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Phytochemical study and anti-inflammatory effect of *Psychotria stenocalyx* (Rubiaceae)

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ABSTRACT

In this study, an *in vivo* anti-inflammatory assay was conducted on *Psychotria stenocalyx* ethanol extract of leaves, evaluating this effect on cell migration and exudate formation in a murine carrageenan-induced model of pleurisy. The extract showed the optimum dose, at 400 mg/kg, inhibiting cellular and vascular parameters: total leucocytes due to polymorphonuclear migration and exudate formation ($p < 0.01$). A phytochemical study was conducted using the hyphenated techniques HPLC-DAD-SPE/NMR and HPLC-DAD-ESI-TOF-HRMS, in which seven monoterpene indole alkaloids were identified: lyaloside **1**, (E)-O-(6′)-cinnamoyl-4”-hydroxy-3”,5”-dimethoxy-lyaloside **2**, strictosamide **3**, pauridianthoside **4**, vallesiachotamine lactone **5**, E-vallesiachotamine **6** and Z-vallesiachotamine **7**, in the alkaloid fraction, obtained by an acid-base extraction on *P. stenocalyx* crude extract. This is the first study developed with this species.

INTRODUCTION

Psychotria L. is the largest genus in the tribe Psychotrieae (Rubiaceae) and is comprised of around 2000 species worldwide, distributed in tropical regions (Nepokroeff *et al.*, 1999).

Many biological and pharmacological investigations of the genus *Psychotria* have shown a significant number of activities, such as antioxidant (Formagio *et al.*, 2014), antibacterial (Moraes *et al.*, 2011), anti-parasitic (Kato *et al.*, 2012), antitumor (Gerlach *et al.*, 2010), anti-inflammatory (Iniyavan *et al.*, 2012), anxiolytic and anti-depressive (Farias *et al.*, 2012), and anti-epileptic (Awad *et al.*, 2009) activities. Studies have also reported the efficacy of *P. poeppigiana* against heart disease, cough, asthma and bronchitis (Guerrero *et al.*, 2010). Species of *Psychotria*was also evaluated for their ability to protect against intracellular amyloid toxicity and induced cell differentiation (Currais *et al.*, 2014). Chemical studies on *Psychotria* species have reported the presence of alkaloids as the main secondary metabolites, particularly monoterpene indole alkaloids (Calixto *et al.*, 2016). Themonoterpene indole alkaloids lyaloside **1**, (E)-O-(6′)-cinnamoyl-4”-hydroxy-3”,5”-dimethoxy-lyaloside **2**, strictosamide **3**, pauridianthoside **4**, vallesiachotamine lactone **5**, E-vallesiachotamine **6**, and Z-vallesiachotamine **7**have previously been reported in *Psychotria* species (Berger *et al.*, 2015; Farias *et al.*, 2009; Passos, *et al.*, 2013a-b; Paul *et al.*, 2003). The genus *Psychotria* can also be characterized as an abundant source of indoles (Gerlach *et al.*, 2010), pyrrolidinoindole (Henriques *et al.*, 2004), quinolin and isoquinolin (Bernhard *et al.*, 2011), and *β*-carboline (Murillo and Castro, 1998) alkaloids. Some of these alkaloids inhibited monoamine oxidase A and B (Passos *et al.*, 2013a), and exhibited potential inhibition of the enzymes acetylcholinesterase and butyrylcholinesterase (Passos *et al.*, 2013b).

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Psychotria stenocalyx Müll. Arg. is a shrub that grows up to two meters, and occurs in coastal tropical forests of South and Southeast Brazil, ranging from Rio de Janeiro to Santa Catarina (Taylor *et al.*, 2015). It is popularly known as "gandiúva-d'anta" and "café-d'anta" (Dillenburg *et al.*, 1985).

In view of the many reports that cite the important biological activity of *Psychotria*, the aim of this study was to carry out an *in vivo* anti-inflammatory assay on the leaf extract, and to isolate and structurally elucidate monoterpene indole alkaloids on *Psychotria stenocalyx*.

