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# Antidepressant-like effect of a *Ginkgo biloba* extract (EGb761) in the mouse forced swimming test: Role of oxidative stress

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ABSTRACT

EGb761 is a well-defined mixture of active compounds extracted from *Ginkgo biloba* leaves. This extract is used clinically due to its neuroprotective effects, exerted probably via its potent antioxidant or free radical scavenger action. Previous studies suggest that oxidative stress, via free radical production, may play an important role in depression and animal models for depression-like behavior. Preclinical studies have suggested that antioxidants may have antidepressants properties. The aim of this study was to investigate the antidepressant-like of EGb761 due to its antioxidant role against oxidative stress induced in the forced swimming test, the most widely used preclinical model for assessing antidepressant-like behavior. Male BALB/c mice were pretreated with EGb761 (10 mg/kg, ip) daily for 17 days followed by the forced swimming test and spontaneous locomotor activity. Animals were sacrificed to evaluate lipid peroxidation, different antioxidant enzyme activities, serotonin and dopamine content in midbrain, hippocampus and prefrontal cortex. EGb761 significantly decreased the immobility time (39%) in the forced swimming test. This antidepressant-like effect of EGb761 was associated with a reduction in lipid peroxidation and superoxide radical production (indicated by a downregulation of Mn-superoxide dismutase activity), both of which are indicators of oxidative stress. The protective effect of EGb761 is not related to excitatory or inhibitory effects in locomotor activity, and was also associated with the modulation of serotonergic and dopaminergic neurotransmission. It is suggested that EGb761 produces an antidepressant-like effect, and that an antioxidant effect against oxidative stress may be partly responsible for its observed neuroprotective effects.



## 1. Introduction

Depression is one of the most prevalent, serious, recurrent, incapacitating and costly psychopathologies worldwide. Depression is characterized by altered mood and cognitive functions, and recurrent thoughts of death or suicide with a lifetime incidence of 15–25% (Paykel, 2006). The World Health Organization predicts that depression will be the second cause of loss in human disability-adjusted life years worldwide. Although the mechanism provoking depression has not been clearly elucidated oxidative stress, via free radical production, may play an important role in its pathophysiology (Ng et al., 2008).

Free radicals are produced constitutively under normal physiological conditions. Organisms have developed various defense mechanisms to protect themselves against injury from free radicals including antioxidant enzymes (catalase, glutathione peroxidase, and superoxide dismutase), free radical scavengers, and metal chelating agents (Reiter, 1995). Normally, there is a balance between the generation of free radicals and antioxidant defense system activity. When this balance is altered to favour production of free radicals due to depletion of antioxidant-system components or increased generation of free radicals, oxidative stress occurs (Halliwel, 1992). Oxidative stress leads to damage of polyunsaturated lipids by lipid peroxidation (LP), a chain-reaction that results in numerous degradation products (Niki et al., 1993). In particular, a number of oxidative disturbances in depressed patients have been reported, including enhanced oxidative damage and decreased antioxidant enzyme levels (Ng et al., 2008; Sarandol et al., 2007). Moreover, preclinical studies have suggested that antioxidants (radical scavengers) may have antidepressant properties (Eren

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et al., 2007; Zafir et al., 2009). It appears reasonable to propose that exogenous antioxidants may be effective in treating depression. Many available synthetic chemical antidepressants have low rates of response, remission and severe adverse effects (Nestler et al., 2002). There are several phytomedicines that have been introduced into psychiatric practice because of greater compliance and milder side effects (Thachil et al., 2007).

The *Ginkgo biloba* extract termed EGb761 has become one of the most widely used medicinal plant products in Europe. It has been used in clinics for the treatment of cerebrovascular insufficiency, degenerative dementia, peripheral vascular disturbances, and neurosensory disorders (DeFeudis and Drieu, 2000). EGb761 is a well-known extract obtained from leaves of the *Ginkgo biloba* tree according to a standardized procedure (Drieu, 1986). The patented extract EGb761 contains two main groups of active compounds, flavonoid glycosides (24%) and terpene lactones (6%) of low molecular weight that permit their penetration of the blood–brain barrier (DeFeudis and Drieu, 2000). The flavonoid fraction is composed of three flavonoids: quercetin, kaempferol, and isorhamnetin, which are linked to a sugar. The terpenoid fraction is composed of ginkgolides A, B, C, M, J and bilobalides (Drieu, 1986). EGb761 exhibits a broad spectrum of pharmacological actions in the central nervous system (DeFeudis and Drieu, 2000). It has been proposed that EGb761 has neuroprotective effects, via its potent antioxidant action (Droy-Lefaix et al., 1995; Rojas et al., 2001, 2008), and as a free radical scavenger (Maitra et al., 1995; Marcocci et al., 1994a; Ni et al., 1996). We have shown that EGb761 could be used as an antioxidant in an animal model of Parkinson's disease (Rojas et al., 2001, 2008).

The forced swimming test (FST) is a well-established preclinical animal model for depression and represents an acute stressful event (Porsolt et al., 1977). The existence of oxidative stress in this depression model has been reported (Akhtar et al., 2005). The aim of this study was to investigate the antidepressant-like effect of EGb761 due to its antioxidant role against oxidative stress induced in the forced swimming test. We analyzed spontaneous locomotor activity, serotonin and dopamine turnover ratio, LP, and different antioxidant enzyme activities such as Mn-superoxide dismutase (Mn-SOD), Cu, Zn-superoxide dismutase (Cu, Zn-SOD), glutathione peroxidase, and glutathione reductase.

## 2. Materials and methods

### 2.1. Materials

EGb761 was provided by Schwabe Pharmaceuticals (Karlsruhe, Germany). Sodium octyl sulfate, sodium metabisulfite, glutathione reductase, NADPH, homovanillic acid, dopamine, serotonin, 5-HIAA, and imipramine hydrochloride were obtained from Sigma–Aldrich (St. Louis, MO, USA). Perchloric acid, thiobarbituric acid, EDTA (Merck, Darmstadt, Germany), polyclonal anti-Cu, Zn-SOD and polyclonal anti-Mn-SOD antibodies (Stressgen Biotechnologies Co., Victoria, BC, Canada), a chemiluminescence detection system (Amersham, Piscataway, NJ, USA) were used for our experiments. All other reagents were reagent-grade and obtained from known commercial sources. Solutions were prepared using deionized water obtained from a Milli R/Q purifier system (Millipore). An adsorbosphere catecholamine analytical column was used (Alltech Associates, Inc., Deerfield, IL, USA).

### 2.2. Animals

Experiments were conducted on male BALB/c mice (Harlan, Mexico) from 11 to 13 weeks of age. The animals were housed five per cage and maintained in standard conditions (12:12 h light/dark cycle, 21 ± 2 °C, relative humidity 40%) and allowed access to food and water *ad libitum* up to the time of the experiment. All experiments were carried out in accordance with the National Institutes of Health Guide (USA) for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978), they were approved by the local ethical committee, and conformed to regulations specified by the Animal Care and Use Committee of our institution and standards of the National Institutes of Health of Mexico (NOM-062-ZOO-1999). All efforts were made to reduce the number of animals used and treated humanely to minimize their pain and discomfort.

