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Antidepressant-like and neuroprotective effects of *Aloysia gratissima*: Investigation of involvement of L-arginine-nitric oxide-cyclic guanosine monophosphate pathway

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ABSTRACT

Ethnopharmacological relevance: *Aloysia gratissima* (Gill. et Hook) Tronc. (Verbenaceae) is used traditionally for the treatment of headache, bronchitis, and nervous systems disorders including depression.
Aim of the study: To investigate the antidepressant-like and neuroprotective effects of *Aloysia gratissima* aqueous extract (AE) and the involvement of L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway.
Materials and methods: The antidepressant-like effect of AE was evaluated through behavioral despair in forced swimming test (FST) and tail suspension test (TST). Swiss albino mice were treated by oral route and after 1 h were analyzed the time of immobility in the FST and TST. In addition, the neuroprotective effect of AE against glutamate excitotoxicity was evaluate through cell viability of hippocampal slices, phosphorylation of Akt, and the immunocontent of inducible oxide nitric synthase (iNOS) were investigated by western blotting.
Results: The immobility time in the FST and TST were reduced by AE (100–1000 and 10–300 mg/kg, respectively). The antidepressant-like effect of AE in the TST was prevented by the pretreatment with N-methyl-D-aspartate (NMDA), L-arginine or sildenafil. The subeffective dose of AE produced a synergistic antidepressant-like effect with MK-801 (an antagonist of NMDA receptor), methylene blue, L-NNA (an inhibitor of NO synthase) or ODQ (an inhibitor of soluble guanylate cyclase). In *ex vivo* experiments, pretreatment with AE prevented the loss of cell viability induced by glutamate, thus affording neuroprotection. Glutamate toxicity caused a decreased Akt phosphorylation and an increased iNOS expression.
Conclusions: The present study provides convincing evidence of neuroprotection and the involvement of the L-arginine-NO-cGMP pathway in the antidepressant-like effect of AE. Therefore, AE could be of potential interest for the treatment of depressive disorders and neurological conditions associated with glutamate excitotoxicity.

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1. Introduction

Aloysia gratissima (Gill. et Hook) Tronc. is an aromatic native plant belonging to Verbenaceae family which is widely distributed in subtropical regions of South America, mainly Brazil, Uruguay, Paraguay, and Argentina. In Brazil it is popularly named “erva santa”, “erva de nossa senhora” and “garupá”, among others. The aerial parts of this plant species are used for the treatment of headache, bronchitis and nervous systems disorders (Souza and Wiest, 2007), digestive and depression (Vendruscolo et al., 2005; Arias Toledo, 2009).

In other species of *Aloysia*, i.e., *Aloysia polystachya*, the hydroethanolic extract of aerial parts has been shown to exhibit anxiolytic (Hellión-Ibarrola et al., 2006) and antidepressant-like effects in mice (Hellión-Ibarrola et al., 2008) and in rats (Mora et al., 2005). Two active diterpenes were isolated from the aerial parts of *Aloysia virgata* exhibiting anxiolytic-like effect in mice (Wasowski and Marder, 2011). However, to the best of our knowledge, there is

Abbreviations: AE, *Aloysia gratissima* aqueous extract; cGMP, cyclic guanosine monophosphate; FST, forced swimming test; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; MTT, (3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; PDE5, phosphodiesterase-5; TST, tail suspension test.

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no scientific report related to antidepressant-like effects of *Aloysia gratissima*.

The essential oil of leaves of *Aloysia gratissima* has been reported to exhibit virucidal (García et al., 2003), nematicidal (Duschatzky et al., 2004), and fungicidal (Dellacasa et al., 2003) activities. The ethanolic extract of aerial parts of *Aloysia gratissima* presents chemical compounds such as kauranes, flavonoids and phenylethanoids (Silva et al., 2006), and sesquiterpens (α-bisabolol), triterpens (α-amirin, betulinic acid, oleanoic acid, and ursolic acid), and flavonoids (genkwanin, 5-hydroxy-7,4′-dimethoxyapigenin, 5-hydroxy-7,3′,4′-trimethoxyluteolin and rutin) were found in the methanolic extract of leaves (Vandresen et al., 2010). The methanolic extract showed antibacterial and antiedematogenic properties (Vandresen et al., 2010) and also antioxidant effect as documented by Rosas-Romero and Saavedra (2005).

Depressive disorders have high incidence in the world population (Berton and Nestler, 2006), with impact in the social function and in the life quality of patients (Nemeroff, 2007). The treatment of depression with conventional antidepressants (monoamine oxidase inhibitors, tricyclics, selective serotonin reuptake inhibitors, selective noradrenaline reuptake inhibitors) provides a complete remission just for 50% of the individuals (Rush et al., 2003) and antidepressant therapy produces side effects (Brunello et al., 2002) that may reduce the adhesion of patients to treatment (MacGillivray et al., 2003). Therefore, additional treatment strategies with favorable side effects profile, credible benefits, and moderate costs are of particular interest (Laakmann et al., 1998).

In the search of new molecules useful for the treatment of neurological disorders, medicinal plant research worldwide has progressed constantly, demonstrating the pharmacological effectiveness of different plant species in a variety of animal models (Zhang, 2004). Plant extracts – including St. John's worth – have been used for the treatment of some psychiatric disorders that is largely studied for the treatment of depression (Linde and Knuppel, 2005) and *Ginkgo biloba* (Sakakibara et al., 2006).

Although the monoaminergic system has been widely studied regarding the mechanism of action of antidepressants, the glutamatergic system has been proposed as an important target for the action of novel antidepressant agents (Skolnick, 1999; Sanacora et al., 2008). Noteworthy, targeting NMDA receptor blockade, particularly with the administration of the NMDA receptor antagonist ketamine produces improvement of the depressive symptoms much faster than conventional monoaminergic agents in patients resistant to conventional drug treatment strategies (Zarate et al., 2006; Maeng and Zarate, 2007; Sanacora et al., 2008). NMDA receptor stimulation induces the activation of nitric oxide synthase (NOS) that converts L-arginine to NO and L-citrulline (Esplugues, 2002). It has been also demonstrated that NOS inhibitors exert antidepressant-like effects in depression models (Dhir and Kulkarni, 2007; Ulak et al., 2008). Moreover, several studies suggest that the inhibition of NO synthesis, with a subsequent decrease in the concentration of cGMP, produce antidepressant-like effects (Kaster et al., 2005). Noteworthy, the NMDA receptors and the L-arginine-nitric oxide (NO)-cGMPc pathway are promising molecular targets for the action of antidepressant drugs.

Therefore, this study was aimed, firstly, to examine the effects of the oral administration of AE in two predictive models of antidepressant activity, the forced swimming test (FST) and the tail suspension test (TST). Secondly experiments were performed to investigate a possible participation of the L-arginine-NO-cGMP pathway in the antidepressant-like effect of *Aloysia gratissima* in the TST. Furthermore, this study analized, by using an *ex vivo* approach, the ability of AE treatment to counteract hippocampal glutamate excitotoxicity and the involvement of Akt phosphorylation and iNOS expression in the neuroprotection afforded by AE.

