

Isolation and Identification of Phenylethanoid Glycosides from *Aloysia polystachya* and Its Activity as Inhibitors of Monoamine Oxidase-A



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ABSTRACT

Aloysia polystachya is used as a sedative and antidepressant by the indigenous populations of Argentina and Paraguay, but the compounds associated with these activities have not been determined. We have separated and identified the main constituents of the hydroethanolic extract of *A. polystachya* by ultra-performance liquid chromatography-mass spectrometry and confirmed the presence of acteoside, isoacteoside, 6'-acetylacteoside, and 4',4''',5,5''-tetrahydroxy-6,6''',3''-trimethoxy-[C7-O-C7'']-biflavone by NMR spectroscopy. Inhibitory activities of the hydroethanolic extract and purified phenylethanoid glycosides against monoamine oxidase-A were assessed using a standard fluorometric assay. The hydroethanolic extract inhibited monoamine oxidase-A activity in a dose-dependent manner with an IC₅₀ of 9.2 µg/mL, while the selective monoamine oxidase inhibitor clorgyline exhibited an IC₅₀ of 0.06 µg/mL (0.22 µM). Acteoside was the strongest inhibitor of monoamine oxidase-A (IC₅₀ value of 5 µM), whereas isoacteoside and 6'-acetylacteoside showed IC₅₀ values of around 10 µM. The results showed that phenylethanoids from a hydroethanolic extract of *A. polystachya* have been found to have inhibitory activity against monoamine oxidase-A. It is likely that the mode of action of the acteosides is multi-targeted, involving the downregulation of inflammatory molecules and neutralization of oxidation reactions as well as inhibition of monoamine oxidase-A.

Introduction

According to a recent report from the World Health Organization [1], more than 320 million people (4.4% of the world population) suffer from depression and anxiety disorders, with around 800 thousands suicides per year being committed as a result of these mental issues. Although treatment with synthetic antidepressants

is available, many patients suffer from side effects such as dry mouth, constipation, dizziness, blurred vision, increased appetite, weight gain, insomnia, and kidney problems [2]. For this reason, approaches using complementary and alternative medicine, including phytotherapy, have been used in the treatment of several types of mental disorders [3].

Various preclinical and clinical studies have provided evidence in support of the benefits of plant-based medicines in the treatment of general and specific anxiety disorders [4, 5]. Of particular interest are investigations into the anxiolytic properties of *Aloysia polystachya* (Griseb.) Moldenke (Verbenaceae), an aromatic species found mainly in Argentina and Paraguay. According to ethnopharmacological studies, local populations use the plant, which is commonly known as burrito, as a digestive, sedative, and antidepressant tonic [6, 7]. Although the anxiolytic and antidepressant properties of hydroethanolic extracts from *A. polystachya* have been confirmed by preclinical studies [8–10], no phytochemical investigations have been performed with the aim of identifying the compounds associated with these activities.

It has previously been shown that the antidepressant activities of some medicinal plants, for example, *Hypericum perforatum* L. (Hypericaceae) and *Peganum harmala* L. (Nitrariaceae), are associated with the inhibition of monoamine oxidase-A (MAO-A) [11–13]. The MAO family is distributed throughout the central and peripheral nervous systems and overexpression of these enzymes promotes the oxidative deamination of monoamines with reductions in the levels of the neurotransmitters serotonin, norepinephrine, and dopamine, which result in the onset of psychiatric disorders. Such deamination processes also generate substances such as hydrogen peroxide, oxygen free radicals, and aldehydes that are responsible for the oxidative stress of cells. MAOs exist in two major isoforms that differ with respect to distribution, substrate specificity, and sensitivity to inhibitors. The MAO-A isoform plays an important role in depression and anxiety disorders, while MAO-B is involved in neurodegenerative diseases [13–17].

In light of the above, we hypothesized that the anxiolytic and antidepressant properties of *A. polystachya* derive, at least in part, from the presence of inhibitors of MAO-A. In order to test this hypothesis, we identified the active principles present in the hydroethanolic extract from leaves of *A. polystachya* and assessed the effects of the crude extract and the main constituents isolated therefrom on MAO-A activity.

