ORIGINAL ARTICLE

Achyrocline satureioides (Lam.) DC. (marcela) reduces brain damage in permanent focal ischemia in rats

Achyrocline satureioides (Lam.) DC. (marcela) reduce el daño cerebral en la isquemia focal permanente en ratas

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ABSTRACT

Introduction: *Achyrocline satureioides* is a plant which has been widely used in popular medicine and experimental studies confirm its antioxidant and anti-inflammatory effects, attributable to the presence of flavonoids, mainly quercetin.
Objectives: to evaluate the neuroprotective effects of a chronic oral pre-administration to rats with an *Achyrocline satureioides* decoction (2 %).
Methods: for decoction, dried flowers of *Achyrocline satureioides* were used. The consumption of food and AS decoction/water of the rats was evaluated daily and weight gain weekly; quercetin content in the decoction and in the plasma of the rats was evaluated by high performance liquid chromatography. The cerebral damage was assessed with a tetrazolium salt (TTC) and a behavioral test was performed previously. Nissl staining and Fluoro-Jade histochemistry were used.
Results: the pre-treatment with *Achyrocline satureioides* in all groups reverted the functional deficit and, during 21 days, the infarction volume also decreased significantly. Nissl staining showed a higher percentage of preserved neuronal population and the Fluoro-Jade showed a decreased of the neurons in degeneration.
Conclusions: the quercetin levels in the decoction and plasma of rats could explain the preventive benefits of *Achyrocline satureioides* due to the antioxidant and anti-inflammatory properties described for this flavonoid.

Key words: *Achyroclines satureioides*, quercetin, permanent focal ischemia.

RESUMEN

Introducción: *Achyrocline satureioides* es una planta que ha sido ampliamente utilizada en la medicina popular y los estudios experimentales confirman sus efectos antioxidantes y antiinflamatorios, atribuibles a la presencia de flavonoides, principalmente quercetina.
Objetivos: evaluar los efectos neuroprotectores de la pre-administración oral crónica a ratas con una decocción de *Achyrocline satureioides* 2 %.
Métodos: para la decocción se utilizaron flores secas de *Achyrocline satureioides*. Se cuantificaron, diariamente, el consumo de alimentos, la decocción y el agua; y cada semana, la ganancia de peso. El contenido de quercetina en la decocción y en el plasma de las ratas se evaluó utilizando la técnica de cromatografía líquida de alta resolución. El daño cerebral se cuantificó con una sal de tetrazolio y antes se realizó una prueba de comportamiento. Se utilizaron la tinción de Nissl y el fluoro Jade.
Resultados: el pretratamiento con *Achyrocline satureioides* en todos los grupos revirtió el déficit funcional, y la decocción durante 21 días también decreció de modo significativo el volumen del infarto. La tinción de Nissl mostró alto porcentaje de población neuronal conservada y el fluoro Jade presentó un decrecimiento en las neuronas en degeneración.
Conclusiones: los niveles de quercetina en la decocción y el plasma de las ratas podrían explicar los beneficios preventivos de *Achyrocline satureioides,* debido a las propiedades antioxidantes y antiinflamatorias descritas para este flavonoide.

Palabras clave: *Achyroclines satureioides*, quercetina, isquemia focal permanente.

INTRODUCTION

Cerebrovascular attacks (CVA) are the second most frequent cause of death world-wide and the third cause of adult disability in the United States.[1] The ischemic CVA (ischemic stroke) results from a transient or permanent reduction in blood flow that affects the territory of a brain artery and accounts for approximately 80 % of all CVA;[2] however, and in spite of this high incidence, there is only one FDA-approved therapy -the thrombolytic tissue plasminogen activator (tPA)- for the acute treatment of ischemic CVA.[3] Ischemia initiates a complex cascade of events, including increased excitotoxicity, loss of membrane permeability with accumulation of intracellular Ca^{2+}, activation of degradative enzymes, activation of inflammatory signals, etc.[4] Oxidative stress is a common final path that could cause lipoperoxidation, nuclear DNA damage and neuronal death[5,6] as well as the production of inflammatory mediators.[7]