2. Materials and methods

2.1. Animals

Swiss male mice weighing 30–40 g were maintained at 21–23 °C with free access to water and food under a 12 h light/dark cycle (lights on at 7:00 h-am). All the manipulations were carried out between 9:00 and 16:00 h, with each animal used only once. These experiments were performed after approval of the protocol by the Ethics Committee of the Institution and all efforts were made to minimize animal suffering.

2.2. Plant material

2.2.1. Botanic material

The aerial parts of *Aloysia gratissima* (Gill. et Hook) Tronc. were collected from the boundaries of Serra do Itajaí National Park, Guabiruba city, Santa Catarina state, Brazil in autumn, 2006. The plant material was identified and authenticated taxonomically at Regional University of Blumenau. A voucher speciment (#2658) of the collected was deposited in the Dr. Roberto Miguel Klein Herbarium for future reference.

2.2.2. Preparation of extract

Aerial parts of *Aloysia gratissima* were washed, and then the dried material was powered. The powered material (2.5 g) was extracted with boiling water (150 ml) for 5 min. The aqueous extract of *Aloysia gratissima* (AE, yield 25.71%) was filtered, lyophilized, stored in freezer at −20 °C and resolubilized in distilled water at the time of administration.

2.3. Drugs and treatment

The following drugs were used: L-arginine, L-glutamate, N^G-nitro-L-arginine (L-NNA), methylene blue, MK-801, NMDA, (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) (ODQ) (Sigma Chemical Co, USA) and sildenafil citrate (Pfizer). All drugs were dissolved in saline, except ODQ which was dissolved in vehicle with 1% DMSO and 7-nitroindazole that was dissolved in vehicle with few drops of Tween-80. All drugs were administered by intraperitoneal (i.p.) route in a constant volume of 10 ml/kg body weight, except NMDA and ODQ which were administered by intracerebroventricular (i.c.v.) route. AE was also administered by oral (p.o.) route by gavage in a volume of 10 ml/kg body weight. Animals were restricted from food 1 h before and after the AE administration.

I.c.v. administration was performed using a microsyringe (25 μl, Hamilton) connected to a 26-gauge stainless-steel needle that was inserted perpendicularly 2 mm deep through the skull according to the procedure originally described by Laursen and Belknap (1986). Briefly, the animals were anesthetized with ether and then gently restrained by hand for i.c.v. injections. The sterilization of the injection site was carried out using gauze embedded in 70% ethanol. Under light anesthesia (i.e. just that necessary for loss of the postural reflex), the needle was inserted unilaterally 1 mm to the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. A volume of 5 μl of sterile saline containing the drugs was injected directly into the lateral ventricle, at the following coordinates from bregma taken from the atlas of Franklin and Paxinos (2001) anterioposterior (AP) = −0.1 mm; mediolateral (ML) = 1 mm; and dorsoventral (DV) = −3 mm. Mice exhibited normal behavior within 1 min after injection. After completion of the experiments, all animals were decapitated and their brains were

examined freshly. Results from mice presenting misplacement of the cannula or any sign of cerebral hemorrhage were excluded from the statistical analysis (overall less than 5% of the total animals used).

The AE was dissolved in distilled water and administered acutely by oral route (p.o.) 60 min before the FST (30–1000 mg/kg, p.o.), TST (3–1000 mg/kg, p.o.) or open-field test. The dissolution of the extract was freshly done from the lyophilized power immediately before its administration by gavage. A control group received distilled water as vehicle. In the experiments designed to study the time–course effect of AE (10 mg/kg, p.o.), the immobility time in the TST and the number of crossings in the open-field test were assessed in an independent group of mice, 1 h, 2 h, 4 h, and 12 h out after the administration of AE (10 mg/kg, p.o.).

To test the hypothesis that the antidepressant-like effect AE is mediated through the inhibition of NMDA receptors, mice were pretreated with NMDA (0.1 pmol/site, i.c.v.) and 15 min after, AE (10 mg/kg) or vehicle was administered. 60 min later the TST was carried out. The dose of NMDA was chosen based on previous studies (Brocardo et al., 2008; Zomkowski et al., 2010).

In another set of experiments, we investigated the synergistic effect of a sub-effective dose of AE (3 mg/kg, p.o.) with a sub-effective dose of MK-801 (0.001 mg/kg, i.p., a non-competitive NMDA receptor antagonist). AE or vehicle was administered 30 min before MK-801. A further 30 min were allowed to elapse before the animals were tested in the TST.

To investigate whether the antidepressant-like effect of AE is mediated through the involvement of the L-arginine-NO pathway in the TST, mice were pretreated with L-arginine, a precursor of nitric oxide (750 mg/kg, i.p., a dose that produces no effect in the tail suspension test). 30 min after L-arginine, AE (10 mg/kg) or vehicle was administered, and 60 min later the TST was carried out.

In another experiment, we also investigated the effect of AE (3 mg/kg, a sub-effective dose) with sub-effective doses of L-NNA (0.3 mg/kg, i.p., a competitive inhibitor of NO synthase with selectivity for the neuronal and endothelial isoforms of the enzyme), methylene blue (20 mg/kg, i.p., an inhibitor of both NO synthase and soluble guanylate cyclase) or ODQ (30 pmol/site i.c.v., a selective inhibitor of soluble guanylate cyclase). AE or vehicle was administered 30 min before the drugs and 30 min later the animals were tested in the TST.

To investigate the role of cyclic GMP (cGMP) in the antidepressant-like action of AE, mice received an injection of sildenafil (5 mg/kg, i.p., a phosphodiesterase 5 inhibitor), or vehicle, 30 min before AE (10 mg/kg), and a further 30 min elapsed before the animals were tested in the TST. The dose of sildenafil was chosen based on previous studies (Kaster et al., 2005; Almeida et al., 2006; Dhir and Kulkarni, 2007; Zomkowski et al., 2010).

2.4. Forced swimming test (FST)

For the FST mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25 ± 1 °C; the total duration of immobility during a 6 min test was scored as described previously by Kaster et al. (2005). Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

2.5. Tail suspension test (TST)

The tail suspension test has become one of the most widely used models for assessing antidepressant-like activity in mice. The test is based on the fact that animals subjected to the short-term, inescapable stress of being suspended by their tail, will develop an immobile posture. The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Briefly, mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6 min period (Rodrigues et al., 2002; Machado et al., 2007). Mice were considered immobile only when they hung passively and completely motionless. The immobility time was recorded by an observer blind to the drug treatment.

2.6. Open-field test

The forced swimming test has some drawbacks represented by the possibility of obtaining false positives or negatives. Drugs enhancing locomotor activity can evoke a 'false' positive effect in these tests, whereas drugs decreasing locomotion may give a 'false' negative result (Borsini and Meli, 1988). Therefore, in order to rule out an interference of the locomotor activity in the interpretation of the results obtained in the immobility tests, the locomotor activity was measured in the open-field test as described previously (Zomkowski et al., 2010). The open field arena used was a wooden box (40 cm × 60 cm × 50 cm) with the floor divided into 12 equal squares. At the start of each trial a mouse was placed in the left corner of the field and was allowed to freely explore the arena. The number of squares crossed with all paws (crossing) was counted in a 6 min session. The arena floor was cleaned between the trials with a 10% ethanol solution and the test was carried out in a temperature, noise and light controlled room.