Results and Discussion

The hydroethanolic extract from leaves of *A. polystachya* was submitted to ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), and the chromatogram so obtained is presented in ► **Fig. 1a**. The main components of the extract were purified by column chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC), and identified as acteoside (syn verbascoside), isoacteoside, 6'-acetylacteoside, and 4',4'',5,5''-tetrahydroxy-6,6'',3'''-trimethoxy-[C7-O-C7'']-biflavone by comparison of their ¹H- and ¹³C-NMR, HSQC, HMBC (► **Table 1S, 2S**, Supporting Information), and MS data (► **Fig. 1b-e**) with values reported in the literature [18–20]. The concentration of acteoside in the hydroethanolic extract, as determined by HPLC, was 108.65 ± 1.3 µg/mg of dried extract. This represents the first record of the constituents of extracts of leaves from *A. polystachya*, although the essential oil of the plant has been analyzed previously and found to contain the monoterpenes carvone and limonene as the major components [21].

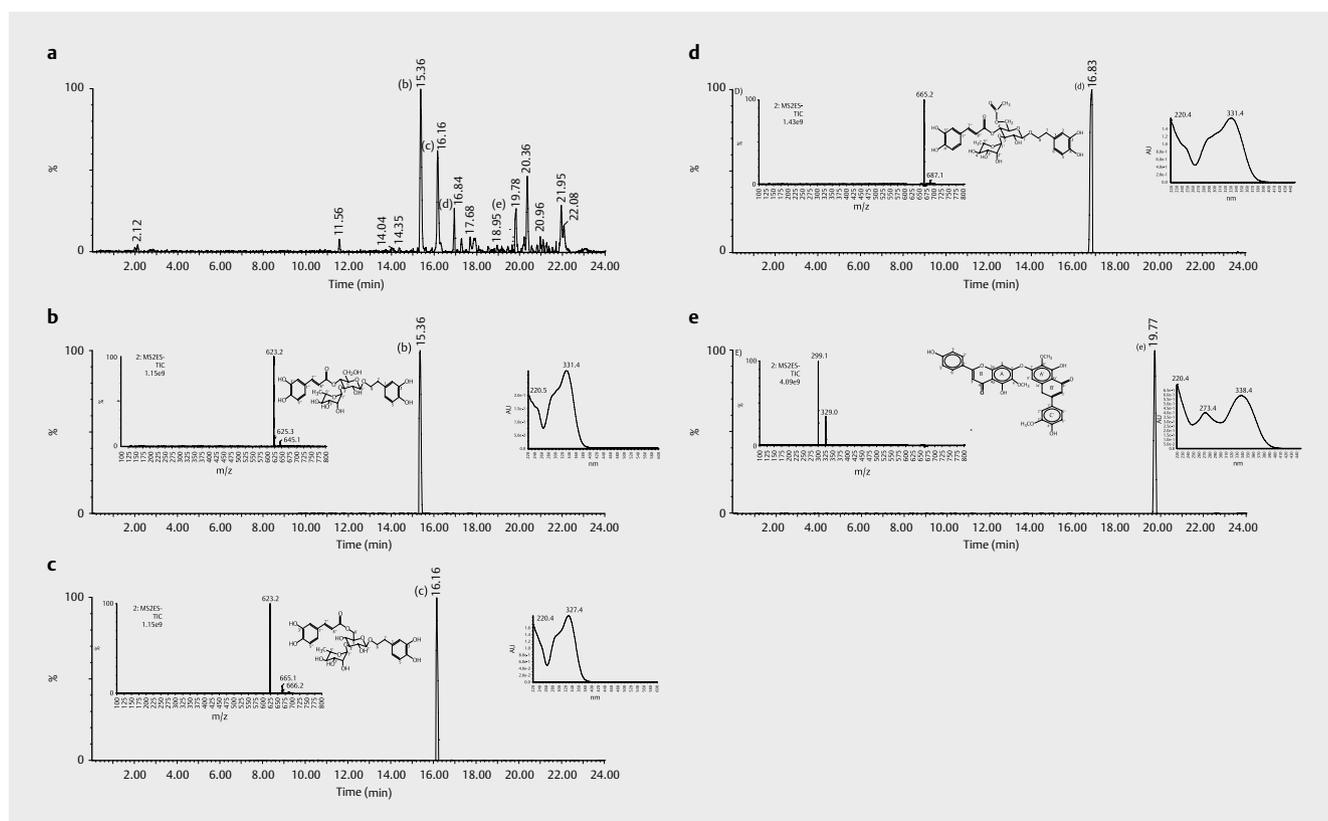
The crude hydroethanolic extract from leaves of *A. polystachya* inhibited MAO-A activity in a dose-dependent manner (► **Fig. 2a**) with an IC₅₀ of 9.2 µg/mL, while the selective MAO inhibitor clorgyline exhibited an IC₅₀ of 0.06 µg/mL (0.22 µM). The purified acteosides also exhibited inhibitory activities against MAO-A (► **Fig. 2b**), with acteoside presenting the lowest IC₅₀ value of 5 µM (3.1 µg/mL) followed by isoacteoside with an IC₅₀ of 10.1 µM (6.3 µg/mL) and 6'-acetylacteoside with an IC₅₀ of 9.5 µM (6.3 µg/mL). Inhibition of MAO-A leads to the reestablishment of the levels of serotonin, norepinephrine, dopamine, and tyramine, which are key neurotransmitters in the control of anxiety and depression [16]. Thus, the presence of diverse MAO-A inhibitors in leaves of *A. polystachya* explains, at least in part, the previously reported antidepressant and anxiolytic activity of the species [8–10].

