

Full-length article

Comparison of antioxidant activities between salvianolic acid B and *Ginkgo biloba* extract (EGb 761)¹

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Key words

antioxidant; *Ginkgo biloba* extract 761; free radical; salvianolic acid B; SH-SY5Y cells;

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Abstract

Aim: To investigate and compare the antioxidant activities of salvianolic acid B (SalB) and *Ginkgo biloba* extract (EGb 761) in aqueous solution, rat microsomes and the cellular system. **Methods:** Superoxide anion ($O_2^{\cdot-}$) was generated using xanthine/xanthine oxidase system and phenazine methosulfate/NADH system, and the effects of SalB and EGb 761 on the generation of $O_2^{\cdot-}$ were achieved by spectrophotometric measurement of the product formed on reduction of nitro blue tetrazolium. Two different methods were used to assess the scavenging effects of the extracts on hydroxyl radical ($\cdot OH$): HPLC method was used for quantitation of $\cdot OH$ by oxy-radical trapping of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) to form DMPO-OH adducts in Fe^{2+} -EDTA- H_2O_2 system. To confirm the HPLC data, $\cdot OH$ was also measured by spectrophotometry using a commercial detection kit. The anti-lipid peroxidation effects of the extracts in microsomes of rat brain, liver and kidney induced by ascorbate-NADPH were determined by thiobarbituric acid (TBA) method. The protective effects of the extracts on peroxide hydrogen (H_2O_2)-induced oxidative damage in SH-SY5Y cells were investigated by assessing cell viability assay, the level of lipid peroxidation, and the lactate dehydrogenase (LDH) release. **Results:** Both SalB and EGb 761 were able to scavenge $O_2^{\cdot-}$ and $\cdot OH$, inhibit lipid peroxidation of microsomes, and protect SH-SY5Y cells against H_2O_2 -induced oxidative damage. However, the concentration of SalB was far lower than that of EGb 761 when a similar effect was obtained. **Conclusion:** The antioxidant efficiency of SalB was greater than that of EGb 761. These results suggest that SalB, like EGb 761, has promising potential in treating oxidative damage-derived neurodegenerative disorders.

Introduction

Oxidative stress is believed to be a primary factor both in neurodegenerative diseases and in the normal process of aging^[1,2]. Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($\cdot OH$), are ascertained to exert some detrimental effects, including lipid peroxidation of cell membranes, alteration of lipid-protein interactions, enzyme inactivation^[3], and DNA breakage^[4]. High oxygen consumption, relatively low antioxidant levels and low regenerative capacity result in brain tissue being susceptible to oxidative damage. Thus, antioxidants and free radical

scavengers might have therapeutic applications in treating ROS-initiated neurodegenerative diseases such as Parkinson's (PD) and Alzheimer's diseases (AD).

Recently, natural antioxidants have received considerable interest owing to their potential health benefits^[5]. *Salvia miltiorrhiza*, commonly known as Danshen, is a well-known Chinese traditional herbal medicine, which has been widely used in oriental medicine for the treatment of heart and brain diseases, hepatitis, hemorrhage, and menstrual abnormalities. Total salvianolic acids are water-soluble components extracted from *salvia miltiorrhiza*. They contain approximately

1% salvianolic acid A (SalA), 57% salvianolic acid B (SalB), 35% rosmarinic acid and other polyphenolic acids^[6]. Clearly, SalB (Figure 1) is the greatest constituent of total salvianolic acids. Our previous study showed that SalB possesses strong antioxidant activities^[7]. It was also demonstrated that SalB could ameliorate learning and memory dysfunctions induced by transient cerebral ischemia in mice^[8], alleviate ischemic damage in rats^[9], and prevent cytotoxicity induced by amyloid β protein ($A\beta$)^[10]. Although the mechanism of action of SalB is not fully understood, some investigators believe that the antioxidant activity of SalB is involved in its pharmacological actions^[8,9].

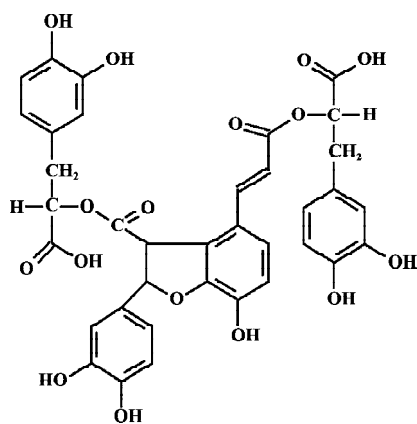


Figure 1. The structure of salvianolic acid B.

