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RESEARCH ARTICLE

Dose–response effect of black maca (*Lepidium meyenii*) in mice with memory impairment induced by ethanol

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Abstract

Previous studies have shown that black variety of maca has beneficial effects on learning and memory in experimental animal models. The present study aimed to determine whether the hydroalcoholic extract of black maca (BM) showed a dose–response effect in mice treated with ethanol 20% (EtOH) as a model of memory impairment. Mice were divided in the following groups: control, EtOH, ascorbic acid (AA) and 0.125, 0.25, 0.50 and 1.00 g/kg of BM plus EtOH. All treatments were orally administered for 28 days. Open field test was performed to determine locomotor activity and water Morris maze was done to determine spatial memory. Also, total polyphenol content in the hydroalcoholic extract of BM was determined (0.65 g pyrogallol/100 g). Mice treated with EtOH took more time to find the hidden platform than control during escape acquisition trials; meanwhile, AA and BM reversed the effect of EtOH. In addition, AA and BM ameliorated the deleterious effect of EtOH during the probe trial. Correlation analyses showed that the effect of BM a dose-dependent behavior. Finally, BM improved experimental memory impairment induced by ethanol in a dose–response manner due, in part, to its content of polyphenolic compounds.

Keywords: Black maca, ethanol, memory and learning, total polyphenols

Introduction

Maca (*Lepidium meyenii* Walp.) grows over 4000 m altitude in the Central Peruvian Andes, particularly in Junin plateau. According to the color of its hypocotyls, ~13 varieties of maca have been described ranging from white to black (Tello, 1992). Also, different biological properties have been observed among varieties of maca (Gonzales et al., 2005; Gonzales et al., 2006; Rubio et al., 2006). From these, black maca (BM) presented the greatest effect on cognitive function (Rubio et al., 2006) in different animal models of memory impairment (Rubio et al., 2007, 2008). In fact, it have been demonstrated by others that maca shows neuroprotective activity (Pino-Figueroa et al., 2010).

One related explanation to the beneficial effect of maca was related to its antioxidant properties *in vitro* and *in vivo* (Sandoval et al., 2002; Lee et al., 2005). For instance, BM was able to reduced malonaldehyde brain levels, a marker related to lipid peroxidation, in ovariectomized mice (Rubio et al., 2008) supporting the fact that maca show the capacity to reduce oxidative stress as suggested previously (Rubio et al., 2008; Pino-Figueroa et al., 2010).

Ethanol administration has been used previously as a model to induce cognitive impairment in experimental animal models in order to describe different mechanisms related to memory processes (de Oliveira and Nakamura-Palacios, 2003; Miller and Mooney, 2004; Izumi et al., 2005; Mameli et al., 2005; Self et al., 2005) and to evaluate the potential beneficial effects of different compounds and drugs (Singh et al., 2003; Bao et al., 2005; Baydas et al., 2005; Khalil et al., 2005; Pinto et al., 2006). In fact, ethanol is a drug that is rapidly absorbed and produces effects only in specific brain regions, including the hippocampus (Matthwes and Silvers, 2004). The neurotoxic effects of ethanol administration are mainly related to its capacity to induce oxidative stress (Pinto et al., 2006).

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To our knowledge, there is no scientific study that evaluates the effect of BM on memory in a dose–response manner. For this reason, the present study aims to determine if the hydroalcoholic extract of BM has a dose–response effect in mice with memory impairment induced by ethanol administration, as a model of memory impairment related to oxidative stress. Ascorbic acid (AA) was used as a positive control (Kumar et al., 2009) to compare the effect of BM. In addition, the total polyphenolic content in BM hypocotyls will be assessed.

Materials and methods

Animals

Three-month-old male mice from the Swiss strain obtained from the animal house of the Universidad Peruana Cayetano Heredia were used for the study. Mice were housed five per cage and maintained at room temperature (22°C) with a 12:12 h light/dark cycle in the animal house at the Universidad Peruana Cayetano Heredia. Mice were fed Purina laboratory chow (Agribrands Purina Peru S.A., Lima, Peru) and tap water *ad libitum*. Purina is a standard laboratory food containing protein 18%, carbohydrates 50%, fat 3.5%, fibre 6%, calcium 0.8%, phosphorus 0.8%, vitamins (A, D, B12, K, E, riboflavine, niacin, panthotenic acid, choline chloride, piridoxine, thiamine, biotin, folic acid) and minerals (copper, Manganese, zinc, iodine and selenium).

