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Original Article

Anxiolytic-like and proneurogenic effects of *Trichilia catigua* ethyl-acetate fraction in mice with cerebral ischemia

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ABSTRACT

Trichilia catigua A. Juss., Meliaceae, known as "catuaba" in Brazil, has been popularly used as a tonic for fatigue, impotence and memory deficits. Previously, we have demonstrated that *T. catigua* ethyl-acetate fraction exerted antidepressive-like effects in mice. Affective-like symptoms are also well recognized outcome of cerebral ischemia in clinical and preclinical settings. Therefore, here we evaluated the effects of ethyl-acetate fraction on the emotional outcomes and its relation with hippocampal neurogenesis in ischemic mice. Male Swiss mice were subject to the bilateral common carotid occlusion during 20 min. The animals received ethyl-acetate fraction (400 mg/kg, orally) 30 min before and once per day during 7 days after reperfusion. Emotional outcomes were assessed using the open field test, elevated zero maze, and the tail suspension test. After the behavioral testing, the animals were sacrificed and their brains were processed to immunohistochemistry and Nissl staining. Ischemic mice exhibited anxiogenic-like behaviors in the elevated zero maze, hippocampal neurodegeneration and decreased hippocampal neurogenesis. The anxiogenic-like effect was counteracted by ethyl-acetate fraction administration. Furthermore, ethyl-acetate fraction restored the number of newborn neurons in the dentate gyrus of hippocampus of ischemic mice. In conclusion, *T. catigua* ethyl-acetate fraction promoted functional recovery and restored hippocampal neurogenesis in ischemic mice.

Introduction

Trichilia catigua A. Juss, Meliaceae, a medium-sized flowering tree that is widely distributed in South American, has been used in folk medicine as a tonic for the treatment of mental fatigue, stress, impotence, and memory deficits (Pizzolatti et al., 2002). Experimental evidences suggested that *T. catigua* ethyl-acetate fraction (EAF) improved memory in the step-down inhibitory avoidance test (Chassot et al., 2011) and promoted antidepressant-like effects in the forced swim test (FST) and tail suspension test (TST) in rodents (Chassot et al., 2011; Campos et al., 2005; Bonassoli et al., 2012). Curiously, the antidepressant-like effects of the EAF were accompanied by an increase in cell proliferation in the dentate gyrus (DG) of the hippocampus (Bonassoli et al., 2012), suggesting that hippocampal plasticity might contribute to the therapeutic effects of EAF.

Cerebral ischemic diseases are among the most prevalent causes of death and the leading cause of adult disability worldwide (Flynn et al., 2008). Patients who survive ischemic brain insult often present long-term deleterious functional outcomes, such as a higher incidence of anxiety, depressive symptoms, and severe cognitive impairments, that negatively affect quality of life (Lilja et al., 2015; De Wit et al., 2017). The Tissue plasminogen activator (The National Institute of Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study Group, 1995) is the only pharmacological therapy that is approved by the Food and Drug Administration (FDA) for the acute treatment of stroke. However, although highly effective, its applicability is limited by the narrow therapeutic window (3–4.5 h) the possible occurrence of hemorrhage (Ginsberg, 2008; Sutherland et al, 2012; Gorelick et al, 2011). Further for global cerebral ischemia (GCI), an effective, approved neuroprotective strategy is therapeutic hypothermia. Clinical evidence has shown that therapeutic hypothermia increases survival rates and improves neurologic outcomes in patients with global ischemic injury (Hoesch and Geocadin, 2007). Nonetheless, the worldwide implementation of

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therapeutic hypothermia remains suboptimal. The main reason for this is the lack of integration of cooling protocols. Therefore, alternative pharmacological treatments are clearly needed, which might increase the number of patient survivors and reverse functional outcomes that are induced by cerebral ischemic diseases.

In this context, Kamdem et al. (2012) showed that a hydroethanolic extract of *T. catigua* (40–100 μg/ml) protected hippocampal slices from the deleterious effects of oxygen and glucose deprivation when it was administered before and during the reperfusion period. To our knowledge, only three studies have investigated the effects of *T. catigua* in *in vivo* animal models of global cerebral ischemia. All of them focused only on the effects of the EAF on cognitive impairments that were induced by cerebral ischemia. Overall, the results showed that the EAF promoted pro-cognitive effects in ischemic rodents in the Morris water maze and 8-arm aversive radial maze (Truiti et al., 2015; Godinho et al., 2018a). These effects were associated with an increase in the levels of endogenous antioxidants, including reduced glutathione and superoxide dismutase, and a decrease in protein carbonyl groups. An antiinflammatory mechanism of action of the EAF was proposed because it decreased the glial response in the hippocampus in ischemic rats (Godinho et al., 2018b). However, remaining to be determined are the effects of the EAF on emotional outcomes of brain ischemia and the possible molecular mechanisms.

The present study evaluated the effects of an EAF on emotional outcomes of bilateral common carotid artery occlusion (BCCAO), a model of cerebral ischemia/reperfusion, in mice. We also evaluated neurodegeneration and the expression of newborn neurons in the hippocampus in ischemic animals using Nissl and doublecortin (DCX) staining, respectively.