Ginkgo biloba is another Chinese plants widely used for its medicinal properties for several thousand years. The standardized extract of *ginkgo biloba* leaves, named EGb 761, contains specific percentages of ginkgo-flavone glycosides (24%) and terpenoids (6%)^[11]. During the past decade, many experiments have demonstrated that EGb 761 exhibits a range of biochemical and pharmacological effects^[12]. It possesses antioxidant and neuroprotective properties, improves blood rheology and tissue metabolism, opposes the detrimental effect of ischemia, attenuates apoptosis in neuronal cultures and inhibits the formation of $A\beta$ aggregates. Recently, EGb 761 has been marketed as a therapeutic dietary supplement to counteract a variety of neurological disorders, including AD. Most of the effects of EGb 761 have been attributed to its antioxidant properties, probably because of the presence of flavonoid or terpenoid constituents in the extract^[13,14].

Although *salvia miltiorrhiza* has been used in traditional medicine for over 1000 years, its antioxidant activity still lacks convincing evidence. Moreover, comparative evaluation of the antioxidant activities of SalB and EGb 761 has not as yet

been conducted. The goal of the present study was to investigate antioxidant and free radical scavenger activity of SalB, and compare the effects of SalB with those of the well-known natural antioxidant EGb 761.

Materials and methods

Drugs and reagents SalB (purity >99%) was purchased from the National Pharmaceutical and Biological Product Inspection Institute Of China (Beijing, China), and EGb 761 was obtained as dry powders from IPSEN (Paris, France). 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,5-dihydroxybenzoic acid (2,5-DHBA), L-ascorbic acid, NADH, NADPH, phenazine methosulfate (PMS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma Chemical (St Louis, MO, USA). High performance liquid chromatography (HPLC) grade acetonitrile were obtained from Merck (Darmstadt, Germany). Other reagents made in China were of analytical grade. SalB and EGb 761 were freshly dissolved in sterilized double deionized water and filtered through a 0.22- μ m filter (Millipore, CA, USA) before each experiment.

In our pilot studies, increasing concentrations of SalB (0.00072, 0.0072, 0.072, 0.72, and 7.2 mg/L) and EGb 761 (1, 5, 10, 50, and 100 mg/L) were studied in scavenging ROS and inhibiting lipid peroxidation in order to select the effective doses for further use in experimental studies.

Superoxide anion assay Two sources were used for the production of the superoxide free radical. The xanthine/xanthine oxidase system^[15]: superoxide anion was detected by spectrophotometry with a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. $O_2^{\cdot -}$ was generated enzymatically using the xanthine/xanthine oxidase system in the absence and presence of different concentrations of SalB or EGb 761. Then the yield of $O_2^{\cdot -}$ could reduce Nitro Blue Tetrazolium (NBT), becoming a colored substance. After reaction, each sample was detected and the absorbance was read at a wavelength of 550 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Prior to performing the study, we examined whether these two plant extracts inhibited xanthine oxidase activity by monitoring uric acid formation spectrophotometrically at 290 nm^[16]. The PMS/NADH system: superoxide radical was generated in a non-enzymic system described by Nishimiki *et al*^[17] with slight modifications. The reaction mixture contained 15 μ mol/L PMS, 78 μ mol/L NADH, 30 μ mol/L NBT, and various concentrations of extracts in phosphate buffer (0.1 mol/L, pH 7.4). After being incubated at ambient temperature for 5 min,

the color was read at 560 nm against blank.

Hydroxyl free radical assay The following two methods were used to assess the scavenging effect on hydroxyl radicals. High performance liquid chromatography with electrochemical detection (HPLC-ECD): a Fenton-type reaction ($\text{Fe}^{2+}\text{-EDTA}+\text{H}_2\text{O}_2\rightarrow\text{Fe}^{3+}\text{-EDTA}+\cdot\text{OH}+\text{OH}^-$) was used to test the ability of SalB and EGb 761 to scavenge $\cdot\text{OH}$, according to a previous study^[18] with slight modifications. The reaction mixture contained 5 mmol/L DMPO, 500 $\mu\text{mol/L}$ FeSO_4 , 500 $\mu\text{mol/L}$ EDTA, and 2 mmol/L H_2O_2 , in the absence and presence of the extracts in a final volume of 0.5 mL. After incubation at 37 °C for 5 min, 50 μL 2,5-DHBA solution was added at a final concentration of 0.5 $\mu\text{mol/L}$. Then 20 μL of the mixture was injected into HPLC and analyzed by using an electrochemical detector. The $\cdot\text{OH}$ was measured by oxy-radical trapping of DMPO to form DMPO-OH adducts in the system. HPLC was carried out on a Spherisorb ODS-2 reversed-phase column (250 mm \times 4.6 mm, 5 μm), with a mobile phase of citric acid/sodium acetate (30 mmol/L citric acid-50 mmol/L sodium acetate-3% acetonitrile, pH 5.1), at a flow rate of 1.2 mL/min and detection potential of 0.6 V with Ag/AgCl as a reference electrode. The quantitation of the DMPO-OH adducts was expressed as the ratio of its peak area to that of 2,5-DHBA used as an internal standard. Spectrophotometry: hydroxyl radicals were also measured by spectrophotometry using a commercially available detection kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. The kit uses a chromogenic substrate, which reacts with the product of $\cdot\text{OH}$, yielding a stable color substance with maximum absorbance at 550 nm.