The use of multicomponent plant mixtures can be advantageous in the treatment of diseases of complex etiology such as anxiety, depression, and other neurological conditions. Currently, the use of phytomedicines in the treatment of diseases that affect the nervous system is based on the paradigm of multi-target-directed ligands, i. e., pharmaceuticals that have multi-target activities resulting from the presence of substances such as polyphenolics with anti-inflammatory, antioxidant, and MAO inhibitory properties that are capable of conferring neuroprotection [20, 22–25].

It is worth noting, however, that the polar character of polyphenolic substances could hinder interactions with their molecular targets. Nevertheless, clinical studies have demonstrated that the contact between polyphenolics and nonpolar secondary metabolites present in the extracts may modify the permeability of cell membranes and facilitate the uptake of polar compounds [23]. Thus, complex plant extracts containing multi-target agents that interact with their receptors in a pleiotropic fashion generate a pharmacological synergism that affects numerous processes, including the movement of polar metabolites across cell membranes. In this context, Li et al. [26] employed a zebrafish model to demonstrate that acteoside could penetrate the blood-brain barrier, and proposed that the phenylethanoid glycoside may have a potential therapeutic effect in Parkinson's disease.

Monoaminergic neurotransmitters are the main targets of modern antidepressants since their deficiencies are responsible for the debilitating symptoms of depression. A recent *in vivo* study demonstrated that the ethanolic and aqueous extracts of *Lippia citriodora* (Verbenaceae) and their main component acteoside exhibited anxiolytic, hypnotic, and muscle relaxant effects, and these properties were attributed, in part, to an interaction with the type A gamma-aminobutyric acid (GABA_A) receptor [27].

Clinical studies have revealed that drugs with the capacity to block inflammatory cytokines, such as TNF-α, or other components of the inflammatory signaling pathway, for example, cyclooxygenase-2 (COX-2), are effective in reducing depressive symptoms in patients with rheumatoid arthritis, psoriasis, and cancer, as well as those suffering from major psychiatric disorders [28]. In this context, it has been reported that acteoside can attenuate the production and release of inflammatory molecules, such as nitric oxide (NO), TNF-α, and interleukin 12 (IL-12) in lipopolysaccharide/interferon-gamma (LPS/IFN-γ)-stimulated macrophages [29], as well as histamine and arachidonic acid in RBL-2H3 mast cells [30]. In addition, acteoside is able to reduce the levels of TNF-α, IL-1β, IL-8, IL-6, and NO, and to activate caspase-1, nuclear factor-kappa-B



► **Fig. 1** UPLC-MS chromatograms of the hydroethanolic extract from *A. polystachya* **a** and standard compounds acteoside **b**, isoacteoside **c**, 6'-acetylacteoside **d**, and 4',4'',5,5''-tetrahydroxy-6,6'',3'''-trimethoxy-[C7-O-C7'']-biflavone **e** with inserts showing the respective UV and negative ion electrospray ionization mass spectra (ESI/MS).

