Aspidosperma pyrifolium Mart: neuroprotective, antioxidant and anti-inflammatory effects in a Parkinson’s disease model in rats


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Keywords
anti-inflammatory action; antioxidant effect; Aspidosperma species; neuroprotection

Abstract

Objectives Aspidosperma species are used for several diseases, especially for malaria in Brazil. Although the genus is object of pharmacological studies, almost none are found on Aspidosperma pyrifolium. We investigate neuroprotective, antioxidant and anti-inflammatory properties of the APSE-Aq fraction (benzoic acid glycosylated derivative) on Parkinson’s disease model.

Methods Male Wistar rats were subjected to a 6-hydroxydopamine injection into the right striatum and treated or not with APSE-Aq (100 or 200 mg/kg, p.o.). The sham-operated group was injected with saline. Two weeks later, animals were subjected to behavioural, neurochemical and immunohistochemical evaluation. The data were analysed by ANOVA and Tukey test.

Key findings The APSE-Aq-treated group shows a partial recovery of behavioural changes as compared with the untreated-6-hydroxydopamine group. A partial recovery was also observed in nitrite contents and lipid peroxidation. APSE-Aq treatments significantly reversed decreases in striatal dopamine and metabolites in the untreated 6-hydroxydopamine group. Immunostainings for markers as tyrosine hydroxylase and dopamine transporter decreased in the untreated 6-hydroxydopamine group and values recovered after APSE-Aq treatments. Similar data were seen for TNF-alpha.

Conclusion APSE-Aq presents neuroprotective, antioxidant and anti-inflammatory activities. Considering that APSE-Aq is chemically related to salicylic acid, it may act on similar targets.

Introduction

The species Aspidosperma pyrifolium Mart. (Apocynaceae) popularly known as ‘pereiro’ is a plant of the caatinga as well as of the Brazilian cerrado biomas. It also occurs in Bolivia and Paraguay. Aspidosperma pyrifolium is widely used in folk medicine and its wood employed in the construction and manufacture of furniture. Species of the genus Aspidosperma are characterized by the presence of indole alkaloids, one secondary metabolite responsible for the defence of plants against predators.

Furthermore, Aspidosperma species, as aqueous crude extracts from the plant bark, are popularly used as anti-inflammatory, antileishmanial and mainly as antiplasmodial in malaria conditions. Although there are many studies on the A. pyrifolium bark chemistry and only one on the chemistry of its seeds, almost none is found on the biological activities of the bioactive components.

In the present work, we focused on the anti-inflammatory, antioxidant and neuroprotective effects of APSE-Aq, a glycosylated derivative of benzoic acid (2-hydroxy-3-O-D-glycopiranosylbenzoic acid) present in A. pyrifolium. This compound is a main component of this species and, as far as we know, was isolated from A. pyrifolium seeds for the first time. Interestingly,
salicylic acid (2-hydroxybenzoic acid) shows many chemical similarities to APSE-Aq and it has been the focus of a number of studies lately.[19–22]

Furthermore, a synthetic derivative of acetylsalicylic acid (aspirin) has been shown to offer a neuroprotective effect in animal models of ischaemic stroke.[23] In addition, another aspirin derivative (5-ASA) presents a broad spectrum of biological activities including anti-inflammatory and neuroprotective, among others.[24] Considering the involvement of inflammatory processes in the pathogenesis of Parkinson's disease[25–27] and the anti-inflammatory action previously shown by us in APSE-Aq,[28] this study aimed to investigate in a model of Parkinson's disease in rats, the behavioural, neurochemical and immunohistochemical effects of APSE-Aq fraction isolated from A. pyrifolium seeds.

Materials and Methods

Plant material
The seeds from A. pyrifolium were collected in the city of Cabrobó, in the state of Pernambuco, Brazil, in June 2010. The voucher is deposited in the Prisco Herbarium Bezerra of the Federal University of Ceará under the number 35524.