### 2.3. Forced swimming test (FST)

Depression-like behavior was assessed in the forced swimming test (FST) with minor modifications by our group (Rojas et al., 2007). Animals were individually placed in a vertical glass cylinder (12 cm in diameter, 23 cm high) filled with tap water (25 ± 1 °C) to a height of 10 cm. Two swimming sessions were conducted: a 15 min pre-test, followed 24 h later by a 6 min test. The total duration of immobility behavior was recorded during the second 6 min test. Immobility was defined as floating passively in an upright position in the water, with only small movements made necessary to keep the head above the water surface. Mice were considered to have stopped swimming when at least three paws were not moving.

### 2.4. EGb761 pretreatment in the FST

EGb761 is a well characterized extract and is the one used in ongoing clinical trials. To test the antidepressant-like effect of EGb761 in the mouse FST, animals were pretreated with EGb761 at different doses (5, 10, 20 or 40 mg/kg, ip) daily for 17 days. In addition, we have reported that EGb761 (10 mg/kg) produces neuroprotection (Rojas et al., 2001, 2004). EGb761 was dissolved in physiological saline and the pH adjusted to 7.4.

There were four groups: Group I: vehicle-treated (saline, ip) + non-FST; Group II: vehicle-treated (saline, ip) + FST; Group III: imipramine (15 mg/kg, ip) + FST; Group IV: EGb761 (5, 10, 20 or 40 mg/kg, ip) + FST. Mice from groups I and II, injected with saline solution, served as controls. Imipramine was used as a classical antidepressant. All groups were treated daily for 17 days and sacrificed after the FST was performed as previously described, their brains were removed immediately, and the prefrontal cortex (PFC), midbrain (MB), and hippocampus (HP) were dissected out on ice for biochemical analysis.

### 2.5. Measurement of locomotor activity

To assess the possible effects of the EGb761 on locomotor activity we used additional groups of mice ($n$ = 8–10 mice per group) that were evaluated as previously reported (Flip and Cunningham, 2003). Locomotor activity was measured by an activity meter Opto-Varimex minor (Columbus Instruments, Ohio, USA). The system uses sensors with high-intensity, modulated infrared light beams to detect animal motion. Locomotor activity associated with ambulatory locomotion was defined as the total distance traveled in 10 min. The mice were placed into the cage activity system, the data-collecting system was immediately activated and locomotor activity was recorded for 10 min. Animals were treated with EGb761 (10 mg/kg), imipramine (15 mg/kg) or with vehicle daily for 17 days, and the last administration day was 60 min before the experimental procedure.

### 2.6. Analysis of dopamine and serotonin turnover

The tissue concentrations of dopamine (DA), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA)

were determined in PFC, MB, and HP according to a method previously described by us (Rojas et al., 2001). The ratios of 5-HIAA/5-HT and HVA/DA were calculated as an index of transmitter turnover. The monoamine content was determined using HPLC system LC 200 (Perkin–Elmer, Shelton, CT, USA) with an electrochemical detector BASi LC-4C (BASi, Inc., West Lafayette, IN, USA). The detector potential was adjusted to 0.8 V vs. Ag/AgCl reference electrode. The mobile phase consisted of aqueous phosphate buffer (pH 3.1), which contained 0.2 mM sodium octyl sulfate, 0.1 mM EDTA, and 15% v/v of methanol. A catecholamine analytical column of 100 × 4.8 mm with 3 μm particle diameter was used (Alltech Associates, Inc., Deerfield, IL, USA). Concentrations of each monoamine were obtained by interpolation of the respective standard curve. The results are expressed as μg of compound per gram of tissue.

### 2.7. Lipid peroxidation analysis

LP was measured by thiobarbituric acid-reactive substances (TBA-RS) assay (Rojas and Ríos, 1993) as an accurate method to characterize oxidative damage to membrane lipids. Four groups of mice ($n$ = 8–10 per group) were assigned as described previously. Brain tissue (PFC, MB or HP) was homogenized in 2 ml of ice-cold 0.05 M phosphate buffer (pH 7.0) containing 0.015 M NaCl and 0.145 M KCl. One ml aliquots of this homogenate were added to 2 ml of the thiobarbituric acid reagent (0.5 g of thiobarbituric acid + 15 g of trichloroacetic acid + 2.5 ml of concentrated HCl in 100 ml water) and the solution heated for 30 min in a boiling water bath. After cooling and centrifugation (2000*g*, 10 min), the absorbance was read on a DU-6 Beckman spectrophotometer at 532 nm. Final amounts of TBA-RS, mostly malondialdehyde, were calculated by interpolation of values in a standard curve and corrected for the content of protein per sample (Lowry et al., 1951). All samples were analysed in duplicate and results are expressed as nmoles of TBA-RS per mg of protein.

### 2.8. Antioxidant enzymes

Eight to ten mice per treatment group were used to analyze different antioxidant enzyme activities such as Mn-SOD, Cu, Zn-SOD, glutathione peroxidase, and glutathione reductase. Four groups of mice ($n$ = 8–10 per group) were assigned as described previously.

#### 2.8.1. Superoxide dismutase (SOD) activity

The production of superoxide radicals was determined by measuring the SOD activity. Total SOD activity was assessed by a competitive inhibition assay previously reported by us (Rojas et al., 2008), using a xanthine/xanthine oxidase system to reduce nitroblue tetrazolium (NBT), which served as the indicator reagent. The amount of protein that inhibited 50% of maximal NBT reduction was defined as one unit of SOD activity. Mn-SOD was differentiated from Cu, Zn-SOD by inhibiting the latter with diethyldithiocarbamate (DDC). Protein concentrations were measured according to Lowry et al. (1951). All samples were analysed in duplicate and results are expressed as units of SOD activity per mg protein.

#### 2.8.2. Glutathione peroxidase (GPx) activity

GPx activity was assayed by a method based on the non-enzymatic oxidation of reduced glutathione according to a method previously described by us (Pedraza-Chaverrí et al., 2000). The reaction mixture consisted of 50 mM potassium phosphate pH 7.0, 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 U/ml of glutathione reductase, and 1 mM glutathione. Tissue homogenate was added to the reaction mixture and allowed to incubate for 5 min at room temperature before initiation of the reaction by the addition of 0.1 ml 1.5 mM $H_2O_2$ solution. Absorbance at 340 nm was recorded with a spectrophotometer, Beckman DU640 (Beckman Coulter, Inc., Fullerton, CA, USA) for 3 min. The activity was calculated from the slope of these lines as μmoles of NADPH oxidized per min, noting that the mM absorption coefficient for NADPH is 6.22. The results are expressed as units/mg protein.