(NF- κ B), NO synthase, and activator protein-1 [31] induced by IL-32 and/or LPS in TH-1 cells and macrophages [32].

Some of these inflammatory mediators, such as IFNs, IL-6, IL-8, and IL-1 β , have been found at abnormal levels in both peripheral and post-mortem tissue samples from depressed individuals, and have been related to the symptoms of depression [33]. Activation of these molecules by psychosocial stressors may promote significant functional changes in the brain, leading to the development of depressive behavior and other psychiatric disorders. IFNs, IL-1 β , and TNF- α , for example, may increase the expression and function of serotonin, noradrenaline, and dopamine receptor pumps, thus reducing the availability of these neurotransmitters in the synaptic cleft [28]. In addition, IFN- γ , IL-6, TNF- α , and oxidative stress can activate indoleamine 2,3 dioxygenase (IDO), an enzyme responsible for the degradation of tryptophan and, thereby, reduce the concentration of the primary precursor of serotonin synthesis [34].

Acteoside, isoacteoside, and 6'-acetylacteoside contain hydroxyphenylethyl and caffeoyl moieties that are known to be associated with antioxidant properties [35, 36], and acteoside itself exhibits considerable antioxidant activity [37]. Studies have shown that acteoside inhibits the aggregation of β -amyloid peptide ($A\beta$) in a dose-dependent manner, functions as a neuroprotective agent, and enhances memory, and these properties have been attributed to the antioxidant activity of the agent [38, 39]. Considering that numerous phenolic-rich species with antioxidant properties are used in the treatment of neurological disorders [15], it has been suggested that the antidepressant and anxiolytic effects of the phenylethanoid gly-

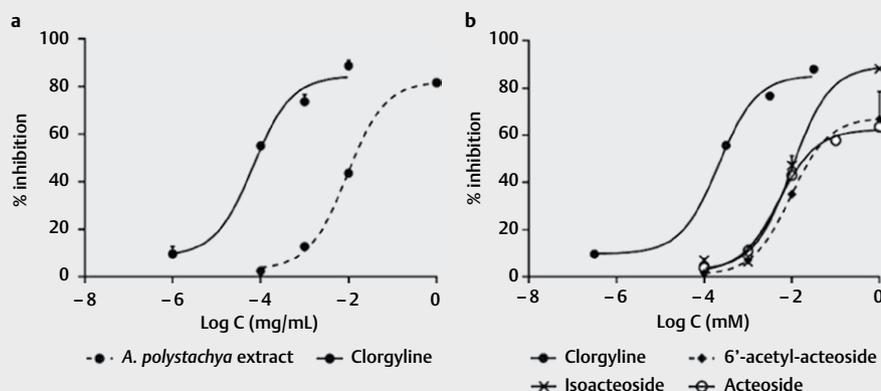
cosides isolated from *A. polystachya* may also result from their antioxidant activities. This supposition was supported by Xu et al. [40], who presented evidence concerning the relationship between increased oxidative stress and depression/anxiety.

Based on the above, we conclude that the antidepressant properties of the hydroethanolic extract from leaves of *A. polystachya*, and of the purified phenylethanoids isolated therefrom, can be explained by multi-target modes of action involving the inhibition of MAO-A, downregulation of inflammatory molecules, and neutralization of oxidation reactions. Hence, the results presented herein support our original hypothesis that the anxiolytic and antidepressant activities of the hydroethanolic extract of *A. polystachya* result, at least in part, from the inhibition of MAO-A. However, the structure-activity relationship of the phenylethanoid glycosides identified in this study requires further attention so that novel molecules can be designed for the treatment of specific neurological disorders.

