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Rosmarinus officinalis L. hydroalcoholic extract, similar to fluoxetine, reverses depressive-like behavior without altering learning deficit in olfactory bulbectomized mice

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ABSTRACT

Ethnopharmacological relevance: Rosemary, *Rosmarinus officinalis* L., has several therapeutic applications in folk medicine for the treatment of a wide range of diseases, including depression.
Aim of the study: To evaluate the ability of *Rosmarinus officinalis* hydroalcoholic extract (ROHE), as compared to the positive control fluoxetine, to reverse behavioral (hyperactivity, anhedonic behavior and learning deficit in water maze) and biochemical alterations (serum glucose level and acetylcholinesterase, AChE, activity) induced by an animal model of depression, the olfactory bulbectomy (OB) in mice.
Materials and methods: Locomotor and exploratory behavior was assessed in the open-field, novel object and novel cage tests, anhedonic behavior was assessed in the splash test; cognitive deficits were evaluated in the water maze task. For the first set of experiments, ROHE (10–300 mg/kg) or fluoxetine (10 mg/kg) was administered once daily (p.o.) for 14 days after OB and the behavioral tests were performed. For the second set of experiments, serum glucose and hippocampal and cerebrocortical AChE activity were determined in OB and SHAM-operated mice treated orally with ROHE (10 mg/kg), fluoxetine (10 mg/kg) or vehicle.
Results: ROHE (10–300 mg/kg), similar to fluoxetine, reversed OB-induced hyperactivity, increased exploratory and anhedonic behavior. OB needed significantly more trials in the training session to acquire the spatial information, but they displayed a similar profile to that of SHAM mice in the test session (24 h later), demonstrating a selective deficit in spatial learning, which was not reversed by ROHE or fluoxetine. A reduced serum glucose level and an increased hippocampal AChE activity were observed in bulbectomized mice; only the latter effect was reversed by fluoxetine, while both effects were reversed by ROHE.
Conclusions: ROHE exerted an antidepressant-like effect in bulbectomized mice and was able to abolish AchE alterations and hypoglycemia, but not spatial learning deficit induced by OB. Overall, results suggest the potential of *Rosmarinus officinalis* for the treatment of depression, validating the traditional use of this plant.

1. Introduction

According to the World Health Organization about 80% of the world's population in developing countries depends essentially on plants for their primary health care (WHO, 2002).

Moreover, the knowledge of medicinal plants often represents the only therapeutic option for many communities and ethnic groups in poor countries. However, few plants have been scientifically studied for the assessment of their quality, safety and efficacy (Calixto, 2005).

Rosemary, *Rosmarinus officinalis* L. (Labiatae), is among a variety of plants used in folk medicine worldwide for the first health care. It is an evergreen perennial shrub native native to Asia Minor and southern Europe; today it has been cultivated in many parts of the world (Al-Sereiti et al., 1999; Heinrich et al., 2006). Several reports in the literature have shown the ethnopharmacological uses of

Abbreviations: AChE, acetylcholinesterase; ANOVA, analysis of variance; OB, olfactory bulbectomy; SSRI, selective serotonin reuptake inhibitor

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Rosmarinus officinalis for the treatment of inflammatory diseases, physical and mental fatigue, improvement of memory and treatment of nervous agitation, hysteria and depression, among other applications (Duke, 2000; Heinrich et al., 2006).

Preclinical studies have demonstrated that the extract of this plant exerts a number of pharmacological activities, such as hepatoprotective (Sotelo-Félix et al., 2002), antibacterial (Del Campo et al., 2000), diuretic (Haloui et al., 2000), antidiabetic (Bakirel et al., 2008), antioxidant (Bakirel et al., 2008), antinociceptive (González-Trujano et al., 2007) and anti-inflammatory (Benincá et al., 2011). We have shown that *Rosmarinus officinalis* hydroalcoholic extract (ROHE) produces an antidepressant-like effect in the forced swim test and tail suspension test, predictive tests of antidepressant activity, by a mechanism dependent on the interaction with the monoaminergic systems (Machado et al., 2009). However, the ability of *Rosmarinus officinalis* to reverse depressive-like behavior induced by a model of depression that mimics several symptoms observed in depressed patients was not reported in the literature.

The olfactory bulbectomy (OB) is an animal model of depression characterized by the bilateral destruction of the olfactory bulbs, which produces behavioral, neurochemical and neuroendocrinological changes that resemble some of the symptoms observed in depressed individuals (Kelly et al., 1997; Leonard, 1984; Song and Leonard, 2005). Therefore, OB provides a good model for studying antidepressant drugs and also may provide neurochemical and neuroanatomical data that are relevant to understand the biological substrates of emotion and the causes of depression in humans (Jesberger and Richardson, 1988). It is also important to mention that OB is one of the few models of depression that mimics the slow onset of antidepressant action reported in clinical studies, since the efficacy of antidepressants in this model is evident almost exclusively after 14 days of treatment (O'Neil and Moore, 2003). Hyperactivity response, the major behavioral change observed in this model, can be reversed by chronic treatments with antidepressants (Kelly et al., 1997; Leonard and Tuite, 1981; Van Riezen and Leonard, 1990). Additionally, OB causes different signs of anhedonia, as well as cognitive deficits (Harkin et al., 2003; Kelly et al., 1997; Song and Leonard, 2005).

OB in rodents has been also associated with biochemical alterations, including a reduction in the brain levels of monoamine neurotransmitters serotonin and norepinephrine (Kelly et al., 1997; Lumia et al., 1992; Song and Leonard, 2005), serum glucose level alterations (Montilla et al., 1984; Perassi et al., 1975), and cholinergic dysfunction (Moriguchi et al., 2006; Nakajima et al., 2007).

Therefore, this study was aimed at investigating the effects of chronic administration of ROHE in behavioral and biochemical alterations induced by OB in mice.

2. Materials and methods

2.1. Plant material and preparation of ROHE

Stems and leaves of *Rosmarinus officinalis* (Labiatae) were collected in Santo Amaro da Imperatriz, Santa Catarina, Brazil, and identified by Dr. Daniel Falkenberg, from Department of Botany, Federal University of Santa Catarina. A voucher specimen (Excicata number 34918) was deposited in the Herbarium of the Department of Botany, Federal University of Santa Catarina, Brazil. The preparation of extract, dried aerial parts of *Rosmarinus officinalis* (600 g) was submitted to maceration in ethanol (96%) during fifteen days at room temperature (25 ± 2 °C). Thereafter, the extract was filtered and then concentrated under reduced pressure (at approximately 60 °C). The maceration was repeated three times. After removing the solvent by lyophilization, this procedure gave 61 g of a green solid and dry hydroalcoholic crude extract (10.2% w/w yield).The ROHE was obtained according to the methodology described by Machado et al. (2009). The extract was kept in closed bottle at 4 °C in a refrigerator for further use.

2.2. HPLC profile of ROHE

The liquid chromatography (HPLC) profile of ROHE was performed according to Benincá et al. (2011). Carnosol used as standard for quantification was obtained according to Benincá et al. (2011). The triterpenes betulinic acid, oleanolic acid, ursolic acid and rosmarinic acid (Sigma-Aldrich, Steinheim, Germany) were also used as standards.