#### 2.8.3. Glutathione reductase activity

Glutathione reductase activity was assayed by using oxidized glutathione as substrate and measuring the disappearance of NADPH at 340 nm with a spectrophotometer, Beckman DU640 (Beckman Coulter, Inc., Fullerton, CA, USA), as previously reported (Carlberg and Mannervik, 1975). The rate of oxidation of NADPH by glutathione disulfide at 30 °C was used as a standard measure of glutathione reductase activity. The reaction mixture of 1 ml contained: 1 mM glutathione disulfide, 0.1 mM NADPH, 0.5 mM EDTA, 0.10 M sodium phosphate buffer (pH 7.6), and a suitable amount of the glutathione reductase sample to give a change in absorbance of 0.05–0.30/min. The oxidation of 1 pmol of NADPH/min under these conditions is used as a unit of glutathione reductase activity. The specific activity is expressed as units/mg protein.

#### 2.8.4. Analysis of SOD protein by western blot

A total of 8–10 mice were studied per treatment group. Tissue (PFC, MB or HP) was homogenized with protease inhibitors in 50 mM phosphate buffer (pH 7.4). Tissue homogenates were centrifuged at 1000*g* and 4 °C for 10 min. Protein (25 μg) was fractionated by reducing 12.5% sodium dodecyl sulfate polyacrylamide gel for electrophoresis and was electroblotted to a nitrocellulose membrane. Immunodetection was performed using primary antibodies specific for Mn-SOD or Cu, Zn-SOD (Stressgen Biotechnologies Co., Victoria, BC, Canada) as reported by our group (Pedraza-Chaverrí et al., 2000). Hybrids were visualized by chemiluminescence using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ, USA), followed by densitometric analysis using SigmaScan Software (Aspire Software International, Ashburn, VA, USA).

### 2.9. Statistical analysis

The data are expressed as means ± SEM. All data were analyzed using one-way analyses of variance (ANOVA), followed by post hoc Duncan's test. Values of $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered to be statistically significant.

## 3. Results

### 3.1. EGb761 produces antidepressant-like effect in the FST

EGb761 (10 mg/kg) and imipramine (classical antidepressant) significantly reduced the duration of immobility (41% and 40%, respectively) in the FST (Fig. 1) as compared to the vehicle-treated control group ($F_{2,109} = 22.865$, $P < 0.001$), suggesting an antidepressant-like effect of EGb761.

In the EGb761 dose-response study the degree of antidepressant-like effect was reduced at 5 mg/kg (30%) when compared to the vehicle-treated control group. However, doses of 20 and 40 mg/kg of EGb761 differences were not significant. Since EGb761 at 10 mg/kg was the most effective dose we used that to analyze LP, spontaneous locomotor activity, serotonin and dopamine turnover ratio, and different antioxidant enzyme activities.

### 3.2. EGb761 has no excitatory or inhibitory effects

To determine whether EGb761 has an antidepressant-like action, locomotor activity was analyzed to exclude excitatory or inhibitory effects after EGb761 administration (Fig. 2). EGb761 did not affect locomotor activity ($F_{2,47} = 0.095$, $P > 0.05$) at the same

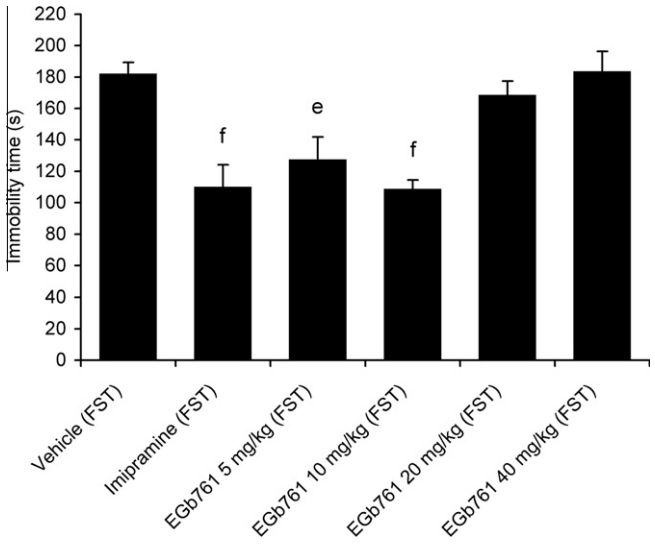


**Fig. 1.** EGb761 produces an antidepressant-like effect in the FST. Mice were administered vehicle, imipramine (15 mg/kg) or EGb761 (5, 10, 20 or 40 mg/kg) daily for 17 days before the FST. Results are expressed as mean (±SEM) of immobility time (in seconds), with eight to ten mice per group. The differences were analysed using one-way ANOVA followed by a *post hoc* Duncan's test. (e) Statistically different from control group (vehicle-FST group), $P < 0.01$, Duncan's test (f) statistically different from control group (vehicle-FST group), $P < 0.001$, Duncan's test. FST = forced swimming test; EGb761 = *Ginkgo biloba* extract.

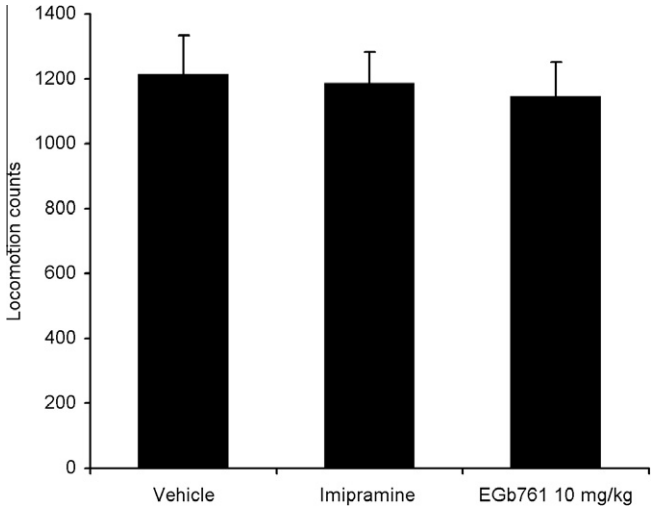


**Fig. 2.** EGb761 has no excitatory or inhibitory effects. Mice were administered vehicle, imipramine (10 mg/kg) or EGb761 (10 mg/kg) daily for 17 days before the FST. The locomotion counts were recorded for 10 min. Results are expressed as mean ± SEM, with eight to ten mice per group. FST = forced swimming test; EGb761 = *Ginkgo biloba* extract.

dose that significantly reduced the immobility response in the FST as compared to the vehicle-treated control group. Imipramine did not produce increases ($F_{2,47} = 0.095$, $P > 0.05$) in locomotor activity as compared to the vehicle-treated control group.

### 3.3. EGb761 produces modulation of serotonergic and dopaminergic neurotransmission in the FST

As shown in Table 1 the antidepressant-like effect of EGb761 can be related to a modulation in 5-HT and DA transmission.

