °C until further analysis. Total proteins (50 μg) were used for Western blot analysis. Western blot analysis was performed using 10% Tris-glycine-sodium dodecyl sulfate-polyacrylamide gels, and the protein was transferred to a nitrocellulose membrane by electroblotting. The membranes were probed with anti-AKT (a rabbit polyclonal antibody), and were visualized using a BCIP/NBT kit according to the manufacturer's instructions. Anti-GAPDH (a mouse monoclonal antibody) was used as a loading control [19].

Statistical analysis

Results were expressed as a means ± S.D and evaluated for statistical significance with an analysis of variance (ANOVA-one way) test. Student's *t* test was used for comparisons of all treatments with the control. Comparison between each chemical inducer treatment vs. OFE pre-treatment and then neurotoxic insults were evaluated by the *t*-test.

Results

Total flavonoid and total phenolic contents of O. fragrans

It well documented that many neuroprotective agents with their anti-oxidative activity decrease the neurotoxicity [20]. Therefore, we evaluated the effects of OFE on DPPH, OH radical scavenging and Fe^{2+} chelating ability, total flavonoid and phenolic contents. The result of OFE by the Folin-Ciocalteu total phenols photometric assay is reported in Table 1. OFE contains a considerable amount (193.53 μg/mg dry weight) of gallic acid equivalents in *O. fragrans*. In addition, OFE was pre-tested with the following screening tests. OFE exerted positive in ferric chloride test, reaction of strong alkali, Fehling's test, Liermann Burchard's test, lead acetate test, and flavones test. On the other hand, OFE exerted negative in Mayer's test. This implies that OFE might have a presence of phenolic hydroxyl chemical structure, triperpenoids, flavone or its glycoside derivatives. However, OFE might not have a presence of alkaloid. Therefore, we further detected the total flavonoid contents of OFE. Interestingly, results found that OFE contained an abundant amount of total flavonoid (101.52 ± 2.18 μg/mg dry weight).

Table 1. Effects of *O. fragrans* on free radical scavenging and antioxidant activities.

O. fragrans	Unit
Total phenol content	193.53 ± 4.48 (μg/mg dry weight)
Total flavonoid content	101.52 ± 2.18 (μg/mg dry weight)
FRAP	IC_{50} = 7.74 ± 0.33 μg/ml
Fe^{2+} chelating ability	IC_{50} = 0.23 ± 1.75 μg/ml
DPPH scavenging ability	IC_{50} = 9.99 ± 2.24 μg/ml
OH^- scavenging ability	IC_{50} = 11.19 ± 3.27 μg/ml

Results are expressed as a percentage of control absorbance. (mean ± S.D., n = 4; and each separate assay was performed in quadruplicate).

Effects of O. fragrans on free radical scavenging and antioxidant ability

In Table 1, the data shows that OFE exerted efficient on the power of Fe^{2+} chelating activity and ferric reducing/antioxidant power with IC_{50} values of 0.23 ± 1.75 μg/ml, and 7.74 ± 0.33 μg/ml, respectively (EDTA with IC_{50} values of 11.37 ± 2.19 μg/ml and trolox with IC_{50} values of 10.75 ± 1.10 μg/ml). OFE also showed dose-dependent bleaching of stable DPPH in 30 min with IC_{50} values of 9.99 ± 2.24 μg/ml (gallic acid with IC_{50} values of 0.74 ± 0.01 μg/ml). Meanwhile, OFE exerted the IC_{50} value as 11.19 ± 3.27 μg/ml on OH radical inhibitory activity (quercetin with IC_{50} values of 2.5 ± 1.4 μg/ml). This also might imply that OFE could affect on Fe^{2+} chelating related reaction by decreasing neurotoxicity. Because of the transition of metal ion, Fe^{2+} possesses the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals. Overall, the ability of OFE on the IC_{50} values of each assay was Fe^{2+} chelating> FRAP> DPPH scavenging $>OH^-$ scavenging. OFE was with the potential anti-oxidative and anti-radical scavenging abilities due to its higher reducing power as well as higher contents of total flavonoid and phenolic content. The result is consistent with the OFE in our laboratory extraction.