2.7. Ex vivo neurochemical experiments

2.7.1. Treatment of mice with AE and preparation of hippocampal slices

The AE (10, 30 and 100 mg/kg) was administered by gavage and after 1 h mice were killed by decapitation and the hippocampus was rapidly removed and placed in ice-cold Krebs–Ringer bicarbonate (KRB) buffer (122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM D-glucose). The buffer was bubbled with 95% O_2–5% CO_2 up to pH 7.4. Slices (0.4 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in KRB at 4 °C and allowed to recover for 30 min in KRB at 37 °C (Oliveira et al., 2002).

2.7.2. Slices incubation

Hippocampal slices were incubated with glutamate (10 mM) (Sigma) for 1 h in KRB. After this period, the medium was withdrawn and replaced by a nutritive incubation medium composed of 50% of KRB, 50% of Dulbecco's modified Eagle's medium (DMEM, Gibco), 20 mM HEPES, and 100 μg/ml gentamicin, at 37 °C in a CO_2 atmosphere for additional 6 h in order to evaluate cell viability (Molz et al., 2008).

2.7.3. Evaluation of cell viability

2.7.3.1. MTT reduction. Hippocampal cell viability was evaluated 6 h after glutamate exposure. Cell viability was determined through the ability of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide; Sigma) (Mosmann, 1983). Hippocampal slices were incubated with MTT (0.5 mg/ml) in KRB for 30 min at 37 °C. The tetrazolium ring of MTT can be cleaved by active dehydrogenases in order to produce a precipitated formazan. The formazan produced was solubilized by adding DMSO, resulting in a colored compound whose optical density was measured in an ELISA reader (550 nm).

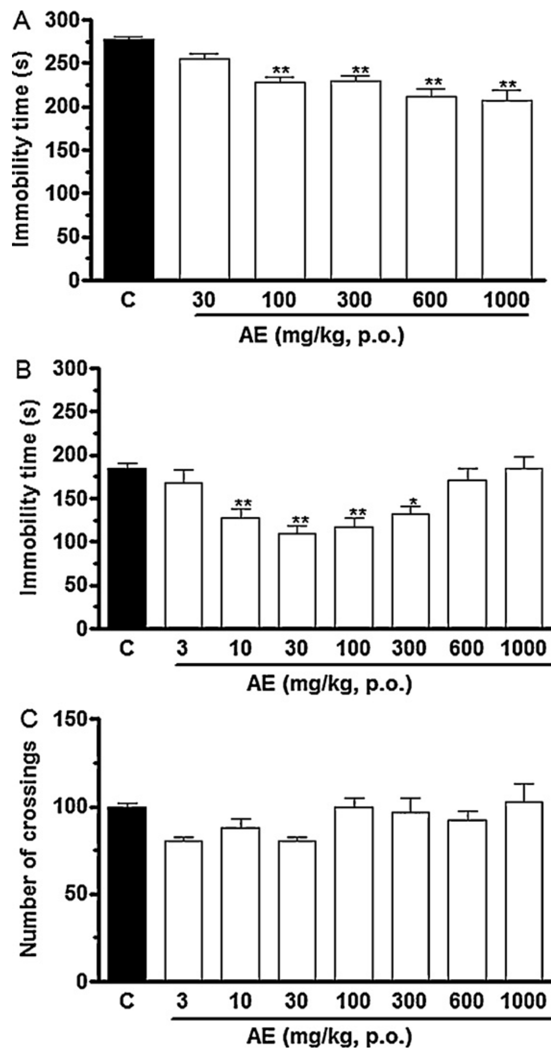

Fig. 1. Effect of *Aloysia gratissima* (AE) (3–1000 mg/kg, p.o.) in the FST (A), in the TST (B) and in the open-field test in mice (C). Values are expressed as mean ± S.E.M. (n = 6–11). *P < 0.05; **P < 0.01 as compared with the vehicle-treated control.

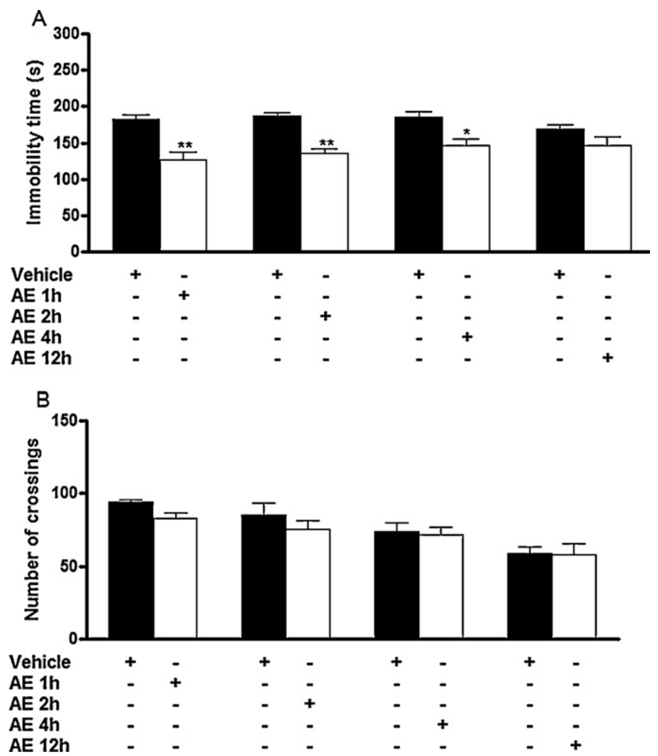

Fig. 2. Time–course effect of the oral administration of EA (10 mg/kg) in the immobility time in the TST (A) and on the number of crossings in the open-field test (B) behavior in mice. AE was administered 1, 2, 4 or 12 h before the test. Values are expressed as mean ± S.E.M (n = 6–7). **P < 0.01,*P < 0.05 compared with the vehicle-treated control group.

2.7.4. Western blot analysis

Slices were solubilized with a SDS-sample solution (4% SDS, 2 mM EDTA, 8% β-mercaptoethanol, and 50 mM Tris, pH 6.8). Samples (60 μg of total protein/track) were separated by SDS-PAGE using a 10% gel. The amount of protein loading was controlled by Ponceau staining of the nitrocellulose membranes. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using a 350 mA, 100 V current (1 h, 4 °C) (Molz et al., 2008). The membranes were blocked with 5% slim-milk (1 h) in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). All steps were followed by three times washing with TBS-T (0.05% Tween-20, 10 mM Tris, 150 mM NaCl, pH 7.5). Primary antibodies were selective for phospho-Akt and total-Akt (Sigma, 1:1000), iNOS (Santa Cruz Biotech, 1:10,000), and β-actin (Santa Cruz Biotech, 1:2000). Immunocomplexes were visualized using the ECL detection system as recommended by the manufacturer. The optical density of bands was quantified by using the Scion Image software (Scion Corporation).

2.7.5. Measurement of protein content

Protein content was evaluated by the method of Peterson (1977) in samples diluted in SDS solution. Bovine serum albumin (Sigma) was used as standard.

