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Strictosidinic acid, isolated from *Psychotria myriantha* Mull. Arg. (Rubiaceae), decreases serotonin levels in rat hippocampus

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ABSTRACT

Psychotria is a complex genus whose neotropical species are known by the presence of glucosidic monoterpene indole alkaloids. These compounds are able to display a large range of effects on the central nervous system, such as anxiolytic, antidepressant, analgesic, and impairment of learning and memory acquisition. The aims of this study were to investigate the effects displayed by strictosidinic acid, isolated from *Psychotria myriantha* Mull. Arg. (Rubiaceae) leaves, on monoamine levels in rat hippocampus and on monoamine oxidase activity. A significance ($p<0.01$) of 83.5% reduction in 5-HT levels was observed after intra-hippocampal injection (20 μg/μl). After treatment by intraperitoneal route (10 mg/kg), a 63.4% reduction in 5-HT levels and a 67.4% reduction in DOPAC values were observed. The results indicate that strictosidinic acid seems to act on 5-HT system in rat hippocampus, possibly inhibiting precursor enzymes of 5-HT biosynthesis. The decrease verified in DOPAC levels suggests a role of strictosidinic acid in the dopaminergic transmission, probably due to an inhibition of monoamine oxidase activity, confirmed by the enzymatic assay, which demonstrated an inhibitory effect on MAO A in rat brain mitochondria.

Introduction

Psychotria (Rubiaceae) is a taxonomically complex genus [1]. In the folk medicine *Psychotria* species are employed all over the world in the treatment of several diseases such as: diarrhea and intestinal parasites [2], snake bites [3], viral and bacterial infections [4,5], hypertension, cardiovascular dysfunctions, mental disturbs and alimentary disorders [6]. *Psychotria viridis* is one of the most cited species, probably due to its effects on central nervous system. This specie, along with *P. carthagenensis* and *Banisteriopsis caapi*, is constituent of the hallucinogenic "ayahuasca" beverage, traditionally used for religious practice in Amazonian region [7].

Neotropical species, belonging to the subgenus *Heteropsychotria*, have been subjected to chemical and pharmacological investigations, revealing the presence of bioactive glucosidic monotepene indole alkaloids (MIAs) [8–14]. Psychollatine, the major MIA isolated from *Psychotria umbellata* Vell., exhibited mild analgesic effects against a number of algogenic stimuli [15], anxiolytic (7.5 and 15 mg/kg) and antidepressant effects (3 and 7.5 mg/kg) in mice models [16,17]. In higher doses (100 mg/kg), psychollatine impaired the acquisition of learning and memory

Abbreviation: MIA, monoterpene indole alkaloids.

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consolidation [16], suggesting the modulation of different neurotransmitter systems, such as glutamate, opioid and serotonergic pathways. Alkaloid extract from *Psychotria myriantha* Mull. Arg., a shrub occurring in southern Brazil, showed dose-dependent analgesic effect (200 mg/kg), partially reversed by naloxone in the hot plate model [18], suggesting the involvement of NMDA receptors in its mechanism of action. Strictosidinic acid, a glycoside indole monoterpene alkaloid isolated from leaves of this specie, is able to inhibit in vitro polymorphonuclear leukocytes (PMN) chemotaxis [13] and it has shown peripheral analgesic and antipyretic activities in mice after oral administration [19]. Moreover, strictosidinic acid (10 mg/kg) seems to act on 5-HT and DA systems in rat striatum, increasing the monoamines metabolism in this brain area [20]. Fractions of *P. suterella* and *P. laciniata* were able to inhibit rat brain monoamine oxidase A (MAO-A) in concentrations ranging from 0.5 to 135 μg/mL. The chemical analysis of the active fractions suggested that the enzymatic inhibition could be attributed to the alkaloids E/Z-vallesiachotamine [21], which were previously described in other *Psychotria* species.