Protective effects of O. fragrans under neurotoxic insults

We further examined the preventive effect of OFE under neurotoxic-induced neurotoxicity with the 10 μg/ml and series concentrations. Pre-treatment with the OFE increased the number of surviving cells under glutamate and 6-OHDA (Figure 1b and c), but not under arachidonic acid-induced neurotoxicity (Figure 1a). OFE was also with a dose-dependent manner against 6-OHDA and glutamate-induced neurotoxicity in rat primary cortical neurons on MTT and LDH assay (EC_{50} = 165.2 and 66.74 μg/ml on MTT, and EC_{50} = 124.6 and 105.44 μg/ml on LDH, respectively). The results indicated that OFE pretreatment attenuated 6-OHDA and glutamate-induced mitochondrial dysfunction and prevent membrane

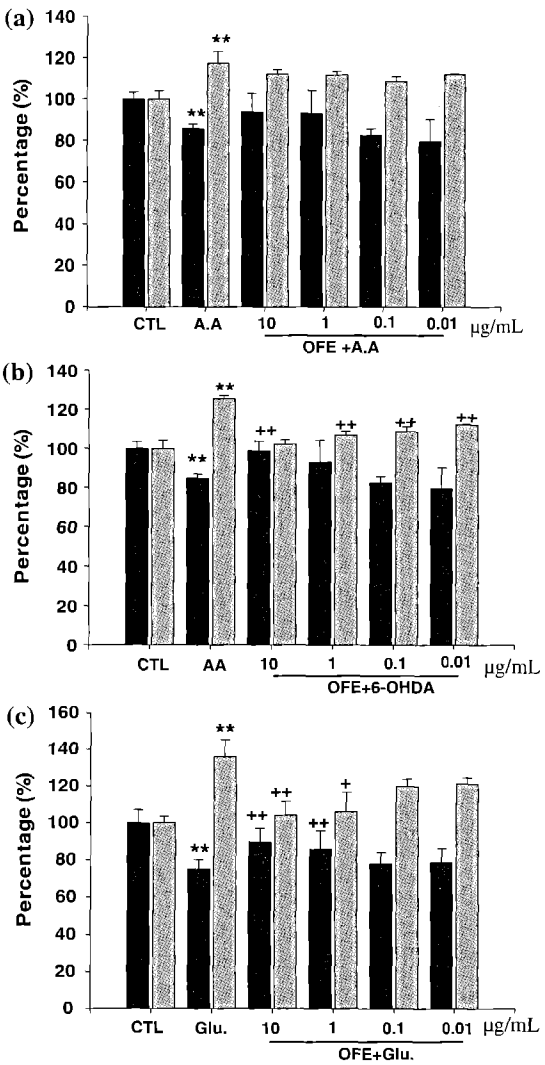

Figure 1. Effects of *O. fragrans* flower extract on 6-hydroxydopamine, arachidonic acid, and glutamate-induced neurotoxicity. Cortical neurons were seeded into 24-well plates and grown for 12 days. Cells were pretreated with OFE for 1 h then treated with plus 50 μM (a) A.A., (b) 6-OHDA or (c) glutamate for 24 h without changing the cultured medium. Cell viability was measured using the MTT and LDH as described previously. Results are presented relative to control cells. Each determination was performed in quadruplicate, * $p < 0.05$, ** $p < 0.01$ compared to control group; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ compared to the respective neurotoxic inducers.

damage, but not significantly under arachidonic acid neurotoxicity. An increased neuronal survival is expressed as a percent of protection of cultures not exposed to any neurotoxins by OFE (data not shown). Treatment with 50 μM 6-OHDA and glutamate resulted in the death of approximately

18% and 26%, respectively, of the cortical neuronal cells when compared with the control cells. However, OFE was not effective in neuroprotection at very low concentrations (0.01–0.001 μg/ml). OFE, at 1.0 and 10 μg/ml showed significant neuroprotection. Maximum neuroprotection (31% ± 5.2) was obtained by pre-treating the cells with 10 μg/ml of OFE.