2.3. Animals

Female Swiss mice (50–55 day old, weighing 35–40 g) were used for this study and maintained at constant room temperature (21 ± 1 °C) with free access to water and food, under a 12:12 h light:dark cycle (lights on at 07:00 h). Mice were allowed to acclimatize to the holding room for 24 h before the behavioral procedure (N=9–11 animals per group). All experiments were carried out between 9:00 and 16:00 h. The procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Institution. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.4. Drugs and treatment

ROHE (10–300 mg/kg, p.o.) and the antidepressant fluoxetine (10 mg/kg, p.o., Sigma Chemical Company, St. Louis, MO, U.S.A.) were dissolved in distillated water and given once a day via oral route (p.o.) by gavage over a period of 14 days (in a constant volume of 10 mL/kg body weight) to mice. Fluoxetine, used here as a positive control, was administered at a dose previously shown to cause antidepressant-like effects (Machado et al., 2009). The dissolution of ROHE was freshly done from the lyophilized power immediately before its administration by gavage. Controls received an identical volume of distilled water (vehicle).The administration schedule and the doses of ROHE were chosen on the basis of experiments previously performed in our laboratory (Machado et al., 2009).

After 14 post-operative days (recovery period), mice were assigned to the following groups:

(I) SHAM-operated/treated with distilled water for 14 days (SHAM/vehicle) as the control group, (II) SHAM-operated/treated with extract for 14 days (SHAM/extract); (III) SHAM-operated/treated with fluoxetine for 14 days (SHAM/fluoxetine); (IV) bulbectomized mice treated with distilled water for 14 days (bulbectomized/vehicle); (V) bulbectomized mice treated with extract for 14 days (bulbectomized/extract); (VI) bulbectomized mice treated with fluoxetine for 14 days (bulbectomized/fluoxetine).

2.5. Bilateral olfactory bulbectomy (OB) surgery

After a 2-week acclimatization period, OB was performed according to the procedure described by Leonard and Tuite (1981). Briefly, mice were anesthetized with xylazin (20 mg/kg; Virbac©, Brazil) in combination with ketamine (100 mg/kg; Virbac©, Brazil), diluted in saline (0.9% NaCl) administered intraperitoneally (i.p.); 10 mL/kg body weight. The skull covering the olfactory bulbs was exposed by skin incision and two burr holes were drilled using a dentist drill. The olfactory bulbs were bilaterally aspirated using a blunt hypodermic needle (1.0–1.2 cm long and with a rounded tip of

0.80–1.2 mm of diameter) attached to a 10 mL syringe (taking care not to cause any damage to the frontal cortex).

Finally, the burr hole was filled with acrylic resin, in order to avoid bleeding and contamination at the surgical site. SHAM-operations were performed in the same way, but the olfactory bulbs were left intact. After being submitted to the surgical procedure, all animals were allowed to recover in a post-operative cage (maintained at 24 °C) for 3 h. After this time period, mice were returned to their home cage. This technique was adapted from previous studies (Leonard, 1984; Leonard and Tuite, 1981; Van Riezen and Leonard, 1990; Zueger et al., 2005).

At the end of the experiments, all animals were sacrificed and the presence of the lesions was verified. The bulbectomized animals that showed incomplete removal of the olfactory bulbs or damage to other brain areas (less than 15% of the total) were excluded from subsequent analysis following the criteria previously described (Jarosik et al., 2007; Kelly et al., 1997).

A 14 day post-surgery period time interval was considered to be sufficient to guarantee an appropriate recovery of the animals, as indicated in literature studies (Jarosik et al., 2007; van Riezen and Leonard, 1990; Zueger et al., 2005).

As depicted in Fig. 1, fourteen days after surgery (1–14° Day, recovery period), drug treatment was started and continued for a period of 14 days (15–28° Day, treatment period).

2.6. Behavioral tests

One day before surgery, locomotor activity and exploratory behavior was analyzed using the open-field. Behavioral changes after OB and/or chronic treatment with ROHE were examined by testing locomotor activity and exploratory behavior in the open-field 4 weeks after OB and 2 weeks after the beginning of chronic drug treatment (Fig. 1). All tests were carried out during the light phase of the light/dark cycle. Light intensity was approximately 200 lx. On the first test day (day 29 of the experiment), 24 h after the last drug treatment, mice were submitted to the open-field. After two hours (time period previously shown to cause no behavioral interference), mice were submitted to the splash test in order to investigate anhedonic behavior (Moretti et al., 2012). On the second test day (day 30 of the experiment), 48 h after the last drug treatment, mice underwent the novel object test. On day 31, 72 h after the last drug treatment, mice were submitted to the novel cage test. On day 32, 96 h after the last drug treatment, mice were subject to training sessions of the water maze task and on day 33, 120 h after the last drug treatment, mice were subject to a test session of the water maze task.

2.6.1. Open-field test

The open-field test was used to investigate locomotor activity and exploratory behavior, since locomotor hyperactivity is the key behavioral feature of bulbectomized rodents. Mice were individually placed in a wooden box ($40 \times 60 \times 50\ cm^3$) with the floor divided into 12 squares. Number of crossings (number of squares crossed by the animal with the four paws) was used to evaluate locomotor activity whereas number of rearings (number of times the mice stood on its hind legs or vertical exploratory activity) to assess the exploratory behavior (Machado et al., 2009; van Riezen and Leonard, 1990; Zueger et al., 2005). All the parameters were registered in a 6-min period.

The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors or clues.

2.6.2. Splash test

The splash test was adapted from Yalcin et al. (2005). This test evaluates grooming behavior, defined as cleaning of the fur by licking or scratching, after the vaporization of 10% sucrose solution on the dorsal coat of mice. The viscosity of the sucrose solution dirties the coat and animals initiate grooming behavior,

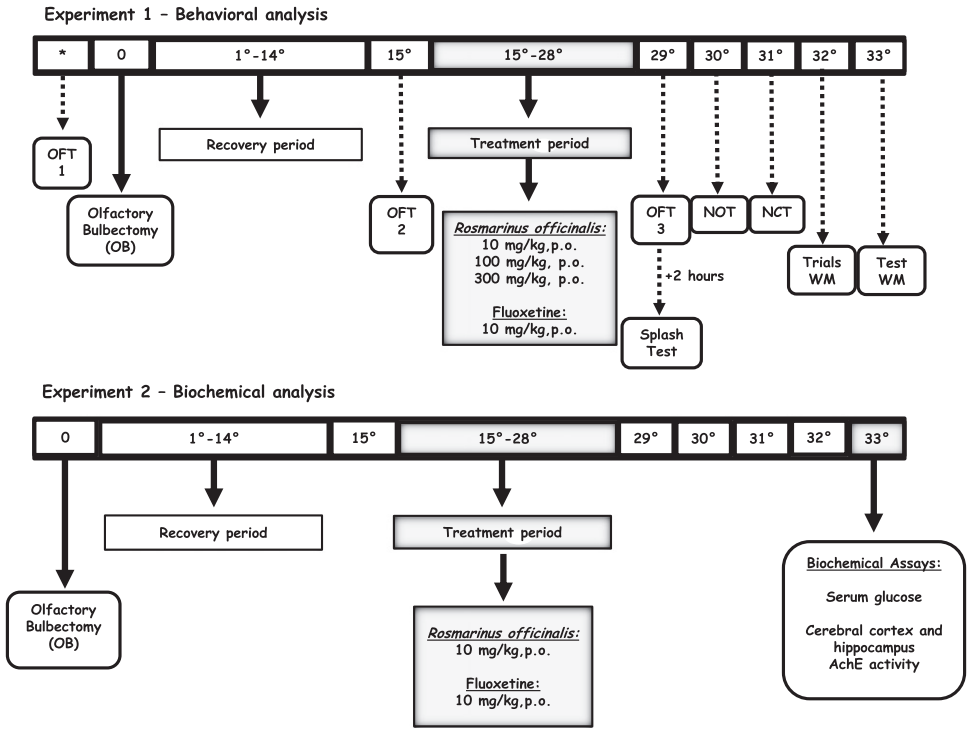

Fig. 1. Schematic representation of the experimental protocol with the treatment period with ROHE and behavioral tests period (Panel A). The animals were killed by decapitation 6 h after the end of the behavioral testing on day 33. Blood samples were collected and hippocampus and frontal cortex dissected, then stored in a freezer at −80 °C for subsequent biochemical analysis (Panel B). (OFT: open-field test, NOT: novel object test, NCT: novel cage test, Trials WM: training session of the water maze and Test WM: test session of the water maze).

with depressive symptoms characterized by an increased latency (idle time between spray and initiation of grooming) and decreased time spent grooming (d'Audiffret et al., 2010). The grooming behavior included nose/face grooming (along the snout), head washing (semicircular movements over the top of the head and behind the ears), and body grooming (body fur licking) (Kalueff and Tuohimaa, 2004). Latency and time spent grooming were recorded for 5 min.