Preparation of the aqueous fraction from Aspidosperma pyrifolium
Aspidosperma pyrifolium seeds (835.0 g) were dried, ground and submitted to maceration in hexane (3 × 2 l) at room temperature resulting in 65.2 g of hexanic extract. The residue of this extraction was submitted to maceration in 2 l ethanol for 72 h. This extraction was repeated two times, and the final ethanol solutions were concentrated and named APSE (dark solid: 162.54 g and yield of 19.47%). The liquid–liquid partition of the ethanolic extract resulted in a final aqueous fraction named A. pyrifolium ethanol-aqueous fraction (APSE-Aq) showing the glycosylated benzoic acid derivative (2 hydroxy-3-O-β-D-glycopiranosylbenzoic acid) as its major component. Below (bottom of the left column) is the 1H NMR spectrum of APSE-Aq, highlighting the characteristic splitting pattern of the three contiguous protons in the trisubstituted benzoic ring.

Animals
Male Wistar rats (250–300 g) were from the Animal Facilities of the Federal University of Ceará. The animals were maintained at a temperature of 24 ± 2 °C with a 12 h light/dark cycle and receiving food and water ad libitum. The study was performed according to the Guide for the Care and Use of Laboratory Animals, National Institutes of Health-EUA, 2011, and was approved by the Ethics Committee for Animals experimentation of the Faculty of Medicine of the Federal University of Ceará under the number 91/2014.

Drugs and reagents
6-Hydroxydopamine (6-OHDA), apomorphine and HPLC standards were from Sigma-Aldrich (St. Louis, MO, USA). Ketamine and xylazine were from Konig do Brasil (Santana de Parnai, São Paulo, Brazil). Antibodies for immunohistochemistry assays were from Santa Cruz Biotechnology (Dallas, TX, USA) or Merck Millipore (Darmstadt, Germany). All other reagents were of analytical grade.

Parkinson's disease model
The neurotoxin 6-OHDA continues to be a valuable tool used as a model for Parkinson’s disease in rats.[29] The animals were anaesthetized with a combination of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and subjected to trichotomy of the superior region of the head. This was followed by the animals’ fixation in the stereotaxic apparatus and intrastriatal injection of 6-OHDA at two points (6 µg/point of a 6-OHDA solution in saline containing 0.2% ascorbic acid) at a final dose of 12 µg/2 µl. The 6-OHDA injections into the right striatum showed the following coordinates[30]: 1st point, AP: +0.5; ML: –2.5; DV: +5.0; 2nd point, AP: –0.9; ML: –3.7; DV: +6.5 from the bregma, and for that a 5 µl Hamilton syringe was used. The syringe was left in the place for 5 min to assure the complete solution diffusion. After the stereotaxic surgery, the incision was sutured and animals returned to their cages for
recuperation. The sham-operated (SO) animals were subjected to all procedures, except that saline was injected into the two points.

**Experimental protocol**

The animals were divided into four groups of 6–15 animals each as follows: SO (control), untreated 6-OHDA group and treated 6-OHDA groups (6-OHDA + APSE-Aq, 100 mg/kg and 6-OHDA + APSE-Aq, 200 mg/kg). The APSE-Aq doses were chosen based on previous experiments.[28] The treatment started 1 h before the intrastriatal injection of 6-OHDA and continued for the next 14 days. At day 15, after the stereotaxic surgery, the animals were subjected to behavioural testing, and at the next day, they were euthanized for brain dissection and neurochemical and immunohistochemical studies (these were performed in slices from three animals per group).

**Apomorphine-induced rotational behaviour**

The test of apomorphine-induced rotational behaviour is widely used for assessing the effects of lesions to the dopaminergic system in rodent models of Parkinson’s disease. The animals were injected with apomorphine (1 mg/kg, s.c.), and the number of rotations/h was determined. This parameter is related to the extent of dopamine depletion, after the unilateral 6-OHDA lesion. The circling behaviour is the result of the unbalance, in the nigrostriatal dopaminergic pathways, between the right (lesioned) and left (unlesioned) brain hemispheres after the 6-OHDA lesion. It is a quantifiable motor deficit and an important paradigm in this model.[31,32]

**Rota rod test**

The rota rod is a standard test of motor coordination, balance and fatigue in rodents and is especially sensitive in detecting cerebellar dysfunction. Motor deficits are usually observed in the Parkinson’s disease model in rodents. Basically, the animal is placed on a rotating bar, under continuous speed (12 rpm/min), and the time latency/min to fall from the bar is recorded.[33] This test was performed at day 15.