Materials and Methods

Plant material

Leaves of *A. polystachya* were harvested at the Farmácia da Natureza da Terra de Ismael (Jardinópolis, SP, Brazil) in January 2016. Plant material was identified by Dr. Lúcia Rossi (Instituto Botânico, São Paulo, SP, Brazil), and a voucher specimen was deposited in the Herbarium of Medicinal Plants at UNAERP with voucher number



► **Fig. 2** Inhibition of monoamine oxidase-A by **a** the crude hydroethanolic extract from leaves of *A. polystachya* and **b** the main constituents of the hydroethanolic extract, namely, acteoside, isoacteoside, and 6'-acetylacteoside. For comparison, the inhibitory activity of the positive control clorgyline is shown in both panels.

HPM-1213. Permission to evaluate the bioactivities of the extracts from Brazilian plants was granted by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (no. 02001.005074/2011-19).

Preparation of the hydroethanolic extract of *Aloysia polystachya*

Leaves (1000 g) were dried for 72 h in a circulating air oven at 45 °C, pulverized, and passed through a 40-mesh sieve. The powdered material was steeped in water:ethanol (20:80; v/v) for 7 days and subsequently filtered through Whatman No. 41 filter paper. The filtrate was reduced to dryness on a rotary evaporator and lyophilized to yield 130.2 g of dry crude extract, resulting in a drug extract ratio of 7.6:1.

Separation and identification of constituents by ultra-performance liquid chromatography-mass spectrometry

Chromatographic analyses were performed using a Waters Acquity UPLC H-Class system equipped with a diode array detector (DAD) and a Waters Xevo TQ-S tandem quadrupole mass spectrometer with a Z-spray source operating in the negative ion mode. Stock solutions containing 1.0 mg/mL of extract or the standards acteoside, isoacteoside, 6'-acetylacteoside, and 4',4''',5,5'''-tetrahydroxy-6,6''',3'''-trimethoxy-[C7-O-C7''']-biflavone in LC-grade methanol were prepared separately, with sonication for 30 min each when necessary, and filtered through 0.45 µm Millipore filters (Merck Millipore). Solutions were diluted to 10 µg/mL with methanol, and aliquots (5 µL) were injected onto a Sigma-Aldrich Ascentis Express C₁₈ column (100 × 4.6 mm i.d.; 2.7 µm particle size). The mobile phase consisted of water containing 0.1 % formic acid (solvent A) and methanol containing 0.1 % formic acid (solvent B) supplied at a flow rate of 0.5 mL/min according to the elution profile: isocratic with 3 % B between 0 and 4 min, followed by linear gradients from 3 to 60 % B between 4 and 19 min and from 60 to 90 % B between 19 and 23 min, and finally returned to 3 % B between 23 and 28 min. The effluent was monitored by DAD in the range of 210 to 720 nm and by MS with the optimized source and operating parameters as follows: capillary volt-

age 2.50 kV, Z-spray source temperature 150 °C, desolvation temperature (N₂) 350 °C, desolvation gas flow 600 L/h, and mass range of m/z 150 to 600 in the full scan mode.

Purification of identified constituents

The crude hydroethanolic extract (10 g) was dissolved in water:ethanol (50:50; v/v) and partitioned consecutively against hexane, ethyl acetate, and n-butanol. The n-butanol fraction was concentrated on a rotary evaporator and a 3 g sample of the fractionated extract was applied to a glass column (10 × 3 cm o.d.) filled with C-18 RP silica gel (230-400 mesh; Sigma-Aldrich) and eluted with water, followed by successive mixtures of water:methanol (90:10, 50:50, and 10:90; v/v). The subfraction obtained by elution with water:methanol (90:10; v/v) was further purified by RP-HPLC using a Shimadzu LC-20AP System coupled to an SPD-20A UV/Vis detector and equipped with a Phenomenex Luna[®] C-18 column (250 × 10 mm i.d.; 5 µm particle size). The mobile phase was water:methanol commencing at 90:10 (v/v) and changing to 30:70 (v/v) in 100 min to yield the four known compounds acteoside (750 mg), isoacteoside (430 mg), 6'-acetylacteoside (42 mg), and 4',4''',5,5'''-tetrahydroxy-6,6''',3'''-trimethoxy-[C7-O-C7''']-biflavone (13 mg). The flow rate of the mobile phase was 1 mL/min, the injection volume was 500 µL, the chromatogram was recorded at 340 nm, and UV spectra were obtained in the range of 240 to 400 nm. The identities of the purified constituents were confirmed from their ¹H- (500 MHz) and ¹³C-NMR (125 MHz) spectra recorded on a Bruker model DPX 500 spectrometer, and comparison of the data with those available in the literature [18, 19, 41]. Purities of the isolated compounds were confirmed by UPLC-DAD-MS (► **Fig. 1b–d**) and NMR (► **Fig. S1–S12**, Supporting Information).