These molecular mechanisms that occur during the ischemic cascade have promoted the search of antioxidant and antiinflammatory molecules that could interfere with the oxidative stress, reducing neuronal damage.[8] Due to their high antioxidant and antiinflammatory actions, early evidences pointed out to plants, fruits, beverages like wine and tea, and their main compounds such as flavonoids, as meaningful candidates in the search for neuroprotective principles.[9]

Diverse populations have utilized plants extracts to treat neurological diseases.[10,11] People of South America used regularly plants extracts for their neurological effects as *Erythroxylon coca* (coca) [12] or *Chondodendron tomentosum* (curare).[13] In this context, the enormous variety of native plants of the South American region has been very

poorly studied on their value in the prevention of nervous system diseases. The search for pharmacological actions useful in CVA is certainly a meaningful endeavor.[14]

In a large Southern South America region that covers Argentina, Uruguay, Brazil and Paraguay there are numerous plants with a great arsenal of molecules with antioxidant capacity. In particular *Achyrocline satureioides* (Lam.) DC. (marcela) is a plant of popular use in this region whose decoctions or infusions have been traditionally used for gastrointestinal disorders, as a sedative and antispasmodic.[15] The antioxidant capacity and free radical scavenging of *Achyrocline satureioides* (AS) has been demonstrated in diverse experimental models[16,17] and it was reported that AS protected cells in culture against an oxidative insult.[18] Furthermore, it has been shown that the aqueous extracts of AS is not toxic in a maxima tolerable dose of 5 g/kg.[19]

Phytochemical analysis of AS shows that its main compounds are polyphenols and flavonoids as: caffeic acid, esters of galangin-3-methyl ether, quercetin;[20] luteolin;[21] 3-methoxy-quercetin[22] and a new chalcone: achyrobichalcone[23] among many other compounds.[24] In this regard, the beneficial effect of AS has been ascribed to its content in flavonoids like quercetin,[9,17] a molecule that has been shown to be neuroprotective in several models *in vitro*[5] and *in vivo,* when delivered in a liposomal preparation after focal ischemia in rat.[6,25] However, there are no reports of the neuroprotective effect of AS preparations *in vivo.*

Taking into account the non-toxicity and high popular consumption of this plant in South America, we decided to explore the putative benefits of a chronic oral pre-treatment with an AS decoction in a model of permanent middle cerebral artery occlusion (pMCAo) in rats. We evaluated the behavioral neurological deficit and the cerebral infarct volume of the rats pre-treated with the decoction and subjected to pMCAo. A histopathological examination using Nissl-staining and the fluorescent marker for neuronal degeneration Fluoro-Jade[26] was conducted. Given the numerous evidences showing the neuroprotective capacity of quercetin[5,8,25,27] and being this flavonoid a conspicuous component of AS,[17] it appeared worthwhile to assess the presence of quercetin in the decoction and its bioavailability in the plasma of the experimental animals to assess the possibility of being the responsible of AS effects.

METHODS

Chemicals and reagents

Ketamina (Vetanarcol), Xylacina-HCl (Vetcross), TTC (2,3,5,-triphenylltetrazolium chloride), and HEPES were obtained from Sigma (St. Louis, MO, USA). Fluoro-jade was obtained from Chemicon International, USA. Other chemicals were of highest commercially available purity and were purchased from Baker (Phillipsburg, PA, USA).

Plant material

Achyrocline satureioides (Asteraceae) was obtained from Institute of Agriculture Research (INIA) "Las Brujas", Canelones, Uruguay. The species were identified by Ing. Agr. P. Davies. A voucher specimen of *Achyrocline satureioides* was kept in the College of Agronomy Republic University, Montevideo, Uruguay (MVFA 32796).

Decoction preparation

Two grams of *Achyrocline satureioides* dried flowers were added to 100 mL of boiling water that was kept boiling for 30 min. After that, the decoction was allowed to reach room temperature for 3 hours when the flowers were drained and separated from the decoction.

Animals and experimental protocol

Experiments were carried out using male Sprague-Dawley rats (280-350 g). Animals had access to food and water *ad libitum*, and were housed in groups of six in a temperature controlled environment (22 ± 3 °C) under a 12 h light/dark cycle. The experiments on animals were approved by the Bioethics Committee of Animal Care from Clemente Estable Institute: Protocol No. 002/5/2010. In some animals water was replaced with the AS decoction during 7, 14, or 21 days (n= 5/groups). The quercetin contained in the AS decoction was evaluated weekly by HPLC.

