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Structure–activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives

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Abstract

Gallic acid and its derivatives are a group of naturally occurring polyphenol antioxidants which have recently been shown to have potential healthy effects. In order to understand the relationship between the structures of gallic acid derivatives, their antioxidant activities, and neuroprotective effects, we examined their free radical scavenging effects in liposome and anti-apoptotic activities in human SH-SY5Y cell induced by 6-hydrodopamine autooxidation. It was found that these polyphenol antioxidants exhibited different hydrophobicity and could cross through the liposome membrane to react with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical in a time and dose-dependent manner. At the same time, the structure–antioxidant activity relationship of gallic acid derivatives on scavenging DPPH free radical in the liposome was also analyzed based on theoretical investigations. Analysis of cell apoptosis, intracellular GSH levels, production of ROS and the influx of Ca^{2+} indicated that the protective effects of gallic acid derivatives in cell systems under oxidative stress depend on both their antioxidant capacities and hydrophobicity. However, the neuroprotective effects of gallic acid derivatives seem to depend more on their molecular polarities rather than antioxidant activities in the human SH-SY5Y cell line. In conclusion, these results reveal that compounds with high antioxidant activity and appropriate hydrophobicity are generally more effective in preventing the injury of oxidative stress in neurodegenerative diseases.

Keywords: Free radicals; Antioxidant; ESR; Hydrophobicity; Partition coefficient; Gallic acid; Oxidative stress; Reactive oxygen species; Glutathione; Apoptosis

1. Introduction

Neurodegenerative diseases are thought to be related to the free radical mediated reactions. For example, Parkinson's disease (PD) is characterised by a selective degeneration of dopaminergic neurons in the *Substantia nigra* (SN) *pars compacta* resulting in a reduction of the dopamine levels in the striatum (Bernheimer et al., 1973; De Erausquin et al., 1994). In almost all these processes, oxidative stress is implicated, where the oxidation of dopamine generates so-called reactive oxygen species (ROS) and an unbalanced overproduction of ROS induces neuronal damage, ultimately leading to neuronal death via apoptosis or necrosis (Fahn and Cohen, 1992; Beal, 1995; Jenner and Olanow, 1998; Igosheva et al., 2005; Qi et al., 2005). Therefore, some antioxidants may hold the keys to preventative measures against neurodegenerative diseases.

Gallic acid (GA, 3,4,5-trihydroxybenzoic acid) and its derivatives are widely present in the plant kingdom and represent a large family of plant secondary polyphenolic metabolites hence natural antioxidants. They are present in the forms of either methylated gallic acids (e.g., syringic acid) or

Abbreviations: DCF-DA, 2′,7′-dichlorofluorescin diacetate; DFT, density functional theory; DG, *n*-dodecyl gallate; DMEM, Dulbecco's modified Eagle's medium; DOPC, 1,2-di[*cis*-9-octadecenoyl]-*sn*-glycero-3-phosphocholine; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; ESR, electron spin resonance; GA, gallic acid; HDMBA, 5-hydroxyl-3,4-dimethoxybenzoic acid; IP, ionization potential; MG, methyl gallate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; OH-BDE, dissociation energy of phenolic O–H bond; OG, *n*-octyl gallate; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; PG, *n*-propyl gallate; ROS, reactive oxygen species; SA, syringic acid; TMBA, 3,4,5-trimethoxybenzoic acid

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galloyl conjugates of catechin derivatives, i.e., flanvan-3-ols, or polygalloyl esters of glucose, quinic acid or glycerol. The latter two groups of polyphenols are also known as vegetable tannins which earned their names from their ability to transform animal skins into leather by interacting with collagen proteins (Tang et al., 2003a,b,c). They are inevitable components of the food and beverages of plant origin, such as tea. In the US, for example, the average GA intake from food is about 1 g/day. In fact, some GA esters are widely used as food additives to prevent food oxidations. GA derivatives have also been found in many phytomedicines with a number of biological and pharmacological activities, including scavenging on free radicals (Kanai and Okano, 1998; Dwibedy et al., 1999), inducing apoptosis of cancer cells (Sakagami et al., 1997; Serrano et al., 1998; Saeki et al., 2000), inhibiting squalene epoxidase (Abe et al., 2000) interfering the signal pathways involving Ca^{2+} and oxygen free radicals (Sakaguchi et al., 1998, 1999; Inoue et al., 2000; Sohi et al., 2003). The mammalian metabolism of gallic acid has been thoroughly studied (Haslam and Cai, 1994; Zong et al., 1999; Manach et al., 2005). In vivo experiments showed that the major GA metabolites are products of methylation (unconjugated and conjugated 4-*O*-methylgallic acid, 2-*O*-methylgallic acid), decarboxylation (unconjugated and conjugated pyrogallol, 4-*O*-methylpyrogallol), and dehydroxylation (resorcinol). Toxicological studies showed that the ''no-observed-adverse-effect-level'' (NOAEL) of gallic acid is at least 120 mg/kg/day for F334 rats and the level of gallates was reported to be as high as 1000 mg/kg for mice (Van et al., 1986; Niho et al., 2001).

It is hypothesized that their bioavailability implicates GA derivatives in the prevention of lipid peroxidation occurring in cell membrane. Whilst the antioxidative activity of GA derivatives has been studied in solutions and such results are extremely useful to provide an index for the intrinsic antioxidative activity, they bear little relevance to the multicellular systems, in which GA derivatives have to be metabolised and transported to the ''reaction sites''. In such cases, one has to answer the questions such as ''how important is the hydrophobicity in enabling the antioxidants to reach the ''reaction sites'' and to scavenge largely hydrophobic free radicals generated from lipid peroxidation?'' For this purpose, a well defined model system has been introduced to mimic the situation of free radical scavenging in the real cell membrane systems by incorporating a stable free radical, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), into the model bilayer membrane prepared from phospholipids (Chen et al., 2000). With such systems, one can evaluate the importance of some molecular properties of antioxidants such as hydrophobicity and steric effect, in scavenging free radicals in the membrane environment (Chen et al., 2000; Hashimoto et al., 1999). More importantly, in conjunction with the computational chemistry, structure–activity relationships can be investigated. From the chemistry point of view, the most relevant parameters for the free radical scavenging activity of antioxidants include the dissociation energy of the phenolic O–H bond (OH-BDE), the energy-eigenvalue of the highest occupied molecular orbital (E_{HOMO}) and ionization potential (IP) of the antioxidant (Van et al., 1993; Zhang, 1998; Cheng et al., 2003; Lien et al., 1999; Bakalbasis et al., 2001; Soffers et al., 2001; Wright et al., 2001; Leopoldini et al., 2004).

Even with the well defined liposome model systems, which are clearly more realistic than ethanol solutions, the relevance to the cell systems remains unknown. Therefore, it is necessary to conduct similar studies in cultured cell systems. In this study, therefore, cell cultures were used and treated with 6-hydroxydopamine (6-OHDA) which is a well known neurotoxin that induces oxidative stress in neuronal cells and leads to cell apoptosis via production of reactive oxygen species (ROS) (Kostrzewa and Jacobowitz, 1974; Kumar et al., 1995).