Serotonin turnover: 5-HT turnover (5-HIAA/5-HT ratio) was decreased after FST in PFC (79%; $F_{3,33} = 127.92$, $P < 0.001$), but an increase in MB (77%; $F_{3,34} = 79.13$, $P < 0.001$) was observed as compared to the non-FST group. Imipramine administration reduced 5-HT turnover in MB (78%; $F_{3,34} = 79.13$, $P < 0.001$), and HP (42%; $F_{3,34} = 18.30$, $P < 0.001$) after FST vs. the vehicle-FST group. However imipramine administered in FST group increased 5-HT turnover in PFC (28%; $F_{3,33} = 127.92$, $P < 0.001$) vs. FST group. EGb761 administration produced a reduction in 5-HT turnover (5-HIAA/5-HT ratio) in PFC (31%; $F_{3,33} = 127.92$, $P < 0.001$), and MB (25%; $F_{3,34} = 79.13$, $P < 0.001$) after FST as compared to the vehicle-FST group. Further, EGb761 in FST group increased 5-HT turnover in MB (238%; $F_{3,34} = 79.13$, $P < 0.001$) and HP (85%; $F_{3,34} = 18.30$, $P < 0.001$) as compared to the imipramine-FST group.

Dopamine turnover: DA turnover (HVA/DA) was decreased in PFC (54%; $F_{3,23} = 3.16$, $P < 0.05$) after FST vs. the non-FST group. In contrast, an enhancement was produced in HP (79%; $F_{3,37} = 36.689$, $P < 0.01$) and MB (40%; $F_{3,37} = 69.365$, $P < 0.001$) as compared to the non-FST group. Imipramine reduced only DA turnover in MB (16%; $F_{3,37} = 69.365$, $P < 0.001$) after FST vs. the vehicle-FST group. EGb761 administration reduced DA turnover (HVA/DA ratio) in PFC (52%; $F_{3,23} = 3.16$, $P < 0.05$) after the FST as compared to the vehicle-FST group. In contrast, EGb761 produced a significant increase in DA turnover in HP (42%; $F_{3,37} = 36.689$, $P < 0.01$), and MB (171%; $F_{3,37} = 69.365$, $P < 0.001$) in the FST as compared to the vehicle-FST group. Further, EGb761 increased DA turnover in MB (171%; $F_{3,37} = 69.365$, $P < 0.001$) and HP (60%; $F_{3,37} = 36.689$, $P < 0.01$) after FST as compared to the imipramine-FST group. In contrast, EGb761 produced a reduction in PFC (73%; $F_{3,23} = 3.16$, $P < 0.05$) after FST vs. the imipramine-FST group.

### 3.4. Antidepressant-like effect of EGb761 is associated with a reduction of lipid peroxidation in mice exposed to FST

The TBA-RS assay was performed as an index of LP to examine whether the antidepressant-like effect of EGb761 was related to the prevention of oxidative stress (Fig. 3).

Significant increases in LP were observed in MB (89%; $F_{3,32} = 5.018$, $P < 0.01$), HP (18%; $F_{3,23} = 5.018$, $P < 0.05$) and PFC (59%; $F_{3,29} = 4.66$, $P < 0.01$) after FST as compared to the non-FST group. Imipramine administration reduced LP in MB (21%; $F_{3,32} = 5.018$, $P < 0.01$) after FST vs. the vehicle-FST group. We demonstrated that EGb761 administration to mice in the FST produces a significant reduction of LP in MB (24%; $F_{3,32} = 5.018$, $P < 0.01$), and HP (20%; $F_{3,23} = 5.018$, $P < 0.05$) as compared to the vehicle-FST group. No changes were observed between imipramine and EGb761 in the FST groups.

### 3.5. Effect of EGb761 on different antioxidant enzymes in mice exposed to FST

To explore the ability of EGb761 to exert part of its protective action via mechanisms involving antioxidant effects in mice exposed to FST, we investigated whether EGb761 treatment is associated with changes in the activity of antioxidant enzymes such as Mn-SOD, Cu, Zn-SOD GPx and glutathione reductase.

We found Cu, Zn-SOD activity was increased in MB (117%; $F_{3,26} = 83.47$, $P < 0.001$), HP (75%; $F_{3,26} = 45.32$, $P < 0.001$), and PFC (49%; $F_{3,26} = 18.14$, $P < 0.001$) after FST as compared to the non-FST group (Fig. 4A). Imipramine administration reduced Cu, Zn-SOD activity in MB (62%; $F_{3,26} = 83.47$, $P < 0.001$), HP (42%; $F_{3,26} = 45.32$, $P < 0.001$), and PFC (43%; $F_{3,26} = 18.14$, $P < 0.001$) after FST vs. the vehicle-FST group. However, EGb761 did not produce changes in Cu, Zn-SOD activity across all treatment groups after FST (Fig. 4A). Further, EGb761 increased Cu, Zn-SOD activity in MB (116%; $F_{3,26} = 83.47$, $P < 0.001$), HP (86%; $F_{3,26} = 45.32$, $P < 0.001$), and PFC (83%; $F_{3,26} = 18.14$, $P < 0.001$) after FST as compared to the imipramine-FST group.

**Table 1**
Effect of EGb761 on DA and 5-HT turnover ratio in different brain regions in mice exposed to forced swimming test.

| Brain structure | Neurotransmitter turnover ratio | Vehicle (non-FST) | Vehicle (FST) | Imipramine (FST) | EGb761 10 mg/kg (FST) |
|---|---|---|---|---|---|
| Midbrain | 5-HIAA/5-HT | 0.3768 ± 0.0179 | 0.6661 ± 0.366[c] | 0.1480 ± 0.0140[f] | 0.5005 ± 0.031[f,i] |
| | HVA/DA | 0.4116 ± 0.0254 | 0.5770 ± 0.293[a] | 0.4823 ± 0.0393[d] | 1.3074 ± 0.073[f,i] |
| Hippocampus | 5-HIAA/5-HT | 0.4194 ± 0.0137 | 0.3417 ± 0.0256[a] | 0.1973 ± 0.0171[f] | 0.3657 ± 0.020[i] |
| | HVA/DA | 0.5102 ± 0.0386 | 0.9130 ± 0.0460[c] | 0.8106 ± 0.0195 | 1.3009 ± 0.071[f,i] |
| Prefrontal cortex | 5-HIAA/5-HT | 0.1804 ± 0.0052 | 0.0377 ± 0.0036[c] | 0.0482 ± 0.0061[f] | 0.0260 ± 0.001[d,h] |
| | HVA/DA | 0.3526 ± 0.0485 | 0.1638 ± 0.0226[a] | 0.2883 ± 0.0670 | 0.0787 ± 0.014[d,g] |

Results are expressed as μg/g tissue weight. Mean ± SEM 8–10 animals per group: The differences were analysed using one-way ANOVA followed by a *post hoc* Duncan's test. FST = forced swimming test. EGb761 = *Ginkgo biloba*.
[a] $P < 0.05$ with respect to the vehicle (non-FST).
[b] $P < 0.01$ with respect to the vehicle (non-FST).
[c] $P < 0.001$ with respect to the vehicle (non-FST).
[d] $P < 0.05$ with respect to the vehicle (FST).
[e] $P < 0.01$ with respect to the vehicle (FST).
[f] $P < 0.001$ with respect to the vehicle (FST).
[g] $P < 0.05$ with respect to the imipramine (FST).
[h] $P < 0.01$ with respect to the imipramine (FST).
[i] $P < 0.001$ with respect to the imipramine (FST).