MATERIAL AND METHODS

Plant Material

The aerial parts of *Psychotria stenocalyx* were collected in June 2012 in Florianópolis, state of Santa Catarina, Brazil. The plant was identified by botanist Ademir Reis and a voucher was deposited in the "Barbosa Rodrigues" Herbarium in Itajaí, state of Santa Catarina, Brazil, under number HBR55428.

Extraction of plant material

Air-dried and powdered leaves of *P. stenocalyx* (449 g) were extracted with ethanol 96% (3 x 72h) at room temperature. After filtration, the extract was concentrated to obtain the crude extract, CE (5.54 g/100g dried leaves) for use in the inflammation study. The CE was dissolved in 0.01 mol/L HCl H_2O/EtOH (80:20 v/v) and then stored in a refrigerator. After 24 h, the extract was filtered and exhaustively extracted with ethyl acetate to remove the nonpolar constituents. The acid extract was alkalinized with 10% NH_4OH in water up to pH 11 and extracted again with ethyl acetate yielding the purified alkaloid fraction (3.24 g/100g CE) which was used for the isolation and characterization of alkaloids.

Inflammation study

Animals

In the present protocol, one month old female Swiss mice weighing 20 to 25 g were housed under standardized conditions (20 ± 2 ºC, 12 h light/dark cycles) with free access to chow and water. The experiments were designed to minimize the animal suffering and the 3R recommendations were followed (replacement, reduction and refinement) (Flecknell 2002). The experiments were carried out on the ethanolic crude extract of *P. stenocalyx* according to the regulations of the Brazilian College of Animal Experimentation (COBEA) and are in accordance with the rules of the Committee for Ethics in Animal Research of the Federal University of Santa Catarina (CEUA - PP00965).

Experimental design of the murine model of pleurisy

The pleurisy induction was performed as previously reported (Saleh *et al.*, 1996). Briefly, on the day of the experiments the animals were randomly divided into different groups (n = 6animals/group) and challenged with Evans Blue dye solution (25 mg/kg) administered intravenously (i.v). Next, the groups received the appropriate treatment as follows: (a) blank control (S), animals pre-treated with intraperitoneal (i.p.) saline (vehicle) 0.5 h before pleurisy induction with saline; (b) negative control (Cg), animals treated with i.p. saline (vehicle) 0.5 h before pleurisy induction with carrageenan; (c) positive control (Dex), animals pre-treated with i.p. dexamethasone 0.5 mg/kg before pleurisy induction with carrageenan; and (d) experimental groups, animals pre-treated with i.p. *Psychotria stenocalyx* crude extract at doses ranging from 100 to 400 mg/kg before pleurisy induction with carrageenan.

After 0.5 h of intraperitneal (i.pl) treatment with saline, each group received a single injection of a λ-carrageenan solution (0.1 mL, 1%) from the right side of the thoracic cavity, to induce pleurisy induction, except for the blank control group (S), which received an i.pl. sterile saline injection (0.1 mL). Four hours after pleurisy induction, the animals were euthanized using an overdose of xylazine and ketamine (50 mg/kg and 250 mg/kg, i.p.), and the pleural cavity was exposed and washed with 1.0 mL of sterile phosphate buffered saline (PBS, pH 7.2) (Laborclin, Pinhais, Paraná, Brazil) containing heparin (20 IU/mL). The pleural fluid was used to measure the inflammatory parameters: total and differential leukocyte count and exudate concentration.

Quantification of the leukocyte content and exudation

To quantify the leukocyte content, pleural fluid samples were submitted to a veterinarian automatic counter (BC-2800 Vet, Mindray, Nanshan, Shenzhen, China). For the differential leukocyte count, cytospin preparations from the exudates were stained with May-Grünwald-Giemsa. The results were expressed as the total number of cells ($\times 10^6$ cells/mL).

The exudate quantification was performed indirectly by measuring the amount of Evans blud dye in the pleural cavity. The fluid samples were centrifuged (300 g for 5 min) (SorvallTMST40, ThermoScientific®, Swedesboro, New Jersey, USA) and 200 µL of the supernatant was transferred to a 96 well-ELISA plate. The amount of dye was estimated by a colorimetric measurement at 620 nm, using an ELISA plate reader (OrganonTeknika, Roseland, New Jersey, USA). The results were expressed in µg/mL by interpolation from an Evans blue dye standard curve ranging from 0.01 to 50µg/mL.