2.6.3. Novel object test

The novel object test was performed in the same arena and test conditions employed for the open-field test, in order to evaluate the exploratory behavior of mice when exposed to an unknown object (50 mL Falcon tube stylized with colorful stripes, placed top down). In this experimental protocol, the novel object was placed in the center of the open-field (wooden box measuring $40 \times 60 \times 50\ cm^3$, with a central area measuring $19.5 \times 18.5\ cm^2$). The area that surrounds this central part is referred to as the peripheral area. The time spent exploring the novel object, as well as number of rearings in the central area of the apparatus, was recorded for 6 min (adapted from Zueger et al., 2005). The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors.

2.6.4. Novel cage test

To investigate the exploratory behavior of the animal in a novel environment, a circular blue plastic arena ($d=44$ cm, $h=22$ cm) with the floor divided into 9 parts was used. The animals were placed in the center of this apparatus in the beginning of the test. The number of crossings and rearings was registered for 6 min (adapted protocol from Zueger et al., 2005). After each test, the apparatus was sprayed with a solution of ethanol 10% and wiped thoroughly to clean and eliminate the residual odor.

2.6.5. Water maze test-memory reference task

The water maze task was performed in a circular swimming pool similar to that described by Morris et al. (1982). The pool was made of black painted fiberglass, 97 cm in diameter and 60 cm in height. For the tests, the tank was filled with water maintained at $23 \pm 2\ ^{o}C$. The target platform ($10 \times 10\ cm^2$) was made of transparent Plexiglas and it was submerged 1–1.5 cm beneath the surface of the water. Starting points for animals were marked on the outside of the pool as north (N), south (S), east (E) and west (W). Four distant visual cues ($55 \times 55\ cm^2$) were placed on the walls of the water maze room. They were all positioned with the lower edge 30 cm above the upper edge of the water tank and in the standard setting, the position of each symbol marked the midpoint of the perimeter of a quadrant (circle = NE quadrant, square = SE quadrant, cross = SW quadrant, and diamond = NW quadrant). The apparatus was located in a room with indirect incandescent illumination. A monitor and a video-recording system were installed in an adjacent room. The experiments were video-taped and the scores for latency of escape from the starting point to the platform during the training sessions and the time spent in the correct quadrant during the probe test session were later measured through the ANY-maze™ video tracking system (Stoelting Co., Wood Dale IL, USA).

Mice were submitted to a spatial reference memory version of the water maze using a protocol that was similar to one described previously (Prediger et al., 2007). The training session consisted of ten consecutive trials during which the animals were left in the tank facing the wall and then allowed to swim freely to the submerged platform. The platform was located in a constant position (middle of the southwest quadrant), equidistant from the center and the wall of the pool. If the animal did not find the platform during a period of 60 s, it was gently guided to it. The animal was allowed to remain on the platform for 10 s after escaping to it and was then removed from the tank for 20 s before being placed at the next starting point in the tank. This procedure was repeated ten times, with the starting points (the axis of one imaginary quadrant) varying in a pseudo-randomized manner. The test session was carried out 24 h later and consisted of a single probe trial where the platform was removed from the pool and each mouse was allowed to swim for 60 s in the maze. The time spent in the correct quadrant (i.e. where the platform was located on the training session) was recorded and the percentage of the total time was analyzed.

2.7. Biochemical analysis

Blood collection was performed by decapitation 6 h after the last behavioral test. Animals fasted for 8 h before blood collection in order to cause no interference in the analysis of serum glucose. The blood samples were collected and allowed to coagulate at room temperature for 30 min and were subsequently centrifuged at 3000*g* for 10 min. Serum was removed and stored at $-80\ ^{\circ}C$ until analysis.

For AChE determination, hippocampus and frontal cortex were homogenized in potassium phosphate buffer (0.1 M, pH 8). The homogenates were centrifuged at 2300*g* for 15 min and the supernatant was separated and stored at $-80\ ^{\circ}C$ until analysis.

2.7.1. Serum glucose determination

The serum glucose levels were measured by the method described by Barham and Trinder (1972), using the commercial kit Kovalent, by enzymatic colorimetric method Glucose GOD-PAP. The principle of this method is based on the determination of glucose after enzymatic oxidation by glucose oxidase. The colorimetric indicator is quinonimine, which is generated from 4-aminoantipipirina and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction).

2.7.2. Determination of AChE activity

AChE activity was measured by the method described by Ellman et al. (1961), using acetylthiocholine iodide as a substrate in homogenates of hippocampus and cerebral cortex. Each sample was assayed in triplicate. The rate of hydrolysis of acetylthiocholine iodide was measured at 412 nm through the release of the thiol compound, which reacts with DTNB producing the colored product thionitrobenzoic acid.

2.7.3. Protein determination

The protein content in hippocampal and frontal cortex homogenate samples was determined using the method of Bradford (1976), using bovine serum albumin as a standard.

2.8. Statistical analysis

Comparisons between the pre-operative and post-operative periods (SHAM X OB groups) and training session of water maze task were performed by repeated one-way-measures analysis of variance (ANOVA), one-way-ANOVA for test session of water maze task and two-way ANOVA for study of the post-treatment period (SHAM X OB-vehicle treatment and SHAM X OB-extract or fluoxetine treatment groups) followed by Duncan test when appropriate. All data are expressed as mean ± standard error of the mean (S.E.M.). Differences with $P<0.05$ were considered statistically significant.

3. Results

3.1. Phytochemical analysis and high-performance liquid chromatographic profile (HPLC)

As shown in Fig. 2 the major compounds identified in ROHE were carnosol, ursolic acid, oleanolic acid, betulinic acid and rosmarinic acid. Ursolic acid (15.71%) and carnosol (10.03%) are the compounds present at higher concentrations in ROHE. Although at lower concentrations, the terpenes betulinic acid (6.21%) and oleanolic acid (5.73%), as well as the phenolic acid, rosmarinic acid (5.99%) was also found in ROHE.

3.2. Effect of chronic treatment with ROHE on OB-induced locomotor and exploratory hyperactivity in the open-field test

The results depicted in Fig. 3A and B show that bulbectomized mice presented an increased number of crossing and rearings in the open-field test, as compared to control group (SHAM-vehicle), indicating that OB induced an enhancement of locomotor and exploratory activities. However, the bulbectomized mice submitted to chronic p.o. treatment with extract (10–300 mg/kg) and fluoxetine (10 mg/kg) demonstrated a significant decrease in locomotor activity and exploratory behavior as compared to the OB-vehicle. The two-way ANOVA revealed a significant main effect of OB [$F(1,91)=37.53$, $P<0.01$] and treatment X OB interaction [$F(4,91)=3.99$, $P<0.01$], but no significant effect of the treatment [$F(4,91)=1.08$, $P=0.37$] in locomotor activity in the open-field test. The two-way ANOVA also revealed a significant effect of OB [$F(1,91)=33.96$, $P<0.01$], treatment X OB interaction: [$F(4,91)=6.82$, $P<0.01$], but no significant effect of the treatment [$F(4,91)=1.36$, $P=0.25$] in exploratory activity in the open-field test.