Inhibitory effects of O. fragrans on ferrous chloride induced lipid peroxidation

The lipids in membrane are continuously subjected to oxidant challenges. Oxidant induced abstraction of a hydrogen atom from an unsaturated fatty acyl chain of membrane lipids initiates the process of LPO, which propagates as a chain reaction [21]. In the process, cyclic peroxides, lipid peroxides and cyclic endoperoxides are generated which ultimately fragment into aldehydes like MDA. Intracellular MDA, a product of lipid peroxidation, was increased by approximately 77% compared to control at the end of a 30 min treatment by Fe^{2+}. Incubation with Fe^{2+} system caused a significant increase in MDA formation in rat mitochrdrias. OFE extract dose-dependently inhibited the amount of MDA generated and shown in Figure 2. Pre-treatment with OFE (10~320 μg/ml) significantly attenuated the levels of malondialdehyde (compared with positive control, Trolox). Decreases in the malondialdehyde levels were 50% by OFE treatment at 46.23 (±3.87), 97.76 (±4.02), 62.5 (±5.26), and 78.3(±5.28) μg/ml in brain, kidney, liver, and heart mitochondrias, respectively. The inhibitory ability order of OFE in the four tissues was liver > brain > heart > kidney.

Effects of O. fragrans under glutamate and 6-hydroxydopamine on AKT protein expression

In oxidative neuronal injury AKT has proven involvement with the neuronal survival pathways. The results showed that glutamate and 6-OHDA significantly decreased the AKT expression in Figure 3. OFE at 10 μg/ml could promote decreasing of AKT expression after glutamate and 6-OHDA. The relative amount of protein expression for OFE on regulating the AKT expression was shown in Figure 3b. These protective effects are related to its ability to attenuate oxidative

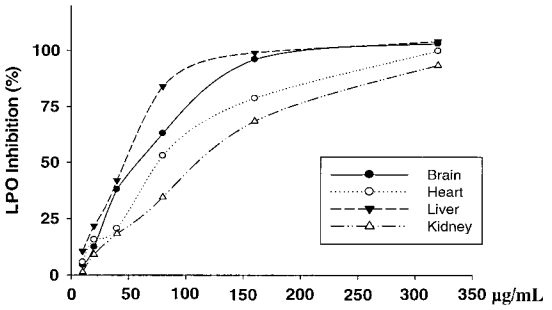

Figure 2. Inhibitory effect of *O. fragrans* flower extract on lipid peroxidation (LPO) in rat tissues mitochodrias induced by ferrous chloride. Each value is the mean of five observations. Levels of TBARS in rat brain, liver, and heart homogenate. The results are expressed as ±SEM of 6 experiments.

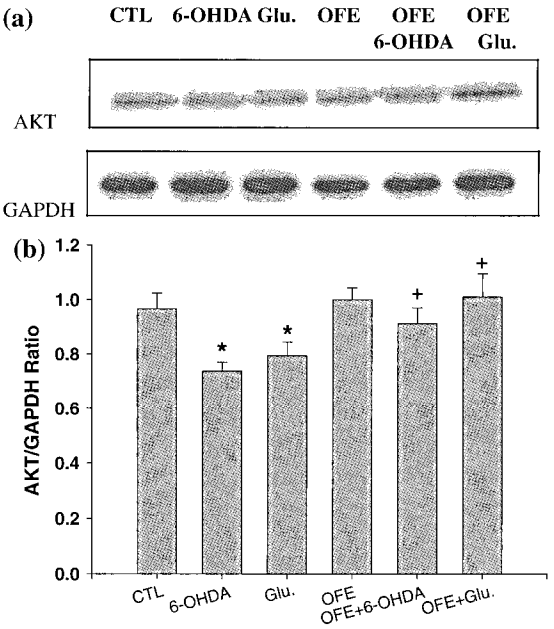

Figure 3. Effects of glutamate and 6-OHDA-induced neurotoxicity on AKT expression in primary cortical neuron cultures by *O. fragrans*. (a) Cortical neurons were then treated with 50 μM glutamate or 6-OHDA with OFE 10 μg/ml for 24 h. Following this incubation period, cells were harvested and lysed, and active or total AKT in equivalent amounts of total lysate protein (50 μg) were visualized by Western blot analysis using an AKT specific antibody. GAPDH was as the internal standard and glutamate or 6-OHDA-induced AKT expression by OFE was observed in three independent experiments. (b) The quantitative analysis of each protein expression level by UVP Biospectrum AC System. $^{*}P<0.05$ compared to control group; $^{+}P<0.05$ compared to the respective neurotoxic inducers

stress. Therefore, OFE exerted its neuroprotective effect on the survival protein level, and detailed mechanisms will be further studied.