2.8. Statistical analysis

In the behavioral experiments, comparisons among treatment groups and control were performed by one-way or two-way ANOVA followed by Tukey's test, when appropriate. In the *ex vivo* experiments a one-way ANOVA was used followed by Duncan's test, when appropriate.

3. Results

3.1. Effect of acute treatment with AE on the immobility time in the FST, TST and locomotor activity in the open-field test

The effect of the acute administration of AE on the immobility time in the FST and TST is shown in Fig. 1A and B, respectively. The administration of 30 mg/kg of AE was ineffective to reduce the immobility time, as compared to control mice. The treatment of mice with AE (effective dose range: 100–1000 mg/kg) by p.o. route significantly decreased the immobility time in the FST (F 5,39 = 17.38, P < 0.01).

AE also caused a reduction in the immobility time in the TST when administered by p.o. route (effective dose range: 10–300 mg/kg) (F 7,47 = 8.34, P < 0.01). However, the administration of lower (3 mg/kg) and higher (600 or 1000 mg/kg) doses of AE was ineffective to reduce the immobility time, showing a bell-shaped curve effect for AE in the TST. AE did not produce any change in ambulation in an open-field test in all doses tested (F 7,52 = 1.89, P = 0.09) in a separate experiment as compared to the control group (Fig. 1C).

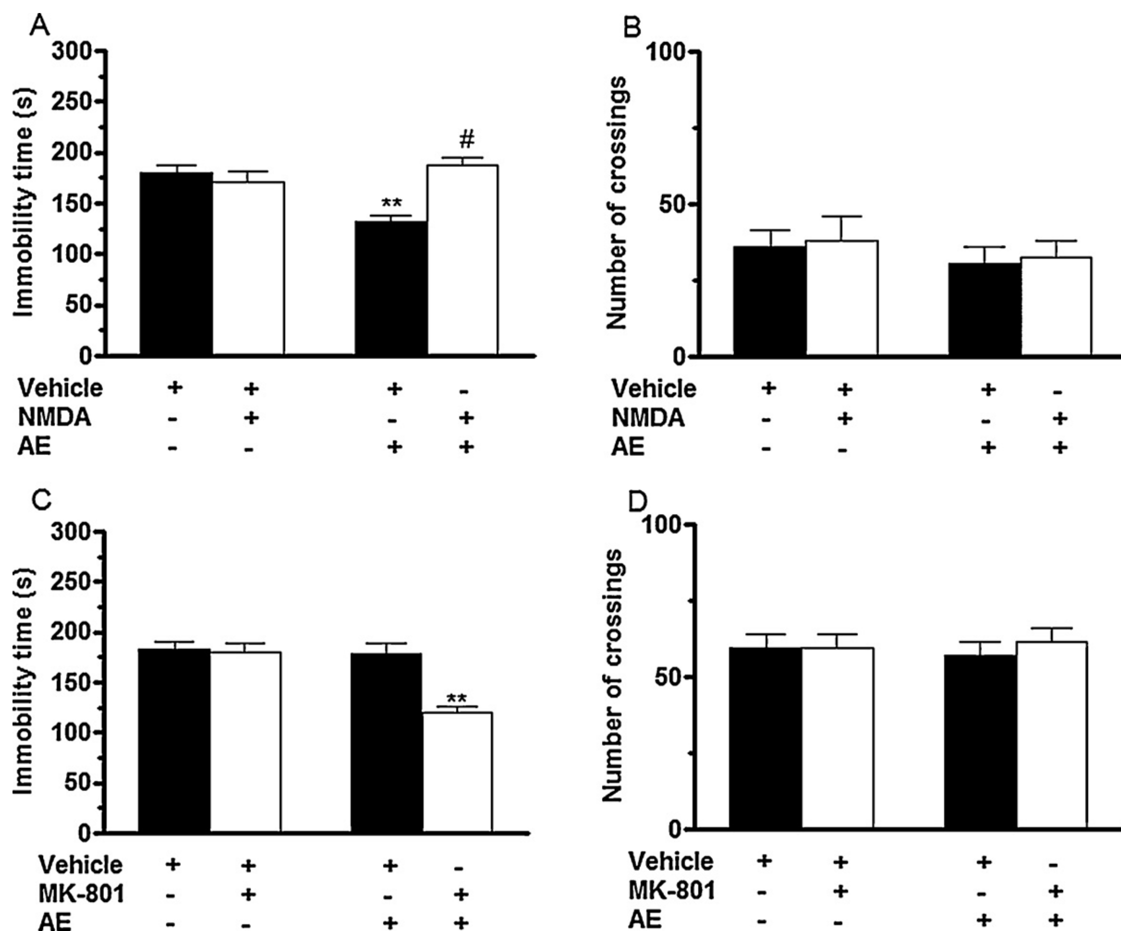

Fig. 3. Effect of the pretreatment of mice with NMDA (0.1 pmol/site, i.c.v.) on the anti-immobility action of AE (10 mg/kg, p.o.) or treatment of MK-801 (0.001 mg/kg, i.p.) in combination with a sub-effective dose of AE (3 mg/kg, p.o.) in the TST (A and C, respectively) and on the number of crossings in the open-field test (B and D, respectively). Values are expressed as mean ± S.E.M. (n = 6–7). $^{**}P < 0.01$ compared with the vehicle-treated control; $^{\#}P < 0.01$ compared with the same group pretreated with vehicle.

3.2. Time–course effect of AE on the immobility time in the TST and locomotor activity in the open-field test

The time–course effect of the oral administration of AE on the immobility time in the TST is shown in Fig. 2A. The treatment of mice with AE (10 mg/kg, p.o.) produced a marked effect in the TST as early as 1 h after its administration, an action that remained statistically significant until 4 h after its administration. Two-way ANOVA showed a significant effect of AE (AE treatment: F 1,43 = 78.49, $P < 0.01$ and interaction AE treatment × time period: F 3,43 = 4.14, $P < 0.05$, but not time period: F 3,43 = 1.21, $P = 0.32$) in the TST. Fig. 2B shows no changes were observed in the locomotor activity of mice as compared to the control group (Treatment: F 1,42 = 2.25, $P = 0.14$; time: F 3,42 = 10.68, $P < 0.01$; interaction treatment × time: F 3,42 = 0.44, $P = 0.72$). Moreover, post hoc analysis showed that AE exhibits similar effect at 1 or 2 h, however its antidepressant-like effect was diminished at 4 h and no longer observed at 12 h.

Since 10 mg/kg of the AE was the lowest acute effective dose (1 h before behavioral analysis), all the experiments regarding the investigation of the mechanisms underlying the antidepressant-like effect of the extract were performed in the TST using this dose.

3.2.1. Effect of treatment of mice with NMDA or MK-801 on the antidepressant-like effect of AE in the TST

Fig. 3A shows that the pretreatment of mice with NMDA (0.1 pmol/site, i.c.v.) was able to reverse the antidepressant-like effect of AE (10 mg/kg, p.o.) in the TST (Pretreatment: F 1,20 = 8.40, $P < 0.01$; treatment: F 1,20 = 4.03, $P = 0.06$; interaction pretreatment × treatment: F 1,20 = 16.29, $P < 0.01$). The administration of NMDA alone or in combination with AE did not affect the ambulation in the open-field (Fig. 3B) (pretreatment: F 1,20 = 0.10, $P = 0.75$; treatment: F 1,20 = 0.74, $P = 0.38$; interaction pretreatment × treatment: F 1,20 = 0.01, $P = 0.96$).