Assay of lipid peroxidation in microsomes of rat brain, liver and kidney Male Wistar rats (Grade II, Certificate No SCXK 2004-0001), weighing 180–200 g each, were purchased from the Experimental Animal Center, Chinese Academy of Medical Sciences. The animals were maintained with free access to food and drinking water under constant temperature and humidity conditions for 1 week before the experiment. Microsomes were prepared according to the method described in a previous study^[19]. Wistar rats were killed by cervical dislocation. Their brains, livers and kidneys were removed and homogenized in 0.25 mol/L sucrose, 0.05 mol/L Tris, 0.025 mol/L KCl, 0.003 mol/L MgCl_2 buffer (pH 7.5). Microsomal fractions were obtained by centrifuging the whole homogenate at 10 000 $\times g$ for 30 min and then centrifuging the supernatant at 105 000 $\times g$ for 60 min. Microsomal pellets were suspended in 0.1 mol/L phosphate buffer (pH 7.4) to yield a final concentration of 10 mg microsomal protein/mL. Protein concentration was determined by the Lowry method using bovine serum albumin as the standard.

The level of malondialdehyde (MDA), a secondary product of lipid peroxidation, is often used as an index of lipid peroxidation by reacting the lipid peroxides with thiobarbituric acid (TBA). In the present study, lipid peroxidation in microsomes prepared from brain, liver and kidney were induced by ascorbate-NADPH^[20] and determined as TBA-reacting products as described in a previous study^[21]. The reaction mixture, containing 1 mg of microsomal protein in 0.1 mol/L phosphate buffer (pH 7.4), 200 $\mu\text{mol/L}$ NADPH, 50 $\mu\text{mol/L}$ L-ascorbic acid, and various concentrations of the tested extracts, was incubated at 37 °C for 30 min (final volume=1 mL). The reaction was terminated by the addition of 2 mL of 0.53% TBA (w/v) and 20% trichloroacetic acid (TCA). This mixture was heated at 100 °C for 30 min, cooled in ice and centrifuged at room temperature for 10 min at 3000 $\times g$. The absorbance of each supernatant at 532 nm was then measured and 1,1,3,3-tetraethoxypropane (TEP) was used as a standard. The results were expressed as percent of control.

Hydrogen peroxide (H_2O_2)-induced oxidative damage in SH-SY5Y cells Human neuroblastoma SH-SY5Y cells were obtained from Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The cells were cultured in DMEM with 10% FBS, 100 U/mL penicillin, 100 mg/L streptomycin, and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 . All experiments were carried out 48 h after cells were seeded at a density of 1.0×10^5 cells/cm². H_2O_2 was added into the cultures from freshly prepared stock solutions with a final concentration of 150 $\mu\text{mol/L}$. The extracts were added to cells 15 min before the treatment with H_2O_2 . Assay for cell viability, lipid peroxidation and enzyme activity were performed 24 h after incubation with H_2O_2 .

MTT is an indicator of the mitochondrial activity of living cells and is generally used as an index of cell survival^[22]. After different treatments, 10 μL of the 5 g/L MTT stock solution was added in 96-well culture plates and maintained for 4 h. Subsequently, the medium was aspirated and 100 μL DMSO was added. The absorbance was measured at 570 nm. The SH-SY5Y cells were washed two times with PBS for harvesting, then scraped from the plates into ice cold PBS (0.1 mol/L, containing 0.05 mmol/L EDTA) and homogenized. The homogenate was centrifuged at 4 °C at 10 000 $\times g$ for 30 min. The resulting supernatant was stored at -20 °C until MDA assay. The content of MDA was measured as described above. LDH, an indicator of cell injury, was detected with an assay kit (Beijing BHK Clinical Reagent, Beijing, China). Culture medium (50 μL) was gently aspirated and saved for LDH determination. LDH could catalyze the synthesis of pyruvic acid from lactic acid, and pyruvic acid then reacted with 2,4-dinitrophenyl hydrazine to form a derivative

of phenylhydrazone. The latter showed a brownish-red color in basic solution. After reaction, each sample was detected and the absorbance was read at a wavelength of 440 nm.