Material and methods

Animals

The experimental procedures were approved by the Ethics Committee on Animal Experimentation of the Universidade Estadual de Maringá (CEUA 103/2014) and were in accordance with the guidelines of the National Institutes of Health and Brazilian College for Animal Experimentation. A total of 64 male Swiss mice (30–40 g) were obtained from the central vivarium of the Universidade Estadual de Maringá, Brazil. All of the animals were housed in groups ($n = 10–15$) under a 12 h/12 h light/dark cycle (lights on from 7:00 PM to 7:00 AM) and had free access to food and water. The room temperature was kept constant at 22 °C ± 1 °C.

Plant material and extract preparation

The bark of *Trichilia catigua* A. Juss, Meliaceae, was acquired in Caetité, Bahia, Brazil, in 2008. A voucher specimen was identified by Dr. Cássia Mônica Sakuragui, Universidade Federal do Rio de Janeiro, RJ, Brazil, and deposited in the Herbarium of Universidade Estadual de Maringá, Maringá, PR, Brazil (HUEM no. 19.434). The extracts were obtained as described by Chassot et al. (2011). Briefly, the crude extract (CE) was produced with acetone-water (7:3, v/v) to yield 101 g from 450 g of *T. catigua* bark. The semipurified *T. catigua* EAF was then obtained after partitioning with ethyl acetate to yield 13 g from 50 g of the CE.

Surgery

Cerebral ischemia was induced by 20 min of BCCAO as described previously (Soares et al., 2013). The mice were first anesthetized with a mixture of isoflurane/oxygen (Isoforine, Cristália, SP, Brazil) that was delivered through a universal vaporizer (Oxigel, São Paulo, Brazil). The mixture that was delivered to the animal was monitored to supply a minimal isoflurane concentration for sufficient anesthesia (evaluated by pinching the animal's tail) and was maintained with 1.3–1.5% isoflurane in 100% oxygen. The animals were then fixed in a stereotaxic frame, and an incision was made in the ventral neck to expose the common carotid arteries. Using aneurysm clips (ADCA, Belo Horizonte, MG, Brazil), both common carotid arteries were occluded. Throughout surgery, rectal temperature was carefully monitored and maintained at approximately 37.5 °C using a heating blanket to prevent hypothermia. At the end of each occlusion, the aneurism clips were removed, the carotid arteries were visually inspected for reperfusion, and the incision was closed with sutures. For 2 h after reperfusion, the mice were maintained in a warming box at 30 °C. The mice were allowed to recover for 7 days until the first behavioral test. Sham-operated animals were subjected to the same anesthetic and surgical procedures, with the exception that the carotid arteries remained intact.

Treatment

Thirty minutes before BCCAO and for 7 consecutive days after BCCAO, the mice received daily oral (*p.o.*) injections of vehicle (0.9% NaCl with 1% propylene glycol) and the *T. catigua* EAF (400 mg/kg; dissolved in NaCl with 1% propylene glycol) in a volume of 10 ml/kg. The dose was based on a previous study that reported memory recovery and a decrease in hippocampal neurodegeneration after BCCAO in mice (Truiti et al., 2015). Four experimental groups were formed: sham + vehicle (Sham + veh, $n = 13$), sham+400 mg/kg *T. catigua* EAF (Sham + EAF, $n = 10$), BCCAO + vehicle (BCCAO + veh, $n = 12$), or BCCAO + 400 mg/kg *T. catigua* EAF (BCCAO + EAF, $n = 10$).

Behavioral tests

Behavioral testing commenced 8 days after BCCAO and 1 day after the cessation of *T. catigua* EAF or vehicle treatment (see Fig. 1 for a global timeline of the behavioral tests). The behavioral tests were conducted in the following order: (*i*) open field test (OFT), elevated zero maze (EZM), and tail suspension test (TST). In order to minimize the environment influences behavioral testing occurred during the light phase between 1:00 PM and 5:00 PM under identical conditions (Fig. 1). The experiments were video-recorded, and the behavioral scores were later analyzed using ANY-maze image analyzer software (Stoelting, Wood Dale, IL, USA).

Open field test

To measure locomotor activity, the OFT was performed. The open field consisted of a wooden square box (70 cm × 70 cm) with 40 cm high walls. The floor was divided into two fields: periphery (20 cm adjacent to the walls) and center (30 cm^2). The mouse was placed in the center of the arena and allowed to move freely for 5 min. After each session, the arena was thoroughly cleaned with a 70% ethanol solution (Soares et al., 2016; Soares et al., 2017; Mori et al., 2017). The distance traveled (in meters) was recorded.