Fig. 3C shows that MK-801 (0.001 mg/kg, i.p.) in combination with a sub-effective dose of AE (3 mg/kg; p.o.) produced an antidepressant-like effect in the TST (pretreatment: F 1,20 = 8.40, $P < 0.01$; treatment: F 1,20 = 4.03, $P = 0.06$; interaction pretreatment × treatment: F 1,20 = 16.29, $P < 0.01$). The administration of MK-801 alone or in combination with AE did not affect the ambulation in the open-field (Fig. 3D) (pretreatment: F 1,20 = 0.10, $P = 0.75$; treatment: F 1,20 = 0.74, $P = 0.38$; interaction pretreatment × treatment: F 1,20 = 0.01, $P = 0.96$).

3.2.2. Effect of pretreatment with L-arginine on the AE induced anti-immobility effect in the TST

The results depicted in Fig. 4A shows that the pretreatment with L-arginine (750 mg/kg i.p., a nitric oxide precursor) prevented the antidepressant-like effect of AE (10 mg/kg, p.o.) in the TST (pretreatment: F 1,20 = 4.73, $P < 0.05$; treatment: F 1,20 = 25.68, $P < 0.01$; interaction pretreatment × treatment: F 1,20 = 1.46, $P < 0.01$). The administration of L-arginine alone or in combination with AE did not affect the ambulation in the open-field (Fig. 4B) (pretreatment: F 1,20 = 0.01, $P = 0.91$; treatment: F 1,20 = 0.04, $P = 0.84$; interaction pretreatment × treatment: F 1,20 = 1.07, $P = 0.31$).

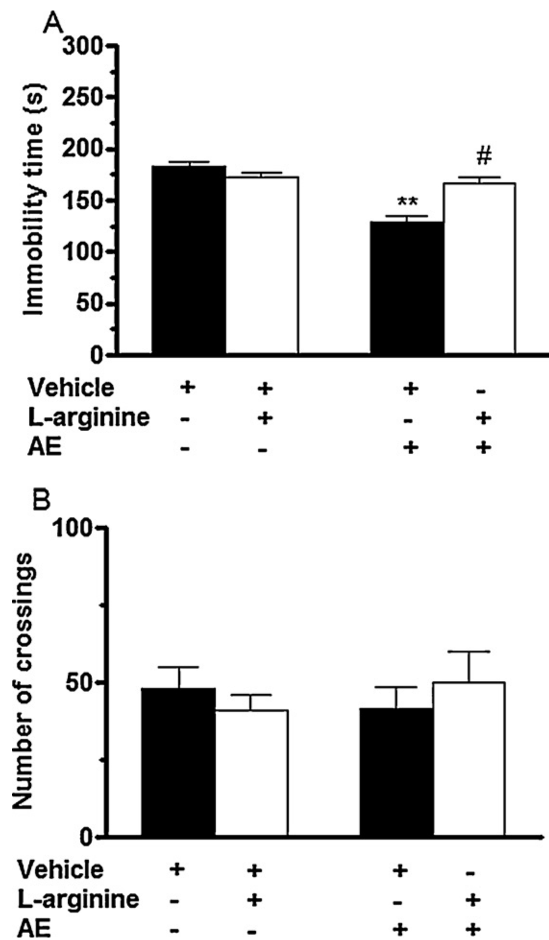

Fig. 4. Effect of the pretreatment of mice with L-arginine (750 mg/kg, i.p.; A) on the anti-immobility action of AE (10 mg/kg, p.o.) in the TST (A) and on the number of crossings in the open-field test (B). Values are expressed as mean ± S.E.M. (n = 6). $^{**}P<0.01$ compared with the vehicle-treated control; $^{\#}P<0.01$ compared with the same group pretreated with vehicle.

3.2.3. *Effects of combined administration of sub-effective doses of the NOS and soluble guanylate cyclase inhibitors and AE in the TST or in the open-field test*

Fig. 5A shows that L-NNA (0.3 mg/kg, i.p., a NOS inhibitor) in combination with AE (3 mg/kg, p.o.) produced an anti-immobility effect in the TST as compared with the administration of either drug alone (pretreatment: F 1,20 = 5.87, $P<0.05$; treatment: F 1,20 = 19.46, $P<0.01$; interaction pretreatment × treatment: F 1,20 = 14.49, $P<0.01$). The administration of L-NNA alone or in combination with AE did not affect the ambulation in the open-field (Fig. 5B) (pretreatment: F 1,24 = 3.03, P = 0.09; treatment: F 1,24 = 0.31, P = 0.58; interaction pretreatment × treatment: F 1,24 = 0.03, P = 0.86).

Fig. 5C shows that methylene blue (20 mg/kg, i.p., direct inhibitor of both nitric oxide synthase and soluble guanylate cyclase) in combination with AE (3 mg/kg, p.o.) also produced an anti-immobility effect in the TST as compared with the administration of either drug alone. The administration of methylene blue (20 mg/kg, i.p.) alone (pretreatment: F 1,23 = 5.82, $P<0.05$; treatment: F 1,23 = 18.22, $P<0.01$; interaction pretreatment × treatment: F 1,23 = 13.1, $P<0.01$) or in combination with AE did not affect the ambulation in the open-field (Fig. 5D) (pretreatment: F 1,24 = 1.72, P = 0.20; treatment: F 1,24 = 0.32, P = 0.58; interaction pretreatment × treatment: F 1,24 = 0.56, P = 0.46).

The results illustrated in Fig. 5E show the administration of ODQ (30 pmol/site i.c.v., a selective guanylate cyclase inhibitor) in combination with AE (3 mg/kg, p.o.) produced an antidepressant-like effect as compared with the administration of either drug alone (pretreatment: F 1,24 = 34.15, $P<0.01$; treatment: F 1,24 = 38.48, $P<0.01$; interaction pretreatment × treatment: F 1,24 = 24.77, $P<0.01$). Fig. 5F shows that the administration of ODQ alone or in combination with AE did not affect locomotor activity in the open-field test (Pretreatment: F 1,24 = 1.09, P = 0.31; treatment: F 1,24 = 0.81, P = 0.38; interaction pretreatment × treatment: F 1,24 = 3.77, P = 0.06).

3.2.4. *Effects of pretreatment with sildenafil in the antidepressant-like effect of AE*

Fig. 6A shows that the anti-immobility effect of AE (10 mg/kg, p.o.) was completely prevented by pretreatment of animals with sildenafil (5 mg/kg, i.p., an inhibitor of phosphodiesterase 5, PDE5), which *per se* produced no effect in the TST (pretreatment: F 1,20 = 14.32, $P<0.01$; treatment: F 1,20 = 17.03, $P<0.01$; interaction pretreatment × treatment: F 1,20 = 10.91, $P<0.01$). The administration of sildenafil (5 mg/kg, i.p.) alone or in combination with AE did not affect the locomotor activity in the open-field (Fig. 6B) (pretreatment: F 1,20 = 0.51, P = 0.48; treatment: F 1,20 = 0.44, P = 0.51; interaction pretreatment × treatment: F 1,20 = 2.43, P = 0.13).