Statistical analysis All results are expressed as means \pm SD. Comparisons between groups were made using an analysis of variance (ANOVA), followed by a least significant difference multiple range test. The differences were considered significant when $P < 0.05$.

Results

Selecting the effective doses of SalB and EGb 761 In our pilot studies, graded concentrations of SalB (0.00072, 0.0072, 0.072, 0.72, and 7.2 mg/L) and EGb 761 (1, 5, 10, 50, and 100 mg/L) were studied on ROS formation and lipid peroxidation. It was not observed that the lower doses of SalB (0.00072 and 0.0072 mg/L) and EGb 761 (1 and 5 mg/L) produced any significant effect compared to the control, although slight reductions appeared (data not shown). Therefore, in the following experiments the higher doses of SalB (0.072, 0.72, and 7.2 mg/L) and EGb 761 (10, 50, and 100 mg/L) were used to compare the antioxidant activities between them. The concentrations 0.072, 0.72, and 7.2 mg/L of SalB used in the following were equivalent to 0.1 μ mol/L, 1 μ mol/L, and 10 μ mol/L in molarity, respectively.

Effects of SalB and EGb 761 on superoxide anion radical

The ability of extracts to scavenge the superoxide anion radical was monitored in two different systems. In Figure 2, the effects of these extracts on superoxide anion radical generated in the xanthine/xanthine oxidase system was presented. Before testing the inhibitory effects of SalB and EGb 761 towards superoxide generation, we examined whether these extracts interact with xanthine oxidase. It was observed that SalB at the concentrations tested here did not exhibit a significant influence on xanthine oxidase, so the reduction in the generation of superoxide anion was the direct effect of SalB on superoxide anion. However, EGb 761 at concentrations above 50 mg/L significantly inhibited xanthine oxidase activity (data not shown), indicating the actual superoxide scavenging action of EGb 761 was lower than that shown in Figure 2. As shown in Figure 2, both of these extracts inhibited $O_2^{\cdot -}$ generation in a dose-dependent manner. At the concentration of 7.2 mg/L, SalB caused 36.6% inhibition of $O_2^{\cdot -}$ generation, and EGb 761 at the concentration of 10 mg/L could achieve a similar inhibitory effect. Although the inhibitory effect strengthened with an increase of the concentration of EGb 761, the result was partly associated with its interaction with xanthine oxidase.

The ability of extracts to scavenge the superoxide anion radical was also assayed in the PMS/NADH system (Figure 3).

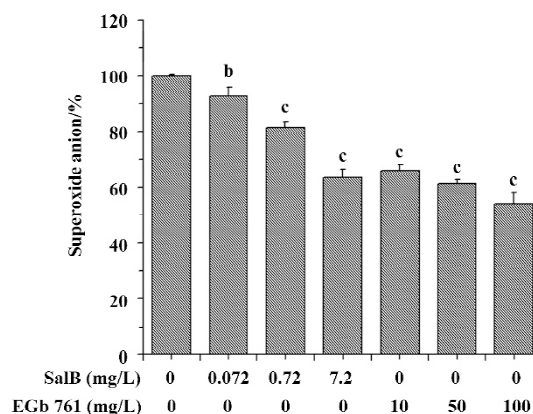


Figure 2. Effects of SalB and EGb 761 on superoxide anion radical generated by xanthine/xanthine oxidase system. $n=8$. Mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs control group.

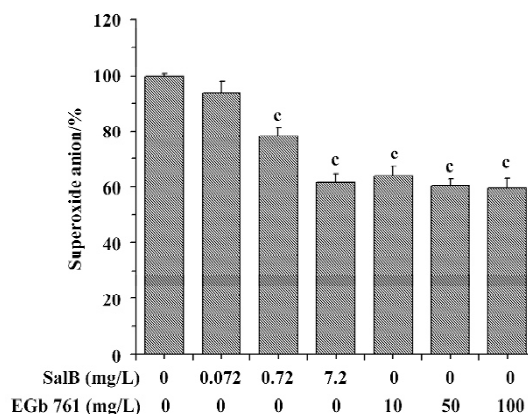


Figure 3. Effects of SalB and EGb 761 on superoxide anion radical generated by PMS/NADH system. $n=8$. Mean \pm SD. ^c $P < 0.01$ vs control group.

It is obvious that SalB exhibited dose-related inhibitory activity towards $O_2^{\cdot -}$ generation, and that the highest concentration (7.2 mg/L) tested here caused about 40% inhibition of $O_2^{\cdot -}$ generation. EGb 761 inhibited the $O_2^{\cdot -}$ generation by 40% at concentrations above 10 mg/L, and the inhibitory activity remained constant at higher EGb 761 concentrations, indicating that the maximal effect of EGb 761 is obtained at approximately 10 mg/L. These results obtained from the two systems indicate that both SalB and EGb 761 are capable of inhibiting $O_2^{\cdot -}$ formation, and the inhibitory activity of SalB is more potent.