In the present study, the antioxidative properties of GA derivatives were studied in ethanol and liposome systems using electron spin resonance (ESR) technique. Density functional theory (DFT) study was also carried out to investigate the relevance of OH-BDE and IP to the antioxidative efficiency of GA derivatives. Then, we focused on the neuroprotective effects of gallic acid derivatives against the 6-OHDA-induced oxidative stress by measuring the intracellular ROS and GSH levels, Ca^{2+} influx and subsequent cell death in human neuroblastoma SH-SY5Y cells. The objectives of these studies are to answer the following questions including:

(1) What is the structure–activity relationship for the antioxidation activity of GA derivatives in model membranes and cell systems?
(2) What is the relevance of the model systems to activities in the cellular level?
(3) What are the major factors in terms of molecular properties in the process of antioxidation of GA derivatives?

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), new-born calf serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) were purchased from Gibco BRL (Grand Island, NY, USA). 1,2-Di[*cis*-9-Octadecenoyl]-*sn*-glycero-3-phosphocholine (DOPC), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 6-hydroxydopamine, 2′,7′-dichlorofluorescin diacetate (DCF-DA), Hoechst 33258, glutathione reduced (GSH), glutathione oxidized (GSSG), trypsin, penicillin, streptomycin and dialysis tubing were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-3 acetoxymethyl ester (Fluo-3 AM) was purchased from Calbiochem (San Diego, CA, USA). Gallic acid, methyl gallate, *n*-propyl gallate, *n*-octyl gallate, *n*-dodecyl gallate, syringic acid, 5-hydroxy-3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid were purchased from Lancaster Synthesis Ltd. (Lancashire, UK).

2.2. Measurement of partition coefficients in octanol–water

The method for measuring partition coefficients was performed according to Andersson and Schrader (1999) with some modifications. For relatively hydrophilic chemicals, a suitable amount of gallic acid, methyl gallate, propyl gallate were, respectively, dissolved in distilled water to make solution of about 20–100 μM. A piece of dialysis tubing of 12 cm length was soaked, thoroughly cleaned with distilled water and dried to remove most of the water. One end of

the dialysis tubing was closed with a tight knot and put it into a 10 ml tube with 5 ml of the solution; while the dialysis tubing was filled with 1 ml of octanol and fixed at the other end. The samples were protected against oxidation by bubbling with nitrogen for 3 min, the set-up was held in an ultrasonic bath (Branson, 1200, Branson ultrasonic corporation, CT, USA) in the dark and agitated over a period of at least 10 h to at a temperature of 25 ± 1 °C. The system was then placed in a water bath at 25 °C overnight.

For the more hydrophobic GA derivatives including syringic acid, 5-hydroxyl-3,4-dimethoxybenzoic acid, 3,4,5-trimethxybenzoic acid, octyl gallate and dodecyl gallate, a suitable amount was dissolved in octanol to form a solution of about 20–100 μM. The experiment was then carried out as before. The concentrations of the gallic acid derivatives in the water and octanol phases were determined with a UV–vis spectrophotometer (Cary 3, Varian). A UV spectrum of water phase (hydrophilic chemicals) or octanol phase (hydrophobic chemicals) was determined before and after partition. The partition coefficients (P) for the chemicals dissolved in distilled water were calculated according to the equation:

$$P = \frac{(A_{\mathrm{Wat1}} - A_{\mathrm{Wat2}})V_{\mathrm{Wat}}}{A_{\mathrm{Wat2}} \times V_{\mathrm{Oct}}}.$$

where A_{Wat1} is the absorption of gallic acid derivatives in water phase before partition, A_{Wat2} the absorption of polyphenols in the water phase after partition, and V_{Wat} and V_{Oct} are the volumes of water phase and octanol phase.

The partition coefficients (P) for the chemicals dissolved in octanol were calculated according to the equation: $P = A_{\mathrm{Oct2}} \times V_{\mathrm{Wat}}/((A_{\mathrm{Oct1}} - A_{\mathrm{Oct2}}) \times V_{\mathrm{Oct}})$, where A_{Oct1} is the absorption of gallic acid derivatives in octanol phase before partition, A_{Oct2} the absorption of polyphenols in octanol phase after partition, and V_{Wat} and V_{Oct} are the volumes of water phase and octanol phase.

2.3. Preparation of liposomes

DOPC and DPPH were dissolved in chloroform to form a uniform solution and transferred to a pear-shaped flask. The concentration of DOPC is 10 mg/ml and the molar ratio of DPPH and DOPC is 0.01. Then the solvents were evaporated off under a dry nitrogen stream for 12–15 min to form a film on the sides of flask. Multilamellar vesicles were prepared by the addition of distilled water. The final concentrations of DOPC and DPPH were 25 mM and 250 μM, respectively. After freezing with liquid nitrogen, followed by thawing and shaking with a vortex mixer five times, the small unilamellar vesicles were prepared by extrusion of the multilamellar vesicle suspension through an Avanti Mini-extruder (Avanti Polar Lipids Inc., Alabaster, AL). The suspension was introduced into the extruder and passed 11 times through two filters (0.1 μm) mounted in tandem. This procedure makes small unilamellar vesicles with an average diameter close to the pore size of the filter. The average size of small unilamellar liposome was about 30 nm as measured by the ALV-5000 photon correlator.

2.4. ESR measurement

Electron spin resonance spectra were recorded on a Bruker ESP 300 Spectrometer operating at X-band frequency with 100 kHz modulation at room temperature. All the initial measurements for DOPC liposome system were carried out 3 min after the reaction mixture was prepared. Five further measurements were made, the last ending 33 min after the preparation of the mixture. The mixtures contained 15 μl liposome and 5 μl distilled water or the polyphenol solution. For the more hydrophobic GA derivatives, distilled water was replaced by ethanol both in control and solution. After the distilled water or polyphenol solution was added to the liposome, the mixture was vortexed for 20 s and then transferred to a glass capillary for ESR measurements. The conditions of ESR measurement were as follows: microwave frequency 9.34 GHz, microwave power 10 mW, time constant 327 ms, conversion time 163 ms, modulation amplitude 1.6 G, sweep width 100 G, two scans. The scavenging effect of polyphenols on the DPPH free radical was calculated from the areas of double integration of ESR spectra of sample with and without the gallic acid derivatives with the ESP 1600 data system. When ethanol was used to replace liposome to study the scavenging effects in solution, the ESR experiment conditions were all the same as before.

In addition, because of the high sensitivity of ESR, relatively low concentration of GA derivatives was used in this experiment.

2.5. Density function theory (DFT) calculations

Molecular geometries were first optimized at the AM1 (Austin Model 1) level (Dewar et al., 1985) then used as initial geometries for DFT calculations. The DFT approach was used in the ab initio calculations in order to properly account for the electron correlation effects which may be particular important in this kind of system. The widely employed hybrid method denoted by B3LYP, which includes a mixture of HF and DFT exchange terms and the gradient-corrected correlation functional of Lee, Yang and Parr, as proposed and parametrized by Becke, was used on the 6–311 G level (Becke, 1993; Hehre et al., 1986; Foresman and Frish, 1993). Vibrational frequencies analysis were performed on each optimized geometries with the same basis set to obtain the zero-point vibrational energies (scaled by a factor of 0.9805) (Foresman and Frish, 1993). All calculations were preformed using Gaussian 98 program (Frisch et al., 1998) on a PC.