Elevated zero maze

The EZM is used to measure anxiety-like behavior in rodents (Braun et al., 2011). The apparatus consisted of a ring-shaped runway (46 cm diameter, 5.5 cm width) that was constructed from gray plastic material and elevated 20 cm above the floor. The runway was divided into two opposing open quadrants with a low border (3 mm height) to prevent the mouse from stepping down and two opposing closed quadrants with sidewalls (11 cm height). Each mouse was individually placed into one of the open quadrants and allowed to explore the arena for 6 min. After each session, the maze was thoroughly cleaned with a 70% ethanol solution (Soares et al., 2016, 2017; Mori et al., 2017). The time spent in the open arms of the maze was recorded and corrected for initial freezing behavior

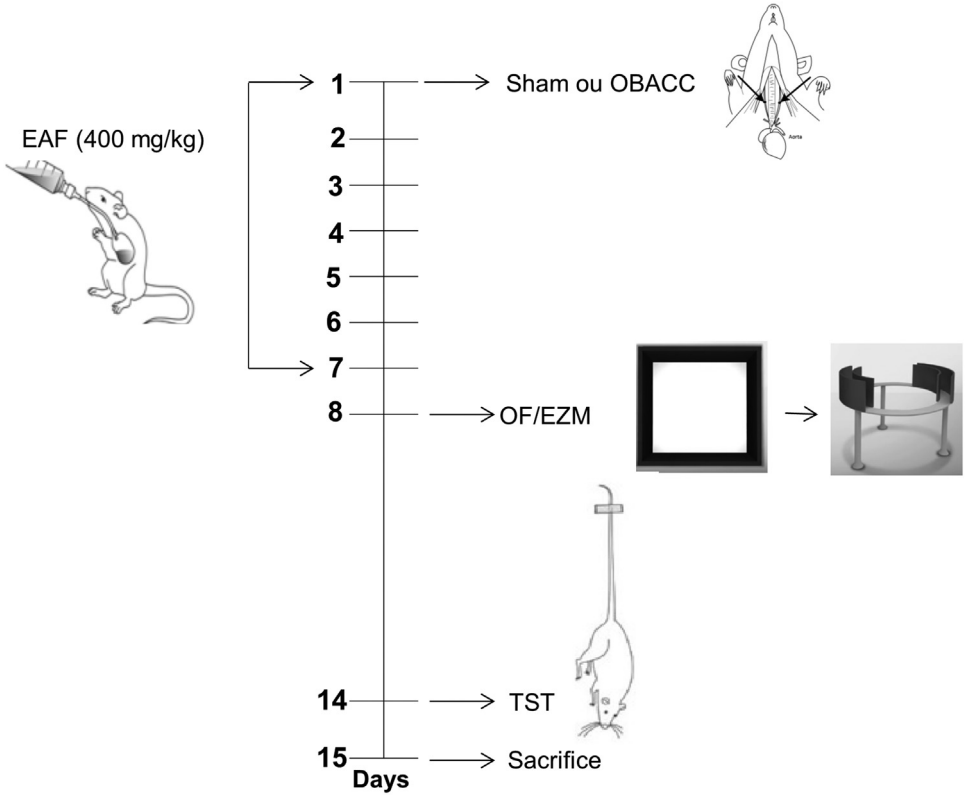

Fig. 1. Experimental design. Vehicle or *Trichilia catigua* EAF 400 mg/kg were *p.o.* administered daily during 7 days. The treatment started at day 1 with two doses, the first one hour before and the second one hour late the sham or BCCAO surgeries. From days 7–14 after reperfusion, mice were subjected to behavioral testing. After the last behavioral test, mice were sacrificed and their brains were processed for histological and immunohistochemical analysis. EZM = elevated zero maze; OF = open field; TST = tail suspension test.

by subtracting the latency to first enter a closed arm from the total time spent in the arena.

Tail suspension test

The TST was conducted as previously described (Bonassoli et al., 2012) with slight modifications. A mouse was individually suspended by its tail with adhesive tape (1 cm from the tip of the tail) in a black wooden box (45 cm × 45 cm × 30 cm) for 6 min. The mouse was considered immobile only when it hung passively and completely motionless. Immobility time was recorded during the last 4 min of the session.

Histology

At the end of the behavioral evaluation, the brains of half of the animals were removed and histologically assessed for neurodegeneration using Nissl staining. The mice were deeply anesthetized with 50 mg/kg sodium thiopental (Thiopentax, Cristália, SP, Brazil) and transcardially perfused with 0.9% saline followed by Bouin's fixative. Following decapitation, the head was immersed in crushed ice (1–2 °C) for 2 h to avoid the appearance of dark neurons, which could confound the actual extent of neurodegeneration. The brains were then carefully removed and postfixed in Bouin's solution for 24 h. Using a rotating microtome (RM2445, Leica, Goettingen, Germany), 7 μm paraffin-embedded coronal sections at a stereotaxic level between −1.70 and −2.70 mm posterior to bregma (Franklin and Paxinos, 1997) were obtained and distributed into four sets of slides that contained four coronal sections, each 28 μm apart. After standard dehydration and diaphanization procedures, slides that contained adjacent sections were immersed in distilled water and submerged in 0.2% Cresyl violet solution (Nissl staining) for 5 min. The slides were then rinsed in distilled water, dehydrated in a graded series of ethanol (70, 80, 90, and 100%), cleared in xylene, and coverslipped with xylene using Permount (Fisher Scientific, São Paulo, Brazil).