Dopamine (DA), serotonin (5-HT) and their main metabolites (3,4-hydroxyindoleacetic acid, DOPAC; homovanilic acid, HVA; and 5-hydroxyindole acetic acid, 5-HIAA) represent important monoamines and metabolites-derived neurotransmitters. DA is one of the most important excitatory neurotransmitters, being widely distributed in the mammalian brain, including the hippocampus. The central dopaminergic transmission is involved in a variety of behaviors and brain functions, including motor activity, cognition, emotion, positive reinforcement, food intake and endocrine regulation [22]. The serotonergic transmission on the CNS has been related with multiple behaviors such as food intake, endocrine regulation, activity rhythm, sexual behavior, sleep, and emotional states [23]. Alterations in 5-HT transmission are related with some neurological and psychiatric illness including migraine, hallucinations, anxiety and depression [24]. In addition, changes on DA transmission are associated with Parkinson disease and schizophrenia [25].

Monoamine oxidases (MAOs) are mitochondrial outer membrane-bound flavoenzymes which catalyze the oxidative deamination of several important neurotransmitters, including 5-hydroxytryptamine (5-HT, or serotonin), histamine and the catecholamines dopamine, norepinephrine and epinephrine [26]. Two subtypes of MAO, MAO-A and MAO-B, are similar in their primary sequences but have different substrate and inhibitor affinities. MAO-A is inhibited by low concentrations of clorgyline and catalyzes the oxidation of 5-HT, whereas MAO-B is inhibited by low concentrations of *l*-deprenyl or pargyline and is active towards benzylamine and 2-phenylethylamine. Dopamine, norepinephrine, tryptamine and tyramine are oxidized by both forms of the enzyme in most species [26,27]. MAO-A inhibitors have been proven to be effective in the pharmacological treatment of depression and further developments have provided reversible inhibitors of MAO-A, which offer antidepressant activity without the serious side effects of the earlier inhibitors. On the other hand, selective inhibitors of MAO-B have found a therapeutic role in the treatment of Parkinson's disease [28].

Considering the relevance of monoamines (DA and 5-HT) in brain functions and the previous results found to *P. myriantha* and strictosidinic acid, the aims of this study were (i) to investigate the effect of intra-hippocampal and acute intraperitoneal (i.p.) strictosidinic acid treatment in the monoamine levels and their metabolites in rat hippocampus, and (ii) to evaluate the effect displayed by strictosidinic acid on MAO-A and MAO-B activities, employing rat brain mitochondria as enzymatic source.

Experimental methods

Chemicals

Dopamine (DA), 3,4-dihydroxyphenyl acetic acid (DOPAC), 3-metoxytyramine (3-MT), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), kynuramine dihydrobromide, pargyline hydrochloride, clorgyline hydrochloride, 4-hydroxyquinoline (4-OH), dimethyl sulfoxide (DMSO), bovine albumin (BSA), HEPES and D-mannitol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3,4-Dihydroxybenzylamine (DHBA) was obtained from Aldrich Chemical Company Inc. (USA). Sucrose, potassium chloride and sodium chloride were acquired from Labsynth (Diadema, SP, Brazil). Sodium phosphate monobasic monohydrate and sodium phosphate dibasic dodecahydrate were purchased from Merck (Darmstadt, Germany). All remaining chemicals used were of analytical grade and were purchased from F. Maia (Cotia, SP, Brazil). Stock solutions of kynuramine, pargyline, clorgyline and 4-OH were prepared in PBS buffer (pH 7.4) and maintained at −20 °C for until six months.

Plant material

P. myriantha was collected in Reserva Estadual do Turvo, Derrubadas, Rio Grande do Sul, Brazil and identified by M. Sobral. A voucher specimen (M. Sobral et al., 8913) was deposited in the ICN Herbarium (Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil).