Effects of SalB and EGb 761 on hydroxyl free radical

The ability of extracts to scavenge the hydroxyl free radical was evaluated by two different methods. Figure 4 shows a typical HPLC chromatograph of DMPO-OH adduct in Fe^{2+} -EDTA- H_2O_2 system. In Figure 5, the effects of these extracts

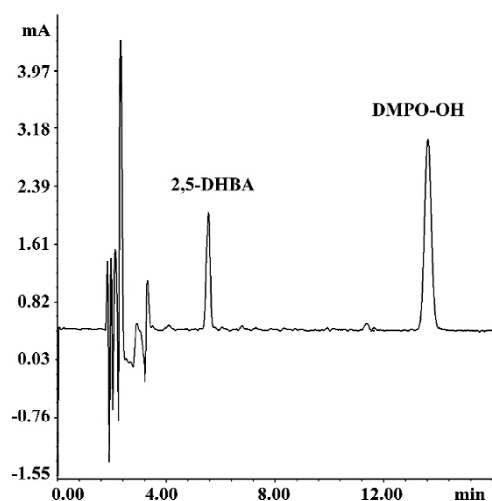


Figure 4. HPLC chromatograph of DMPO-OH adduct in Fe^{2+} -EDTA- H_2O_2 system.

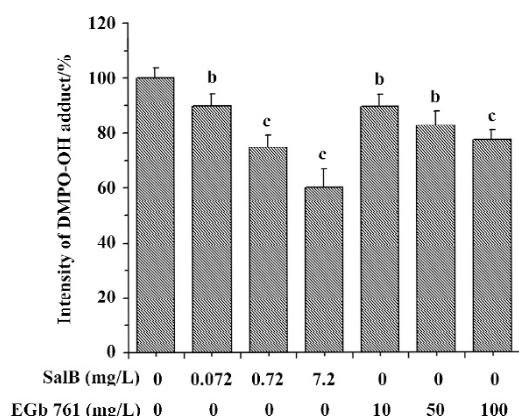


Figure 5. Effects of SalB and EGb 761 on hydroxyl free radical generated by Fe^{2+} -EDTA- H_2O_2 system and monitored by HPLC-ECD. $n=5$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control group.

on hydroxyl radical monitored by HPLC-ECD was presented. All the concentrations of the two extracts significantly inhibited the formation of DMPO-OH and exhibited dose-related inhibitory activity. SalB exerted a maximal inhibitory effect of 40% at a level of 7.2 mg/L, and EGb 761 could not achieve the same inhibitory effect even at the highest concentration (100 mg/L) tested here.

The ability of extracts to scavenge the hydroxyl radical was also assayed by spectrophotometry using a commercial detection kit. All the concentrations of the two extracts significantly inhibited $\cdot\text{OH}$ generation and the inhibition profile was consistent with the results obtained by HPLC-ECD. These results obtained from the two methods indicate that both SalB and EGb 761 are capable of inhibiting $\cdot\text{OH}$ forma-

tion, and the inhibitory activity of SalB is much more potent (Figure 6).

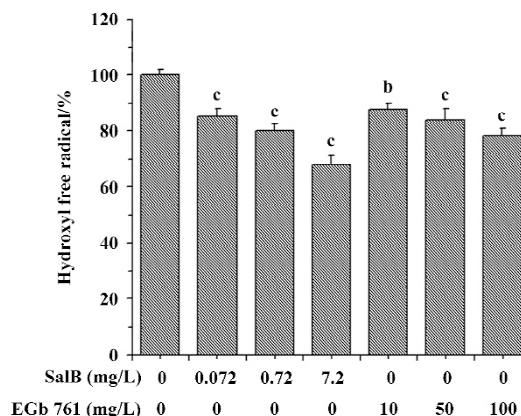


Figure 6. Effects of SalB and EGb 761 on hydroxyl free radical measured by spectrophotometry. $n=8$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control group.

Effects of SalB and EGb 761 on lipid peroxidation in microsomes The effects of these two extracts on microsomal MDA formation are shown in Table 1. SalB and EGb 761 at all the concentrations tested here significantly decreased the formation of MDA and had a dose-dependent inhibitory activity. Consistent with the ROS scavenging activity presented above, SalB was more efficient than EGb 761 in inhibiting lipid peroxidation in microsomes.

Table 1. Effects of SalB and EGb 761 on lipid peroxidation in microsomes of the rat brain, liver and kidney induced by ascorbate-NADPH. $n=6$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control group.