In each hemisphere, the number of cells that presented a well-delimited, spherical form with a distinct nucleus and nucleolus was counted throughout the CA1, CA2, CA3, and CA4 subfields of the hippocampus (400× magnification; Olympus BX-41 microscope). Neurons that had shrunken cell bodies or surrounding empty spaces were considered destined to die and excluded from the counting. The results are presented as the mean ± SEM of three sections per animal.

Immunohistochemistry

The other half of the mice were deeply anesthetized with 50 mg/kg of sodium thiopental, *i.p.* (Soares et al., 2013) (Thiopentax, Cristália, São Paulo, Brazil), and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.2 M phosphate buffer (PB). The brains were removed, post-fixed in the same fixative for 24 h, and cryoprotected by immersion in 30% sucrose for 48 h. The brains were then quickly frozen and kept at −80 °C until the immunohistochemical analysis. Frozen tissue was serially sectioned on a cryostat (Criocut 1800, Reichert-Jung, Heidelberg, Germany) into 30 μm coronal sections at −1.22 to −2.70 mm posterior to bregma (Franklin and Paxinos, 1997) and collected into six Eppendorf tubes that contained 0.1 M PBS plus 0.1% sodium azide and stored at 4 °C.

Free-floating sections were quenched in 1% H_2O_2 for 30 min and then blocked with 2% bovine serum albumin in 0.1 M PBS for 60 min at room temperature. The sections were incubated overnight with goat polyclonal anti-DCX antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.1 M PBS that contained 0.3% Triton X-100 at room temperature. The sections were then incubated with

the respective biotinylated secondary antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h and incubated in ABC solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The peroxidase reaction was performed using 3-3′-diaminobenzidine (DAB; Sigma) and 0.05% H_2O_2. $NiCl_2$ was added to the DAB solution to increase the staining contrast. The sections were mounted on gelatin-coated slides and coverslipped with Permount.

The quantitative measurement of DCX-immunoreactive (IR) neurons was performed in the DG of the hippocampus. Using a light microscope (Olympus BX-41 microscope) with a 40× objective, the number of DCX-IR neurons was manually quantified in the subgranular zone (SGZ) and inner granular cell layer (GCL) of the DG. The SGZ was defined as the two-cell-body width of the hilus along the border of the GCL. The results are presented as the mean ± SEM of three sections per animal.

Statistical analysis

SAS 9.3 software was used for the analysis. Behavioral and histological data were examined for assumptions of normality and homoscedasticity (between-group and within-group homogeneity of variance). The behavioral data satisfactorily fit both a normal distribution and homoscedasticity. One-way analysis of variance (ANOVA) was used for between-group comparisons. If a main effect of *Group* was found, then Tukey–Kramer's multiple-range test was used to distinguish between them. The histological data were normalized to the mean values of the sham group. The data were analyzed using the generalized linear model with a Poisson distribution to model the count data (i.e., number of cells, Nissl-stained cells, and DCX-stained cells). Values of $p < 0.05$ were considered statistically significant.

Results and discussion

Overall, 64 animals entered the experiment, nineteen (29.7%) died after complete recovery from anesthesia. The rate of mortality was 0% in sham operated animals (Sham + veh and Sham + EAF.), 45.4% in BCCAO + veh group (10/22, 45.4%) and 47.3%. BCCAO + EAF mice (9/19, 47.3%), reflecting the fatal effect of cerebral ischemia.

The outcomes of brain ischemia include motor, sensory, and visual deficits and the development of neuropsychiatric symptoms. In the present study, we found no differences in the total distance traveled ($F_{3,44} = 0.22$, $p = 0.8$; Fig. 2A) in the OF test among the experimental groups, indicating that no locomotor alterations were caused by cerebral ischemia.

On the other hand, ANOVA revealed a difference in the time spent in the open arms of the EZM ($F_{3,44} = 13.15$, $p < 0.0001$; Fig. 2B). The BCCAO + veh group spent less time in the open arms of the EZM compared with the Sham + veh ($p = 0.0001$) and Sham+EAF ($p = 0.0004$) groups. This result is consistent with our previous studies that showed that BCCAO increased anxiety-like behavior in ischemic mice in the elevated plus maze (EPM) and EZM 7, 14, and 28 days after reperfusion (Soares et al., 2013, 2016, 2017; Mori et al., 2017). Other groups also reported that BCCAO caused anxiogenic-like behavior in the EPM and social interaction test in mice 2 and 7 days after reperfusion (Nakashima et al., 2003; Neigh et al., 2009). Interestingly, *T. catigua* EAF increased the time spent in the open arms of the EZM in the BCCAO + EAF group compared with the BCCAO + veh group ($p = 0.02$), indicating an anxiolytic-like profile for this compound. At this point our results contrast with another study which reported that a single oral administration of EAF (100–400 mg/kg) did not alter anxiety-like behavior in naive animals in the EPM (Chassot et al., 2011). One plausible explanation for this discrepancy is the regimen of EAF administration (i.e., acute vs. repeated) and the presence of a pathological condition (*i.e.*, naive vs. BCCAO). Repeated administration of the EAF might act by reversing anxiety-like symptoms that are triggered by cerebral ischemia.