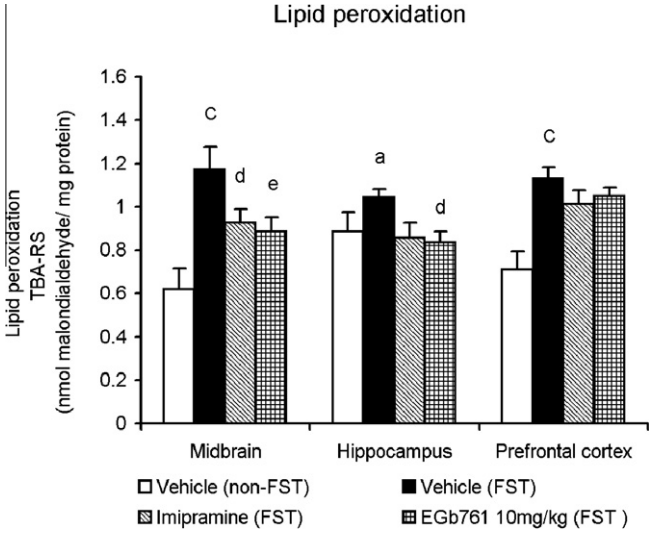


**Fig. 3.** The antidepressant-like effect of EGb761 (10 mg/kg) is linked to reduction of lipid peroxidation in mice exposed to FST. Mice were treated with vehicle (non-FST), vehicle (FST), imipramine (FST) or EGb761 (FST). Lipid peroxidation was measured by thiobarbituric acid-reactive substances (TBA-RS) assay in different brain regions. Results are expressed as mean ± SEM, with eight to ten mice per group. The differences were analysed using one-way ANOVA followed by a *post hoc* Duncan's test. (a) Statistically different from non-FST group, $P < 0.05$, Duncan's test, (c) statistically different from non-FST group, $P < 0.001$, Duncan's test, (d) statistically different from vehicle-FST group, $P < 0.05$, Duncan's test, (e) statistically different from vehicle-FST group, $P < 0.01$, Duncan's test. FST = forced swimming test; EGb761 = *Ginkgo biloba* extract.

FST reduced Mn-SOD activity in MB (50%; $F_{3,26} = 7.16$, $P < 0.001$), but an increase in HP (33%; $F_{3,27} = 17.192$, $P < 0.001$) as compared to the non-FST group (Fig. 4B). Imipramine administration increased Mn-SOD activity in MB (66%; $F_{3,26} = 7.16$, $P < 0.001$), after FST vs. the vehicle-FST group. EGb761 administration reduced Mn-SOD activity (Fig. 5B) in PFC (55%; $F_{3,29} = 18.09$, $P < 0.001$), and HP (66%; $F_{3,27} = 17.192$, $P < 0.001$) in mice exposed to FST as compared to the vehicle-FST group. EGb761 reduced Mn-SOD activity in MB (28%; $F_{3,26} = 7.16$, $P < 0.001$), HP (58%; $F_{3,27} = 17.192$, $P < 0.001$) and PFC (61%; $F_{3,29} = 18.09$, $P < 0.001$) after FST compared to the imipramine-FST group.

The protein content of Mn-SOD and Cu, Zn-SOD in MB, HP and PFC, measured by Western blot, is shown in Fig. 5. The content of Mn-SOD and Cu, Zn-SOD remained unchanged in all groups of mice studied except for Cu, Zn-SOD in MB of the "vehicle-treated + FST" group that was enhanced (23%; $F_{3,7} = 4.74$, $P < 0.05$) as compared to the non-FST group.

We found GPx activity was reduced after FST in MB (20%; $F_{3,28} = 18.168$, $P < 0.001$) and PFC (42%; $F_{3,24} = 25.20$, $P < 0.001$) except in HP as compared to the non-FST group (Fig. 4C). Imipramine increased in MB (20%; $F_{3,28} = 18.168$, $P < 0.001$) but reduced in HP (37%; $F_{3,24} = 9.929$, $P < 0.001$) and PFC (35%; $F_{3,24} = 25.20$, $P < 0.001$) vs. the vehicle-FST group. However, EGb761 treatment reduced GPx activity (Fig. 4C) in MB (35%; $F_{3,28} = 18.168$, $P < 0.001$) in mice exposed to FST as compared to the vehicle-FST group. EGb761 in FST group showed a reduction of GPx in MB (46%; $F_{3,28} = 18.168$, $P < 0.001$) and increased in HP (52%; $F_{3,24} = 9.929$, $P < 0.001$) and PFC (85%; $F_{3,24} = 25.20$, $P < 0.001$) as compared to the imipramine-FST group.

We found glutathione reductase activity was reduced after FST in MB (22%; $F_{3,27} = 39.393$, $P < 0.001$), HP (38%; $F_{3,27} = 38.420$, $P < 0.001$) and PFC (36%; $F_{3,31} = 26.908$, $P < 0.001$) as compared to the non-FST group (Fig. 4D). Imipramine increased glutathione reductase activity in MB (49%; $F_{3,27} = 39.393$, $P < 0.001$), HP (47%; $F_{3,27} = 38.420$, $P < 0.001$) and PFC (19%; $F_{3,31} = 26.908$, $P < 0.001$). EGb761 did not produce changes in glutathione reductase activity across all treatment groups (Fig. 4D) except to a reduction in PFC (20%; $F_{3,31} = 26.908$, $P < 0.001$) in mice exposed to FST vs. vehicle-FST group. EGb761 in FST group showed a reduction of glutathione reducatse activity in MB (38%; $F_{3,27} = 39.393$, $P < 0.001$), HP (41%; $F_{3,27} = 38.420$, $P < 0.001$) and PFC (32%; $F_{3,31} = 26.908$, $P < 0.001$) as compared to the imipramine-FST group.

## 4. Discussion

There is an increasing interest in the study of the antidepressant effect of herbs, since treatment of depression with conventional antidepressants results in complete remission in almost 50% of individuals (Nestler et al., 2002). Several research groups have shown that *Ginkgo biloba* extracts have diverse effects on improvement of mood and cognitive performance, and protection against memory deficits and central nervous system disorders (DeFeudis and Drieu, 2000; Polich and Gloria, 2001; Trick et al., 2004). However, an antidepressant effect of *Ginkgo biloba* extracts has not been clearly demonstrated.