For behavioral and morphometric studies (Infarct volume) thirty six animals were divided into six groups: Group 1: AS decoction during 7 days; Group 2 during 14 days; Group 3 during 21 days (n= 5/groups) all followed by pMCAo during 24 hours. Group 4: pMCAo during 24 h. (n= 9). Group 5: AS decoction only during 7, 14 and 21 days (n= 3/groups). Group 6: sham operated (n= 3). In a second set of rats, the parietal cortex and striatum of the animals were used for histological and histochemical analysis: Nissl (n= 4) and Fluoro-Jade (n= 6).

The body weight of the all animals was assessed weekly and the controls of the intake of food and AS decoction and/or water (in the case of the control animals) were carried out daily.

Behavioural testing

Twenty four hours after pMCAo, and previous to the sacrifice, the animals of all experimental groups were subjected to a neurological examination as described by Menzies *et al*.[28] Briefly, the behavior of animals was scored as 0: no apparent deficits; 1: contra-lateral forelimb flexion when suspended by the tail; 2: decreased grip of the contra-lateral forelimb while tail pulled; 3: spontaneous movement in all directions, contra-lateral circling only if pulled by tail and 4: spontaneous contra-lateral circling. Tests were conducted blinded.

Ischemic surgery

Control and experimental animals were anaesthetized with ketamine (80 mg/kg) and Xylazine (5 mg/kg), anesthetics frequently utilized in ischemic experimental procedures.[29,30] Since there are some reports of a neuroprotective action of ketamine,[31] a sham operated group was performed to control this putative effect on the global final assessment of protection. Body temperature of the animals was continuously monitored throughout the surgical procedure with a rectal thermometer, and maintained at 37.5 °C with a heating pad. The focal cerebral ischemia was induced by pMCAo model as described by Sydserff and co-workers[32] with minor modifications. In brief, a surgical midline incision was made to expose the left common, internal and external carotid arteries. The external carotid and the common carotid arteries were closed by a ligature, the occipital artery was cut by diathermy using a coagulator and the internal carotid artery was temporarily occluded using a

micro-aneurysm clip. A small incision was then made in the common carotid artery, and a 19-mm length of 4-0 monofilament nylon sutures, its tip rounded by heating, was introduced into the internal carotid artery. This tip is soft and flexible and reduces the risk of vessel injury and intracranial bleeding. The filament was advanced up to the appearance of a mild resistance to this advancement indicating that the intraluminal occluder has entered the anterior cerebral artery and occluded the origin of the anterior cerebral artery, the middle cerebral artery (MCA) and posterior communicating arteries.[33] The filament was left in place, fixed to the left carotid artery, at its incision point of entrance by a diathermic point using a coagulator. Skin was finally closed with four suture points with regular suture thread. The filament was in place for the next 24 h. In the groups of sham-operated rats all surgical procedures except the pMCAo were performed. The animals were then allowed to recover from the anesthesia and returned to their cages after the surgery with free access to food and water.

Quantification of the cerebral infarct volume

Twenty-four hours after the pACMo, the animals of all experimental groups were re-anaesthetized with urethane (1.2 g/mL) and intracardially perfused with 0.9 % NaCl (200 mL).[34] Brains were quickly dissected and sectioned at 2, 5, 8, and 11 mm from the frontal lobe. Five coronal sections from each brain (from 2.20, 1.20, 0.20, -0.26, and -1.30 mm with respect to *Bregma*[35] were obtained. After sectioning, slices were incubated for 30 min in a 2 % solution of TTC in 0.9 % NaCl at 37 °C and then fixed in 10 % formalin.[36] The stained slices were photographed with a digital camera and the zones of infarction and brain total areas were outlined.[6,37] These areas were quantified by the image processing program, (Image Pro Plus) and the total infarct volume was calculated by integrating the infracted area of all sections (area of infarct in mm^2 x section thickness). To compensate for swelling, the following formula was applied: infarct size x contralateral hemisphere size/ipsilateral hemisphere size, according to *Chan et al*.[38] Results were expressed as infarct volume in mm^3.