Treatment	Content of MDA (%)		
	Brain	Liver	Kidney
Untreated (control)	100.0 \pm 1.2	100.0 \pm 1.8	100.0 \pm 2.1
SalB (mg/L)			
0.072	86.3 \pm 1.5 ^b	80.4 \pm 1.2 ^b	73.0 \pm 1.8 ^c
0.72	68.4 \pm 1.2 ^c	36.8 \pm 0.6 ^c	50.5 \pm 2.1 ^c
7.2	31.4 \pm 0.4 ^c	24.8 \pm 0.2 ^c	35.3 \pm 1.8 ^c
EGb 761 (mg/L)			
10	71.9 \pm 1.7 ^b	43.4 \pm 0.3 ^c	62.9 \pm 3.1 ^c
50	62.7 \pm 1.6 ^c	31.2 \pm 0.9 ^c	42.5 \pm 1.9 ^c
100	57.4 \pm 0.6 ^c	27.8 \pm 0.4 ^c	37.1 \pm 2.4 ^c

Effects of SalB and EGb 761 on viability of SH-SY5Y cells induced by H_2O_2 The effects of SalB and EGb 761 on the cytotoxicity induced by H_2O_2 in SH-SY5Y cells were

evaluated in this study (Figure 7). Treatment with 150 $\mu\text{mol/L}$ H_2O_2 for 24 h resulted in a significant inhibition of MTT reduction (the cell viability decreased to 65.2% with respect to untreated cells). However, pre-treatment of cells with SalB (0.072–7.2 mg/L) or EGb 761 (10–100 mg/L) concentration-dependently protected cells against the toxicity induced by H_2O_2 . The lowest concentration of SalB or EGb 761 tested here did not enhance significantly viability, although a slight increase was found. In addition, the concentration of SalB was far lower than that of EGb 761 when the similar protective effect was obtained.

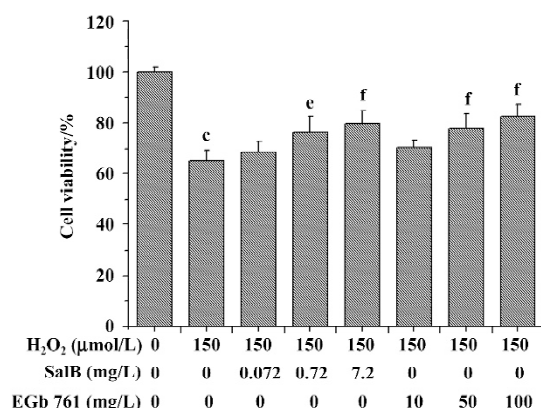


Figure 7. Effects of SalB and EGb 761 on H_2O_2 -induced decrease in SH-SY5Y cell viability when added 15 min prior to H_2O_2 treatment. Cell viability was estimated by MTT assay after cells were pretreated with indicated concentrations of SalB or EGb 761 prior to incubation with H_2O_2 (150 $\mu\text{mol/L}$). $n=6$. Mean \pm SD. ^c $P<0.01$ vs untreated group. ^e $P<0.05$, ^f $P<0.01$ vs group treated with H_2O_2 alone.

Effects of SalB and EGb 761 on the content of MDA and the activity of LDH in SH-SY5Y cells induced by H_2O_2 The treatment of SH-SY5Y cells with 150 $\mu\text{mol/L}$ H_2O_2 caused an increase in the intracellular MDA level by 137.2%. However, preincubation of cells with SalB or EGb 761 markedly attenuated the increase in the MDA level in a dose-dependent manner, and SalB at a lower concentration could achieve a similar inhibitory effect (Figure 8).

The effects of these two extracts on LDH activity induced by H_2O_2 are shown in Figure 9. There was a marked increase of LDH leakage after the addition of H_2O_2 to the culture media, and this response was attenuated by EGb 761 in a dose-dependent manner. Although SalB markedly attenuated the increase in the LDH activity only at the highest concentration, this effective concentration of SalB was far lower than that of EGb 761 when a similar inhibitory effect was obtained.

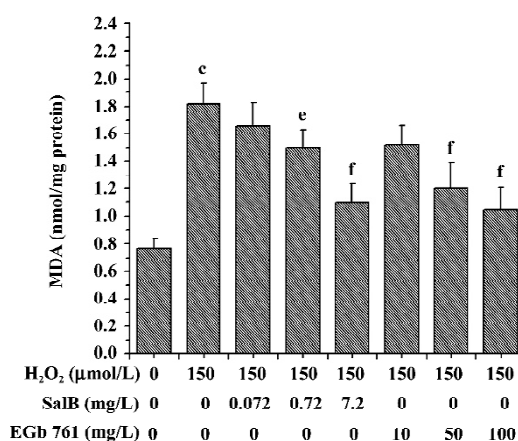


Figure 8. Effects of SalB and EGb 761 on H_2O_2 -induced lipid peroxidation in SH-SY5Y cells. Cells were pretreated with indicated concentrations of SalB or EGb 761 prior to incubation with H_2O_2 (150 $\mu\text{mol/L}$) and lipid peroxidation was assessed by TBA method. $n=4$. Mean \pm SD. ^c $P<0.01$ vs untreated group. ^e $P<0.05$, ^f $P<0.01$ vs group treated with H_2O_2 alone.