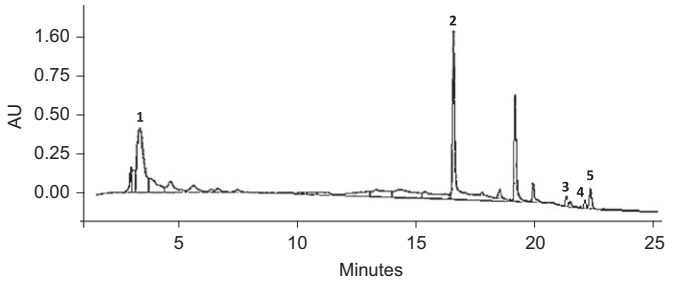

Fig. 2. Chromatographic profiles (HPLC) of crude extract from *Rosmarinus officinalis* L. Peaks represent rosmarinic acid (1), carnosol (2), betulinic acid (3), oleanolic acid (4), and ursolic acid (5).

3.3. Effect of chronic treatment with ROHE on OB-induced hyperactivity as assessed with the novel object and novel cage tests

Fig. 4 shows that OB caused a significant increased time spent exploring the novel object and an increased number of rearings in the central area of the apparatus as compared to the control group (SHAM-vehicle) (Fig. 4A and B, respectively). These results indicate an OB-induced hyperactivity in the novel object test. Furthermore, chronic treatment with ROHE (10–300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) in bulbectomized mice caused a reversal of the hyperactivity induced by novelty, since it decreased the time spent exploring the novel object and the number of rearings in the central area when compared with the OB-vehicle group (Fig. 4A and B). The two-way ANOVA revealed a significant effect of OB [$F(1,90)=11.73$, $P<0.01$] and treatment X OB interaction [$F(4,90)=3.06$, $P<0.05$], but not of treatment [$F(4,90)=0.90$, $P=0.46$] in the time exploring the novel object. Regarding the number of rearings around the object, a significant effect of OB [$F(1,90)=9.65$, $P<0.01$] and treatment X OB interaction [$F(4,90)=3.21$, $P<0.05$], but not of treatment [$F(4,90)=1.64$, $P=0.17$] was observed.

As shown also in Fig. 4C–D, bulbectomized mice showed locomotor and exploratory hyperactivity induced by the novel environment when compared to SHAM-vehicle group (147.5% and 187.0% of increase, respectively) in the novel cage test. However, the bulbectomized mice submitted to chronic treatment with ROHE (10–300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) demonstrated a significant decrease in locomotor activity and exploratory behavior as compared to the OB-vehicle. The two-way ANOVA revealed a significant effect of OB [$F(1,90)=26.61$, $P<0.01$], treatment X OB interaction:[$F(4,90)=4.60$, $P<0.01$], but no significant main effect of treatment [$F(4,90)=0.61$, $P=0.65$] in the locomotor activity in the novel cage test. The two-way ANOVA also revealed a significant main effect of OB [$F(1,90)=32.03$, $P<0.01$], treatment X OB interaction [$F(4,90)=3.71$, $P<0.01$], but no significant effect of treatment [$F(4,90)=1.14$, $P=0.343$] in the exploratory activity in the novel cage test.

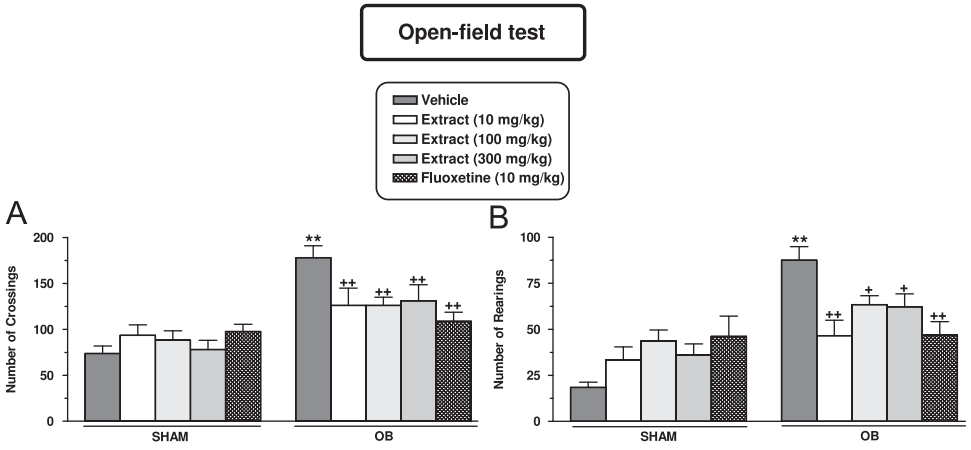

Fig. 3. Effect of the chronic treatment of mice (14 day) with ROHE (10–300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) on the number of crossings (panel A) and rearings (panel B) in bulbectomized mice in the open-field test. Each column represents the mean + S.E.M. of 9–11 animals. $^{**}P<0.01$ as compared to control group (SHAM-vehicle); $^{+}P<0.05$, $^{++}P<0.01$ when compared with OB-vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