Processing of the tissue

The animals treated during 21 days with the AS decoction and within 24 hours of the pMCAo, were anesthetized; the brains were perfused through the left heart ventricle with heparinised saline solution (200 mL), followed by 4 % paraformaldehide (300 mL). After that, a cryoprotection procedure with 15 and 30 % sucrose (w/v) was carried out. The brains were rapidly frozen for 3 min afterwards and coronal brain cryosections (20 µm) were serially cut.[39]

Nissl stain

The sections were mounted on slides, washed three times for 5 min with PBS-Triton X-100 (0.4 %) and subsequently stained with Toluidine blue 0.1 %, for approximately 2 min. Finally, dehydration of the sections was done and mounted with Canada balsam. In order to correct for edema differences in brain volumes in each experimental group, stereological procedures were used.[40] For this purpose a homothetic transformation of areas in tissue sections of each group was performed.

In order to asses the effects of AS treatment on the survival of the neurons in striatum, in sections ipsilateral to the occluded medial cerebral artery (0.20 µm with respect to Bregma), photographs of 4 fields adjacent to the lateral ventricle were taken with a Nixon microscope (Magnification 20X). On the photographs, the total population of survival neurons (those with echromatic nucleus and conserved

cytoplasm in the 4 experimental groups (Sham; AS; Ischemia and treatment 21 days AS + ischemia) (n= 4/group) were quantified. Number of neurons on sham operated animals was taken as 100 % and the percentage of viable cells was quantified accordingly.

Histochemistry of Fluoro-Jade

Degenerating neuronal somata and their processes were detected with Fluoro-Jade staining as originally described by *Schmued et al*.[41] Brain sections were serially incubated in the following solutions for the time indicated: 100 % alcohol, 3 min; 70 % alcohol, 1 min; distilled water, 1 min; 0.06 % potassium permanganate, 15 min; H_20, 1 min; 0.001 % Fluoro-Jade in 0.09 % acetic acid, 30 min; H_20, 2 x 1 min. Stained sections were allowed to dry at room temperature protected from light and mounted with Canadian Balsam. Sections were examined using an Olympus IX81 fluorescent microscope equipped with a DP71 Olympus camera.

Quercetin quantification

To evaluate the stability of the decoction of AS, quercetin concentrations were assessed weekly according to a standard procedure in a Waters modular HPLC system (Waters Associates, Milford, MA.).[42] Separation of constituents was achieved by reverse-phase HPLC using a C18 column (Phenomenex,USA) with 5 mm particle size. A binary HPLC pump (Waters 1525) with a 717 plus autosampler Waters and a photodiode array detector Waters 2998 linked to Empower 2 (Waters) chromatography data software was utilized. The temperature of the column was set at 30 °C. The mobile phase used was: (A) 100 % MeOH, (B) 0.5 % H3PO4 pH= 2,5 % MeOH, at 0.7 mL/min. The gradient system consisted of (min/%B): 0/80, 40/0, 41/80, 47/80. The eluant was monitored by photodiode array detection at 375 nm and spectra of products obtained between 210-600 nm.

Plasma sample preparation

Control rats treated with decoction during 7, 14, and 21 days (n= 2/groups) were anesthetized with urethane (2.4 g/kg) and whole blood sample was obtained from the left ventricles, centrifuged at 1500 rpm, for 15 min at 4 °C and supernatants were injected in a Waters modular HPLC system using the same protocol mentioned above.

Statistical analysis

All data are presented as mean ± SD. Data on the behavior, infarction volume and cellular counts were statistically analyzed and the differences between the means of different experimental groups were analyzed by one way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.* Statistical significance was accepted at $p< 0.05$.