2.6. Assessment of cell viability

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium supplemented with heat-inactivated new-born calf serum (10%, v/v), glucose (4.5 mg/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml) in humidified 5% CO_2/95% air at 37 °C. Cells were cultured at a density of 2×10^5 cells/ml on 96-well plates and cultured 24 h before treatment. The culture medium was then replaced with fresh medium containing various concentrations of 6-OHDA and incubated for 24 h to investigate the neurotoxicity of 6-OHDA. Gallic acid derivatives were added 1 h before insult with 6-OHDA for a subsequent 24 h.

Cell viability was measured by quantitative colorimetric assay with MTT, showing the mitochondrial activity of living cells as described in the literature (Mosmann, 1983; Denizot and Lang, 1986). After treatment with 6-OHDA for 24 h, the medium was removed and fresh medium contain 0.5 mg/ml MTT was added to each well, followed by incubation for 3 h at 37 °C. Finally the medium containing MTT was removed, and cells were lysed with dimethyl sulfoxide (DMSO). The absorbance at 595 nm was measured using a Bio-Rad 3350 microplate reader. Control cells were treated in the same way without 6-OHDA, and the value of different absorbance was expressed as a percentage of control.

2.7. Morphological changes

The changes in nuclear morphology of apoptotic cells were observed by labelling the cells with the nuclear stain Hoechst 33258 and examining them under fluorescent microscopy. After being treated with 6-OHDA and/or gallic acid derivatives for 24 h, the cells were fixed with Carnoy's fixative consisting of methanol and glacial acetic acid (3:1, v/v), and incubated with Hoechst 33258 (3 μg/ml) for 30 min, the nuclear morphology was then observed under a fluorescence microscopy (Olympus, kx14e). Cells that exhibited reduced nuclear size, chromatin condensation, and intense fluorescence, and nuclear fragmentation were considered as apoptotic.

2.8. HPLC electrochemical analysis of the intracellular GSH and GSSG

To measure the amount of GSH and GSSG, we used a HPLC system (ESA delivery system) equipped with a Supelcosil LC18 reversed-phase column (250 mm × 4.6 mm; 5 μm particle size, Supelco Inc., Bellefonte, PA, USA) and Coularray electrochemical detector (ESA Inc., Chelmsford, MA, USA). The mobile phase consisted of 50 mM sodium dihydrogen phosphate and 2% acetonitrile (pH 2.7). The analysis was carried out at a column temperature of 25 °C with 20 μl sample injection and a flow rate of 1.0 ml/min. The potential of channels 1, 2, 3 and 4 were set at 400, 600, 800 and 1000 mV. The peak area was used to calculate the concentration. Calibration was achieved by simultaneously measuring authentic GSH and GSSG dissolved in 10 mM HCl.

After treatment, about 10^6 cells were collected with two PBS washes, then the cells were homogenized with 100 μl aqueous TCA (5%, w/v) to precipitate

proteins. After centrifugation at 12,000 × *g* for 10 min at 4 °C, the supernatant was diluted with 10 mM HCl and the amount of GSH and GSSG were measured with an HPLC-ECD. The chromatograms were analyzed and the amounts of GSH and GSSG were calculated with Coularraywin software (ESA Inc., Bedford, MA).

2.9. Measurement of intracellular ROS

The level of intracellular ROS was quantified by fluorescence with 2′,7′-dichlorofluorescin diacetate (DCF-DA) as described by Bass et al. (1983). H_2DCF-DA is a cell permeant and nonfluorescent. After entering live cells, the diacetate groups are cleaved by intracellular esterases. Oxidation of the reduced dyes occurs in the presence of ROS, changing the dyes to a fluorescent 2′,7′-dichlorofluorescin (DCF). The subsequent fluorescence change is used to measure the level of cellular ROS. Cells were collected and incubated with 5 μM DCF-DA (dissolved in DMSO) for 30 min at 37 °C in the dark. Then cells were washed three times with PBS (pH 7.4) and the relative levels of fluorescence were quantified in a fluorescence spectrophotometer (Hitachi F-4500, Tokyo, Japan, 485-nm excitation and 535-nm emission). The measured fluorescence values were expressed as a percentage of the fluorescence in control cells.

2.10. Intracellular calcium concentration ($[Ca^{2+}]_i$)

The concentration of intracellular Ca^{2+} was measured with Fluo-3 AM by the method of Aoshima et al. (1997) with modification. After treatment, cells were harvested, washed and resuspended in a standard medium (containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1.5 mM $CaCl_2$ and 20 mM HEPES with the final pH of 7.4. This was incubated with added Fluo-3 AM (5 μM) for 30 min at 37 °C. After washing three times, cells were resuspended in the standard medium and transferred to a fluorometer cuvette. The fluorescence intensity of Fluo-3 was measured by a fluorescence spectrophotometer (Hitachi F-4500) at an excitation wavelength of 490 nm and an emission wavelength of 526 nm. $[Ca^{2+}]_i$ was calculated from the Fluo-3 fluoresce intensity using the equation:

$$[Ca^{2+}]_i = \frac{K_d(F_0 - F_{min})}{(F_{max} - F_0)(\text{nmol l}^{-1})}$$

Assuming the K_d remains constant between 10 and 25 °C (K_d = 400 nmol l^{-1} at 25 °C), and increases linearly up to 42 °C.

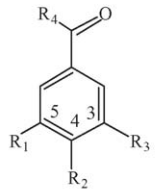
The maximal Fluo-3 fluorescence intensity (F_{max}) was determined by adding 0.1% Triton-X 100 and the minimal fluorescence (F_{min}) was determined by quenching Fluo-3 fluorescence with 5 mM EGTA. F_0 is the fluorescence measured without adding Triton-X 100 or EGTA.

2.11. Statistical analysis

All experiments were performed in triplicate. One-way ANOVA was used to estimate overall significance followed by post hoc Tukey's tests corrected for multiple comparisons (Miller, 1981). Data are presented as mean ± S.E.M. A probability level of 5% ($P < 0.05$) was considered significant.

3. Results

The structures of the gallic acid derivatives studied in this work (shown in Fig. 1) include methylated gallic acid (GA) derivatives, namely, 5-hydroxyl-3,4-dimethoxybenzoic acid (HDMBA), syringic acid (SA), 3,4,5-trimethoxybenzoic acid (TMBA), and gallic acid esters, namely, methyl gallate (MG), *n*-propyl gallate (PG), *n*-octyl gallate (OG) and *n*-dodecyl gallate (DG). These two sets of compounds provide a good distribution of defined molecular properties such as hydrophobicity, positioning of phenolic hydroxyl groups and steric effects.