Experimental design

For the present study, 66 male mice (initial body weight: 31.88 ± 0.57 g) were randomized divided in seven groups (10 or 9 per group) according to treatment: 1) mice treated with vehicle (Control group); 2) mice treated with vehicle and a 20% ethanol solution (EtOH); and mice that received a 20% ethanol solution plus 3) AA (250 mg/kg) (Kumar et al., 2009); 4) 0.125 g/kg (EtOH + BM 0.125); 5) 0.25 g/kg (EtOH + BM 0.25); 6) 0.50 g/kg (EtOH + BM 0.50), and 7) 1.00 g/kg (EtOH + BM 1.0) of a hydroalcoholic extract of BM.

Vehicle (distilled water), EtOH, AA and BM were orally administered for 28 days using an intubation needle No 18 (Fisher Scientific, Pittsburgh, Pennsylvania). EtOH concentration and administration pathway used in this study were chosen from a preliminary dose–response study performed previously by the researchers where three different concentrations (10, 15 and 20% of ethanol solution dissolved in distilled water) of ethanol were studied (data not shown). Also, previous studies demonstrated that a 20% ethanol solution produced negative effects on memory and learning tasks (Lukoyanov et al., 2003; Assunção et al., 2007). Ethanol was administered to rats, from groups 2 to 7, 1 h before the open field (day 22) test and water Morris maze (from day 23 to 28).

All animal procedures were conducted in compliance with "Guide of the care and use of laboratory animals" (National Research Council, 1996). The Institutional Review Board of the Scientific Research Office from the Universidad Peruana Cayetano Heredia approved the study (SIDISI-UPCH: 52763, 2007).

Plant material

The dried hypocotyls of BM were obtained in 2007 from Carhuamayo, Junin at 4000 m altitude in the Central Peruvian Andes where it is traditionally cultivated (Valerio and Gonzales, 2005). Irma Fernandez, a Botanist of the Department of Pharmaceutical Sciences, Universidad Peruana Cayetano Heredia, authenticated the identity of the plant. The voucher (IFV 1885) was deposited at the Department.

Preparation of hydroalcoholic extract of BM

Hydroalcoholic extract of BM was prepared with aqueous ethanol (60%, v/v) by percolation at room temperature for 24 h and concentrate at low pressure to constant weight. The extract was prepared by Eng. Alfonso Higa from Agroindustrial Chanchamayo (Lima, Peru). One gram of dried BM hypocotyls produced 0.22 g of hydroalcoholic of BM. This extract was further diluted in distilled water to obtain different concentrations in 1 ml. Solutions were placed in vials and kept in a refrigerator at 4°C until use.

Determination of polyphenol content

Total polyphenol content was assessed according to Folin-Ciocalteu described previously by Kähkönen et al. (1999). The analysis was made by triplicate. Briefly, 1.5 ml of Folin-Ciocalteu reagent (1:10 v/v) and 1.2 ml of 7.5% sodium carbonate solution were mixed with 300 µl of the extract and kept in a dark room for 30 min at room temperature. Pyrogallol (50 µg/ml) was used as a standard. The absorbance of the sample was measured at 760 nm. The results are expressed as g pyrigallol/100 g of hydroalcoholic extract.

Open field test

The open field test was performed to evaluate the influence of treatments on locomotor activity. The apparatus consisted in a square with 90 × 90 cm white floor, which was divided into 81 equal squares (10 × 10 cm) by black lines, and surrounded by white walls, 10 cm high. On day 22, mice were placed in the middle of the arena and the number of crossings with four paws (from one square to another) was recorded for 5 minutes as a measure for locomotor activity.

Water Morris maze

This task was adapted for mice from the paradigm originally described by Morris (1984). The water maze was a circular pool (65 cm in diameter, 25 cm high), filled with water (26 ± 1°C) and made opaque with black ink, to the depth of 20 cm. The pool was divided into four quadrants. An escape platform (6 cm in diameter, 19 cm high) was placed in the middle of one quadrant, 1.0 cm below the water surface, equidistant from the sidewall

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and middle of the pool. The platform providing the only escape from the water was located in the same quadrant on every trial. Three different starting points for mice were placed around the perimeter of the pool. On each of the four training days, all three start points were used once each in a pseudo-random sequence so the starting point was different every session. The water maze was always located in a large room with a number of extra-maze visual cues including (lights, desks, personal computer and video equipment, etc.). The experimenter was always sat at the same position. All experiments were carried out between 10:00 h and 16:00 h.