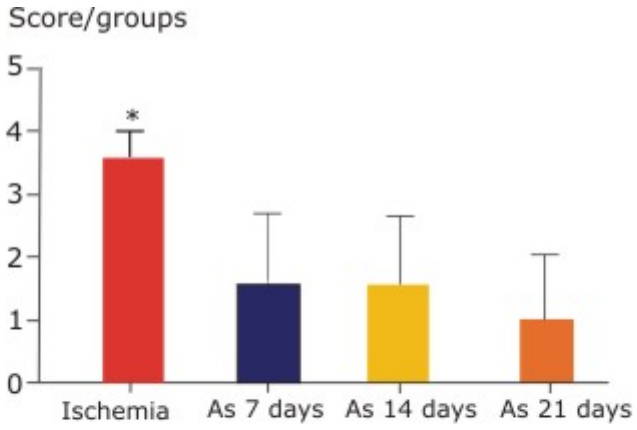
RESULTS

There was no difference in the daily volume intake of the decoction or water neither in weekly body weight increase in experimental and control animals along the experiment. During the first week of treatment animals receiving AS showed a

significant increase in food intake that was not reflected in body weight increases and lasted only one week (data not shown).

Neurological test

Before the pMCAo the neurological score was normal (score = 0) in all animals according to the procedure of Menzies *et al.*[28] Vehicle-treated ischemic rats presented scores corresponding to severe behavioral impairments 24 h after pMCAo (3.4 ± 0.5) (Fig. 1 - Ischemia). The animals pre-treated with the AS decoction during 7 (AS 7 days), 14 (AS 14 days), and 21 (AS 21 days) days before the permanent occlusion showed a significantly improvement in neurobehavioral performance compared to vehicle treated rats: (1.4 ± 1.1); (1.4 ± 0.9), and (1.2 ± 1.1), respectively (Fig. 1).



As: *Achyrocline satureioides*

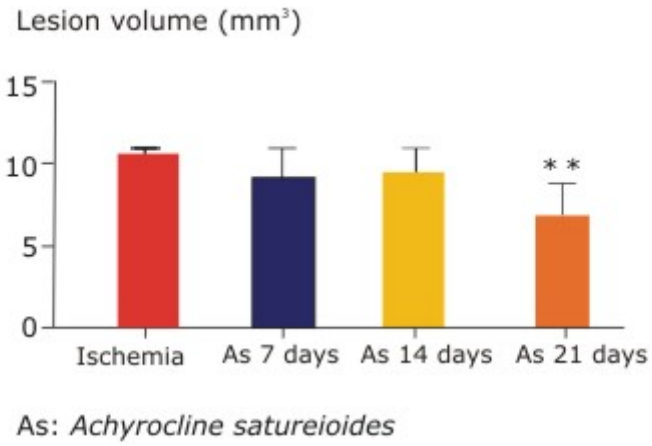
Values are expressed as mean ± SD. Comparisons were performed by ANOVA followed by Tukey-Kramer test.
* Significantly different from the untreated ischemic group ($p < 0.05$).

Fig. 1. Total neurological score in ischemic groups (ischemia) (n= 9) and pre-treated with *Achyrocline satureioides* (AS) decoction during 7 (AS 7 days), 14 (AS 14 days) or 21 (AS 21days) (n= 5 in each group) days before of 24 h of permanent middle cerebral artery occlusion (pMCAo).

Cerebral Infarct Volume

The infarct volume assessed after 24 h of pMCAo was reduced in the ischemic group pre-treated with AS during 21 days (6.9 ± 1.3 mm^3) compared to the ischemic group (Ischemia) that took only water (10.9 ± 3.2 mm^3). The animals pre-treated during 7 (AS 7 days) and 14 days (AS 14 days) with AS showed infarct volume values similar to the ischemic group: 9.2 ± 1.8 mm^3 and 8.8 ± 1.9 mm^3 respectively, (Figs. 2 and 3). No lesion was observed in the groups without pMCAo (control, AS decoction for 7, 14 and 21 days) and in sham operated animals (data not shown).

Fig. 2. Tetrazolium salt (TTC)-stained coronal brain sections obtained after 24 h of permanent occlusion of the medial cerebral artery. In the only ischemic (no treatment) group (A) the lesion (white area) is clearly defined, as well as in the following section (B) corresponding to rats receiving a pre-treatment with *Achyrocline satureioides* (AS) decoction during 7 days. In sections C and D (14 and 21 days of treatment with AS respectively) the non colored area is much smaller, a difference that is clear cut in section D.



Values are expressed as mean ± SD. * Different from ischemic group by ANOVA followed by Tukey-Kramer test ($p < 0.05$).