In addition to anxiety, depressive behavior is a common outcome of cerebral ischemia (Yan et al., 2007). However, here no difference was found in immobility time in the TST among groups ($F_{3,44} = 0.38$, $p = 0.7$; Fig. 2C). In this, context should be considered that Yan et al. (2007) found that mice which were subjected to BCCAO exhibited depressive-like behavior in the TST 3 days after reperfusion, and in other studies, an increase in immobility time in the FST appeared only 21 days after reperfusion (Soares et al., 2017; Mori et al., 2017). Thus, would be possible that the difference between our study and these previous studies may reflect different experimental conditions, such as the time after reperfusion, animal species, and behavioral tests that are used to evaluate antidepressant-like activity. In addition, it should be noted that the lack of an experimental group treated with antidepressant drug reference as a positive control makes difficult the interpretation of TST data. Thus, more experiments should be conduct in order to better understand this preliminary result.

BCCAO leads to reductions of oxygen and glucose in brain tissue, which in turn result in neuronal death (Kumaran et al., 2008; Soares et al., 2013). Indeed, as shown in Fig. 3, significant differences were found in the number of intact-appearing neurons in the CA1 ($\chi^2 = 2130.80$, $p < 0.0001$), CA2 ($\chi^2 = 325.79$, $p < 0.0001$), CA3 ($\chi^2 = 792.62$, $p < 0.0001$), and CA4 ($\chi^2 = 121.23$, $p < 0.0001$) subfields of the hippocampus. Overall, BCCAO decreased the number of intact neurons compared with the Sham+veh ($p < 0.0001$) and Sham+EAF ($p < 0.0001$) groups. Intriguingly no effect of the *T. catigua* EAF was observed in the BCCAO + EAF group ($p > 0.05$). This result is discordant with a previous study that showed that an EAF prevented hippocampal neurodegeneration following BCCAO in mice (Truiti et al., 2015). However, in this previous study, the animals were sacrificed 8 days after BCCAO (i.e., 1 day after the last EAF administration), in contrast to the present study in which the animals were sacrificed 15 days after BCCAO (i.e., 8 days after the last EAF administration). Therefore, one possibility is that ischemia-induced neuronal death was not totally suppressed by the EAF, but rather only postponed. In order to confirm this hypothesis more experiments evaluating the effects of *T. catigua* EAF on a time-course of a neurodegeneration induced by brain ischemia should be conduction.

As a compensatory phenomenon, hippocampal neurogenesis has been shown to increase after cerebral ischemia in rodents (Kawai et al., 2004). Several studies have shown that hippocampal cell proliferation peaks 7–10 days after ischemia, but severe decreases occur within 2–4 weeks (Kawai et al., 2004; Schiavon et al., 2010; Soares et al., 2013). Accordingly in the present study a significant decrease in the number of DCX-positive neurons was detected 15 days after reperfusion (Fig. 4; $\chi^2 = 21.81$, $p < 0.0001$) in the BCCAO+veh compared with the Sham+veh ($p = 0.0004$) and Sham+EAF ($p = 0.005$) groups. *T. catigua* EAF treatment increased the number of DCX-positive cells in the BCCAO + EAF group compared with the BCCAO + veh group ($p < 0.0001$). Curiously, aversive experiences or such stressors as brain ischemia lead to an increase in anxiety-related behavior and a decrease in adult hippocampal neurogenesis. Newborn neurons in the DG enhance behavioral adaptability to changes in the environment (Cameron and Schoenfeld, 2018). Thus, the recovery of hippocampal neurogenesis, reflected by an increase in the number of DCX-positive neurons in the DG in ischemic mice, may be involved in the anxiolytic-like effect of the EAF that was observed in the present study.

Several molecular mechanisms have been proposed for the therapeutic actions of *T. catigua*. Campos et al. (2005) associ-