Gallic acid and derivatives

1. Gallic acid R_1= R_2 =R_3= R_4= OH
2. 5-Hydroxy-3,4-dimethoxybenzoic acid R_2= R_3= OCH_3; R_1=R_4=OH
3. Syringic acid R_1= R_3= OCH_3; R_2 =R_4=OH
4. 3,4,5-Trimethoxybenzoic acid R_1= R_2 =R_3= OCH_3; R_4=OH
5. Methyl gallate R_1= R_2 =R_3= OH; R_4=CH_3
6. *n*-Propyl gallate R_1= R_2 =R_3= OH; R_4=$CH_2CH_2CH_3$
7. *n*-Octyl gallate R_1= R_2 =R_3= OH; R_4=$(CH_2)_7CH_3$
8. *n*-Dodecyl gallate R_1= R_2 =R_3= OH; R_4=$(CH_2)_{11}CH_3$

Fig. 1. The structures of gallic acid and its derivatives.

3.1. Hydrophobicity of the gallic acid derivatives

The hydrophobicity of molecules were evaluated by the logarithmic scale of the standard partition coefficient (log *P*) (Andersson and Schrader, 1999); the greater the value of log *P*, the greater the molecular hydrophobicity.

It has been observed in this work that incubation for 400–500 min was required for polyphenols under study to reach partition equilibrium in water/octanol. Fig. 2 shows the time–concentration plot for the aqueous phase of GA. The calculated equilibrium time is in good agreement with literature observation (Andersson and Schrader, 1999). To ensure a good equilibration in this work, we allowed 800 min for the partition process and the measured results are tabulated in Table 1. It is apparent that the log *P* values increased almost linearly as a function of the degree of methylation to the phenolic hydroxyl groups of GA. It is also evident that, for the gallic acid esters, the log *P* values increase exponentially with the length of the acyl chains. Comparing the log *P* values of SA,

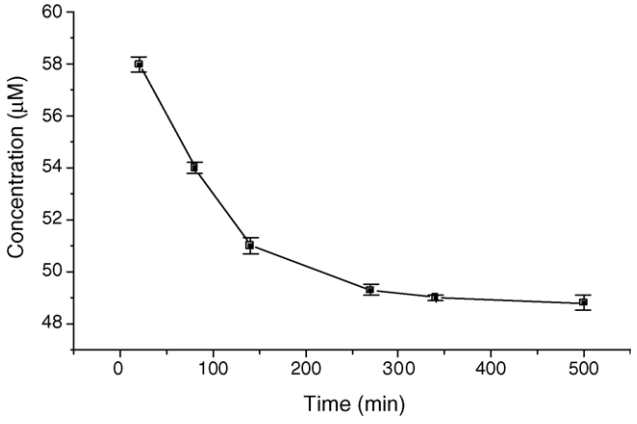


Fig. 2. Partition process of gallic acid in octanol/water system measured as the gallic acid concentration in water as a function of time.

Table 1
Partition coefficients of polyphenols in octanol–water system

Polyphenol	*P*-values	log *P*
Gallic acid	0.29 ± 0.04	−0.53 ± 0.05
Syringic acid	0.91 ± 0.05	−0.04 ± 0.02
5-Hydroxyl-3,4-dimethoxybenzoic acid	1.18 ± 0.05	0.07 ± 0.02
3,4,5-Trimethoxybenzoic acid	1.64 ± 0.10	0.21 ± 0.03
Methyl gallate	6.29 ± 0.15	0.79 ± 0.01
n-Propyl gallate	8.6 ± 0.32	0.93 ± 0.02
n-Octyl gallate	131.3 ± 24.7	2.11 ± 0.08
n-Dodecyl gallate	179.7 ± 25.4	2.25 ± 0.06

HDMBA with MG, esterification of the carboxylic group of gallic acid appears to have stronger effects on the log *P* values than methylation of its hydroxyl groups.

3.2. DPPH scavenging efficiency of gallic acid derivatives

The lipophilic DPPH is a stable free radical and its solution ESR spectrum shows a distinct symmetric quintet owing to spin splitting resulting from two adjacent ^{14}N spins, reflecting that DPPH undergoes fast tumbling with a rotational correlation time of about 0.1 ns. In liposome, the DPPH spectral lines are broadened to some extent showing some typical anisotropic broadening effects (Fig. 3) probably due to the restricted motions in liposome. Nevertheless, the ESR spectrum indicates that DPPH is solubilized in the bilayer of the DOPC.

Addition of the gallic acid reduces the signal intensity of DPPH to an extent (Fig. 3) which is dosage dependent: the DPPH scavenging efficiency increases almost linearly as a function of GA concentration (Fig. 4).

Table 2 shows the scavenging efficiency of gallic acid derivatives on DPPH in both ethanol solution and liposome systems. Comparing the results for GA, SA, HDMBA and TMBA, the order for the DPPH scavenging efficiency is GA > SA > TMBA = HDMBA. Thus, in this group the hydroxyl groups are important for free radical scavenging efficiency. In particular, the hydroxyl group at the *para*-position to the carboxylic group appears essential to maintain the scavenging activity; methylation of this OH group diminishes the scavenging activity as in TMBA and HDMBA. However, an inverse correlation is observable with the log *P* in both systems. Moreover, although MG, PG, OG and DG have the same number and distribution of hydroxyl groups in their molecules, their scavenging efficiency on DPPH varies with the length of ester chain and shows an order: MG > PG > OG > DG. This suggests that the steric effects also play an important role. Yet again, an inverse correlation is present with log *P* values.

3.3. Theoretical investigation

To complement the above experimental observations regarding the effects of OH-groups, position and the length of ester chain on the scavenging capacity of free radicals, a theoretical study was carried out. The O–H bond disassociation

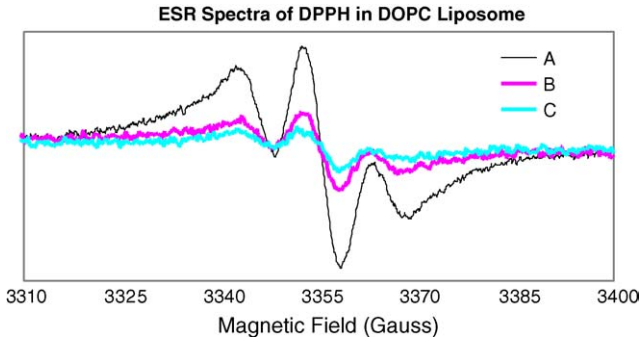


Fig. 3. ESR spectra of DPPH free radical in DOPC liposome without antioxidants (A), after mixing with 6.81 μM GA for 3 min (B) and 30 min, respectively.

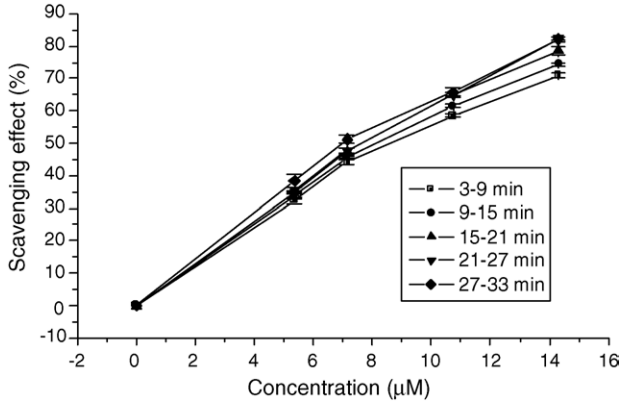


Fig. 4. GA scavenging effects on DPPH in DOPC liposome as a function of GA concentration.