Fig. 3. Infarct volume (mm3) at 24 h after pMCAo (permanent middle cerebral artery occlusion). Ischemic rats (ischemia) received water (n= 9) or *Achyrocline satureioides* (AS) decoction during 7 (AS 7 days), 14 (AS 14 days) and 21 (AS 21 days) (n= 5/groups) days previous to the pMCAo.

Nissl staining

The figure 4 shows the neuronal population in the striatum of the different experimental groups, stained with Nissl. Striatal neurons of the control animals (Fig. 4A, [Sham], Fig. 4C [As]) showed a conservation of the general histoarchitecture of the white/grey matter, with neurons with round body and clear cytoplasm with Nissl granules plus defined nucleus.

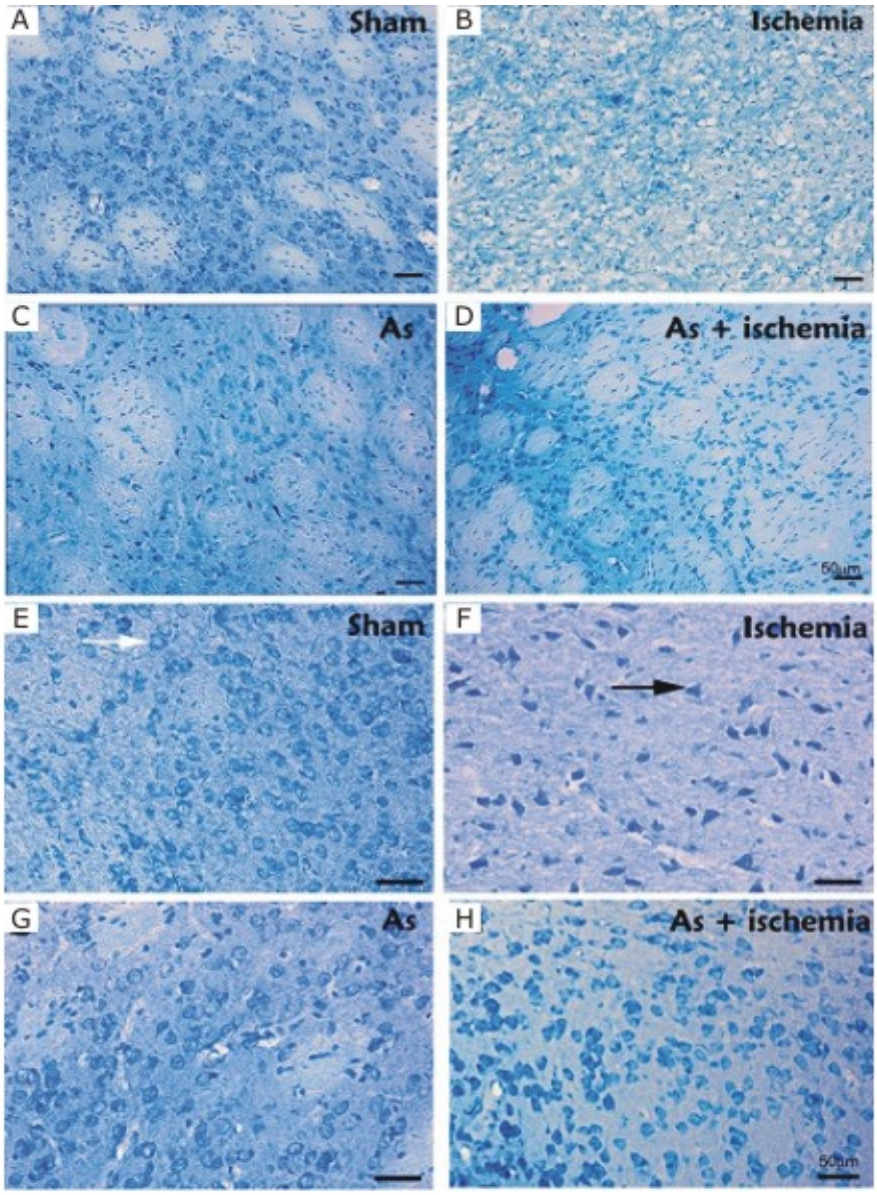
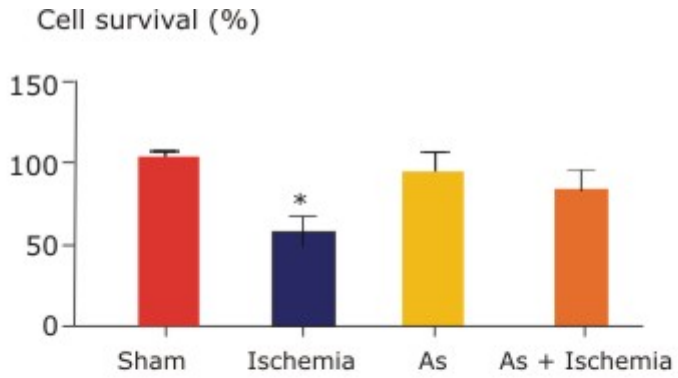