Escape acquisition

A trial began by placing the animal in the water facing the wall of the pool at one of the starting points. If the animal failed to escape on the platform within 120 s it was gently placed there by the experimenter and allowed to stay for 15 s. The inter-trial interval was 5–10 min. Three escape trails were given to all mice per day for five consecutive days (days 23–27 of each treatment). The escape latency was recorded during these trials.

Spatial memory test

Twenty-four hours after the last training trial (day 28) in the escape acquisition test, mice were submitted to the probe trial in which the platform was removed. In the 60-s probe trial, the time in the target quadrant (s) was obtained as a measure for spatial memory.

Escape latency is defined as the time (s) that mice required to reach the hidden platform during the escape acquisition sessions; meanwhile, the target quadrant is referred to the quadrant in which the platform was located during the escape acquisition sessions.

Statistical analyses

Data were analyzed using the statistical package STATA (version 8.0) for personal computers (Stata Corporation, 702 University Drive East, College Station, TX). Homogeneity of variances was assessed using a Bartlett test. If variances were homogeneous, differences between groups and treatment were assessed by one-way or two-way analysis of variance (ANOVA). If the p value, in the ANOVA test, was significant, the differences between pair of means were assessed by the Scheffe's test. Body weight and the time in the target quadrant were analyzed using one-way ANOVA; meanwhile, escape latency during the water Morris maze was analyzed using two-way ANOVA. Data are presented as mean ± standard error of the mean (SEM). When variances were not homogeneous, differences between groups were assessed using Mann–Whitney U nonparametric test. Data are presented as Median and interquartile range. Data from the open field test was analyzed using this nonparametric test. Data are presented as Median and interquartile range. In general, a value of $p < 0.05$ was considered to be statistically significant.

Results

Total polyphenols content in the hydroalcoholic extract of BM

The total polyphenols content found in the hydroalcoholic extract of BM was 0.65 g pyrogallol/100 g. Thus, the doses of polyphenols were 3.13, 6.25, 12.5 and 25 mg pyrogallol/kg body weight for 0.125, 0.25, 0.50 and 1.00 g hydroalcoholic extract of BM/kg body weight, respectively.

Body weight

At the beginning, no differences between groups were observed regarding to body weight (Control: 31.90 ± 1.64 g; EtOH: 31.50 ± 1.99 g; AA: 31.56 ± 0.85 g; EtOH + BM 0.125: 31.11 ± 0.87 g; EtOH + BM 0.25: 32.78 ± 1.82 g; EtOH + BM 0.50: 30.78 ± 0.81 g; EtOH + BM 1.00: 33.10 ± 0.89 g; $p > 0.05$). Also, there were no statistical differences at end of the study between control (33.90 ± 1.377 g), EtOH (30.30 ± 1.21 g), AA (32.33 ± 0.88 g) and BM-treated mice plus EtOH (0.125 g/kg: 32.33 ± 0.94 g; 0.25 g/kg: 33.78 ± 1.53; 0.50 g/kg: 31.44 ± 0.84; and, 1.00 g/kg: 34.10 ± 0.89; $p > 0.05$).

Effect of hydroalcoholic extract of BM on locomotor activity during the open field test

No statistical differences between control and EtOH groups were found (194.5 [160.3–235.5] vs. 235.5 [202.5–270.0]; $p = 0.615$) regarding to the number of crossings. In addition, there were no differences between control, EtOH, AA (201.0 [181.0–239.0]; $p > 0.05$) and 0.125 (253.0 [188.5–273.0]; $p > 0.05$), 0.25 (210.0 [191.0–253.0]; $p > 0.05$), 0.50 (227.0 [211.0–266.5]; $p > 0.05$) and 1.00 (215.5 [164.8–271.3]; $p > 0.05$) g/kg of BM. No differences between BM-treated groups were observed.