Table 2
DPPH scavenging efficiency of polyphenols in ethanol solution and DOPC liposome

Polyphenols	IC_{50} (μM) in liposome	IC_{50} (μM) in ethanol solution	Number of OH
Gallic acid	7.29 ± 0.23	6.0 ± 0.1	4
Syringic acid	7.44 ± 0.17	7.3 ± 0.1	2
5-Hydroxyl-3,4-dimethoxybenzoic acid	NE*	NE*	2
3,4,5-Trimethoxybenzoic acid	NE*	NE*	1
Methyl gallate	8.41 ± 0.16	7.2 ± 0.1	3
n-Propyl gallate	9.43 ± 0.14	8.2 ± 0.1	2
n-Octyl gallate	11.92 ± 0.15	11.8 ± 0.2	2
n-Dodecyl gallate	14.6 ± 0.56	13.2 ± 0.2	2

* NE: no scavenging effects against DPPH (190 μM) both in ethanol solution and liposome.

Table 3
Theoretical descriptors of antioxidant by DFT calculations

Phenol	H_p (Hartree)	H_r (Hartree)	BDE (kJ/mol)	IP (kJ/mol)	ΔBDE (kJ/mol)	ΔIP (kJ/mol)
Gallic acid	−646.33576	−645.72182	299.1404	796.6057	−47.5056	−6.5474
Syringic acid	−724.85935	−724.23788	318.8732	770.8033	−27.7728	−32.3498
5-Hydroxyl-3,4-dimethoxybenzoic acid	−724.85994	−724.22860	343.1655	771.7914	−3.4805	−31.3617
3,4,5-Trimethoxybenzoic acid	−764.12266			732.4949		−70.6582
Methyl gallate	−685.60922	−684.99617	296.7935	783.3631	−49.8525	−19.79
n-Propyl gallate	−764.18342	−763.57066	296.0266	778.3739	−50.6194	−24.7792
n-Octyl gallate	−960.60389	−959.99112	298.0444	778.4251	−48.6016	−24.728
n-Dodecyl gallate	−1117.74128	−1117.12776	298.0116	767.3509	−48.6344	−35.8022

H_p is the enthalpy of phenol and it is the sum of electronic energy and scaled zero-point energies calculated by B3LYP/6-311G. H_r is the enthalpy of the phenoxyl radical generated after H-abstraction and it is the sum of electronic energy and scaled zero-point energies calculated by B3LYP/6-311G. BDE is the O–H bond dissociation enthalpy and it was calculated as follows: $BDE = H_r + H_h - H_p$. H_h is the enthalpy of hydrogen atom (−0.50000 Hartree). BDE and IP value for phenol are 346.646 and 803.1531 kJ/mol, respectively, Leopoldini et al. (2004). $\Delta BDE = BDE_{antioxidant} - BDE_{phenol}$ $\Delta IP = IP_{antioxidant} - IP_{phenol}$

enthalpy (OH-BDE) and ionization potential (IP) were calculated (Table 3) since it has been verified that H-atom transfer and electron transfer were two oxidation pathways in which antioxidants can play a preventive role (Wright et al., 2001; Leopoldini et al., 2004; Zhang et al., 2003). In the former, the antioxidants (ArOH) react with a radical to form an $ArO^{\bullet}$ radical, therefore, the BDE of the O–H bonds is an important parameter determining the antioxidative bioactivity. In the second mechanism, in contrast, the antioxidants gave electrons to the free radicals to form $AROH^{\bullet}$ radicals which are in turn ionised to form $ArO^{\bullet}-$ radical. In such cases, the ionization potential is also important for the scavenging activity.

In the second mechanism, in contrast, the antioxidants gave an electron to the free radicals to form $AROH^{\bullet}$ radicals which are in turn ionised to form $ArO^{\bullet}-$ radicals. In such cases, the ionization potential is also important for the scavenging activity.

In aqueous solutions, the differences of BDE and IP compared to phenol, ΔBDE and ΔIP, provide mechanistic indicators for two antioxidation mechanisms. If $\Delta IP \leq -151$ kJ/mol and $\Delta BDE \approx -42$ kJ/mol, the mechanism is dominated by H-atom transfer, whereas if $\Delta IP < -189$ kJ/mol, the antioxidant mechanism is often dominated by single electron transfer (SET) mechanism (Wright et al., 2001; Leopoldini et al., 2004). For all the GA derivatives studied, IP values are far greater than BDE values; ΔIP values are all greater than −151 kJ/mol and ΔBDE are lower than −42 kJ/mol except HDMBA. For methylated gallic acids (GA, SA, HDMBA), the BDE is inversely whereas the IP is conversely correlated with the scavenging efficiency. With regards to gallic acid esters, no obvious difference in the O–H BDE and IP values was found. The steric effect may be plausible explanation for the difference scavenging effect of gallic acid esters in liposome where the lipid matrix of liposome restricts the movement of antioxidants, especially when the molecule has long side chain. The influence of side chain on antioxidant activity has also been found in α-tocopherol homologs during (Fe^{2+} + ascorbate) or (Fe^{2+} + NADPH)-induced lipid peroxidation in rat liver microsomes (Kagan et al., 1990).

To investigate this effect, the parameter 1/Mw or the number of carbons in the acyl chains (CN) were introduced to assess the steric effect and two good linear relationships between steric parameters and the scavenging effect were found:

$$\frac{1}{IC_{50}} = \frac{0.00402 + 21.93579}{Mw}, \quad R = 0.99288, \quad n = 5, \quad P < 0.001$$

$$IC_{50} = 7.55926 + 0.57724 \times CN, \quad R = 0.99643, \quad n = 5, \quad P < 0.001$$

3.4. Gallic acid derivatives prevent 6-OHDA-induced loss of SH-SY5Y cell viability

SH-SY5Y cells were incubated with different concentrations (1–200 μM) of 6-OHDA and/or gallic acid derivatives, and the cell viability was determined. 6-OHDA-induced cell death in a dose-dependent manner (Fig. 5A). The survival rate of SH-SY5Y was about 45–50% when the cells were treated with 100 μM of 6-OHDA for 24 h. When cells were treated with various concentrations of gallic acid derivatives alone for 24 h, the survival rate varied little at low concentrations (less than 150 μM) and was reduced to 80% or even lower at high concentrations (data not shown).

When cells were pre-treated with different concentrations of gallic acid and its esters for 1 h followed by 6-OHDA treatment, they showed protecting effects on the cell viability against the damage caused by 6-OHDA in a dose-dependent manner (Fig. 5B). Cells were pre-incubated with different gallic acid derivatives (100 μM) in the presence of 100 μM 6-OHDA for 24 h, and cell viability was influenced by the molecular structure of the gallic acid derivatives as shown in Fig. 5C. TMBA and HDMBA exerted no obvious protective effects at 100 μM concentration. GA and SA increased the cell viability rate by 10–15%. Gallic acid esters, however, all showed significant protective effects. PG exhibited the most protective ability, and the increased cell viability to 90%.