Fig. 4. Histological appearance of striatum ipsilateral to the occluded artery in all experimental groups stained with Nissl: (4A and 4E) Sham operated animals showing a normal distribution of healthy stiatal neurons with euchromatic nucleus and a conserved cytoplasm (arrows). (4B and 4F) Ischemic striatum with predominance of dead and dying neurons as shown by dark cytoplasm and picnotic nucleus plus areas with no neurons and oedema (arrows). (4C and 4G) As pretreatment alone: no images of neuronal death are observed and the global appeareance is close to sham (4A and 4E). (4D and 4H) Ischemia in *Achyrocline satureioides* (AS) treated group. A recovery in the total number of neurons with normal appearance can be seen, as well as a decrease of the oedema.

In ischemic animals (Fig. 4B), there is an almost complete loss of tissue histo-architecture that does not allow even to differentiate the white matter from the surrounding parenchyma. Neurons appear hyperchromatic and have lost the round form of normal ones and phantom nuclei appear scattered in the field.

As it can be observed in figure 4D, the global appearance of the tissue in ischemic groups pre-treated with AS during 21 days was closer to normal (Fig. 4A) with apparent increased number of normal neurons compared to ischemic rats. Nevertheless, general histoarchitecture of the white/grey matter difference is not observed (Fig. 4D). The striatal sections of animals treated with the AS decoction, and no ischemia, did not show evident damage (Fig. 4C).

When the number of striatal neurons with normal structural features were counted in every experimental group and compared with the number of neurons in sham operated animals (Fig. 5), a significant reduction in the number of cells was observed in the ischemic group (50 %) (Fig. 5, [Ischemia]). A significant recovery in the neuronal population was observed with AS pre-treatment during 21 days (80 %) (Fig. 5, [AS + Ischemia]).



As: *Achyrocline satureioides*

Values are expressed as mean ± SD. Comparisons were performed by ANOVA followed by Mann-Whitney test. (N= 6/groups). * Significantly different ($p < 0.05$).

Fig. 5. Number of normal neurons, as seen in previous pictures (Fig. 4), in striatum ipsilateral to the occluded artery, counted in four fields adjacent to the lateral ventricle fields, expressed as percentage of the number of neurons in the same fields in striatum of sham operated animals. There was a significant decrease in the number of neurons in striatum of ischemic animals (ischemia) that recovered in *Achyrocline satureioides* (AS) treated animals during 21 days before ischemia (AS + ischemia), AS treatment alone (AS) did not change the number of neurons.

Fluoro-Jade staining

Since the Fluoro-Jade technique is a neuron-degenerating staining, striatal sections of controls animals show no reactivity. In contrast, the ischemic striatum showed marked staining indicating degenerating neurons. Fewer reactive neurons were seen the groups pre-treated with AS during 21 days). Reactivity is not present in the AS treated groups without ischemia (data not shown).

Quercetin quantification in decoction and plasma of rats

Quercetin concentration in the decoction (mg/mL) measured by HPLC was of 1.14 ± 0.19 mg/mL and showed a significant stability during the 21 days of the experiment. Every rat showed an intake of 43.6 ± 6.64 mL which were equivalent to 50.2 ± 17.9 mg/mL of flavonoids expressed as equivalents of quercetin. Quercetin levels in the rat plasma was 60.4 ± 58.2 ng/mL) after 21 days of administration of the decoction. Treatment alone did not show differences with sham-operated animal.