3.3. *Neuroprotective effect of AE against glutamate-induced toxicity*

The incubation of hippocampal slices for 1 h with glutamate (10 mM, *in vitro*) significantly reduced the cell viability, assessed by MTT reduction, when compared to control slices. Glutamate-induced hippocampal slice damage was not observed in hippocampal slices obtained from mice previously treated (1 h before decapitation of mice and slices preparation) with AE as an *ex vivo* evaluation of the neuroprotective effect of AE (10 mg/kg) (Fig. 7).

3.4. *AE prevents glutamate-induced decreased of Akt phosphorylation and increase of iNOS immunocontent*

Fig. 8 shows that glutamate induced a decrease in $^{\text{Ser-473}}$Akt phosphorylation in hippocampal slices obtained from mice previously treated with vehicle (*ex vivo* experiment). On the contrary, glutamate was unable to cause this effect in slices obtained from mice previously treated with AE (10 mg/kg, p.o., 1 h before slices preparation). These results suggest that the activation of the PI3K/Akt pathway is implicated in the protection afforded by AE. No significant alteration was observed in total Akt protein immunocontent.

In addition, Fig. 9 shows a glutamate-induced increase in the iNOS expression in hippocampal slices obtained from mice previously treated with vehicle. Also, glutamate was unable to cause this effect in slices obtained from mice previously treated with AE (10 mg/kg, p.o., 1 h before slices preparation).

4. Discussion

In the present study, we demonstrated for the first time that acute AE orally administered is effective in producing significant antidepressant-like effect in FST and TST, without modifying the motor performance of mice. Moreover, an *ex vivo* evaluation of glutamatergic toxicity in hippocampal slices obtained from pretreated mice showed that AE is also neuroprotective to hippocampal damage induced by glutamate.

The FST and TST are widely accepted stress models of depression used to screen new antidepressant drugs, as they are sensitive to all major classes of antidepressant drugs including

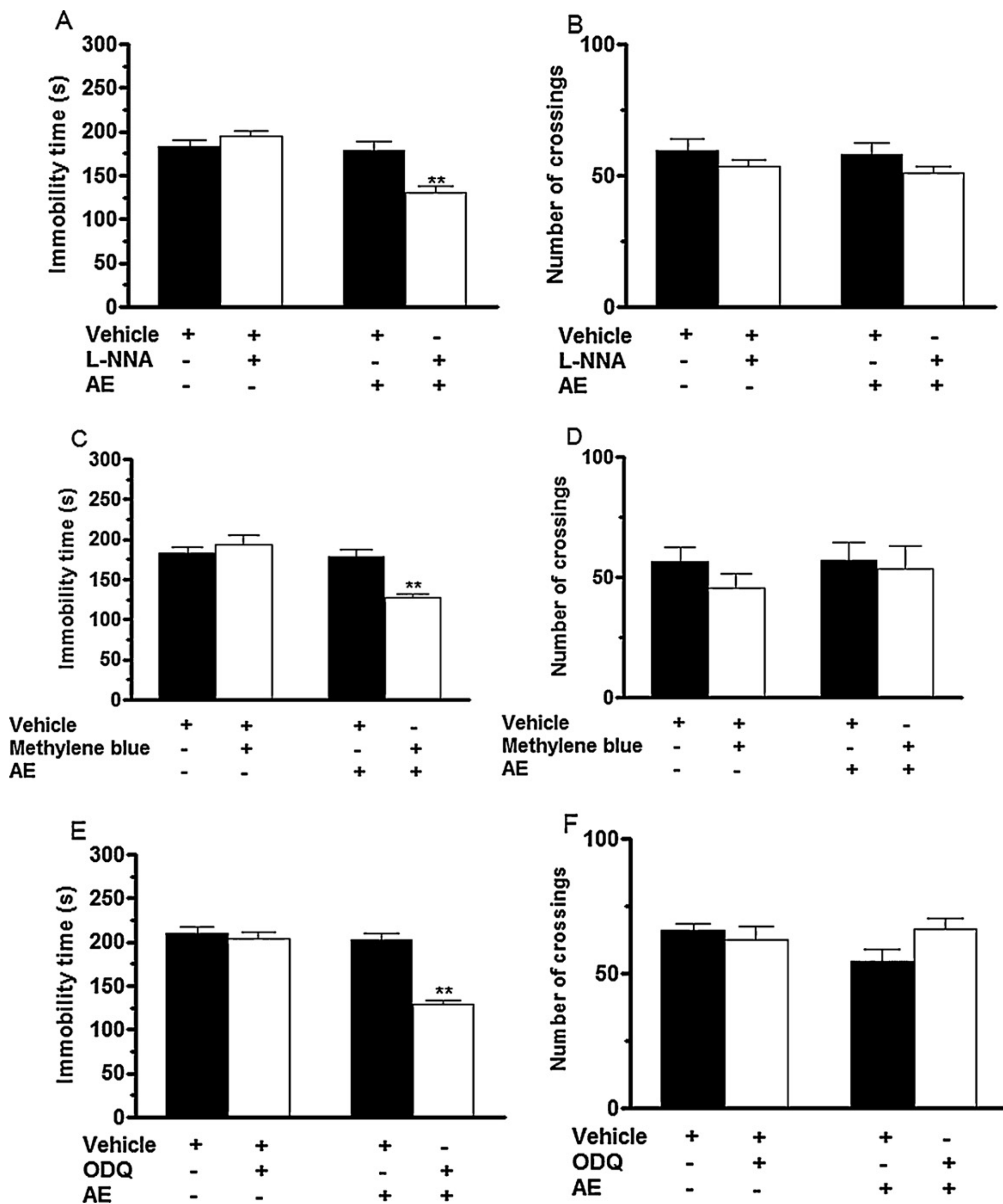

Fig. 5. Effect of L-NNA (0.3 mg/kg, i.p.), methylene blue (20 mg/kg, i.p.) or ODQ (30 pmol/site, i.c.v.) in combination with a sub-effective dose of AE (3 mg/kg, p.o.) in the TST in mice (A, C and E, respectively) and in the open-field test (B, D, and F, respectively). Values are expressed as mean ± S.E.M. (n = 6–7). **P < 0.01 compared with the vehicle-treated control group.

tricyclics, serotonin-selective reuptake inhibitors, monoamine oxidase inhibitors, and atypical (Porsolt et al., 1977; Steru et al., 1985). However, these tests do not have an identical neurochemical basis (Bai et al., 2001). Therefore, the fact that AE administration is active in both tests reinforces the assumption that AE might play a role in the modulation of depression. However, AE caused a biphasic ('bell-shaped') dose–response curve in the TST, but did not produce this effect in the dose range tested in the FST. Indeed, a bell-shaped dose–response profile for several compounds has been reported in the TST, such as for 5,6-dibromo-N,N-dimethyltryptamine, a marine natural compound, and nortriptyline hydrochloride (Oliveira et al., 1990; Diers et al., 2008). Therefore, the FST is generally recommended for a screen of antidepressant-like effects, and a drug showing positive effects should then be further tested in the TST (Cryan et al., 2002). It has also been proposed that the TST is less stressful than the FST and possesses greater pharmacological sensitivity to lower doses of antidepressant (Thierry et al., 1986). Accordingly, the antidepressant-like effect of AE was observed at lower doses in TST than FST. Moreover, the antidepressant-like effect of 10 mg/kg AE in the TST was observed until 4 h of administration, an interesting prolonged effect as compared *Tabebuia avellanedae*, another plant extract which presents antidepressant-like effect in TST (Freitas et al., 2010).