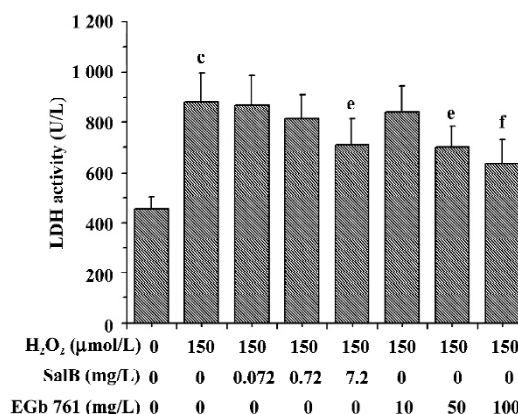


Figure 9. Effects of SalB and EGb 761 on H_2O_2 -induced LDH activity in SH-SY5Y cells. Cells were pretreated with indicated concentrations of SalB or EGb 761 prior to incubation with H_2O_2 (150 $\mu\text{mol/L}$) and LDH activity was assessed according to the manufacturer's instructions. $n=6$. Mean \pm SD. ^c $P<0.01$ vs untreated group. ^e $P<0.05$, ^f $P<0.01$ vs group treated with H_2O_2 alone.

Discussion

Oxidative stress, defined as the imbalance between the formation of reactive oxygen species (ROS) and antioxidative defence mechanisms, has been suggested to be involved in the pathogenesis of most of the neurodegenerative disorders including AD^[1]. Antioxidants that react preferentially with ROS to inactivate them have excited great interest because of their potential for therapeutic use. In this study, we

investigated and compared the antioxidant activities of SalB and EGb 761 in aqueous solution, rat microsomes and the cellular system. Our results showed that SalB is more efficient than EGb 761 in scavenging ROS, especially hydroxyl radical ($\cdot\text{OH}$). For lipid peroxidation inhibition activity in microsomes, we also obtained similar results. Furthermore, both SalB and EGb 761 protected SH-SY5Y cells against H_2O_2 -induced oxidative damage, while the concentration of SalB was far lower than that of EGb 761 when a similar protective effect was obtained.

Superoxide anion ($\text{O}_2^{\cdot-}$) has been of intense interest owing to its increased dominance *in vivo* in different disease conditions. Although $\text{O}_2^{\cdot-}$ is far less active than $\cdot\text{OH}$, it can generate $\cdot\text{OH}$ by superoxide dismutase (SOD) in the biological system. EGb 761 has been previously reported to scavenge $\text{O}_2^{\cdot-}$ generated by using the water radiolysis method, and at concentrations above 100 mg/L completely scavenge superoxide^[14]. But in the present study, our data showed that EGb 761 only inhibited $\text{O}_2^{\cdot-}$ formation by approximately 40% at a concentration of 100 mg/L. The great difference between them may be the result of different systems used for the production of $\text{O}_2^{\cdot-}$. Our data also showed that SalB inhibited $\text{O}_2^{\cdot-}$ formation by approximately 40% at concentrations as low as 7.2 mg/L, suggesting that SalB is an efficient scavenger for $\text{O}_2^{\cdot-}$. However, EGb 761 inhibited the activity of xanthine oxidase (XOD), an important biological source of $\text{O}_2^{\cdot-}$ radical, whereas SalB did not exhibit such inhibitory activity at the concentrations tested here. In addition, EGb 761 appears to have a SOD activity^[13]. Whether SalB possesses enzyme activity requires further investigation.

$\cdot\text{OH}$ is the most active radical causing lipid oxidation and enormous biological damage. Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$), a well known $\cdot\text{OH}$ generator, was often used to test the ability of antioxidants to scavenge this free radical. When catalysed by iron-EDTA, this reaction occurs more rapidly^[23], and more importantly, EDTA is able to weaken the interference effects from some compounds that chelate or bind to iron ions. For these reason, we generated $\cdot\text{OH}$ -radicals from a Fenton system containing EDTA. The radicals formed were scavenged and quantified by oxy-radical trapping of DMPO to form DMPO-OH adducts using the HPLC method. The DMPO-OH adduct peak was separated successfully and identified by HPLC-ECD. To confirm the HPLC data by an alternative method, $\cdot\text{OH}$ was also measured by spectrophotometry. Our results are consistent with the previous reports that EGb 761 could scavenge $\cdot\text{OH}$ -radicals^[14], and demonstrate that SalB is more potent than EGb 761 in scavenging this radical, because EGb 761 even at the highest concentration (100 mg/L) could not achieve the same

level of inhibition as could SalB at a level of 7.2 mg/L.