Extraction and isolation

The alkaloid was extracted from leaves of *P. myriantha* as previously described by Simões-Pires et al. [13]. Briefly, dried leaves were extracted with EtOH at room temperature. The extract was concentrated under vacuum at 40 °C and the alkaloids fraction was obtained by acid/base extraction, being the alkaline extracts partitioned with dichloromethane and n-butanol. The butanolic extract was purified by semi-preparative HPLC using Symmetry-Prep column (7 μm, 19×150 mm,

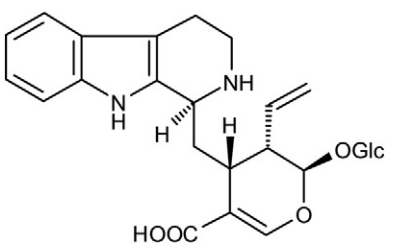

Fig. 1. Chemical structure of the alkaloid strictosidinic acid.

Waters), MeOH:H_2O (30:70) with Et_3N 2 mM, flow rate 10 mL/min, UV 254 nm) providing strictosidinic acid (Fig. 1), whose structure was further elucidated by 1 and 2D NMR and HRMS [7]. Before the biological experiments, strictosidinic acid was submitted to UHPLC/HR-TOF-MS analyses in order to confirm its identity and purity. The HRMS spectra of strictosidinic acid afforded a pseudo-molecular ion at $[M+H]^+$ m/z 317.2181, calculated for C26H33N2O9 (Supplementary Data).

Animals

Experiments for determination of monoamine levels in hippocampus were performed with male Wistar rats, weighing 200–250 g, acquired from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS). The brain mitochondrial fractions used in the enzymatic assays were obtained from male Wistar rats (120 days old, 300–400 g) purchased from Biotério Central of Universidade Federal do Rio Grande do Sul (UFRGS). Animals were housed for one week in our animal facility, under controlled environmental conditions (22 ± 1 °C, 12 h light/dark cycle, free access to food [commercial standard rodent cube diet Nuvilab, CR1] and water), until the beginning of experiments. All animal experiments were carried out in accordance with the guidelines for care and use of experimental animals contained in the National Research Council Guide for the Care and Use of Laboratory Animals. The experiments were performed after approval by the University Ethics Committee (# 2003139).

Brain monoamines

Treatments

For intra-hippocampal treatment, the animals were divided in control (n = 5) and treated (n = 8) groups. The administration was realized by the infusion of a 20 μg/μl solution of strictosidinic acid through canules implanted bilaterally 1 mm above the pyramidal cells of the CA1 hippocampus sub-region. The rats were euthanized by decapitation 15 min after the injection and the hippocampus were removed, weighed and stored at −80 °C until analysis for monoamine levels content.

The intraperitoneal (i.p.) treatment was performed in control (n = 4) and treated (n = 5) groups; the last one received strictosidinic acid 10 mg/kg. Sixty minutes after treatment administration, rats were euthanized by decapitation, brains were rapidly removed and hippocampus were dissected from the surrounding tissue, weighed, frozen in liquid nitrogen and stored at −80 °C until analysis for monoamine levels content.

HPLC-ED analysis

Hippocampus were homogenated in perchloric acid 0.1 M with sodium bisulphite 1 mM (1:10 m/V) containing internal standard 3,4-dihydroxybenzylamine (DHBA) in a concentration of 1.6 μM, using a homogenizer (Ultra-Turrax T8, IKA Labortechnik, Germany). The samples were then centrifuged at 14000 × *g*, for 40 min at 4 °C and the supernatants, after filtration, were injected onto high performance liquid chromatography coupled with an electrochemical detection system (HPLC-ED).