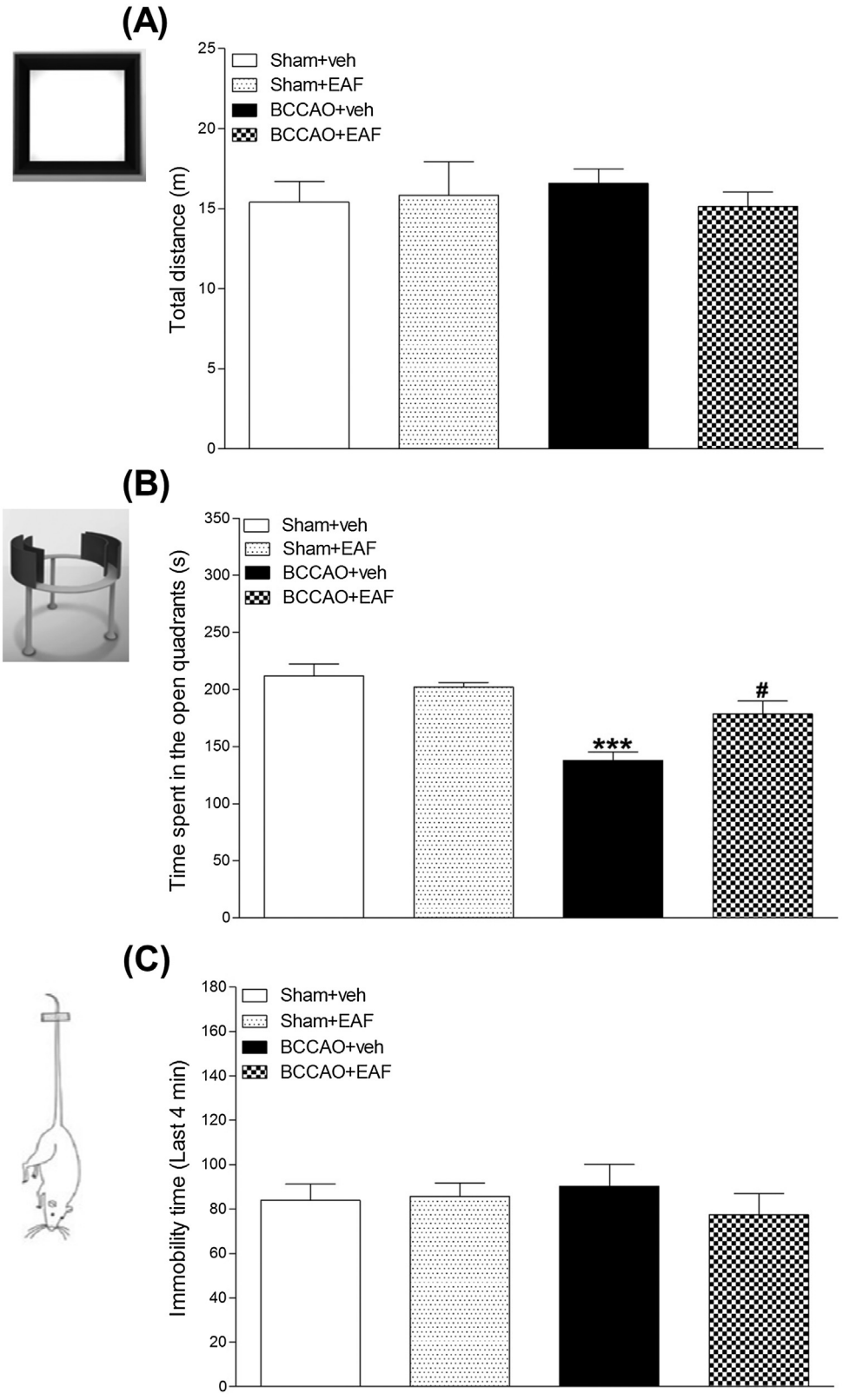

Fig. 2. *Trichilia catigua* EAF ameliorates affective impairments in BCCAO mice. (A) Total distance in the OF; (B) Time spent in the open arms of EZM; (C) Immobility time in the TST. Bars represent the means ± S.E.M. per experimental group. Sham + veh ($n = 13$); Sham + EAF ($n = 10$); BCCAO + veh ($n = 12$), BCCAO + EAF ($n = 10$). $^{***}p < 0.001$ compared to the Sham+veh group. $^{\#}p < 0.05$ compared to the BCCAO+veh group (ANOVA followed by the Tukey's test). EZM = elevated zero maze; OF = open field; TST = tail suspension test.