The involvement of NMDA receptors and the L-arginine-NO-cGMP pathway in antidepressant-like activity of AE and the ability

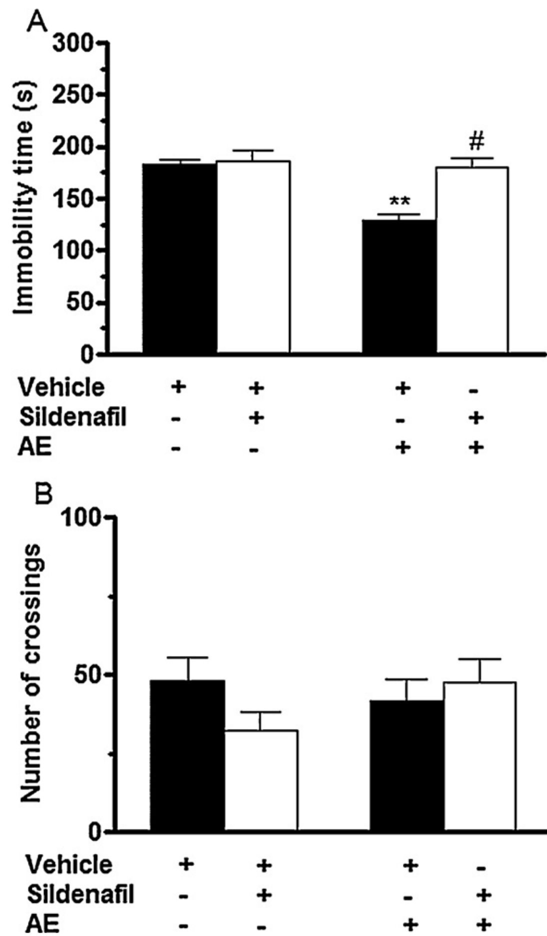

Fig. 6. Effect of the pretreatment of mice with sildenafil (5 mg/kg, i.p.) on the anti-immobility effect of AE (10 mg/kg, p.o.) in the TST (A) and on the number of crossings in the open-field test (B). Values are expressed as mean ± S.E.M. ($n = 6$). $^{**}P < 0.01$ compared with the vehicle-treated control; $^{\#}P < 0.01$ compared with the same group pretreated with vehicle.

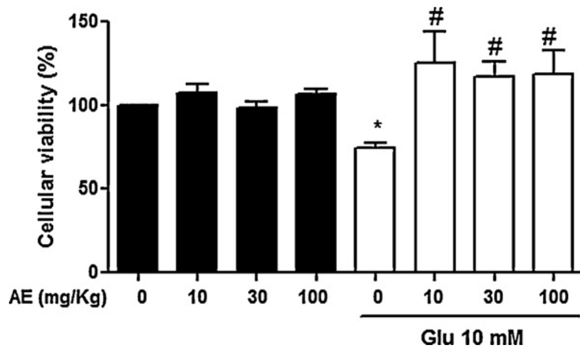

Fig. 7. *Ex vivo* cell viability analysis in hippocampal slices incubated with glutamate (Glu, 10 mM) for 1 h. When present, AE (10, 30 or 100 mg/kg) was pre-administered in mice by gavage (1 h before slices preparation). After this period, incubation media was withdrawn and replaced for fresh culture medium without Glu and maintained for additional 6 h. Control group (C, first black bar) was considered as 100% and other black bars represents cell viability of slices incubated only in culture medium. White bars represents the cell viability of hippocampal slices subjected to glutamate. The values represent mean ± SD of at least 5 experiments carried out in triplicates. Mean significantly different from control group (100%); $P < 0.05$. $^{\#}$Mean significantly different from Glu group; $P < 0.05$.

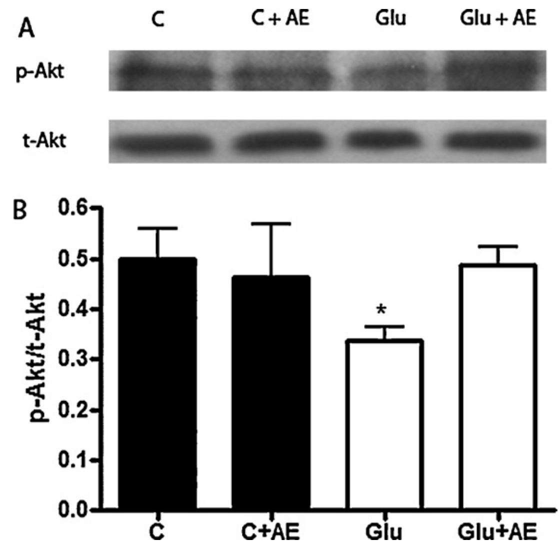

Fig. 8. AE treatment increases Akt phosphorylation in hippocampal slices. AE treatment (10 mg/kg) 1 h before glutamate (10 mM) significantly increases Akt phosphorylation at Ser^{473}. (A) Representative Western blot of phosphorylated (p-Akt) and total content of Akt protein (t-Akt), 56 KDa; (B) The ratio of phosphorylated Akt/total Akt signal (p-Akt/t-Akt). The optical densities of bands were measured by Scion Image software. Data in bars are presented as mean ± SD of 4 experiments carried out in triplicate. $^{*}P < 0.05$ compared with control (C) group.

of AE treatment (*ex vivo* experiments) to prevent glutamate-induced toxicity in hippocampal slices were evaluated.

The involvement of glutamate in the pathophysiology of depression has been suggested in several studies (for review, see Sanacora et al., 2008). Increased levels of glutamate in the frontal cortex in postmortem samples (Hashimoto et al., 2007) and in the plasma of patients with depression (Mauri et al., 1998) have been reported. Additionally, NMDA receptor antagonists possess antidepressant properties and conventional antidepressants have been reported to reduce the binding, expression and function of NMDA receptors (Skolnick, 1999; Sanacora et al., 2008). The reversal of the antidepressant-like effect of AE by NMDA suggests its effect is