The *Ginkgo biloba* extract named EGb761 is a complex mixture of ingredients with a unique broad spectrum of pharmacological activities in the central nervous system. Therefore, it appears to act through several different mechanisms. The present study introduces the multifunctional non-toxic drug EGb761 (DeFeudis,

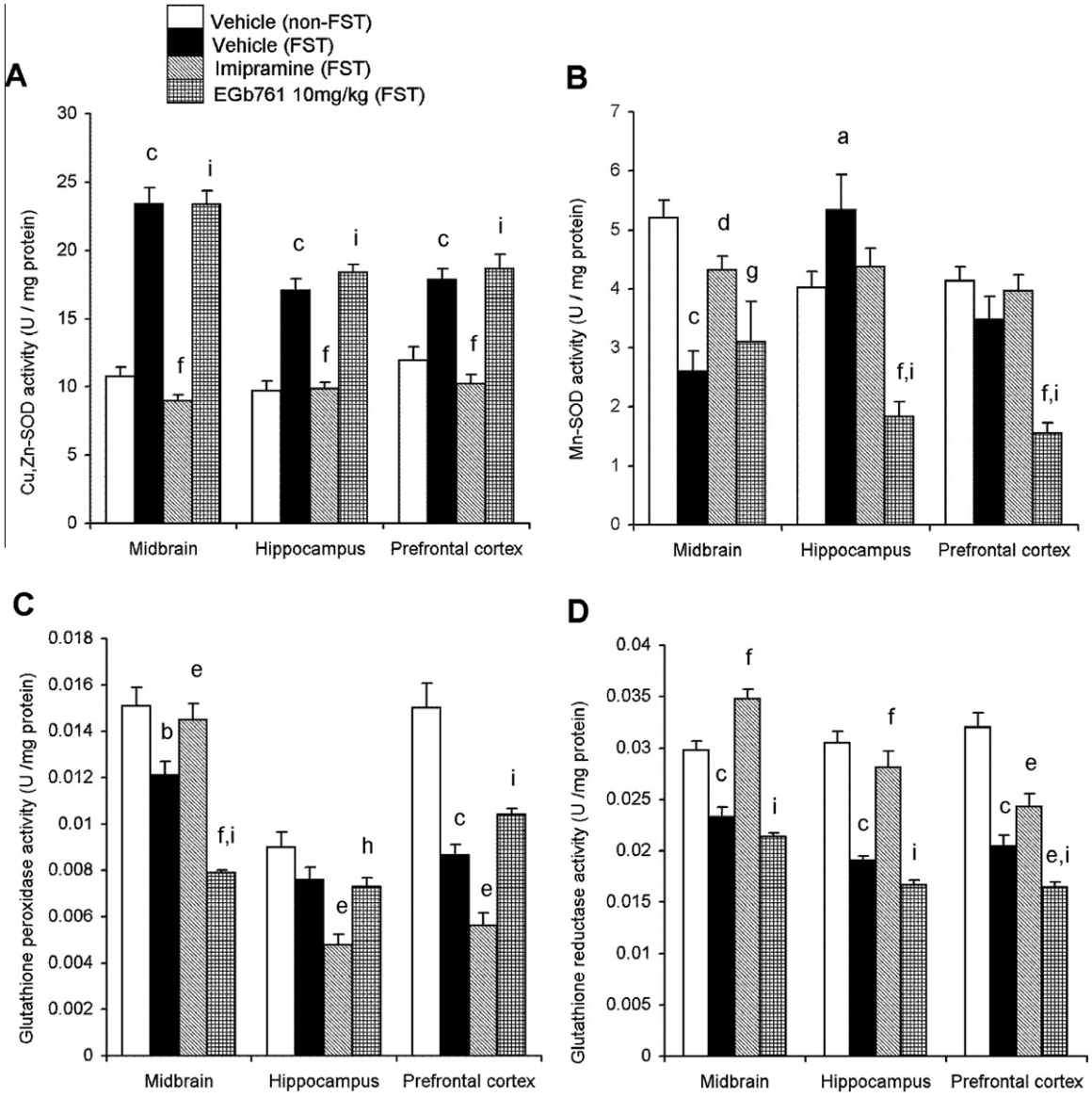


**Fig. 4.** The antidepressant-like effect of EGb761 (10 mg/kg) in the FST is possibly associated with a reduction of superoxide radical production indicated by a downregulation of Mn-SOD activity. Mice were treated with vehicle (non-FST), vehicle (FST), imipramine (FST) or EGb761 (FST). Cu, Zn-SOD (A), Mn-SOD (B), GPx (C), and glutathione reductase (D) activity was analysed in different brain regions. Results are expressed as mean ± SEM, with eight to ten mice per group. The differences were analysed using one-way ANOVA followed by a *post hoc* Duncan's test. (a) Statistically different from non-FST group, $P < 0.05$, Duncan's test, (b) statistically different from non-FST group, $P < 0.01$, Duncan's test, (c) statistically different from non-FST group, $P < 0.001$, Duncan's test, (d) statistically different from vehicle-FST group, $P < 0.05$, Duncan's test, (e) statistically different from vehicle-FST group, $P < 0.01$, Duncan's test, (f) statistically different from vehicle-FST group, $P < 0.001$, Duncan's test, (g) statistically different from imipramine-FST group, $P < 0.05$, Duncan's test, (h) statistically different from imipramine-FST group, $P < 0.01$, Duncan's test, (i) statistically different from imipramine-FST group, $P < 0.001$, Duncan's test. Mn-SOD = Mn-superoxide dismutase; Cu, Zn-SOD = Cu, Zn-superoxide dismutase; GPx = glutathione peroxidase; FST = forced swimming test; EGb761 = *Ginkgo biloba* extract.

1998), which exerts potent antidepressant-like effect in the FST. This protective action might be directly related with the antioxidant actions that EGb761 exerts in the brain.

We used a standardized extract of *Ginkgo biloba* named EGb761 (Drieu, 1986). The isolated constituents of EGb761 have been found to be pharmacologically active in a variety of assays. The therapeutic benefits of this extract may reside in the synergistic effect of all characterized components. It is very important to characterize the composition of the extract preparations used as the presence or absence of specific components could lead to different results; these would essentially be different preparations of *Ginkgo biloba* extract.

In the present study, our results demonstrate that the EGb761 (10 mg/kg) treatment produced a significant reduction (39%) in recorded immobility time in the FST, with a comparable profile to the classical antidepressant imipramine (38%). There are several herbal medicines that have been introduced into psychiatric practice because of greater compliance, milder side effects, and less toxicity (Thachil et al., 2007) and EGb761 has these features.

To avoid false positives in the FST it is important to rule out the possibility that the reduction in immobility time produced by EGb761 did not merely result from a psychostimulant effect that could have induced a marked motor stimulation. Our results showed that EGb761 treatment did not increase or reduce locomotor activity, suggesting that EGb761 exerts a selective antidepressant-like effect.