Effect of hydroalcoholic extract of BM on escape acquisition and spatial memory

Figure 1 shows the escape latency of mice with memory impairment induced by ethanol and treated with 0.125, 0.25, 0.50 and 1.00 g/kg of hydroalcoholic extract of BM. Two-way ANOVA analyses revealed an effect of the number of days ($F_{4,295} = 193.29$, $p < 0.001$) and groups ($F_{6,295} = 33.23$, $p < 0.001$). In addition, an effect of the interaction days x groups was observed ($F_{24,295} = 2.17$, $p < 0.01$). No differences between groups were observed in the first day of the escape acquisition trial. From day 2 to day 5, control group showed a better performance than ethanol-treated mice ($p < 0.01$). In addition, mice treated with 0.125, 0.25, 0.50 and 1.00 g/kg of BM reach shorter escape latencies than ethanol group in days 2 ($p < 0.05$), 3 ($p < 0.001$), 4 ($p < 0.001$) and 5 ($p < 0.001$). There were no differences in mice treated with any dose of BM ($p > 0.05$) or control group ($p > 0.05$) regarding to escape latency.

Figure 2 shows the time in the target quadrant during the probe trial of the water Morris maze. Ethanol resulted in a reduction in the time spent by the mice in the target quadrant when compared to control group ($p < 0.001$). Mice treated with ethanol also spent a significantly shorter time than those mice treated with AA ($p < 0.001$) and BM at 0.125 ($p < 0.05$), 0.25 ($p < 0.01$), 0.50 ($p < 0.001$) and 1.00 g/kg ($p < 0.001$). In addition, mice treated with 0.125 g/kg showed lower values than controls and those treated with AA ($p < 0.01$) and 1.00 g/kg of BM ($p < 0.01$). No differences between control and with 0.25, 0.50 and 1.00 g/kg BM groups were observed ($p > 0.05$).

No correlation between the number of crossings in the open field test and time in the target quadrant ($r = 0.29$,

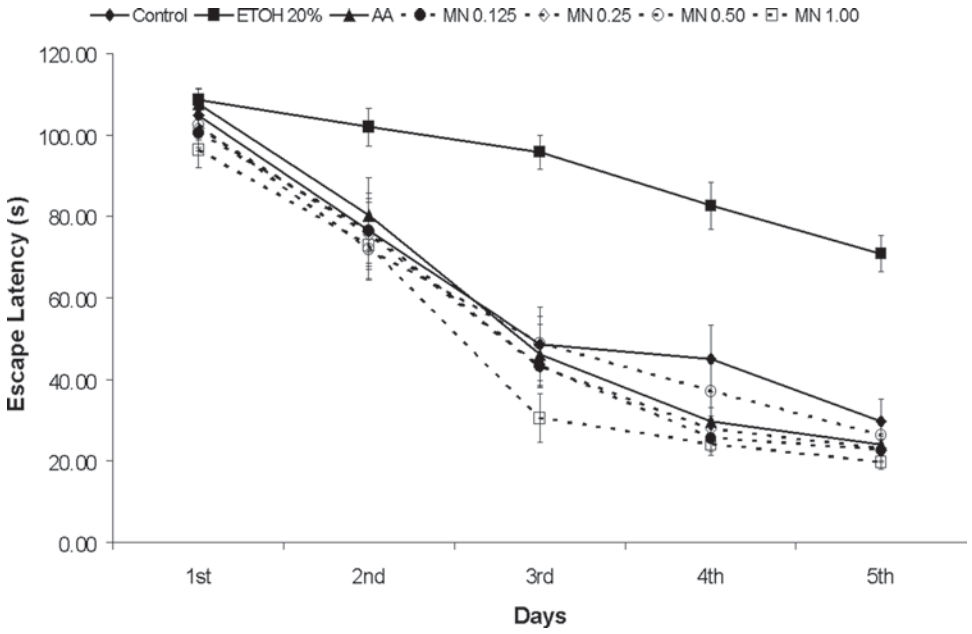

Figure 1. Effect of black maca on escape latency (s) in mice with ethanol-induced memory impairment in the water Morris maze. Male mice received vehicle (Control), ethanol (EtOH), ascorbic acid (AA), and 0.125, 0.25, 0.50 and 1.00 g/kg of BM plus EtOH (BM 0.125, BM 0.25, BM 0.50, BM 1.00, respectively). All treatments were orally administered for 28 days. Escape acquisition trials were performed from days 23 to 27. Mice were submitted to three trials for 5 consecutive days. Ethanol was administered 1 h before each acquisition session. Data are mean values ± SEM.