Discussion

A variety of plant products have been used for prevention and treatment of a broad range of diseases. Some of these plants are useful in the neurodegenerative diseases, such as the Parkison's disease model, and in preventing the effect of Crataegus flavonoids on ischemic-reperfusion damage to the brain of the Mongolian gerbil (stroke model) [22]. In this study, we examined the effects of OFE on neuronal cells due to the screening in our investigation on antioxidant properties and found out that OFE exerted potential neuroprotective effects. The possibilities of its effects are discussed in the following paragraphs.

Our results demonstrate that the OFE was capable of anti-radical and anti-oxidative abilities. OFE was marginally more effective in the Fe^{2+} reducing ability in the metal chelating power with an IC_{50} value of 0.23 μg/ml. Similarly, in the other assays, OFE scavenged hydroxyl radical and DPPH radical. Table 1 clearly demonstrates that the OFE reacts with and neutralize stable free radicals independent of any enzymatic activity. Moreover, neuroprotection by OFE could, in part, be a result of the ethanol extract increasing the activity of FRAP. These also could support the fact that OFE could attenuate the 6-OHDA and glutamate-induced neurotoxicity. This also indicates that lipid peroxidation induced by oxidative stress was reduced under OFE treatment. OFE was the most effective in inducing activity and also in preventing the formation of malondialdehyde in ferrous chloride-treated tissues. Furthermore, increasing the production of reactive oxygen species causes oxidation of proteins, damage of DNA, and lipid peroxidation.

OFE contained abundant total flavonoid and polyphenol components in our extraction. In our laboratory, we have collected over 300 species of ethnobotany. *O. fragrans* exerted the most potential antioxidant, free radical scavenging abilities. In addition, natural products and their synthetically developed active components have been widely used in medicine to counter the deleterious effects of ROS. For example, *Ginkgo biloba* [23], *Salviae miltiorrhizae* [24], *Panax quinquefolius* [25] and *Withania somnifera* [26] were shown to have antioxidant properties. In addition, natural polyphenols and flavonoid have many pharmacological activities, especially on their antioxidant functions on neuroprotection [27, 28].

Otherwise, the neuroprotective effects of OFE were observed in the concentration range of 0.01–10 μg/ml and exerted the protective effects at higher concentrations. Also OFE exerted in preventing cellular damage against oxidative stress produced by glutamate and 6-OHDA, but not under arachidonic acid. Therefore, the results also imply that OFE might via anti-ROS associated pathway to protect neurotoxic insults. Pre-treatment with OFE increased cell survival from 10 to 45% (neuroprotection EC_{50} = 66–165 μg/ml) in our observation. Meanwhile, *in vitro* studies examining mechanisms of neuroprotection fail to include biologically relevant metabolites of the flavonoids known to enter the circulation, and thus most likely to be bio-available to cells and tissues [28]. This possibility also could explain that OFE would protect cortical neurons at much higher concentration than cell-free bioassays and also implies that OFE exerted its neuroprotective effects at micro molar concentration.

The protective effects of OFE on AKT expression might involve their interaction with cell signaling cascades, their influence on gene expression and the down regulation of pathways leading to cell death or survival. Since information regarding the mode of action of OFE in the nervous system is lacking, and the neuronal survival or protection related proteins has been found in different pathways [29]. We will focus on and clarify the detail neuroprotective mechanisms of OFE in the future study. Therefore, we found the dried flower of *O. fragrans* with a high total flavonoid and polyphenolic content in this study. OFE would be beneficial for the neurodegenerative diseases by its anti-oxidative stress ability to regulate the neuronal survival mechanisms.

In conclusion, our results suggest that OFE protected rat cortical neuron against 6-OHDA and glutamate-induced neurotoxicity. Most likely, the OFE act as an ROS scavenger and anti-lipid peroxidation against neurotoxicity. This study is being continued attempting to isolate the principle components from OFE by biological assay guided fractionation. Thus, the flower of *O. fragrans* is a potential neuroprotective agent that may be useful in preventing neurodegenerative diseases.

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