Monoamine determination in the hippocampal supernatants were carried out using an HPLC-ED technique previously reported [29] with some modifications and previously validated. The chromatographic system consisted of a Waters 510 pump (Waters, Milford, MA, USA) equipped with a 464 Pulse Electrochemical Detector (Waters, Milford, MA, USA) and a Rheodyne injector (loop 20 μl). The glassy carbon electrode potential was 800 mV (vs Ag/AgCl reference electrode). Empower software was used for data acquisition and peak integration. A Waters Spherisorb ODS1 column (5 μm, 4.6 × 250 mm) was employed to separate the peaks. The mobile phase consisted of 0.36 mM heptanesulfonic acid, 100 mM formic acid, 1 mM citric acid, 0.1 mM EDTA, diethylamine (0.25% V/V), acetonitrile (2.2% V/V) adjusted to pH 4.2–4.5 and filtered by vacuum thorough a 0.45 μm filter, and degassed by vacuum prior to ultrasound bath to eliminate air bubbles, which interfere with the electrochemical assay. Analyses were performed in isocratic mode at a flow rate of 0.8 ml/min and at room temperature for a 35 min run time.

Student's *t*-test was employed for comparisons between control and experimental groups and the results were expressed as mean ± S.E.M (standard error of the mean) from three determinations. When the *P* value was <0.01 (*), the difference was considered significant.

Monoamine oxidase inhibition assay

Preparation of brain mitochondria

Animals were euthanized by decapitation. Brains were immediately removed and washed in ice-cold isolation medium with the following composition: 10 mM HEPES; 68 mM sucrose; 10 mM KCl; 220 mM D-mannitol; and 0.1% BSA (pH 7.4). Brain tissues were mechanically homogenized in a Potter-Elvehjem tissue grinder in 10 vol. of the ice-cold buffer. Then, tissue homogenates were centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 11500 × *g* for 15 min at 4 °C. After, the supernatant was removed and the resulting pellet was suspended in 3 ml of phosphate buffered saline solution (PBS, pH 7.4) and stored at −80 °C. Protein concentrations were determined by Peterson's modification of the procedure proposed by Lowry and co-workers.

Enzymatic incubations

MAO inhibition assays were conducted with a fluorescence method based in endpoint reading, using kynuramine as non-selective substrate for MAO A and MAO B. The incubations were performed according to previously reported by Van Diermen et al. [30], with minor modifications. Briefly, assays were carried out in black polystyrene 96-well microtiter plates in a final volume of 200 μl. The concentration of kynuramine was maintained in 50 μM, and the final protein concentration was adjusted to 0.1 mg/ml.

For the MAO A inhibition assays, the wells containing 120 μl of PBS (pH 7.4), 5 μl of pargyline 10 μM (to get a final concentration of 250 nM), 25 μl of the sample solution prepared in PBS and DMSO (to get a final concentration of 1% DMSO), and 40 μl of the mitochondrial suspension (to get a final protein concentration of 0.1 mg/ml) were preincubated at 37 °C for 30 min. The MAO B inhibition assays were performed in the same way that the MAO A incubations, except by the use of 5 μl of clorgyline 10 μM to replace the pargyline solution. As positive control for MAO activity (without inhibition), 5 μl of a 40% DMSO solution (in PBS) were used in place of the inhibitor, in the absence of samples.

As positive control for MAO A and MAO B activities, 5 μl of a 40% DMSO solution (in PBS) and 5 μl of pargyline 10 μM (or clorgyline 10 μM) were used in the absence of the samples. Strictosidinic acid was tested in concentrations ranging from 0.1 to 500 μg/ml (10 concentrations). Data analysis was performed with Prism 5.0 (GraphPad Software, Inc., CA, USA). The degree of inhibition IC_{50} was assessed by a sigmoidal dose–response curve. The standard deviation was calculated for sigmoidal regression.

Results and discussion

In the present study, the concentrations of DA (and their metabolites DOPAC and 3-MT) and 5-HT (and its metabolite 5-HIAA) were quantified by HPLC-ED in rat hippocampus after two treatment schemes with strictosidinic acid: (1) 20 μg/μl by intra-hippocampal injection, and (2) 10 mg/kg by acute intra peritoneal administration. Complementary, the effect of strictosidinic acid on MAO A and MAO B activities was evaluated in mitochondrial fractions obtained from rat brain.