Quantification of acteoside by an HPLC-diode array detector

A sample (1 mg) of the dried hydroethanolic extract was redissolved in 1 mL of a mixture (80:20; v/v) of methanol (J.T. Baker HPLC grade) and Milli-Q Ultrapure water (Merck Millipore), sonicated for 30 min, and filtered through a 0.45-µm Millipore filter. Aliquots (20 µL) of

this solution were analyzed on a Shimadzu LC-10APvp system coupled to an SPD-M10Avp DAD and fitted with a Phenomenex Luna C18 column (250 × 4.6 mm i.d., 5 μm) protected by a Phenomenex C18 precolumn (4.0 × 3.0 mm i.d., 5 μm). Separations were carried out at room temperature (22 ± 1 °C) using a mobile phase comprising acetic acid 0.1 % in water (solvent A) and methanol (solvent B; J.T. Baker HPLC grade) supplied at a constant flow rate of 1.0 mL/min according to the program: linear gradient from 10 to 70 % B between 0 and 32 min, from 70 to 10 % B between 32 and 35 min, and a final isocratic elution with 10 % B between 35 and 40 min. The detection wavelength was set at 330 nm.

The content of acteoside in the extract was estimated using acteoside (Sigma-Aldrich; CAS no. 61276-17-3) as the external standard. Solutions containing 500, 250, 125, 62.5, 31.2, and 15.6 μg/mL of the reference standard were prepared, and calibration curves were constructed by subjecting each solution to HPLC analysis in triplicate [42]. The ratio of peak area of standard acteoside to the corresponding concentration of analyte was established through linear regression of the standard curves (► **Fig. S13**, Supporting Information). Analytical data were validated with respect to linearity, precision, and accuracy according to the guidelines issued by the Agência Nacional de Vigilância Sanitária [42]. Limits of detection (LoD) and quantitation (LoQ) of acteoside were 0.30 and 0.92 μg/mL, respectively.

Monoamine oxidase-A inhibition assays

Recombinant human MAO-A, tyramine, clorgyline, vanillic acid, 4-aminoantipyrine, and horseradish peroxidase were purchased from Sigma-Aldrich. MAO-A inhibition assays were performed using 96-well plates following a modified version of the method described by López et al. [43]. Each well was loaded with 50 μL of chromogenic solution (0.8 mM vanillic acid, 2.5 mM 4-aminoantipyrine, and 4 U/mL horseradish peroxidase in phosphate buffer at pH 7.6), 100 μL of 3 mM tyramine, and a 50-μL aliquot of the crude hydroethanolic extract or one of the purified phenylethanoids dissolved in methanol. Finally, 50 μL aliquots of 8 U/mL MAO-A were added to each of the wells and the plate was incubated at 37 °C for 30 min, during which time absorbances were recorded every 5 min using a microplate reader. Clorgyline (≥ 97 % GC; Sigma-Aldrich) and methanol were included as positive and negative controls, respectively, on each assay plate.

Statistical analysis

Three independent assessments of MAO-A inhibitory activities were performed and the acquired data were analyzed using GraphPad Prism software. The IC₅₀ values of the hydroethanolic extract, purified phenylethanoids and the positive control were calculated by nonlinear regression, simulating plots of log (inhibitor concentration) versus normalized percentage inhibition.

Supporting information

H- and ¹³C-NMR spectra of acteoside, isoacteoside, 6'-acetylacteoside, and 4',4''',5,5'''-tetrahydroxy-6,6'',3''''-trimethoxy-[C7-O-C7''']-biflavone and HSQC/HMBC correlation analyses for the three phenylethanoids are available as Supporting Information.

Conflict of Interest

The authors declare no conflict of interest.

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