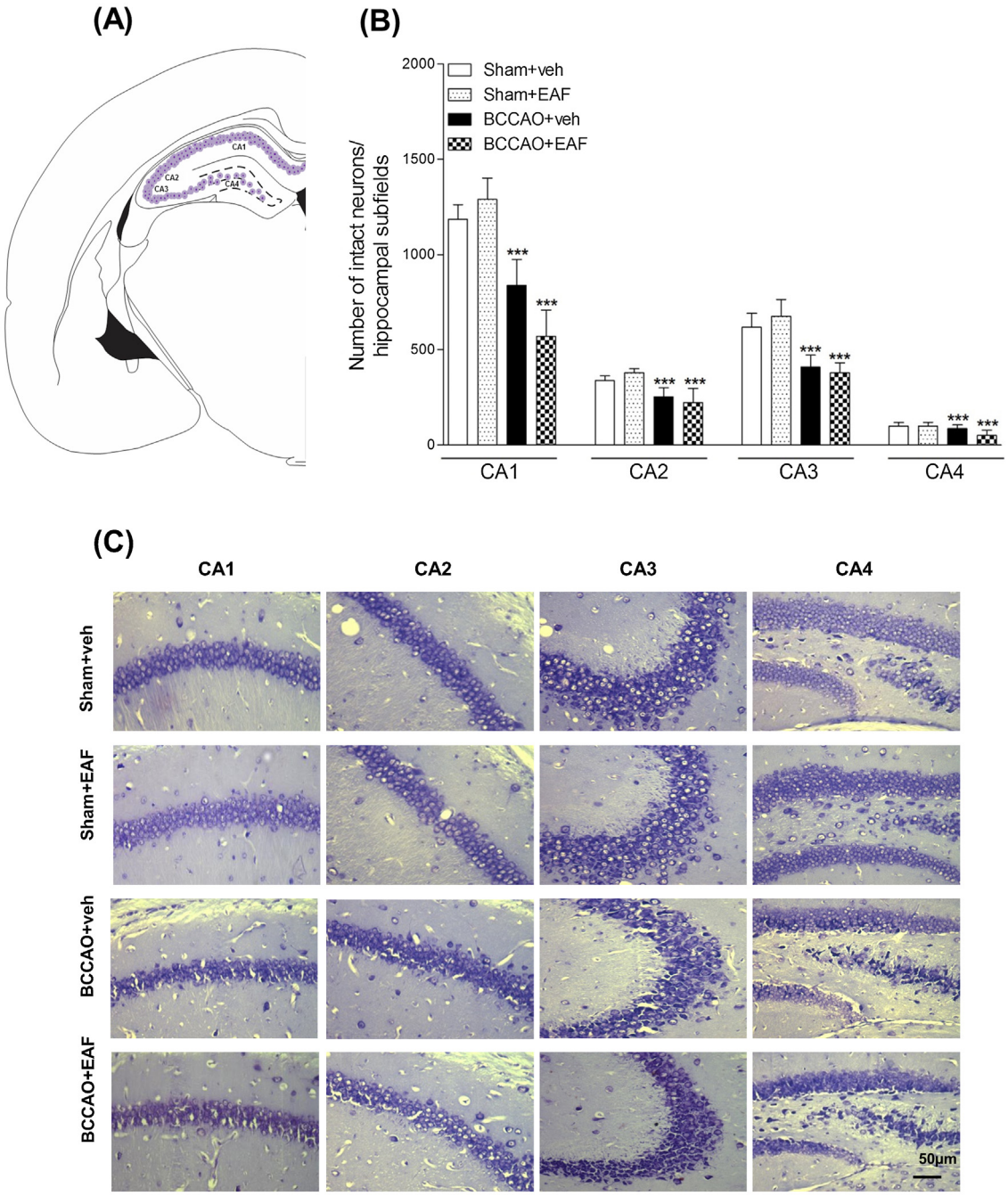

Fig. 3. *Trichilia catigua* EAF did not change hippocampal neurodegeneration in BCCAO mice. (A) Representative diagram illustrating a coronal brain section containing the CA1, CA2, CA3 and CA4 hippocampal subfields (Franklin & Paxinos, 1997) where the intact appearing neurons were counted. (B) Number of intact neurons in the CA1, CA2, CA3 and CA4. Bars and values represent mean ± SEM per experimental group. Sham + veh ($n = 6$); Sham + EAF ($n = 6$); BCCAO + veh (n = 7), BCCAO + EAF ($n = 6$). $^{***}p < 0.001$ compared to the Sham+veh group (Generalized linear model with a Poisson distribution). (C) Representative photomicrographs of Nissl staining in the CA1, CA2 CA3 and CA4.

ated the antidepressant-like effects of *T. catigua* with modulation of the dopaminergic system, in which haloperidol and chlorpromazine completely reversed the effects of *T. catigua* in the FST in rats. Accordingly a recent study found that *T. catigua* inhibited the activity of monoamine oxidase and acetylcholinesterase in rat brain homogenates (Bernardo et al., 2017). Bonassoli et al. (2012) reported that the antidepressant-like effect of an EAF was accompanied by an increase in bromodeoxyuridine-positive cells in the DG. Moreover, experimental evidence suggests that the effects of *T. catigua* involve the neuroimmunoendocrine system. Once, *T. catigua* treatment in female rats during pregnancy and lactation resulted in modulation of the production of immunoglobulin G antibodies in their offspring (Fernandes et al., 2017). Most recently the treatment with the hydroalcoholic extract of *T. catigua* reduced the fatigue induced by treadmill in mice for promoting an acetylcholinesterase inhibition and antioxidant actions (Martins et al., 2018). Indeed, the antioxidant and antiinflammatory actions may have at least partially contributed to the therapeutic effects of *T. catigua* (Barbosa et al., 2004; Tang et al., 2007). *T. catigua* exerted an inhibitory effect on xanthine oxidase activity and scavenging activity of the superoxide anion radical (Bernardo et al., 2017). In the field of ischemic cerebral disease, the treatment with EAF reversed