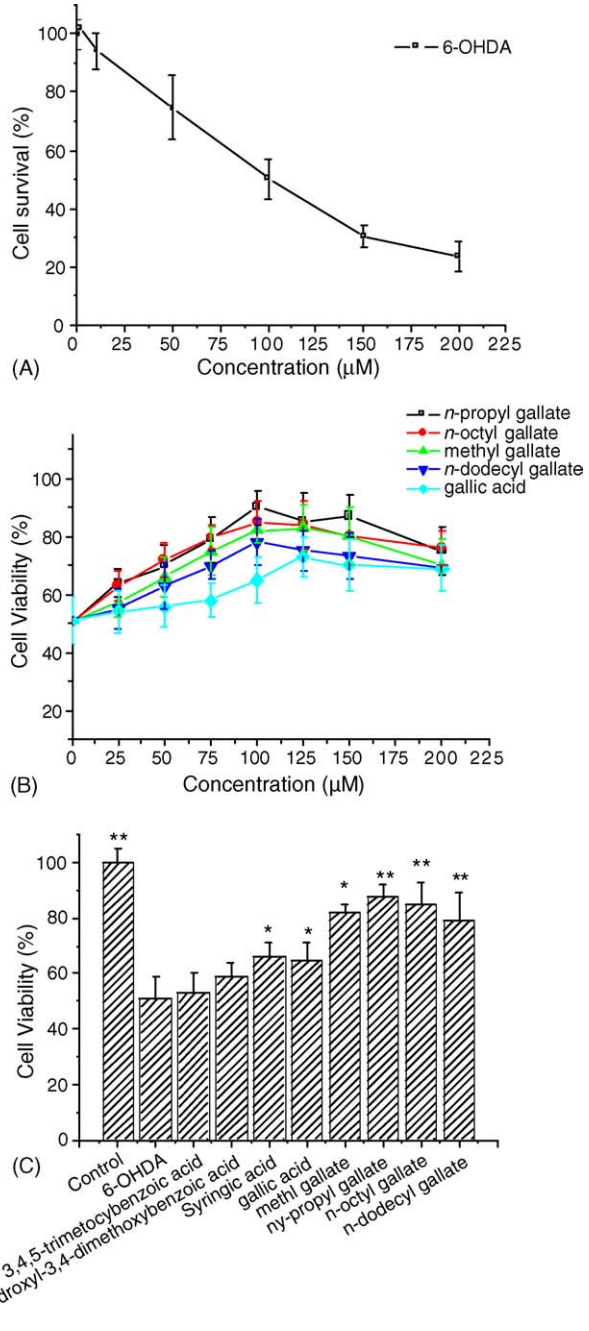


Fig. 5. Effect of 6-OHDA and gallic acid derivatives on SH-SY5Y cell viability. Cells were incubated in drug-free medium or medium containing different concentrations of 6-OHDA (A) for 24 h. (B) Cells were pre-incubated with different concentrations of gallic acid esters for 1 h, then co-incubated with 100 μM 6-OHDA for an additional 24 h. (C) Cells were pre-incubated with 100 μM gallic acid derivatives for 1 h, then co-incubated with 100 μM 6-OHDA for an additional 24 h. Data are expressed as percentage of the untreated control ± S.E.M., $n = 8$. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with 6-OHDA treated cells by ANOVA.

3.5. Gallic acid derivatives rescue 6-OHDA-induced changes in nuclear morphology

Apoptotic nuclei, as indicated by condensed nuclei and nuclear fragmentation, were apparent after exposure to 100 μM 6-OHDA (Fig. 6B). The apoptotic nuclear changes were reduced significantly in the cells pre-treated with 100 μM gallic acid esters PG, OG and DG but not MG (Fig. 6D–F). The other four compounds did not exert any protective effects (Fig. 6C and H–J).

3.6. Intracellular ROS level

Intracellular ROS level was examined by using DCF-DA (Fig. 7). SH-SY5Y cells treated with 100 μM 6-OHDA for 24 h exhibited a significant increase in the DCF signal as compared with the control group. An increase in DCF fluorescence was attenuated significantly by pre-treatment with gallic acid esters, in the order of PG > OG > MG > DG. The decreasing effects of other compounds in the first group were much weaker (Fig. 7). Treatment with gallic acid derivatives alone in our experimental concentration had no obvious effects on the intracellular ROS level despite strong scavenging effects on DPPH in the liposome model (data not shown).

3.7. Intracellular glutathione

To examine the endogenous glutathione levels in apoptotic cells, the contents of both reduced GSH and oxidized glutathione (GSSG) were measured using HPLC-ECD and quantified by ESA software (Fig. 8). The retention time of GSH and GSSG were 3.98 and 9.87 min, respectively, in our experimental conditions. After exposure to 100 μM 6-OHDA for 24 h, the intracellular level of GSH was decreased to 64% of control level and the GSSG level was increased to 180% of the control. Pre-incubation with GA and SA for 1 h slightly improved the GSH level, but not significantly. The difference in GSSG was also not significant. TMBA and HDMBA had no obvious effect on intracellular glutathione. When the gallic acid esters were pre-incubated with the cells, the GSH level increased while GSSG decreased significantly. In agreement with the cell viability assay, PG exerted the most obviously protective effect; the intracellular level of GSH increased to about 90% and GSSG decreased to about 125%. The protective effects of other eaters were as follows: OG > MG > DG. When gallic acid derivatives were incubated alone (100 μM), the level of GSH slightly increased (3–5%), but did not reach statistical significance (data not shown).

3.8. Gallic acid derivatives block 6-OHDA-induced elevation in $[Ca^{2+}]_i$

To examine the intracellular calcium concentration in apoptotic cells, SH-SY5Y cells were incubated with Fluo-3 AM. As shown in Fig. 9, exposure of cells to 6-OHDA (100 μM) resulted in a more than two-fold elevation of $[Ca^{2+}]_i$. Pre-treatment with gallic acid esters could induce a significant decrease in $[Ca^{2+}]_i$, compared with the 6-OHDA treated group, suggesting that gallic acid esters protect the cells against the