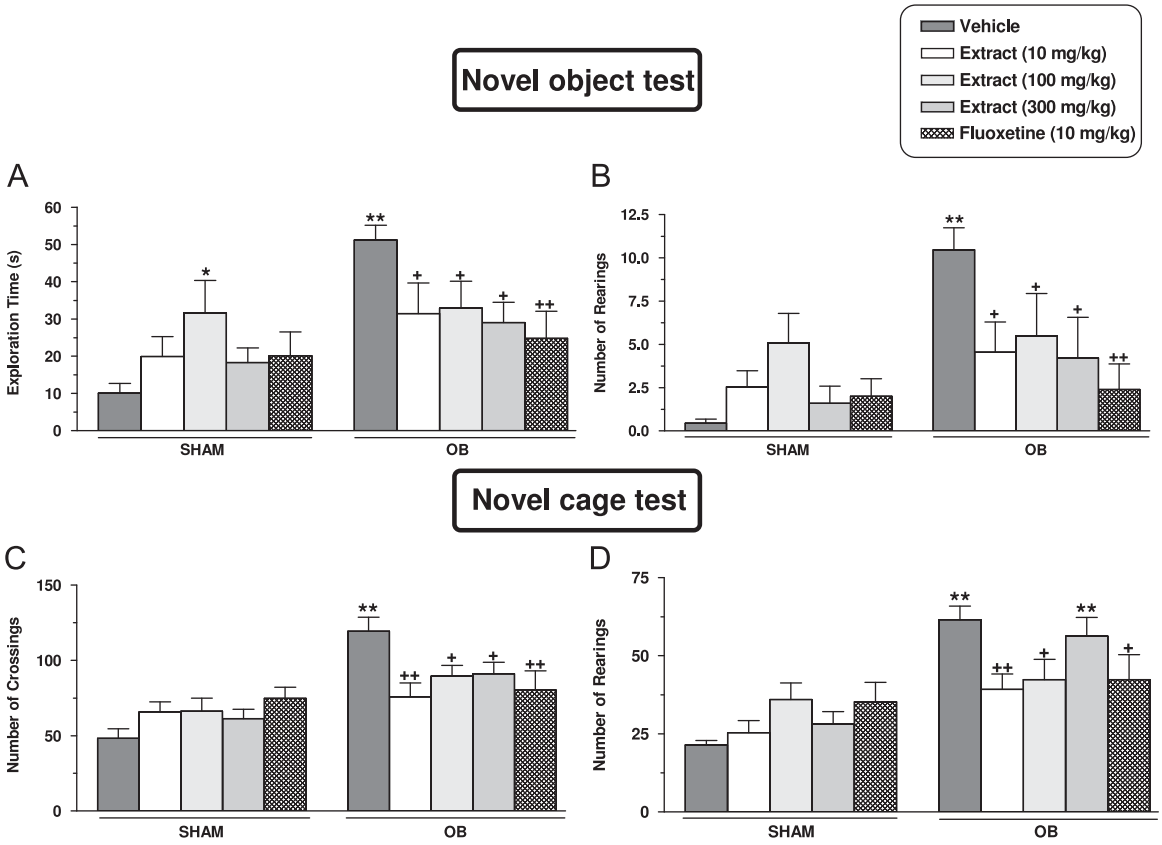

Fig. 4. Effect of chronic treatment of mice (14 day) with hydroalcoholic extract of *Rosmarinus officinalis* (10–300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.), on the exploratory activity of bulbectomized mice in the novel object test and novel cage test. The exploratory behavior was assessed monitoring the time spent exploring the novel object (panel A) and the number of rearing responses in the central area of the open-field (panel B) in the novel object test; and the number of crossings (panel C) and rearings (panel D) of bulbectomized mice in the novel cage test. Each column represents the mean + S.E.M. of 9–11 animals. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group (SHAM-vehicle) and $^{+}P < 0.05$, $^{++}P < 0.01$ when compared with OB-vehicle group. Results were analyzed by two-way ANOVA, followed by Duncan's multiple range post-hoc test.

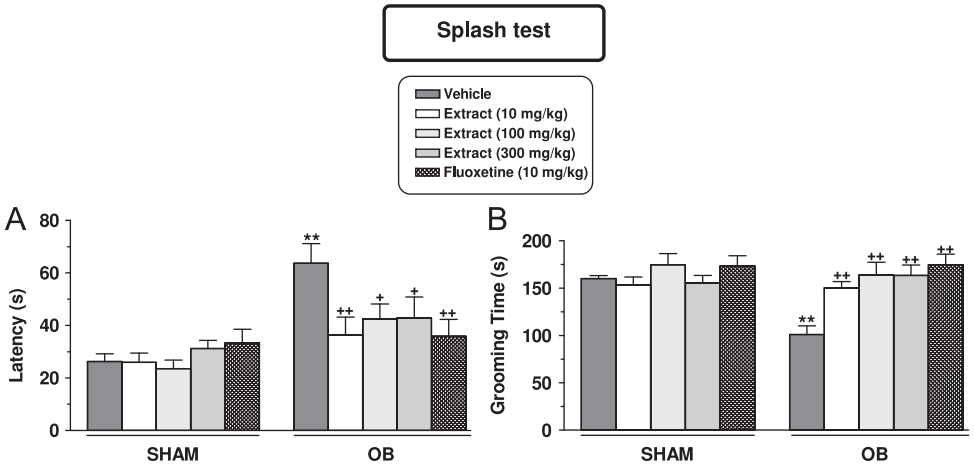

Fig. 5. Effect of chronic treatment mice (14 day) with ROHE (10–300 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.), in bulbectomized mice in the splash test. The anhedonic behavior of bulbectomized mice was analyzed through latency for initiation of grooming behavior (panel A) and time spent grooming (panel B). Each column represents the mean + S.E.M. (n = 9–11). $^{**}P < 0.01$ compared with the control group (SHAM-vehicle); $^{+}P < 0.05$, $^{++}P < 0.01$ when compared with OB-vehicle group. Results were analyzed by two-way-ANOVA, followed by Duncan's post-hoc test.

3.4. Effect of chronic treatment with ROHE on OB-induced anhedonic-like behavior assessed by the splash test

The effects of chronic treatment with ROHE in the anhedonic-like behavior induced by OB were inferred through the latency and time spent grooming in the splash test, as shown in Fig. 5A and B, respectively. The results show an increased latency (idle time between spray and initiation of grooming) and decreased time spent grooming (anhedonic-like behavior) in bulbectomized mice. Noteworthy, chronic p.o. treatment with extract (10–300 mg/kg) or fluoxetine (10 mg/kg) significantly reversed the increased latency (Fig. 5A) and the decreased time spent grooming (Fig. 5B) elicited by OB. These results indicated that ROHE and fluoxetine were able to abolish the anhedonic-like

behavior induced by OB. A two-way ANOVA revealed a significant main effect of OB [$F(1,94)=20.48$, $P<0.01$] and significant treatment X OB interaction [$F(4,94)=2.96$, $P<0.05$], but no significant main effect of treatment [$F(4,94)=2.00$, $P=0.10$] in the latency to exhibit grooming behavior. A two-way ANOVA also revealed a significant main effect of OB [$F(1,94)=4.23$, $P<0.05$] and treatment [$F(4,94)=6.64$, $P<0.01$] as well as treatment X OB interaction [$F(4,94)=4.10$, $P<0.01$] in the time spent grooming.

3.5. Effect of chronic treatment with ROHE in the cognitive performance of SHAM and bulbectomized mice in the water maze task

We tested the ability of SHAM-operated and bulbectomized mice to acquire (training session) and retrieve (test session) spatial information in the water maze paradigm as indicative of learning and memory functions. Firstly, to rule out a possible per se effect of ROHE tested in the spatial learning and memory of mice, additional groups of mice were evaluated in the water maze 33°days after surgery and after the repeated administration (14 days) of ROHE (10, 100 and 300 mg/kg, p.o.) or fluoxetine (10 mg/kg, p.o.). The results illustrated in Fig. 6A and B suggest

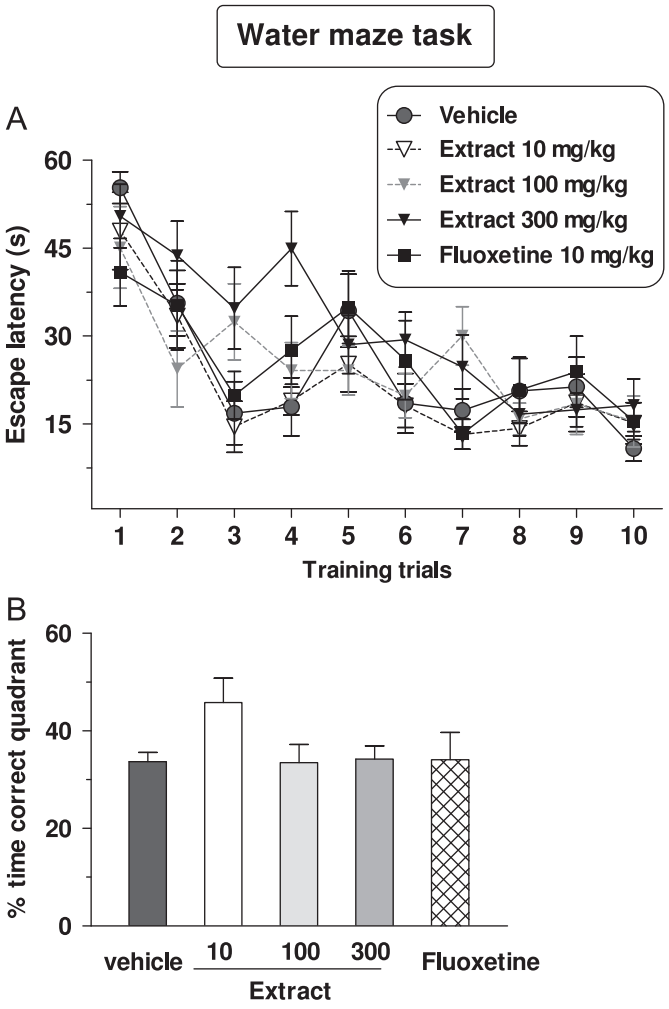

Fig. 6. Effects of chronic administration (14 day) with ROHE (10–300 mg/kg) or fluoxetine (10 mg/kg) on the spatial learning and memory of SHAM-operated mice evaluated in the water maze task (Panels 6A and 6B). Data are presented as means+S.E.M. latency, in seconds, for escape to a submerged platform (6A) ($n=9$–11 animals in each group) and % time in the correct quadrant (6B). The probe test session was performed 24 h after the training trials. Data are presented as means+S.E.M. of the time spent in the correct quadrant.