Porsolt et al. (1990) reported that administration of EGb761 had no effect on activity in the FST. However, our results showed that

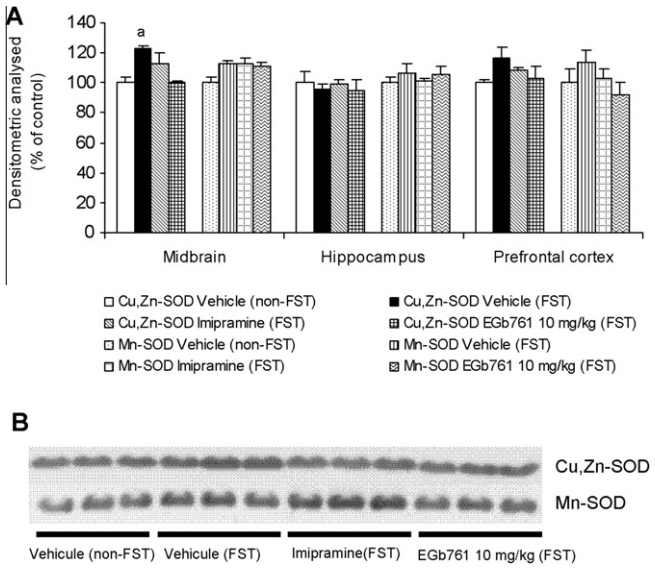


**Fig. 5.** Densitometric analysis of western blot of Mn-SOD and Cu, Zn-SOD in the antidepressant-like effect of EGb761 (10 mg/kg) in the FST (A) in different brain regions. Representative western blots of Mn-SOD and Cu, Zn-SOD protein levels in the antidepressant-like effect in the FST (B) in midbrain. Eight to ten mice were used per group. Values are the mean ± SEM. The differences were analysed using one-way ANOVA followed by a *post hoc* Duncan's test. [a]Statistically different from the control group ("saline + saline"), $P < 0.05$, Duncan's test. Mn-SOD = Mn-superoxide dismutase; Cu, Zn-SOD = Cu, Zn-superoxide dismutase; FST = forced swimming test; EGb761 = *Ginkgo biloba* extract.

EGb761 has an antidepressant-like effect, but we administered it for 17 days and not 5 days as in that study. Montgomery (1995) proposed that a treatment period of 14 days is an appropriate period for demonstrating antidepressant efficacy. Consequently, the present results clearly demonstrate that EGb761 possesses antidepressant-like activity in the FST.

Monoamine neurotransmitters (DA and 5-HT) are involved in the pathogenesis of depression and play important roles in mediating the effects of antidepressants (Savegnago et al., 2007). It has been suggested that an increase in the level of monoamines at the synapse is the first step of antidepressant activity (Piñeyro and Blier, 1999). Classical antidepressants such as tricyclic antidepressants exert their action by enhancing synaptic monoamine levels (Kulkarni et al., 2008; Xu et al., 2005). In our study, we found that FST induces neurochemical alterations in 5-HT and DA turnover in different brain regions as previously reported (Connor et al., 2000). In particular, in our study a variety of neurochemical alterations were elicited mainly, in 5-HT and DA transmission, in PFC, HP and MB after FST exposure, and that EGb761 regulated these alterations.

Cortical and limbic areas are implicated in dysfunctional changes in depression and antidepressant action (Krishnam and Nestler, 2008). Besides, depression can be alleviated by increasing the levels of monoamine neurotransmitters in the central nervous system (Nestler et al., 2002). Tricyclic antidepressants such as imipramine, inhibit 5-HT and noradrenaline reuptake and increasing neutrotransmitter levels. As the ratio of neurotransmitter compared to its metabolites can be used as an index of neurotransmitter metabolism (turnover), the reduction of turnover suggests a slow down in the metabolism of neurotransmitters. We showed that FST enhanced 5-HT turnover (5-HIAA/5-HT) in MB and HP during FST suggesting an enhancement of 5-HT metabolism. Imipramine in FST group showed reduction of 5-HT turnover in MB and HP as previously reported. However, EGb761 in FST group reduced 5-HT turnover but in MB and PFC. This suggests that a reduction of 5-HT turnover produced by EGb761 may be related, at least in part, to a serotoninergic activation which produces an enhancement of 5-HT levels. Our data show that EGb761 as well as the tricyclic antidepressant imipramine reduces serotonin turnover (Connor et al., 2000; Kelliher et al., 2003) in depression-like behavior.

Moreover, the dopaminergic system is involved in the pathogenesis and treatment of depression (Renard et al., 2001). It is now evident that DA plays a non-negligible role in depression and in the action of antidepressants. Recent studies have demonstrated that tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRIs) act on the dopaminergic system (D'Aquila et al., 2003; Esposito, 2006). We showed that FST increases DA turnover (HVA/DA) in the mesolimbic system (MB and HP). Imipramine reduced DA turnover in MB and EGb761 reduced it in PFC in the FST group. This suggests that a reduction of DA turnover produced by EGb761 in FST group may be related, at least in part, to a dopaminergic activation which produces an enhancement of DA levels. It has been suggested that antidepressants preferentially potentiate DA transmission (D'Aquila et al., 2000) as shown in the current study. The potentiation of DA might be responsible for the therapeutic effect of antidepressant treatments, given the involvement of the mesocortical DA pathway in the control of motivation and emotion, which are impaired in depression (Yadid and Friedman, 2008).

We showed that serotonergic and dopaminergic systems were activated in cortex after EGb761 administration in FST. However, imipramine effects are mainly limited to serotoninergic system in limbic area. This suggests that EGb761 could be a better candidate to treat antidepressant-like effect where 5-HT and DA are involved. In particular, the PFC and HP are essential brain regions involved in cognition, emotion and mood (Krishnam and Nestler, 2008). Damage to these brain regions may lead to symptoms commonly present in depressed patients.

These findings suggested that the antidepressant-like effects of EGb761 were mediated through the modulation of serotonergic and dopaminergic neurotransmission. It has been shown that EGb761 regulates the dopaminergic and serotonergic uptake systems (Ramassamy et al., 1992). The antidepressant-like effect of EGb761 in this study might be due, in part, to the stabilization of the uptake systems. In addition, FST induces oxidative stress (Akhtar et al., 2005) through free radical production, which can decrease synaptosomal membrane fluidity and modify the function of the DA- and 5-HT-transporters, and EGb761 can counteract these effects since this extract prevents the modification of membrane fluidity (Ramassamy et al., 1993).

The current data are in accord with the finding that strains of mice, dose-administration, and drug metabolism are factors that might affect results. These factors affect not only the behavioral responses to FST exposure but also neurochemical responses to this stressor (López-Rubalcava and Lucky, 2000).

LP, an index of oxidative stress, may damage the cell membrane (membrane fluidity, receptors, and ion channels) (Mattson, 1998), which may result in calcium influx and cause cell death. With regard to the oxidative stress hypothesis, enhancement of oxidative damage and decreased antioxidant enzyme levels in depressed patients has been reported (Ng et al., 2008; Sarandol et al., 2007). Moreover, preclinical studies have suggested that antioxidants may have antidepressant properties (Eren et al., 2007; Zafir et al., 2009).