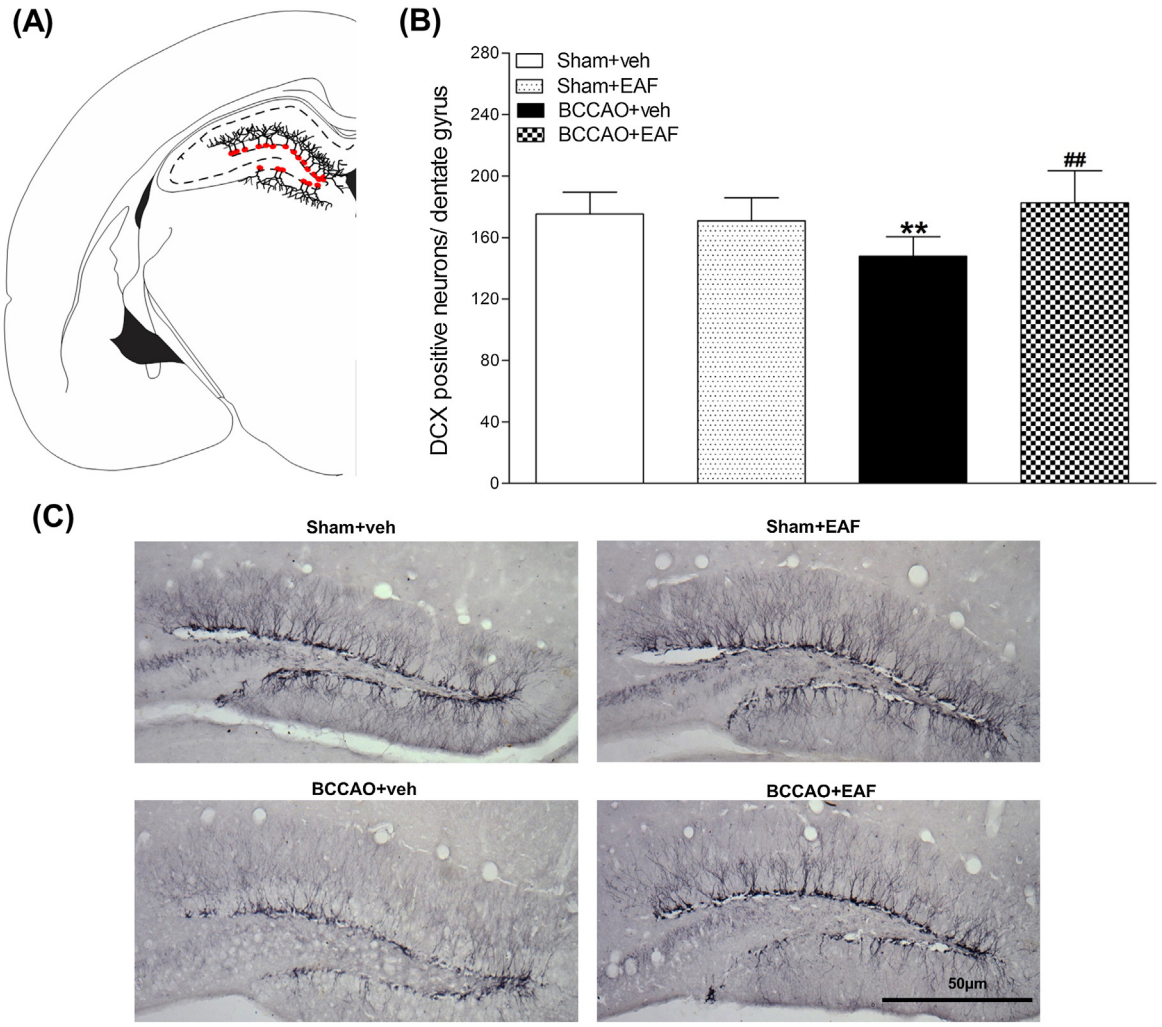

Fig. 4. *Trichilia catigua* EAF increases the number of DCX-positive neurons in BCCAO mice. (A) Diagram of the hippocampal dentate gyrus (Franklin & Paxinos, 1997). (B) Number of DCX-positive neurons per section of the hippocampal dentate gyrus. Bars and values represent mean ± SEM per experimental group. Sham + veh ($n = 6$); Sham + EAF ($n = 6$); BCCAO + veh ($n = 7$), BCCAO + EAF ($n = 6$). $^{**}p < 0.01$ compared to the Sham + veh group. $^{\#\#}p < 0.01$ compared to the BCCAO + veh group (Generalized linear model with a Poisson distribution). (C) Representative photomicrographs of DCX-IR neurons.

the content of protein carbonyl groups and activity of myeloperoxidase and decreased the expression of glial fibrillary acidic protein (GFAP) and OX42 in the CA1, CA3, and DG of ischemic rats (Godinho et al., 2018a; Godinho et al, 2018b). Together these data show that the pleiotropic effects of *T. catigua* may contribute to the functional recovery in ischemic mice.

Conclusion

The present study examined the effects of repeated EAF administration in mice that were subjected to BCCAO and its influence on emotional outcomes, hippocampal neurodegeneration, and neurogenesis in the DG. Ischemic mice exhibited anxiety-like behaviors in the EZM, severe neuronal loss in the CA1, CA2, CA3, and CA4 subfields of the hippocampus, and a decrease in the number of newborn neurons in the DG. The EAF attenuated anxiety-like behavior that was caused by ischemia and increased the number of newborn neurons in the DG in BCCAO mice. These results indicate a partial neuroprotective effect of the EAF against brain ischemia.

Contributors

LMS (postdoctoral fellowship) conducted the surgery and wrote the manuscript. JPCF (Graduation student fellowship) conducted the behavioral tests and neurohistological experiments. RL and JCPM worked on obtaining the EAF and its chromatographic characterization. CVN helped with some laboratory infrastructures. HM and RMWO designed the study and reviewed the manuscript.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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