that the treatment with ROHE did not interfere, at least at the present doses, with the spatial learning and memory of the animals, since no alterations in the escape latency (training session) were observed. A one-way ANOVA with repeated measures revealed no significant effect of treatment in the escape latencies during the training trials [$F(4,45)=2.75$, $P=0.39$] (Fig. 6A). Moreover, a one-way ANOVA revealed no significant effect of treatment in time spent in the correct quadrant in test session [$F(4,45)=1.89$, $P=0.13$] (Fig. 6B).

As can be seen from Fig. 7A, OB resulted in a significant decline in spatial learning, as indicated by longer latencies to find the platform [$F(1,18)=8.33$, $P<0.05$]. Subsequent Duncan post-hoc tests indicated that SHAM/vehicle mice learned quicker than bulbectomized mice, since although both groups displayed similar final escape latencies to find the platform, the learning curve of bulbectomized mice was clearly offset to the right, i.e. they needed a greater number of trials to satisfactorily acquire the spatial information (Fig. 7A). The one-way ANOVA revealed no significant effect of OB in time spent in the correct quadrant (test session) [$F(1,18)=0.06$, $P=0.81$], as observed in the water maze (Fig. 7B).

Moreover, repeated administration (14 day) by p.o. route of ROHE (10, 100 and 300 mg/kg) or fluoxetine (10 mg/kg) did not promote any significant effect on the spatial learning [$F(4,48)=0.36$; $p=0.83$] and memory [$F(4,48)=0.38$; $p=0.81$] of bulbectomized mice (Fig. 7C and D).

3.6. Effect of chronic treatment with ROHE on the serum glucose level in bulbectomized animals

The results depicted in Fig. 8A show a decreased serum glucose level in bulbectomized mice as compared with the control group (SHAM-vehicle). This effect was abolished by p.o. treatment with ROHE (10 mg/kg), but not by fluoxetine (10 mg/kg). A two-way ANOVA indicated a significant effect of treatment X OB interaction [$F(2,46)=4.11$, $P<0.05$], but not of OB[$F(1,46)=1.57$, $P=0.21$] and treatment [$F(2,46)=0.20$, $P=0.81$].

3.7. Effect of chronic treatment with ROHE on cerebrocortical and hippocampal AChE activity in bulbectomized mice

Fig. 8 also shows the effect of chronic p.o. administration of ROHE (10 mg/kg) and fluoxetine (10 mg/kg) on the activity of the enzyme AChE in the cerebral cortex (Fig. 8B) and hippocampus (Fig. 8C) of bulbectomized animals. As demonstrated in Fig. 8B, the activity of AChE in the frontal cortex was not changed in the group of bulbectomized mice treated with ROHE as compared with the control (SHAM-vehicle) and with BO-vehicle groups. However, as shown in Fig. 8B, the activity of AChE in the frontal cortex was lower in the group of bulbectomized mice treated with fluoxetine (10 mg/kg, p.o.) as compared with the bulbectomized/vehicle group. A two-way ANOVA revealed a significant effect of treatment X OB interaction [$F(2,38)=3.49$, $P<0.05$], but not of OB [$F(1,38)=2.29$, $P=0.13$] and treatment [$F(2,38)=1.91$, $P=0.16$] in the activity of AChE in the frontal cortex. However, Fig. 8C shows a significant increase on hippocampal AChE activity in bulbectomized/vehicle group, as compared with control group (SHAM-vehicle), an effect reversed by ROHE (10 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.). A two-way ANOVA revealed a significant main effect of OB [$F(1,38)=4.28$, $P<0.05$], treatment [$F(2,38)=6.19$, $P<0.01$] and treatment X OB interaction [$F(2,38)=3.96$, $P<0.05$] on the activity of AChE in the hippocampus.

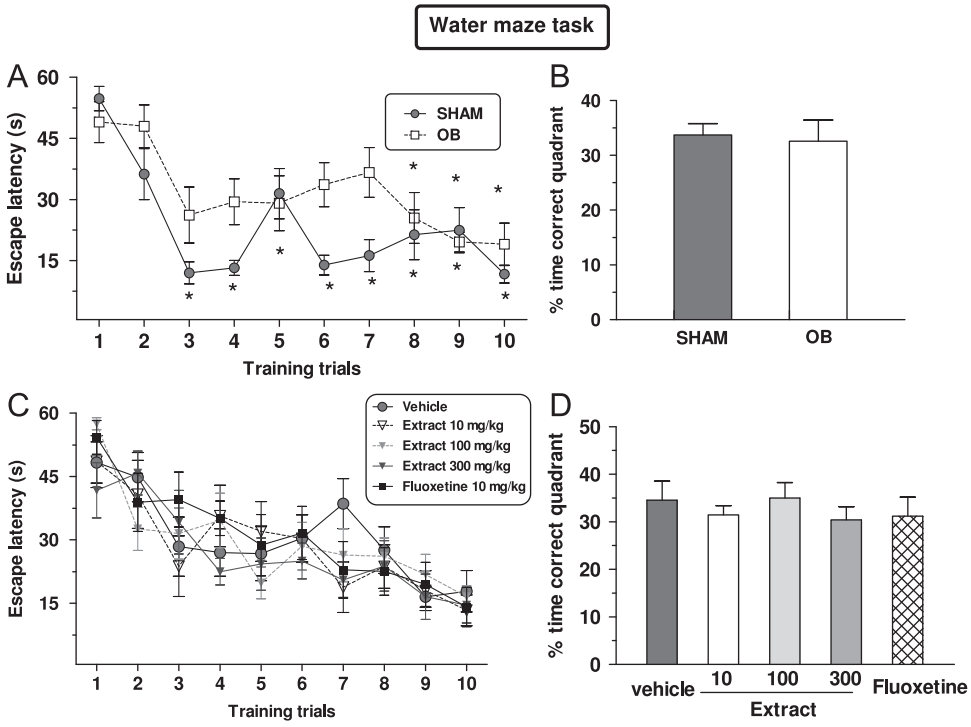

Fig. 7. Effects of chronic administration (14 day) with ROHE (10–300 mg/kg) or fluoxetine (10 mg/kg) on the spatial learning and memory of olfactory bulbectomized mice evaluated in the water maze task. Training trials were carried out on day 32 after OB. Data are presented as means+S.E.M. latency, in seconds, for escape to a submerged platform (A,C) (n=9–11 animals in each group). The probe test session was performed 24 h after the training trials. Data are presented as means+S.E.M. of the time spent in the correct quadrant (B,D). $^{*}P<0.05$ compared to the first trial of the same group (Duncan post-hoc test).