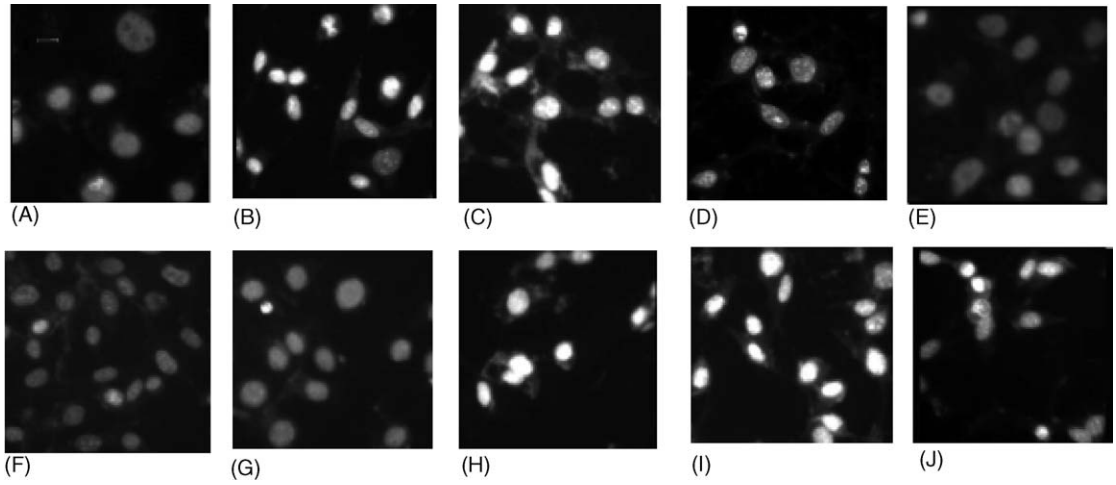


Fig. 6. Fluorescence micrographs of SH-SY5Y cell nuclei from untreated cells (A); cells exposed to 100 μM 6-OHDA (B) or pre-treatment with 100 μM gallic acid derivatives for 1 h and then exposed to 100 μM 6-OHDA for 24 h. The sequence is (C) gallic acid, (D) methyl gallate, (E) *n*-propyl gallate, (F) *n*-octyl gallate, (G) *n*-dodecyl gallate, (H) syringic acid, (I) 5-hydroxy-3,4-dimethoxybenzoic acid and (J) 3,4,5-trimethoxybenzoic acid. The cells were stained with the DNA-binding fluorochrome Hoechst 33258. Scale bar = 25 μm.

elevation of $[Ca^{2+}]_i$ caused by 6-OHDA. GA and SA could also attenuate the elevation of $[Ca^{2+}]_i$, although the effect was weaker than that of gallic acid esters. Gallic acid derivatives alone (100 μM) partly increased $[Ca^{2+}]_i$ by 10–15% (data not shown).

4. Discussion

The present study was aimed to evaluate the structure–activity relationship for the antioxidant activity of gallic acid derivatives in a well defined system of liposomes with the help of theoretical investigation and neuron protective effect against the 6-OHDA-induced stress human SH-SY5Y cells, including improvement in cell viability, intracellular GSH level, maintainance of nuclear morphology, and reduction of ROS level and the influx of Ca^{2+}.

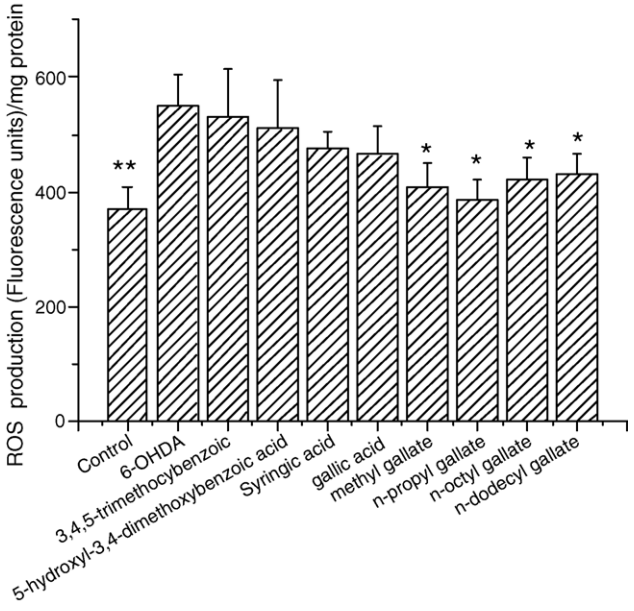


Fig. 7. Gallic acid derivatives attenuated 6-OHDA-induced ROS production in SH-SY5Y cell. Cells were exposed to 6-OHDA for 24 h without or with pre-incubated with 100 μM gallic acid derivatives for 1 h. Data are expressed as percentage of the untreated control ± S.E.M., $n = 6$. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with 6-OHDA treated cells by ANOVA.

4.1. Structure–antioxidant activity relationship in liposomes

In ethanol solution and liposome, the DPPH scavenging efficiency of GA derivatives is critically dependent on the presence of the phenolic hydroxyl groups; methylation of hydroxyl groups showed detrimental effects on the antioxidative activity. The OH group at *para*-position to the carboxylic group is essential for the activity. This is also reflected in the results of variation in values of BDE and IP by theoretical studies. Furthermore, gallic acid esters, having exactly the same number of hydroxyl groups, showed a clear decline of antioxidant activity with an increase of the acyl chain length, indicating that the steric freedom is also important for the activity of antioxidants. Since antioxidants have to diffuse into liposomes to react with lipophilic free radicals such as DPPH, it is reasonable to expect that the hydrophobicity of antioxidants is only relevant to their radical scavenging efficiency in liposomes and cells. However, the results obtained in this study are consistent in both solution and model membrane systems. It is surprising to note that the hydrophobicity failed to show any positive correlation to the scavenging efficiency towards the lipophilic free radical DPPH. For example, gallic acid has much lower *P*-values than its methyl and propyl esters have (Table 1), their DPPH scavenging efficiency in liposome systems was not drastically different. In fact, gallic acid showed an extremely rapid DPPH scavenging reaction in liposomes (Fig. 3) even though gallic acid is perceived as pretty hydrophilic with a log *P* value of −0.5 (being similar to that of acetone). No diffusion bottleneck

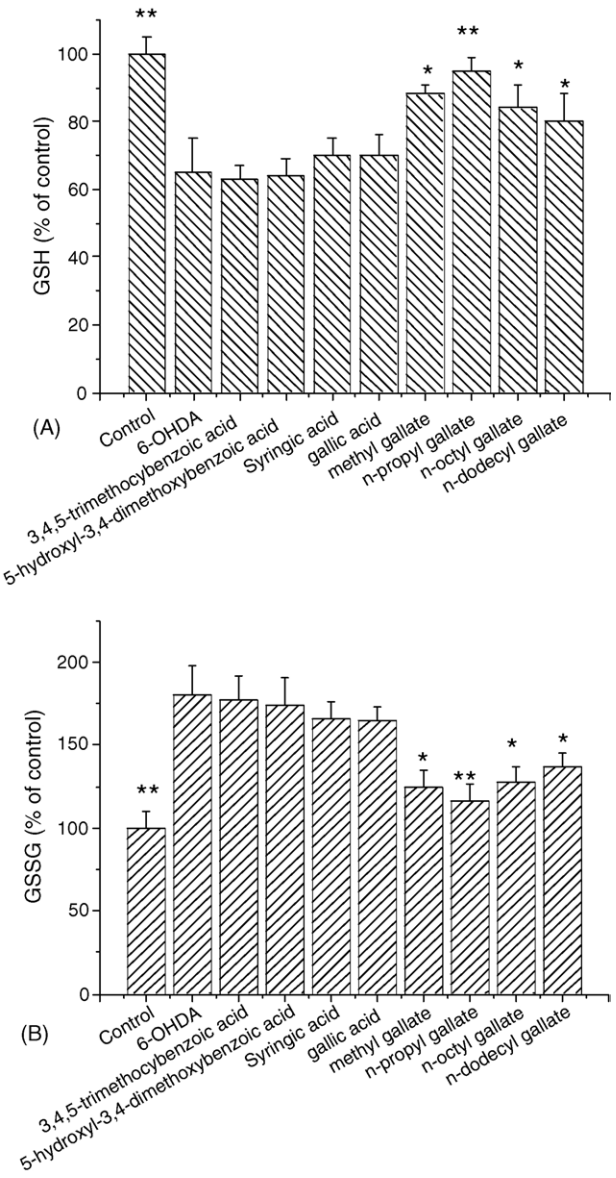


Fig. 8. Gallic acid derivatives attenuated 6-OHDA-induced intracellular glutathione level variation in SH-SY5Y cell. (A) GSH and (B) GSSG. Cells were exposed to 6-OHDA for 24 h without or with pre-incubated with 100 μM gallic acid derivatives for 1 h. Data are expressed as percentage of the untreated control ± S.E.M., $n = 6$. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with 6-OHDA treated cells by ANOVA.