**Neurochemical determinations of DA, DOPAC and HVA by HPLC**

At the next day after the behavioural tests, the animals were euthanized for decapitation and tissue dissection. The striatal contents of DA, DOPAC and HVA were determined by HPLC. Homogenates were prepared in 10% HClO₄ and centrifuged at 4 °C (15,339 x g, 15 min). The supernatants were filtered, and 20 μl was injected into the HPLC column. For that, an electrochemical detector (model L-ECD-6A, from Shimadzu, Kyoto, Japan) coupled to a column (Shim-Pack CLC-ODS, 25 cm) with a flow of 0.6 ml/min was employed. A mobile phase was prepared with monohydrated citric acid (150 mM), sodium octyl sulphate (67 mM), 2% tetrahydrofuran and 4% acetonitrile, in deionized water. The mobile phase pH was adjusted to 3.0 with NaOH (10 mM). Monoamines were quantified by comparison with standards, processed the same manner as the samples. The results are expressed as ng/g tissue.

**Determination of nitrite contents**

In this assay, the Griess reagent (1 part 0.1% naphthylethylenediamine dihydrochloride in distilled water plus 1 part 1% sulphanilamide in 5% H₃PO₄) indicates the presence of nitrites in the sample. Striatal homogenates (10% in KCl buffer) were centrifuged (11,269 x g for 10 min), 100 μl supernatants were added to 100 μl Griess reagent, and this mixture stayed on RT for 10 min. The standard NaNO₂ curve was obtained (in spectrophotometer, at 520 nm) and used for calculating the results expressed as μmol nitrite per g tissue.[34]

**Determination of lipid peroxidation by thiobarbituric acid reactive substances**

Lipid peroxidation expresses oxidative stress induced by ROS reactivity. A largely used method for measuring it is the determination of malondialdehyde (MDA) in biological samples.[35] Although the lipid peroxidation products are MDA and 4-hydroxy-2-nonenal (4-HNE), MDA is a good biomarker of oxidative stress and end product of lipid peroxidation.[36] Striatal homogenates (10%) in 1.15% KCl were added (250 μl) to 1 ml 10% TCA, followed by addition of 1 ml 0.6% thiobarbituric acid. After agitation, this mixture was maintained in a water bath (95–100 °C) for 15 min. Then, the mixture was cooled on ice and centrifuged (4000 rpm/5 min). The thiobarbituric acid reactive substances (TBARS) content was determined in a plate reader, at 540 nm, with results expressed in μmol MDA per g tissue. A standard curve with several MDA concentrations was also performed.

**Immunohistochemistry assays**

Brain striatal sections (5 μm) from three animals/group were fixed in 10% buffered formol, for 24 h, followed by a 70% ethanol solution. The sections were embedded into
paraffin wax for slice processing on appropriate glass slides. These were placed in the oven at 58 °C, for 10 min, followed by deparaffinization in xylol and rehydration in alcohol at decreasing concentrations, and washed in distilled water and PBS (0.1M sodium phosphate buffer, pH 7.2), for 10 min. The endogenous peroxidase was blocked with a 3% hydrogen peroxide solution, followed by incubation with the appropriate primary antibody, for tyrosine hydroxylase (TH), dopamine transporter (DAT) or TNF-alpha, and diluted according to the manufacturer’s instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Millipore, Billerica, MA (USA), for 2 h, at room temperature in a moist chamber. The glass slides were then washed with PBS (three times, 5 min each) and incubated with the biotinylated secondary antibody, for 1 h, at room temperature. Then, they were washed again in PBS and incubated with streptavidin peroxidase, for 30 min, at room temperature. After another wash in PBS, they were incubated in 0.1% DAB solution (in 3% hydrogen peroxide). Finally, the glass slides were washed in distilled water and counterstained with Mayer’s haematoxylin, washed in tap water, dehydrated in alcohol (at increasing concentrations), diaphanized in xylol and mounted on Entelan® for optic microscopy examination.