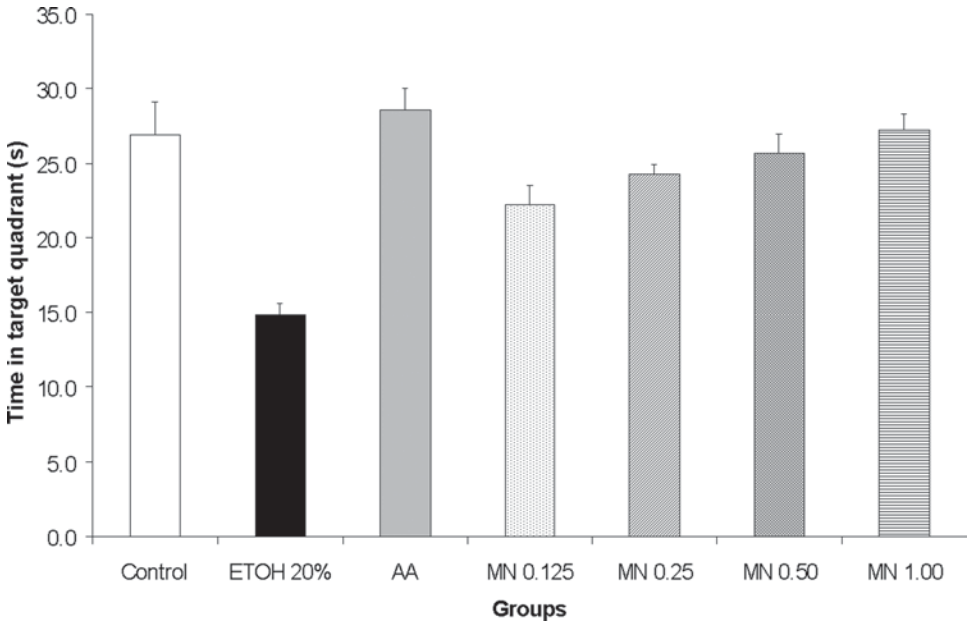

Figure 2. Effect of black maca on the time in the target quadrant during the probe trial in the water Morris maze. Groups: vehicle (Control), ethanol (EtOH), ascorbic acid (AA) and 0.125, 0.25, 0.50 and 1.00 g/kg of BM plus EtOH (BM 0.125, BM 0.25, BM 0.50, BM 1.00, respectively). All treatments were orally administered for 28 days. Probe trials were performed on day 28. Ethanol was administered 1 h before the probe trial. Data are mean values ± SEM. *$p < 005$ vs. Control group, [a]$p < 005$ vs. EtOH group, [b]$p < 005$ vs. AA group and [c]$p < 005$ vs. BM 0.125 group.

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$p = 0.137$) and escape latency during the last day of the escape acquisition session ($r = -0.04$, $p = 0.854$) were found. Moreover, there was a positive correlation between treatment and the time spent by the mice in the target quadrant during the probe trial ($r = 0.42$, $p < 0.01$) and negative correlation between treatment and the escape latency at the 5th day of the escape acquisition session ($r = -0.43$, $p < 0.01$) suggesting a dose–response effect of BM on spatial memory.

Discussion

It is known that ethanol is able to alter cognitive and behavioral performance in both humans and laboratory animals (Gönenç et al., 2005). In fact, one of the principal cognitive effects of ethanol is disruption of learning and memory (Gönenç et al., 2005) by inducing oxidative stress in brain (Pinto et al., 2006) due its capacity to cross cell membranes, including the blood-brain barrier (Mansouri et al., 2001). Ethanol preferentially impairs hippocampal-dependent learning and memory tasks (Acheson et al., 2001). In fact, both ethanol and hippocampal lesions impair water maze performance on spatial learning and memory tasks (Matthews et al., 1999). Furthermore, ethanol administration produces lipid peroxidation, which is an indicator of oxidative stress, in the brain (Mansouri et al., 2001; Assunção et al., 2007). The outcomes of this study support the fact that ethanol consumption can cause memory impairment during the water Morris maze by increasing escape latency and reducing the time in the target quadrant during the acquisition trials and probe trial, respectively. The results observed during the water maze in ethanol-treated mice may be due to its oxidative capacity in brain as mentioned above.