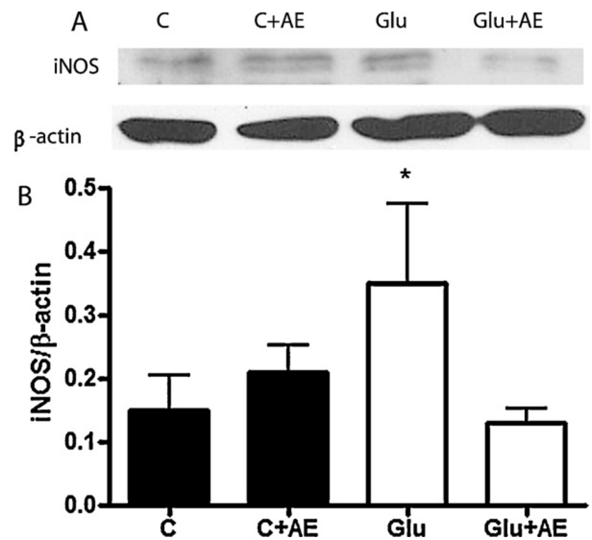

Fig. 9. AE treatment decreases iNOS expression in hippocampal slices. AE treatment (10 mg/kg) 1 h before glutamate (10 mM) significantly decreases iNOS expression. (A) Representative Western blotting of iNOS (130 kDa) and β-actin (30 kDa) immunocontent used as loading control to western blot analysis. (B) The ratio of iNOS/β-actin signal (iNOS/β-actin). The optical density of bands was measured by Scion Image software. Data in bars are presented as mean ± SD of 4 experiments carried out in triplicate. $^{*}P < 0.05$ compared with control (C) group.

dependent on the inhibition of NMDA receptor activation. Besides, we observed that the co-administration of MK-801 and AE produced a synergistic antidepressant-like effect. The dose of MK-801 used in the present study was previously shown to cause no effect in the open-field test, but it was able to cause a synergistic antidepressant-like effect with folic acid in the FST (Brocardo et al., 2008). Moreover, it has been shown that fluoxetine and desipramine inhibit NMDA receptors at clinically relevant concentration range (Szasz et al., 2007), a finding that indicates that glutamatergic, noradrenergic and serotonergic systems interact in the mechanism of action of antidepressants (Forray et al., 1999; Maura et al., 2000; Bonanno et al., 2005; Szasz et al., 2007). In addition, it was recently reported that the antidepressant-like effect of escitalopram in the FST was reversed by NMDA pretreatment in mice (Zomkowski et al., 2010).

Due to the existence of data indicating that the NO-cGMP pathway is involved in the pathophysiology of depression (Harkin et al., 1999; Kaster et al., 2005; Zomkowski et al., 2010) and also the fact that the activation of NMDA receptors is directly associated with the activation of NOS in the central nervous system, the involvement of this pathway in the antidepressant-like effect of AE was investigated. We showed that the pretreatment with L-arginine, a substrate for NOS, significantly inhibited the anti-immobility effect caused by AE. Our results are in accordance with studies that have shown that NOS inhibitors (Wegener et al., 2003) and reduction of NO levels within the hippocampus can induce antidepressant-like effects, thus implicating endogenous hippocampal NO in the neurobiology of depression (Joca and Guimarães, 2006). Similarly to our results, the antidepressant-like effects of escitalopram, paroxetine, and venlafaxine were also blocked by pretreatment with L-arginine (Dhir and Kulkarni, 2007; Ghasemi et al., 2009; Zomkowski et al., 2010). These results indicate that the effect of AE in the TST may be dependent on, at least in part, the inhibition of NO synthesis.

The assumption that the L-arginine-NO-cGMP pathway is involved in the reduction in the immobility time elicited by AE is reinforced by the finding that L-NNA (an inhibitor of NOS), ODQ (a selective, irreversible, heme-site inhibitor of sGC) or methylene blue (an inhibitor of both NOS and sGC), produced a synergistic antidepressant-like effect with AE. Accordingly, it has been reported that inhibitors of NOS or sGC potentiated the effect of adenosine, venlaxine (Kaster et al., 2005; Dhir and Kulkarni, 2007), escitalopram and memantine (Almeida et al., 2006; Zomkowski et al., 2010) in the FST. Hence, our results also suggest that the antidepressant-like effect of AE may be mediated through the reduction of cGMP, as a consequence of the reduction of NO synthesis, reinforcing the notion that cGMP is an important molecular target for antidepressant action.

Noteworthy, the reversal of the antidepressant-like effect of AE caused by pretreatment with sildenafil, a selective PDE5 inhibitor, further indicates that AE exerts its effect in the TST by decreasing cGMP levels. The intracellular cGMP concentrations are regulated not only by sGC, but also by PDE, which catalyses the hydrolysis of the second messengers cAMP and cGMP. The duration and magnitude of a NO-induced cGMP signal are determined by the activity of PDE5 (Beavo, 1995). Accordingly, the antidepressant-like effect of memantine (Almeida et al., 2006), venlafaxine (Dhir and Kulkarni, 2007), folic acid (Brocardo et al., 2008), and escitalopram (Zomkowski et al., 2010) was prevented by sildenafil.

Moreover, the results obtained in the *ex vivo* experiments with hippocampal slices show that AE exerts neuroprotection against the glutamate excitotoxicity. Hippocampal slices exposed to 10 mM glutamate presented reduced cell viability, which was not observed in slices prepared from mice previously treated with AE (10 mg/kg, p.o.). According to Molz et al. (2008), glutamate at this concentration causes hippocampal cells death by apoptosis, which was completely blocked by MK-801.

Several signaling pathways have been reported to be involved in the mechanism of action of neuroprotective agents, with special attention to the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. Akt is a serine–threonine kinase activated by PI3K phosphorylation implicated in cell growth, migration, and survival (Cantley, 2002), and directly related to neuroprotection against glutamate-induced toxicity (Piermartiri et al., 2009). In the present study, the neuroprotection afforded by AE (10 mg/kg) against excitotoxicity seems to be mediated by activation of Akt signaling and recovery of cell viability in hippocampal slices. In addition, Akt signaling has been shown to be implicated in the mechanism of action of antidepressant drugs (Beaulieu et al., 2009).

Furthermore, we observed that glutamate (10 mM) also increased the iNOS expression in hippocampal slices. Noteworthy, this effect was not observed in slices prepared from mice previously treated with AE. Since AE is rich in kauranes, flavonoids, and phenylethanoids (Silva et al., 2006) and possesses antioxidant activity (Rosas-Romero and Saavedra, 2005) we cannot rule out an action of these molecules in the mechanism of neuroprotection afforded by AE and further investigations will be carried out to clarify this issue.

Therefore, considering the effects of AE herein shown, it is feasible to suggest that the antidepressant-like action of AE might be related to its neuroprotective action in the hippocampus, which is consistent with the relationship between hippocampal damage and depressive disorders (Sapolsky, 2000). Altogether, our results positively support the traditional use of *Aloysia gratissima* in South America folk medicine. Studies are being carried out in our laboratories to discriminate additional activities of aqueous extract and the constituents of *Aloysia gratissima* upon the central nervous system, as well as to clarify their mechanisms of action.

5. Conclusion

The results of the present study revealed that AE exerted an antidepressant-like effect in the FST and TST. In addition, the antidepressant-like effect was shown to be dependent on its interaction with NMDA receptors and L-arginine-NO-cGMP pathway. Moreover, AE protected hippocampal slices against glutamate excitotoxicity through activation of Akt pathway and decrease of iNOS expression. Altogether, the results indicate that *Aloysia gratissima* may be of interest as a plant species source for therapeutic agent for the treatment of depressive disorders and neurodegeneration.

Acknowledgements

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