The immunostaining intensity was quantified by the ImageJ software (National Institute of Health, Bethesda, MD, USA), and the results were expressed as relative optical density using the entire image area.

Statistical analyses
For statistical analyses, One-way ANOVA, followed by Tukey as the post hoc test, was used for multiple comparisons. The photomicrograph data were quantified by the ImageJ software (NIH). Differences were considered significant at *P* < 0.05.

Results
Apomorphine-induced contralateral rotations
The untreated 6-OHDA group presented around 209 rotations/h as related to the SO group which showed no circling behaviour. On the other hand, the 6-OHDA groups after APSE-Aq treatments (100 and 200 mg/kg) presented 124 and 70 contralateral rotations/h, respectively, suggesting a neuroprotective effect of this *A. pyrifolium* fraction (Figure 1).

Rota rod test
As expected, the animals of the untreated 6-OHDA group felt more than five times/min from the rotating bar, indicating motor incoordination. This motor alteration was partially reversed in the 6-OHDA+APSE-Aq100 and 6-OHDA+APSE-Aq200 groups that showed 1.00 and 0.67 falls/min as compared with the SO group (Figure 2).

Nitrite/nitrate measurements in rat ipsilateral striata
There was a two times increase in nitrite/nitrate concentrations in the ipsilateral striata (lesioned) from the
untreated 6-OHDA group as compared with the SO group. On the other hand, increases of only 30% and 11% were observed in the 6-OHDA groups after treatments with APSE-Aq at the doses of 100 and 200 mg/kg, respectively (Figure 3).

**TBARS determination in rat ipsilateral striata**

An almost two times increase in lipid peroxidation was demonstrated in the untreated 6-OHDA group as compared with the SO group. This value went down to 64% and 63% after treatments with APSE-Aq at the doses of 100 and 200 mg/kg, respectively (Figure 4).

**DA and its metabolites measurements in rat ipsilateral striata**

Dopamine concentrations decreased around 90% in the right lesioned striatum of the 6-OHDA group as compared to the SO group. Much lower decreases were observed in the 6-OHDA groups after treatments with the doses of 100 mg/kg (45%) and 200 mg/kg (only 13%, which was not significantly different from the SO group). Although similar decreases were observed in DOPAC contents in the untreated 6-OHDA group (93%), the decreases were of 77% and 61% for the 6-OHDA + APSE-Aq100 and 6-OHDA + APSE-Aq200, respectively, and also significantly different from the SO group. As far as HVA concentrations are concerned, decreases of 75% were observed in the untreated 6-OHDA group, while 58% and 47% decreases were demonstrated in the 6-OHDA groups after treatments with APSE-Aq at the two doses. These values were significantly different only from the SO group (Figure 5).

**Immunohistochemical assays for tyrosine hydroxylase and the dopamine transporter in the rat ipsilateral striata**

A 95% reduction in the TH immunostaining was demonstrated in the right lesioned striata from the untreated 6-OHDA group. A partial reversion and then lower reductions in TH immunostaining were observed in 6-OHDA groups after APSE-Aq treatments with the 100 (79%) and 200 mg/kg (66%). DAT immunostaining decreased by 97% in the untreated 6-OHDA group as related to the SO group, and this value was greatly reversed after 100 (37% decrease) and 200 mg/kg (20% decrease) APSE-Aq treatments (Figure 6).

**Immunohistochemical assay for TNF-alpha in the rat ipsilateral striata**

A 20 times increase in TNF-alpha was observed in the untreated 6-OHDA group as related to the SO group. However, only a two times increase was observed in the 6-OHDA after treatment with APSE-Aq (100 mg/kg). The 6-OHDA + APSE-Aq200 showed a value even lower that shown by the SO group (Figure 7).