The HPLC-ED method was optimized and validated for the parameters of linearity, limits of detection and quantification (Table 1) and precision (Table 2), appearing to be appropriated for monoamine determination in hippocampus homogenates. When control and experimental group were compared, the hippocampal levels of 5-HT were significantly ($p<0.01$) different in intra-hippocampal treatment (Table 3), while the DOPAC e 5-HT were significantly ($p<0.01$) altered in i.p. treatment (Table 4).

There was an 83.5% reduction in the 5-HT levels after intra-hippocampal injection. When rats received strictosidinic acid by i.p. route, a 63.4% reduction in 5-HT levels, and also a 67.4% reduction in DOPAC values were observed. In spite of the fact that it is not possible to quantify DOPAC levels in intra-hippocampal treatment, these results indicate that the administration of strictosidinic acid by both routs share the same profile. It is possible to believe that the metabolism of strictosidinic acid can contribute to the differences found in both routes of administration. The decrease verified in the DOPAC levels after i.p. injection with strictosidinic acid could also suggest the effect of this alkaloid on monoamine oxidase (MAO) activity in hippocampus. MAO A and MAO B are mitochondrial enzymes that catalyze the oxidative deamination of monoamines to the correspondent aldehyde and free amine, with generation of hydrogen peroxide. The aldehyde is rapidly metabolized by aldehyde dehydrogenase to acidic metabolites. DOPAC is the acidic metabolite from dopamine and it is commonly used as the measure of MAO activity in vitro and in vivo [21].

The effect of strictosidinic acid on MAO A and MAO B was evaluated in mitochondrial fractions from rat brain. In these assays, strictosidinic acid was able to inhibit MAO A ($IC_{50} = 150 \pm 1.25$ μg/ml, maximum inhibition of 68.67%) in a concentration dependent way (Fig. 2). On the other hand, this MIA did not show to inhibit MAO B on the tested concentrations. The inhibition observed on MAO A activity is in agreement with the reduction in DOPAC levels in hippocampus, since that DA is a non-selective substrate for MAO-A and B, being converted to DOPAC by both enzymes. The brain mitochondrial fraction was chosen for the enzymatic experiments because it presents high protein content, allowing testing a great number of samples in multiple concentrations.

Table 1
Summarization of results of the calibration curves for monoamine quantification.

Monoamine	Equation of calibration curve	r^2 Value[a]	Linearity[b] (ng)	LOD[c] (ng/mL)	LOQ[d] (ng/mL)
DA	y = 9394.2 x − 5820960	0.9998	30–54	11.97	36.26
DOPAC	y = 6175.6 x + 95084	0.9995	1–5	10.22	30.96
3-MT	y = 6785.9 x − 461299	0.999	10–20	17.57	52.28
HVA	y = 6577 x + 116318	0.9999	1–5	1.6	4.86
5-HT	y = 6878.8 x + 252832	0.9999	1–10	0.23	0.7
5-HIAA	y = 3472 x + 40463	0.9996	1–5	7.62	23.1

[a] y = Peak area ratio; x = monoamine concentration.
[b] Correlation coefficient of calibration curve.
[c] Limit of detection.
[d] Limit of quantification.

Table 2
Monoamine levels in hippocampus after intra-hippocampal injection of strictosidinic acid 20 μg/μl.

Monoamine	Coefficients of variation (%)	
	Intra-day	Inter-day
DA	1.52	3.09
DOPAC	1.34	2.26
3-MT	0.79	0.88
HVA	–	–
5-HT	3.79	3.33
5-HIAA	3.23	3.5

Table 3
Monoamine levels in hippocampus after intra-hippocampal injection of strictosidinic acid 20 μg/μl.

	Control (n = 5) (nmol/g tissue)	Treated (n = 8) (nmol/g tissue)
DA	20.40 ± 0.95	20.04 ± 0.84
3-MT	9.75 ± 1.24	9.86 ± 1.24
5-HT	1.35 ± 0.11	0.22 ± 0.05*
5-HIAA	3.27 ± 0.24	3.11 ± 0.29

Results expressed as mean ± standard error of the mean.
* $p<0.01$, Student's *t* test.