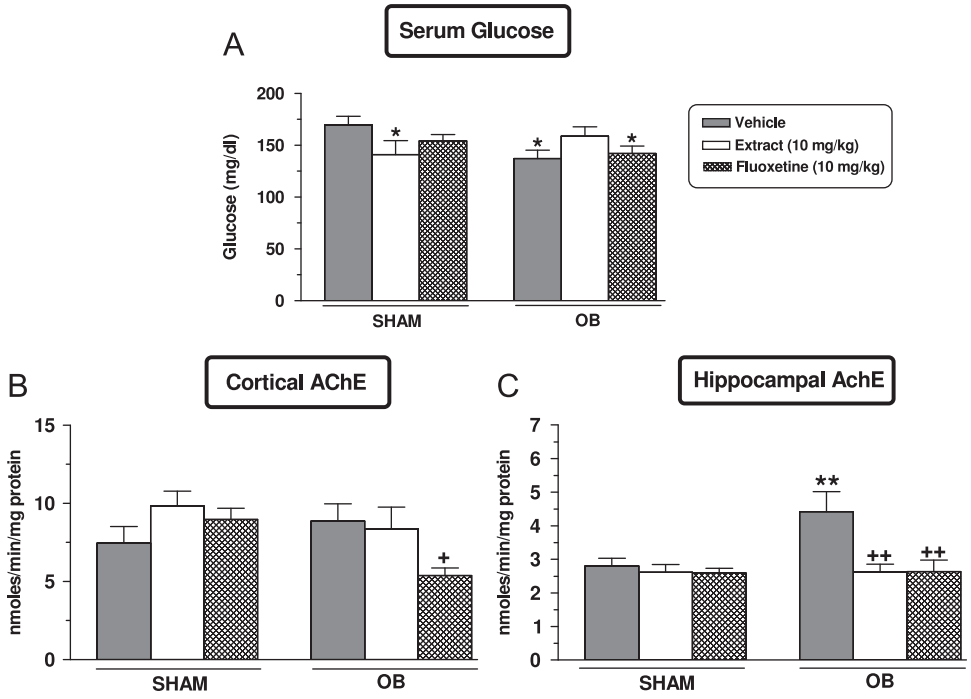

Fig. 8. Effect of the chronic treatment of mice (14 day) with ROHE (10 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) on serum glucose level (expressed as mg/dl) (panel A) and on AChE activity in frontal cortex (panel B) and hippocampus (panel C) of bulbectomized mice. Enzyme activity was expressed as nmol/min/mg protein. Each column represents the mean+S.E.M. of 7–9 animals. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group (SHAM-vehicle) and $^{+}P<0.05$, $^{++}P<0.01$ when compared with OB-vehicle group. Results were analyzed by two-way-ANOVA, followed by Duncan's post-hoc test.

4. Discussion

To gain a better understanding of the potential antidepressant of *Rosmarinus officinalis*, this study evaluated the effects of the extract of this plant in the OB model, since the removal of olfactory bulbs in rodents result in several behavioral, neurochemical and neuroendocrinological alterations, comparable to those seen in depressed patients (Kelly et al., 1997; Song and Leonard, 2005). Indeed, the antidepressant-like effect of ROHE was firstly investigated by our group in two behavioral models

predictive of antidepressant activity, the forced swimming test (FST) and tail suspension test (TST) in mice. ROHE produced an antidepressant-like effect, since the acute treatment of mice with the extract by p.o. route significantly reduced the immobility time in the FST (100 mg/kg) and TST (10–100 mg/kg). Moreover, the repeated administration (14 day) of the ROHE by p.o. route also produced an antidepressant-like effect in the TST (100–300 mg/kg). However, the behavioral tests with predictive validity are designated with this terminology because they are based exclusively on the behavioral effects of drugs used clinically, but do not mimic the symptoms of disease. In addition, another drawback of these tests is the fact that they are sensitive to the acute administration of antidepressants, but the action of classical antidepressants in the existing therapies can take up to several weeks to show their full therapeutic effect (Cryan et al., 2002, 2005). Interestingly, the OB model used in the present study has been suggested to possess a good face validity with human depressive disorder, especially agitated depression (Harkin et al., 2003; Kelly et al., 1997; Romeas et al., 2009).

The behavioral abnormalities induced by OB appear after 2 weeks in rodents; probably because the lesion caused by OB induces reorganization processes in the limbic and cortical areas (Jarosik et al., 2007; van Riezen and Leonard, 1990; Zueger et al., 2005). In the present study bulbectomized mice showed a significant increase in locomotor and exploratory activities in the open-field, novel object and novel cage tests and also an anhedonic-like behavior. These behavioral alterations are indicative of a depressive-like profile of these animals (Harkin et al., 2003; Kelly et al., 1997; Zueger et al., 2005). Noteworthy, ROHE, similarly to the antidepressant fluoxetine, used here as a positive control, was able to abolish these behavioral alterations.

The hyperactivity in bulbectomized animals is the main alteration reported in literature. Various classes of clinically active antidepressants abolish the hyperactivity of bulbectomized rodents in the open-field, such as: serotonin reuptake inhibitors-SSRIs (citalopram, fluoxetine, paroxetine, sertraline, venlafaxine), noradrenaline reuptake inhibitor—NRI (reboxetine), tricyclic antidepressant (amitriptyline, desipramine, imipramine) (Butler and Leonard, 1990; Connor et al., 2000; Jarosik et al., 2007; Kelly et al., 1997; Possidente et al., 1996; Rodríguez-Gaztelumendi et al., 2009; Song and Leonard, 2005). Additionally, it was reported that curcumin, the active component of *Curcuma longa*, was able to reverse hyperactivity induced by OB in rats (Xu et al., 2005).

Furthermore, another relevant behavioral change triggered by the OB is the increased vulnerability and responsiveness to environmental stress (Mar et al., 2000; van Riezen and Leonard, 1990). In our investigation, the bulbectomized mice showed locomotor and exploratory hyperactivity induced by novelty, indicated by an increase in behavioral reactivity when mice were submitted to the novel object test (decreased latency for entering the central arena, increased time spent exploring and increased rearing responses in the central area where the novel object was located) and to the novel cage test (increased number of crossings and rearings in the new environment). These results are in agreement with literature data that report that the hyperactivity induced by OB is directly related to a greater reactivity to novel environments or deficit in habituation to new situations (Mar et al., 2000; van Riezen and Leonard, 1990; Zueger et al., 2005). The chronic treatments with ROHE (10–300 mg/kg, p.o.) and fluoxetine (10 mg/kg) were able to reverse all the OB-induced behavioral alterations in the novel cage test. These results are in accordance with the fact that antidepressants such as fluoxetine and amitriptyline are effective to restore normal responding by permitting more effective adaptation to novel stimuli in the bulbectomized rodents (Mar et al., 2000, 2002), reinforcing the notion that ROHE has an antidepressant -like action.

Anhedonia, or hyposensitivity to pleasure, is one of the key symptoms for a diagnosis of depression (WHO, 1992). In the present study this behavior was inferred by the decreased grooming time and increased latency to grooming in the splash test in bulbectomized mice as compared to those exhibited by control mice (SHAM). This result is in accordance with some studies that have shown an anhedonic-like behavior in bulbectomized rats (Romeas et al., 2009; Stock et al., 2000). Noteworthy, in our study, the anhedonic-like behavior induced by OB was abolished by ROHE and fluoxetine. This result is in line with the ability the compounds with antidepressant properties, such as the classical antidepressants fluoxetine, imipramine and desipramine (David et al., 2009; Detanico et al., 2009;Yalcin et al., 2005) as well as *Ptychopetalum olacoides* Bentham (marapuama) extract (Piato et al., 2008), which were capable of reversing anhedonic behavior induced by stress models of depression. Moreover, this result also reinforces the notion the ROHE has antidepressant properties.