DISCUSSION

Results obtained in the present study give, for the first time, consistent evidence for a neuroactive capacity of aqueous preparation of AS when administered chronically, in the lesions provoked by permanent focal ischemia in the rat. The decrease in the extension of the lesion demonstrated by TTC, the decrease of degenerative neurons shown by Fluoro-Jade, the increase in the number of surviving neurons and the recovery of motor behaviour are a clear support for the protective capacity.

Importantly, no significant changes between the control animals and the AS- treated group in terms of the increase of body weight or intake of food and decoction were quantified and the preparation was well accepted by the experimental animals. No significant effects on either of the assessment procedures utilized were observed. Additionally, another important distinctive aspect of the present study is the chronicity of the intake of the decoction. Several studies on the effects of plants or even isolated flavonoids have shown that much better bioavailability can be obtained after prolonged administration.[43] An addtional strenght of these results is that they are obtained in the striatum, the brain region most affected in the experimental pMCAo.[8] Previous studies and our own results (Fig. 2) have shown that other brain areas reached by the main artery occluded, the medial cerebral artery, such as the parietal cortex, are less lesioned.[37] This could be an explanation for the apparent discrepancy between the histological and motor behavioral results. The parietal cortex, less lesioned, could get a quicker recovery as shown by improvement in motor deficits.

Morphometric analysis of the infarct volume using TTC staining has been commonly employed to determine the efficacy of cerebroprotective compounds in pre-clinical trials. TTC is a sensitive histochemical indicator of mitochondrial respiratory enzyme function.[44] Therefore, brain lesion identified by TTC staining indicates that mitochondrial function and oxidative respiratory enzyme systems in those tissues are impaired.[6,37] Figure 2 showed a possible influence of the AS pretreatment during 21 days in the neuronal oxidative system.

With these results, AS would join the selected group of plant with demonstrated action on the central nervous system. Only a small group of plants are recognized in Western world as neuroactive, including *Ginkgo biloba, St John's wort, Kava-kava, Valerian, Bacopa monniera and Convolvulus pluricaulis.* Nevertheless, with the exception of *Gikngo biloba* and likely *Bacopa monniera*[10] most of them are effective in anxiety and insomnia and not in protective or anti neurodegenerative actions.[45-47]

Although AS is not utilized popularly for nervous system ailments, previous studies in cells in culture have already shown its protective capacity.[18] Other studies, including data from our group, show that the flavonoids quercetin, luteolin and 3-O-methylquercetin are the main chemical constituents of the AS preparations[9,16,18] and precisely; quercetin and structural related flavonoids are neuroprotective. Our group

was the first to demonstrate that there are structural features of flavones specifically related to the neuroprotection.[48] It is then very likely that the results on neuroactive capacity exerted by AS decoction could be at least partially explained by the actions[49,50] ascribed to its constituent flavonoids: quercetin and luteolin.[17] Studies *in vivo* describe the neuroprotective effects of the luteolin in the ischemia models in rats[51,52] and addition to our group[6,25] recent experimental evidence confirms the neuroprotective effects of quercetin against cerebral ischemia.[53]

Ishisaka et al., using High Performance Liquid Chromatography (HPLC) and mass spectrometry showed that the quercetin can accumulate enough to exert biological activity in rat brain during a chronic oral administration.[54] Chronic flavonoids intake in the diet or after use of plants with ethno-pharmacological profile could reach pharmacological levels in the brain by an accumulative effect.[55] However the mechanisms globally supporting the beneficial effects of these flavonoids in the brain *in vivo* remain to be elucidated. Our result showed that plasma bioavailability of quercetin was increasing along the experiment and it is likely that the same happens in the brain. Nevertheless, these aspects together with experiments with a neutral fraction (flavonoid) of AS are part of our present work, which is intended to provide more data on the role played by flavonoid in the observed effects. Taking into account the broad popular use of AS, there is no toxicity described in experimental study and its high content of flavonoids, makes possible to propose that a chronic oral administration with AS preparation may offer preventive benefits in people aged and with risk of stroke.

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