All doses of hydroalcoholic extract of BM showed inhibitory effects against ethanol-induced memory impairment during the escape acquisition trials; that is, mice treated with BM showed shorter escape latency than mice treated with ethanol. In addition, BM-treated mice showed a similar behavior than those treated with AA in the escape acquisition sessions. During the probe trial, AA- and BM-treated mice showed an increase in the time spent in the target quadrant in a dose–response manner. It is important to notice that the water Morris maze investigated spatial learning and memory (D'Hooge and De Deyn, 2001) and it is especially sensitive to impaired hippocampal function (Gage and Bjorklund, 1986). The latter suggests that BM improved spatial learning and memory in male mice treated with ethanol. Also, this is the first study that demonstrated that BM effect on spatial learning and memory follows a dose–response behavior.

Outcomes from the present study showed that polyphenol content in BM was 0.65 g pyrogallol/100 g of hydroalcoholic extract. Polyphenolic constituents in plants enhance the cognitive performance of rats during memory tasks, especially those related to spatial learning and memory including water Morris maze (Kim et al., 2004; Barros et al., 2006). The neuroprotective effects of plants containing polyphenolic compounds such as quercetin (Naidu et al., 2004; Andres-Lacueva et al., 2005) and anthocyanins (Ramirez et al., 2005; Barros et al., 2006; Shin et al., 2006) have been previously reported. Moreover, the antioxidant effects on quercetin (Naidu et al., 2004; Farombi and Onyema, 2006) and anthocyanins (Cho et al., 2003; Shih et al., 2010) on brain have been previously demonstrated. Previous studies demonstrated that maca hypocotyls contain quercetin (Lee et al., 2004) and anthocyanins (Valerio and Gonzales, 2005). For these reasons, the effect of BM on memory and learning may be due to its content of polyphenolic compounds and their antioxidant activity as suggested previously (Rubio et al., 2006, 2007, 2008; Pino-Figueroa et al., 2010). In fact, black was able to reduce brain lipid peroxidation in ovariectomized mice, a model related to estrogen deficiency. Another possibility related to the effect of maca may be related to the antiapoptotic capacity of its polyphenolic compounds (i.e., quercetin and anthocyanins). In fact, other authors found that quercetin could blockade the activation of caspase cascade in neurons exposed to amyloid beta toxicity in vivo and in vitro (Wang et al., 2001).

Also, epidemiological studies found a significant correlation between dietary intake of vegetables and improvement in cognitive function in elderly people (Lee et al., 2001). In fact, aging women consuming cruciferous vegetables (e.g., broccoli and cauliflower) showed less cognitive decline than those not consuming them (Kang et al., 2005). Members of the genus *Lepidium*, including maca, belong to the cruciferous (Brassicaceae) family and it is possible that this plant may have effects on cognitive functions.

In addition, some novel compounds have been recently identified, as two new imidazole alkaloids (lepidine A and B) (Cui et al., 2003). Also, a benzylated product, named Macaridine, derivative of 1,2-dihydro-*N*-hydroxypyridine, together with the benzylated alkamides (Macamides), *N*-benzyl-5-oxo-6E,8Eoctadecadienamide and *N*-benzylhexadecanamide, as well as the acyclic keto acid, 5-oxo-6E,8E-octadecadienoic acid have been described (Muhammad et al., 2002). However, the effect of these compounds has not been assessed for any of the functions described for maca including learning and memory. For these reasons, future studies are necessary to elucidate the chemical composition of BM and the components related to its neuroprotective effect.

Correlation analyses showed that the effect of BM on escape latency and time in the target quadrant is no related to locomotor activity. These outcomes demonstrated that the effect of BM on memory and learning is not related to locomotor activity as showed by others (Rubio et al., 2006, 2007, 2008).

Conclusion

Finally, the hydroalcoholic extract of BM inhibits the ethanol-induced memory impairment in a dose–response manner in male rats. Also, the total content of polyphenols such as quercetin and anthocyanins may be related to the effect of BM on cognitive function.

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Declaration of interest

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