Table 4
Monoamine levels in hippocampus after acute intraperitoneal injection of strictosidinic acid 10 mg/kg.

	Control (n = 4) (nmol/g tissue)	Treated (n = 5) (nmol/g tissue)
DA	9.32 ± 0.94	7.89 ± 0.37
DOPAC	1.72 ± 0.09	0.56 ± 0.09*
3-MT	8.52 ± 0.82	7.19 ± 0.97
5-HT	1.44 ± 0,12	0.52 ± 0.04*
5-HIAA	0.56 ± 0.10	0.70 ± 0.07

Results expressed as mean ± standard error of the mean.
* $p<0.01$, Student's *t* test.

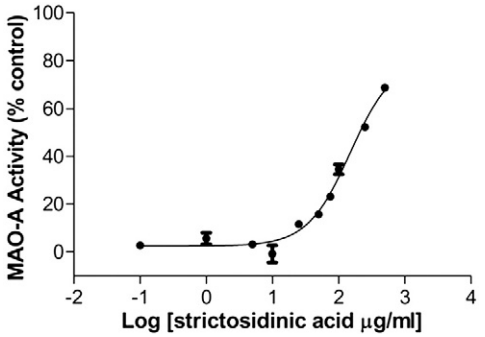

Fig. 2. Effects displayed by strictosidinic acid on MAO-A activity. Strictosidinic acid was tested in ten different concentrations ranging from 0.1 to 500 μg/ml. The degree of inhibition IC_{50} was assessed by a sigmoidal dose–response curve. Each point represents the mean ± SMD of the sigmoidal regression for two independent determinations. ($IC_{50} = 150.1 \pm 1.25$ μg/ml; maximum inhibition = 68.67; Hill Coefficient 1.341; $R^2 = 0.9816$).

The ratio 5-HIAA/5-HT is frequently employed as a serotonin metabolism indicator, since it establishes the 5-HT consume and the formation of its metabolization product [31]. The results obtained in the hippocampus analyses of rats treated with 20 μg/μl strictosidinic acid by intra-hippocampal injection and 10 mg/kg strictosidinic acid i.p. resulted in the reduction of 5-HT tissue levels, without the consequent increase of 5-HIAA values, indicating no increase of 5-HT metabolism in this structure. This may be due to the block of 5-HT synthesis, which is in accordance with previous findings where the inhibition of 5-HT synthesis by *p*-chloroamphetamine reduced the levels of this indolamine in brain structures evaluated [32].

Tryptophan is the biosynthetic precursor of 5-HT through the tryptophan hydroxilase action. The disponibility of this essential amino acid and the activity of this enzyme are the mainly regulatory processes of 5-HT formation. In this way, the tryptophan lack can lead to a 5-HT deficiency in the brain [31]. The monoterpene indole alkaloids are biosynthesized through tryptophan decarboxylation, showing the existence of a common precursor between 5-HT and strictosidinic acid. Therefore, it is possible to imagine an action of strictosidinic acid in the precursor enzymes of 5-HT, which can justify the reduction observed in the hippocampus. The role of 5-HT in learning and memory is not clearly known, but there are many reports indicating the relationship between the use of 5-HT antagonists and the facilitation of learning and memory processes [33].

Broadly, the monoterpene indole alkaloid strictosidinic acid seems to act on 5-HT system in rat hippocampus, reducing the 5-HT levels. Regarding the importance of monoamines to central nervous system performance and their involvement in neurodegenerative diseases, the results presented in this work indicate that strictosidinic acid plays a role in 5-HT system towards a decrease of its levels. This suggests that the further investigation of *P. myriantha* and strictosidinic acid, using pharmacological models of depression, learning and memory tasks, is worthwhile.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.fitote.2012.04.013.

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