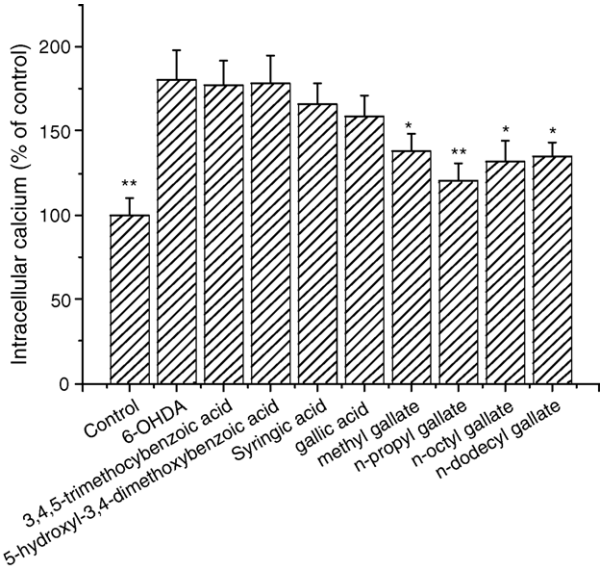


Fig. 9. Gallic acid derivatives blocked 6-OHDA-induced intracellular elevation of $[Ca^{2+}]_i$ in SH-SY5Y cell. Cells were exposed to 6-OHDA for 24 h without or with pre-incubated with 100 μM gallic acid derivatives for 1 h. Data are expressed as percentage of the untreated control ± S.E.M., $n = 6$. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with 6-OHDA treated cells by ANOVA.

was observable for any of the polyphenolic antioxidants. This is probably own to high concentration of antioxidants was used. So it is reasonable to conclude that multiple mechanisms regulate antioxidant action significantly, although they contribute to the antioxidant activity at different degrees. Bias is inevitable when analysis is limited to only one mechanism.

4.2. Structure–antioxidant activity relationship in cell systems

How relevant are these results from model systems to real cells? Such questions are reasonable when taking into consideration the differences between structures, biochemistry of cells and the structure/chemistry in model systems (Azzi et al., 2004; Ishige et al., 2001). Therefore, it is vital to address the above raised question by extending such studies to cells.

6-OHDA has long been used to establish the experimental model of PD in cultured cell and animal systems. Oxidative stress is believed to play an important role in 6-OHDA-induced dopaminergic cells apoptosis. 6-OHDA-induced ROS will initially deplete intracellular glutathione and increase cellular vulnerability (Kostrzewa and Jacobowitz, 1974; Kumar et al., 1995; Cohen and Heikkia, 1974; Graham, 1978; Hastings, 1995; Hara et al., 2003). ROS formation can also trigger the elevation of Ca^{2+}, and excessive mitochondrial Ca^{2+} accumulation may lead to cytochrome *c* release and superoxide anion production (Jha et al., 2000). Cytochrome *c* can bind to the apoptotic protease-activating factor-1 (APAF-1), which activates procaspase-9 in the presence of ATP, resulting in caspase-mediated execution of apoptosis (Janus et al., 2000; Chakraborti et al., 1999; Castillo and Babson, 1998; Kruman and Mattson, 1999; Fiskum et al., 2003; Li et al., 1997); therefore, cellular viability and morphology, ROS level, GSH level and the stabilizing Ca^{2+} homeostasis in SH-SY5Y cells exposed to 6-OHDA were choosen to measure the neuroprotective effect of the gallic acid derivatives.

Judged from these parameters, this study showed that pre-treatment with gallic acid derivatives does suppress 6-OHDA-induced oxidative stress to various degrees. Amongst the methylated gallic acids, only GA and SA showed some moderate activity against 6-OHDA stress. Little activity is observable for TMBA and HDMBA. This is in excellent agreement with the free radical scavenging efficiency of the polyphenols in solution and liposomes. Being much more

hydrophilic than its esters (MG, PG, OG and DG, see Table 1), gallic acid showed much weaker protective effects than its esters (MG, PG, OG and DG). This implies that the hydrophobicity of antioxidants has considerable importance in cell systems. Among the gallic acid esters, a clear optimum is observable for PG, suggesting the importance of a balance between hydrophobicity and antioxidant activity for good antioxidants in multicellular systems. Our previous study on the distinct effects of tea catechins on 6-OHDA-induced apoptosis in PC12 cells is in agreement with the present study (Nie et al., 2002). (−)-Epigallocatechins gallate (EGCG) and (−)-epicatechin gallate (ECG) have good scavenging effects and relatively high hydrophobic properties and thus both compounds showed much higher protective efficiency than, (−)-epicatechin (EC), (+)-catechin or (−)-epigallocatechin (EGC). As for EGC, its poor antioxidant activity and low hydrophobicity may prevent its passage through the cell membranes; thus, EGC confers the less antioxidant protection.

Based on these results and analysis, it can be inferred that hydrophobicity is a particularly important factor in determining antioxidant activity within cell systems. A hydrophobic antioxidant may easily enter the cytoplasm and attenuate ROS formation and accumulation in 6-OHDA toxicity. The importance of hydrophobicity in determinating antioxidant capacity is also found in other models of oxidative stress (Ishige et al., 2001; Behl et al., 1997).

The purpose of this work was to study the relationship of structure with function for a series of antioxidants in protecting cells against oxidative stress damage. It is also an example to evaluate the antioxidants' free radical scavenging capacity by combining the results obtained from chemical solution, liposomes, cell models and theoretical prediction. The results from this study suggest that the neuroprotective effect of a compound on a cell against oxidative stress damage not only depends on its capacity to scavenging free radicals, but also depends on its hydrophobic property that allows it to cross cell membranes to reach its targets. It is, therefore, inadequate to evaluate an antioxidant only by theoretical calculation or chemical methods and its protective effects against oxidative stress damage should also be evaluated in the cellular systems. The combination of the theoretical prediction with experimental screening is probably of some value in designing antioxidants with good efficacy.

5. Conclusions

In summary, we have studied the structure activity relationship of gallic acid derivatives in solution, liposome and cell systems. BDE and IP obtained from ab initio calculations were used to elucidate the antioxidant activity of gallic acid derivatives. Some other molecular properties of the antioxidants should also be taken into account to interpret the experimental results. The different performance of gallic acid derivatives in liposome and cell systems under oxidative stress was compared and it was found that molecular polarity is an essential factor in determining the activity of antioxidants in cell systems. Antioxidants with high antioxidant activity and proper hydrophobicity may be the best choice for preventing the oxidative stress injury in neurodegenerative diseases.

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