**Discussion**

The genus *Aspidosperma* is a most important one from the Apocynaceae family occurring in Brazil, with species...
presenting medicinal and economical value. Chemically, species from this genus show a predominance of indole alkaloids, which constitute good chemotaxonomic markers for the botanical classification of the Aspidosperma genus.

Popularly, the aqueous infusion from the bark of Aspidosperma species is used as antinociceptive, anti-inflammatory and antidiabetic, in the treatment of leishmaniasis and mainly against fever in the treatment of malaria. This antimalarial effect is common among species of the Aspidosperma genus. However, considering the great variety of chemical constituents shown in the seeds in the present work, we decided to investigate the neuroprotective, antioxidant and anti-inflammatory properties of a

Figure 5 Effects of the benzoic acid glycosylated derivative fraction (APSE-Aq) from Aspidosperma pyrifolium seeds on striatal dopamine (DA) and its metabolites (DOPAC and HVA) contents. DA: a. vs SO, \(q = 8.657, P < 0.001\); b. vs 6-OHDA + APSE-Aq100, \(q = 4.120, P < 0.05\); c. vs 6-OHDA + APSE-Aq200, \(q = 5.970, P < 0.01\); d. vs SO, \(q = 4.116, P < 0.05\). DOPAC: a. vs SO, \(q = 11.65, P < 0.001\); b. vs 6-OHDA + APSE-Aq200, \(q = 4.240, P < 0.05\); c. vs SO, \(q = 10.39, P < 0.001\); d. vs SO, \(q = 8.348, P < 0.001\). HVA: a. vs SO, \(q = 11.76, P < 0.001\); b. vs 6-OHDA + APSE-Aq200, \(q = 4.116, P < 0.05\); c. vs SO, \(q = 9.123, P < 0.001\); d. vs SO, \(q = 6.784, P < 0.001\) (one-way ANOVA and Tukey as the post hoc test).

Figure 6 Effects of the benzoic acid glycosylated derivative fraction (APSE-Aq) from Aspidosperma pyrifolium seeds on the striatal tyrosine hydroxylase (TH) and dopamine transporter (DAT) immunoreactivities. TH: a. vs SO, \(q = 51.39, P < 0.001\); b. vs 6-OHDA + APSE-Aq100, \(q = 9.872, P < 0.001\); c. vs 6-OHDA + APSE-Aq200, \(q = 15.85, P < 0.001\); d. vs SO, \(q = 42.25, P < 0.001\); e. vs SO, \(q = 33.25, P < 0.001\). DAT: a. vs SO, \(q = 39.46, P < 0.001\); b. vs 6-OHDA + APSE-Aq100, \(q = 23.32, P < 0.001\); c. vs 6-OHDA + APSE-Aq200, \(q = 29.34, P < 0.001\); d. vs SO, \(q = 14.88, P < 0.001\); e. vs 6-OHDA + APSE-Aq200, \(P < 0.01\); f. vs SO, \(q = 8.540, P < 0.001\) (one-way ANOVA and Tukey as the post hoc test). [Colour figure can be viewed at wileyonlinelibrary.com]
glycosylated derivative-rich fraction (APSE-Aq) extracted from *A. pyrifolium* seeds in a Parkinson’s disease (PD) model in rats. We showed that APSE-Aq partly blocked the behavioural changes observed in the untreated 6-OHDA group decreasing the apomorphine-induced rotation and motor incoordination, important parameters demonstrated in this experimental model of PD. This fraction also partly blocked the reductions in DA and metabolite contents, a hallmark of PD, as demonstrated in the lesioned right striatum of the untreated 6-OHDA group. In PD, besides the degeneration of dopaminergic neurons in the substantia nigra pars compacta, inflammatory responses are also present pointing out to its important contribution to the pathogenesis of the disease and progressive loss of nigral dopaminergic neurons.\[25,26\]

Interestingly, the NF-\(\kappa\)B transcription factor is considered a prototypical proinflammatory signalling pathway and represents a convergence point important to neurodegeneration.\[46\] Furthermore, knowing that salicylates derivatives elicit protection via NF-\(\kappa\)B blockade opens new windows for pharmacological interventions.\[47,48\] Another work\[49\] showed neuroprotective effects of aspirin and salicylic acid in a model of MPTP-induced dopamine depletion that, according to the authors, could be related to their hydroxyl radical scavenging activity. Later, another work\[50\] demonstrated a similar action of sodium salicylate in a chronic rotenone model of Parkinson’s disease. The antioxidant and anti-inflammatory properties of sodium salicylate and its derivatives could probable be responsible for its neuroprotective potential.