Increased LP has been reported in depressed patients (Galecki et al., 2009; Sarandol et al., 2007). In the current study the presence of elevated LP in MB, HP and PFC was detected after FST as reported by others (Akhtar et al., 2005). However, EGb761 after FST reduced LP in MB and HP, suggesting that an antidepressant-like effect of EGb761 is due, in part, to its antioxidant properties (Marcocci et al., 1994b; Sastre et al., 1998). But LP was reduced only in MB

after imipramine administration in the FST group. This shows that EGb761 was effective in reducing LP in the FST group. Imipramine reduced LP in a brain region no related to mood, cognition and motivation. Several studies in the central nervous system have reported that the protective actions of EGb761 are related with its antioxidant and free radical scavenger properties (DeFeudis and Drieu, 2000; Rojas et al., 2004, 2008). We have reported that EGb761 blocks LP in an animal model of Parkinson's disease (Rojas et al., 2001, 2008).

It has been shown that an enhancement of SOD activity in depressed patients (Sarandol et al., 2007) is correlated with the severity of depression. Increased SOD activity probably reflects an upregulated SOD system in defence against increased free radicals in depression. In support to the free radical scavenger hypothesis, FST increased Cu, Zn-SOD activity in the different brain regions analysed after FST. But only imipramine was able to reduce significantly Cu, Zn-SOD activity. Mn-SOD activity was enhanced in response to FST, and EGb761 reduced significantly this effect. Imipramine has no effect on Mn-SOD activity.

This suggests indirectly that an early mechanism of EGb761 protection in this depressive-like behavior is acting as a superoxide radical scavenger, and preventing enhancement of Mn-SOD activity. It has been proposed that EGb761 has beneficial neuroprotective effects, probably via its antioxidant action, which involves scavenging both superoxide and hydroxyl radicals (Marcocci et al., 1994b; Pincemail et al., 1989; Rojas et al., 2008). We suggest that changes in Mn-SOD activity by EGb761 are due to a superoxide radical scavenger action of this enzyme but not to a reduction in the amount of protein, as shown by the lack of changes in the western blot analysis. We may suggest that changes in Cu, Zn-SOD and Mn-SOD activity are due to a post-translational modifications of SOD protein but not to a reduction in the amount of the enzyme, as showed by the lack of changes in the western blot analysis. This kind of biological modifications in proteins may be due to the toxic action of reactive oxygen species (Yamakura and Kawasaki, 2010).

EGb761 is a potent superoxide anion scavenger and has SOD activity (Pincemail et al., 1989). EGb761 also inhibits superoxide production in human postmortem brain tissue and inhibits SOD (Gsell et al., 1995). This conclusion is consistent with a genome-wide monitoring of the neuromodulatory actions of EGb761, a phenomenon described for many proteins characterized by significant over-transcription of their genes during oxidative stress. Such proteins include Mn-SOD (Rimbach et al., 2003). Our results show that Mn-SOD activity is regulated by EGb761 (Soulié et al., 2002) in mice exposed to FST, showing that EGb761 acts only on Mn-SOD activity.

EGb761 regulates other antioxidant enzymes including GPx and glutathione reductase (Sasaki et al., 2002). Our findings suggest that imipramine and EGb761 in mice exposed to FST regulate GPx and glutathione reductase activity an some protection had provided but this is less significant as compared to Cu, Zn-SOD and Mn-SOD activity.

Several studies indicate the significance of SOD activity and LP levels in various psychiatric disorders including depression. The elevated concentration of LP with a concomitant elevation in SOD activity has been reported in major depression (Bilici et al., 2001). Accordingly, in the present study the FST caused an increase in Mn-SOD activity and LP, and EGb761 reversed that effect. FST produced an enhancement of Cu, Zn-SOD and Mn-SOD activity, LP, but imipramine and EGb761 reversed both oxidative stress indexes in different brain regions.

Our study suggests that EGb761 could be better to relief antidepressant-like effect than imipramine because is able to regulate serotonergic and dopaminergic transmission in cortical and limbic regions. The antioxidant effect of EGb761 in the FST could be due to reduced oxidative stress, regulating, in part, DA and 5-HT neurotransmission. We would like to propose that the antioxidant effect of EGb761 in mice exposed to FST is due primarily to the scavenging of superoxide free radicals.

Other interesting studies have reported that acute administration of EGb761 (50 mg/kg) produces antidepressant-like effect (Kalkunte et al., 2007). However, the authors used different route of drug administration (oral intubation), time administration of EGb761 (single dose) and a higher dose of EGb761 as compared to our study. In addition, Kalkunte et al. (2007) used carboxymethyl cellulose suspension to administer the *Ginkgo biloba* extract, but in our study we used physiological saline as vehicle. This could affect the availability of bioactive compounds of EGb761 and explain why in our study EGb761 had antidepressant-like effect at 10 mg/kg, since we used intraperitoneal administration and physiological saline as vehicle.

On the other hand, diverse types of *Ginkgo biloba* extracts are now commercialized around the globe for diverse therapeutic purposes. However, extraction procedures used to obtain the favorable compounds of herbal material to use for health benefits are an important factor. In particular, EGb761 is designed to eliminate ginkgolic acid because its derivatives may cause allergic reactions (DeFeudis, 1998). This particular extract is the best analytically characterized extract, the best studied with regard to pharmacological and toxicological characteristics, as well as regarding clinical efficacy, and is well tolerated (DeFeudis and Drieu, 2000).

Subchronic and chronic toxicity in rodents has shown the remarkable safety of EGb761. Concerning animal toxicological data, the oral $LD_{50}$ in mice was more than 9600 mg/kg which represents 2100 times more than the recommended daily dose (for a review see DeFeudis, 1998). No mutagenic, carcinogenic, teratogenic or embryotoxic effects have been demonstrated for the extract in clinical or preclinical studies. EGb761 seems to be very safe, and side effects are rare.

In 98% of the clinical studies, the tolerability was good or very good; the adverse effects of EGb761 are mild, transient, and reversible: in only a very few cases, gastrointestinal upsets like nausea and vomiting and vascular problems such as headaches and dizziness, were reported. In addition, neither blood pressure and heart rate nor cholesterol and tryglycerid levels seem to be altered with the intake of EGb761 (for a review see DeFeudis, 1998).

One of the important considerations in developing drug therapies for patients with depression is to prevent low rates of response, remission, and potential side-effects during or after long-term administration as seen in available synthetic chemical antidepressants such as imipramine used in this study. There are several herbal medicines that have been introduced into psychiatric practice because of greater compliance and milder side effects (Thachil et al., 2007). EGb761 is a good candidate to treat depression because it has a very impressive clinical safety record, and rapidly cross the blood–brain barrier when peripherally administered.

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