In accordance with previous studies reporting a reduced performance of OB rodents in different paradigms used to investigate learning and memory processes (Harkin et al., 2003; Kelly et al., 1997; Mucignat-Caretta et al., 2006) our results show a poor performance of OB compared to SHAM-mice in the spatial version of the Morris water maze. Interestingly, it was shown in our study that OB mice needed significantly more trials in the training session to acquire the spatial information, but they displayed a similar profile to that of SHAM-groups in the test session (24 h later), demonstrating a selective deficit in spatial learning in water maze task. Important to note, this results is opposed to some reports that have demonstrated impairment of OB in both spatial learning and memory in the water maze. This discrepancy with early data may be explained by differences between the protocols utilized to evaluate the spatial learning and memory in the water maze. In these previous studies, each mice was given 4 trials per day for 4–5 consecutive days to find the platform (Mucignat-Caretta et al., 2006), while in the current study each mice was given 10 consecutive trials during the training session (only 1 day) and the test session occurred 24 h later, similar to study reported by Prediger et al. (2005). Thus, it is possible that a training schedule with a higher number of consecutive trials instead of repeatedly training over a number of days promotes equivalence in the learning performance for both strains, which can be observed in the similar pattern of the escape latencies of the latter training trials.

The Morris water maze is a test of hippocampal function (Morris et al., 1982) that does not depend upon olfactory cues, but more on visual cues. A study reported by Van Rijzingen et al. (1995) showed that two weeks after OB, the Morris maze performance was severely impaired. However this alteration is a transient cognitive deficit since the recovery occurs spontaneously approximately 6 weeks following surgery. Thus, the Morris maze performance of OB animals and SHAM- controls 6 weeks after surgery did not show a difference in escape latency neither during acquisition nor during the probe trial.

In the present study the chronic treatment with ROHE (10–300 mg/kg, p.o.) or fluoxetine did not abolish the deficit in spatial learning induced by OB. This model of depression induced an impairment of learning and memory in the three-panel runway task in rats (Yamamoto et al., 1997) and in passive avoidance task in mice on the 7th and 14th day after the surgery (Hozumi et al., 2003). The impairment of learning and memory induced by OB in these tests, on the 14th day was improved by administration of the cholinesterase inhibitor physostigmine. Additionally, the ability of several compounds, including curcumin (Xu et al., 2005), nobiletin (Nakajima et al., 2007) and an active ginseng metabolite (20(S)-protopanaxadiol) (Xu et al., 2010) to reverse

cognitive deficit in olfactory bulbectomized rodents in the step down was reported. However, there are few data dealing with the effects of *Rosmarinus officinalis* on cognitive performance, but it has a history of usage as cognitive enhancer (Kennedy and Scholey, 2006). A study by Hosseinzadeh et al. (2004) reported that the essential oil of *Rosmarinus officinalis* injected intraperitoneally to rats 0.5 h before training for 5 consecutive days improved the intact memory and scopolamine-induced learning deficits in rats performing the Morris water maze task. A recent study in an elderly population has reported that this plant when administered at a low dose caused an improvement on cognitive function but at a high dose caused an impairing effect (Pengelly et al., 2012). Moreover, in one study of 144 healthy individuals, airborne *Rosmarinus officinalis* essential oil significantly enhanced cognitive performance and mood (Moss et al., 2003). These studies reported that the *Rosmarinus officinalis* is a promising candidate for the improvement of memory in healthy people or for the treatment diseases associated with cognitive deficit, an effect that may be due to, at least in part to its anticholinesterase properties (Duke, 2007; Ingole et al., 2008; Kennedy and Scholey, 2006; Singh et al., 2011). Regarding the present study, it remains to be established if a more prolonged treatment with ROHE, as well as fluoxetine, would be able to abolish the OB-induced cognitive deficit.

In a second experimental phase of this study, we evaluated some biochemical parameters that could be changed by OB procedure, as serum glucose level, and activity of the enzyme AChE in the hippocampus and frontal cortex. The ability of the ROHE and fluoxetine (positive control) to blunt some of the alterations induced by OB on these parameters was also evaluated.

This study showed a decrease in the serum glucose level in bulbectomized animals, as compared with the control-SHAM, similarly to results previously reported in the literature (Belló and Rummler, 1980; Perassi et al., 1975). Moreover, a recent study showed that acute hypoglycemia causes depressive-like behaviors (increased immobility in the forced swim test and reduced saccharin preference, an indicative of anhedonic-like behavior) in mice which were prevented by the antidepressants fluoxetine and desipramine (Park et al., 2012). However, in present study the chronic treatment with fluoxetine was not able to alter the reduction of serum glucose level induced by OB. Interestingly, treatment with ROHE decreased serum glucose level in SHAM mice, but abolished the decrease on glucose levels induced by OB, since it was able to restore serum glucose level to normal. Thus, the effects of ROHE and fluoxetine on serum glucose level in SHAM and OB mice are quite different. Indeed literature data have reported that *Rosmarinus officinalis* exerts notable hypoglycemic or anti-hyperglycemic activity (Abu-Al-Basal, 2010; Bakirel et al., 2008). Although interesting the effects of ROHE and fluoxetine on serum glucose level do not seem to be associated with the behavioral alterations described in our study.

Taking into account the well known implication of the cholinergic system with the behavioral alterations elicited by OB (Moriguchi et al., 2006; Nakajima et al., 2007) and in the pathophysiology of depression (Dagytė et al. 2011), the present study also dealt with the determination of AChE activity in the hippocampus and frontal cortex. AChE is an important constituent of cholinergic neurotransmission that catalyzes the hydrolysis of acetylcholine in the synaptic cleft and neuromuscular junctions (Soreq and Seidman, 2001). Interestingly, in the present study an increase on AChE activity in the hippocampus, but not in the frontal cortex, in the bulbectomized mice was reversed by chronic treatment with ROHE (10 mg/kg) and fluoxetine (10 mg/kg). In line with this, the in vitro anticholinesterase effect of *Rosmarinus officinalis* was reported (Adsersen et al., 2006). Moreover, treatment with the antidepressant fluoxetine decreased the activity of AChE in human serum and erythrocyte membrane (Müller et al., 2002). Our results suggest that an increased hippocampal activity of this enzyme may be associated with the depressive-like behavior observed in the bulbectomized mice. Regarding the ability of ROHE and fluoxetine to reduce the activity of AchE, consequently increasing acethylcholine levels, we may raise the hypothesis that this could lead to a desensitization of nicotinic acethylcholine receptors. Indeed, it has been proposed that a fine balance between the activation and desensitization of nicotinic receptors is required to yield relevant antidepressant-like effects (Mineur and Picciotto, 2010). However, in our study an absence of alteration of AChE in cerebral cortex was observed in olfactory bulbectomized mice, a result that is similar to a finding reported by Yamada et al. (2011) in bulbectomized mice.

5. Conclusion

The present study shows that OB mice exhibited hyperactivity and anhedonic-like behavior associated with an increased hippocampal AChE activity, parameters that were abolished by chronic treatment with ROHE, similar to the effects produced by fluoxetine. These results suggest that *Rosmarinus officinalis* may be further investigated as an effective therapeutic alternative for the treatment of agitated depression associated with anhedonia.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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