Lipid peroxidation is one of the reactions induced by oxidative stress and is especially active in those tissues having membranes rich in polyunsaturated fatty acid. The microsomal fraction has been shown to be particularly sensitive to peroxidation and as such has been used extensively to investigate the antioxidant activity of many naturally occurring and synthetic compounds^[24]. EGb 761 has been reported to reduce the free radical-induced lipid peroxidation generated by NADPH- Fe^{3+} systems in rat microsomes, and to protect human liver microsomes from lipid peroxidation induced by cyclosporin A^[25]. In the present study, we investigate the anti-lipid peroxidation effect of SalB and EGb 761 in microsomes of rat brain, liver and kidney induced by ascorbate-NADPH. The results presented here confirmed the inhibitory effect of EGb 761 on lipid peroxidation and showed that SalB was more efficient than EGb 761, which was consistent with their greater ROS scavenging ability. Therefore, the ability of these plant extracts to inhibit lipid peroxidation seems to be related to their ability of scavenging ROS.

Human neuroblastoma SH-SY5Y cells were used in this study because this is a widely used cell line for modeling neuronal characteristics. H_2O_2 is one of the major components of ROS which has been extensively used as an inducer of oxidative stress in an *in vitro* model^[26]. Moreover, as a hydroxyl radical donor, H_2O_2 has been reported to mediate A β toxicity^[27]. The exposure of cultured cells to free radicals results in an imbalance in energy metabolism in addition to the deleterious effects of hydroxyl and peroxy radicals on membrane lipids and proteins. Therefore, cellular function is impaired, which finally causes cell death. Our present studies confirmed that cells treated with H_2O_2 caused a marked decrease in cell survival and elevation of oxidative stress characterized by accumulation of MDA production and LDH release. Lipid peroxidation may cause the damage of the cell membrane, which alters the biophysical characteristics of the cell membrane such as membrane fluidity and the conformation of protein sulphhydryl groups. The release of LDH into the culture medium was regarded as an indicator of cellular damage and cytotoxicity^[28]. However, when SH-SY5Y cells were preincubated with SalB or EGb 761, the H_2O_2 -induced increase in the level of MDA and the release of LDH was remarkably attenuated as well as H_2O_2 -induced reduction in cell survival. Our results are consistent with a previous study on EGb 761 obtained in rat cerebellar granule cells^[29], and show that SalB is more efficient than EGb 761 in protecting SH-SY5Y cells against H_2O_2 -induced oxidative damage. However, EGb 761 could upregulate the activity of SOD, the

key antioxidant enzyme in the brain^[30], and thus enhance the antioxidant capacity of the neuronal system. However, the influence of SalB on antioxidant enzyme activities in H₂O₂-treated SH-SY5Y cells remains unclear.

SalB is a water-soluble single compound isolated from the traditional Chinese drug *Salvia miltiorrhiza*. Our previous studies suggested that SalB has strong antioxidative activity^[7]. Analysis of the molecular structure of SalB also indicated that it possesses a potent capacity for scavenging free radicals. The phenolic hydroxyl group in the structure of the SalB may be related to its antioxidant activity. The EGb 761 extract is a relatively complex mixture that contains flavonoid and terpene components. EGb 761 displays, mainly via its flavonoid constituents, free radical scavenging and antioxidant actions that could account for much of the pharmacological effects of the total extract^[13,14]. Interestingly, EGb 761 is becoming one of the most popular dietary supplements in the USA for enhancing memory. In European countries it is a commonly prescribed drug for treatment of age-related disorders, including AD. Kose and Dogan^[31] have shown that the antioxidant potency of EGb 761 appears to be greater than that of ascorbic acid, glutathione and uric acid, and comparable with that of lipid-soluble antioxidants such as retinol acetate and α -tocopherol. Our results presented herein demonstrated that SalB is more potent than EGb 761 in scavenging O₂⁻ and \cdot OH, inhibiting lipid peroxidation of microsomes, and protecting SH-SY5Y cells against H₂O₂-induced oxidative damage. Moreover, for drugs to be the effective treatment of neurodegenerative diseases they must be capable of penetrating the blood-brain barrier, and SalB can satisfy the requirement. These data suggest that there is promising potential for the natural antioxidant SalB to be used in treating neurodegenerative diseases that involve free radical and oxidative damage.

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