Oxidative stress contributes to dopaminergic neurons degeneration in PD and is closely linked to other components of the degenerative process as nitric oxide toxicity and inflammation.\[51\] In the present work, we showed that APSE-Aq significantly decreased the nitrite contents and lipid peroxidation seen in the right lesioned striatum of the untreated 6-OHDA groups. Similarly, antioxidant effects of aspirin and salicylic acid were seen in rat brain slices subjected to hypoxia.\[52\] Oxidative stress has been implicated to both initiation and progression of PD, and it is believed that oxidative and nitrosative stress plays an important role in the pathogenesis of this disease.\[53–55\]

The intrastriatal injection of 6-OHDA is known to decrease the immunoreactivities for tyrosine hydroxylase (TH), the rate-limiting enzyme for DA biosynthesis and to the dopamine transporter (DAT), both important biomarkers in PD.\[32,56,57\] Our data corroborated with these findings and showed that APSE-Aq significantly protected the untreated 6-OHDA group from dopaminergic neurons loss partly avoiding the TH and DAT immunoreactivity decreases.

Although aspirin irreversibly inhibits COX1 and COX2, naturally occurring salicylates and salicylic acid (SA) are only weak COX1 and COX2 inhibitors, although SA presents most of the pharmacological effects of aspirin. This suggests that some additional SA targets must exist besides cyclooxygenases as recently suggested by Klessig et al.\[58\] Several of these SA/aspirin targets are associated with inflammation (as TNF-alpha and NF-\(\kappa\)B) and energy metabolism, and evidences show that SA binds and alters the activity of multiple plant proteins and these findings...
represent a shift from the paradigm that hormones mediate their functions via one or a few receptors.\textsuperscript{(23)} In contrast, low micromolar SA levels in vivo alter the disease-associated activity of high mobility group box 1 (HMGB1)\textsuperscript{(59)} and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).\textsuperscript{(60)}

High mobility group box-1 is a ubiquitous and abundant nonhistone DNA-binding protein and is also an important proinflammatory molecule once released into the extracellular space from the nuclei. HMGB1 is involved in a diverse range of CNS diseases, including Parkinson’s disease, and anti-HMGB1 antibody has been shown to improve neurological deficits in rats.\textsuperscript{(61)}

Glyceraldehyde 3-phosphate dehydrogenase is a cytosolic enzyme that plays a central role in the production of energy, via glycolysis and energy metabolism. In addition, other functions of this protein have come to light in the past few decades, including its participation in DNA repair\textsuperscript{(62)} and transcription.\textsuperscript{(63)} Furthermore, GADPH has been assumed to be a target for the neuroprotective effects of some antiparkinsonian compounds as deprenyl.\textsuperscript{(64)} Thus, GADPH is a major suspect in neurodegenerative diseases, including Parkinson’s disease.\textsuperscript{(65)} Others have revealed the effect of salicylates on GADPH-mediated cell death and its central role in neurodegeneration.\textsuperscript{(66,67)}

In conclusion, we showed that the APSE-Aq fraction isolated from \textit{A. pyrifolium} seeds offers neuroprotection in a model of PD which is probably associated with its anti-inflammatory and antioxidant properties. Although in the present work no investigation focusing on HMGB1 or GAPDH was carried out, it is possible that these molecules be also a target for APSE-Aq, considering its chemical similarities with other salicylates derivatives as aspirin. Nevertheless, other investigations have to be pursued in order to clarify this issue.

**Declarations**

**Conflict of interest**

The Authors declare that there is no conflict of interest.

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