Statistical analysis

All data were expressed as mean ± SEM. Statistical differences between groups were tested by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. The results were analyzed using GraphPad Prism v5.0 software (GraphPad Software Inc., San Diego, California, USA) and values of $P < 0.05$ were considered significant.

HPLC analyses and isolation of compounds

The alkaloid fraction of *P. stenocalyx* crude extract (50 mg) was dissolved in 1 mL MeOH. The solution was filtered through a PVDF membrane syringe filter (25 mm, 0.45 µm; Tedia Brazil) before HPLC analysis. A reversed-phase Luna C18 (Phenomenex, 150 × 4.6 mm, 3 µm, 100 Å) was used for the

chromatographic separation. Gradient elution was performed using a combination eluent (A) consisting of H_2O/MeCN (95:5, v/v), and organic eluent (B) consisting of H_2O/MeCN (5:95, v/v), both acidified with 0.1% formic acid (Tedia Brazil). The gradient method was: 0 min, 15% B; 60 min, 65% B; 65-75 min, 100% B; 76-85 min, 15% B. Elution rate 0.5 mL/min. The injection volume was 10 µL and UV traces were monitored at 254, 280, and 330 nm. HPLC-DAD-SPE/NMR analysis was carried out on an Agilent 1200 HPLC. Compounds **3-7** were adsorbed on solid-phase extraction cartridges (HySphere Resin GP, 10 mm × 2 mm, 10 µm spherical polydivinylbenzene stationary phase) using an automatic cartridge exchanger (Bruker Biospin GmbH). Twenty consecutive chromatographic runs were performed, with 10 µL injections and a flow rate of 1.0 mL/min. After the adsorption process, the cartridges were dried with nitrogen for 30 min to remove residual solvent. Deuterated MeOH-d4 (99.8% D) was used to elute the compounds from the SPE cartridges directly into NMR tubes (Bruker, 3 mm o.d.). NMR experiments were performed with a Bruker Avance III system (^{1}H operating frequency of 600.13 MHz) equipped with a Bruker SampleJet sample changer and a cryogenically cooled gradient inverse triple-resonance 5.0 mm TCI probe-head (Bruker Biospin) optimized for ^{1}H and ^{13}C observation. Icon NMR (ver. 4.2, Bruker Biospin) was used to control the automated acquisition of NMR data, which were then processed using Topspin (version 3.0, Bruker Biospin). The HRMS and UV spectra were obtained in positive mode in a HPLC-DAD-ESI-TOF-MS instrument consisting of a Shimadzu Prominence LC-20AD UFLC system (Kyoto, Japan), using a T-piece splitter to direct 1% of the HPLC eluate to a micrOTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionization interface. Mass spectra were acquired in positive-ion mode, using a drying temperature of 200 °C, capillary voltage of 4500 V, nebulizer pressure of 3.0 bar, and drying gas flow of 12 L/min.

RESULTS AND DISCUSSION

Anti-inflammatory effect

The inflammatory model adopted in the present work uses carrageenan as the phlogistic agent, to trigger an inflammatory reaction in the pleural cavity and the consequent damage associated with this process. In this specific model, it is possible to evaluate the cell migration, mainly polymorphonuclear, and exudate formation that is present in several acute inflammatory conditions (Luz *et al.*, 2016; Saleh *et al.*, 1996; Schmid-Schönbein, 2006).

It was reported that the alkaloid extract of *Psychotria myriantha* was able to inhibit *in vitro* polymorphonuclear leukocyte chemotaxis, and that this activity was attributed to its alkaloids myrianthosine and strictosidinic acid (Simões-Pires *et al.*, 2006). A decrease in carrageenan-induced inflammation was also reported for the crude extract of *Psychotria nilgiriensis* fruits (Iniyavan *et al.*, 2012). Regarding cell migration, *P. stenocalyx* extract significantly reduced total leukocyte migration to the pleural cavity of animals challenged with a 1 % carrageenan solution at all the tested doses (% inhibition: 400 mg/kg: 53.6 ± 5.6; 200 mg/kg: 57.1 ± 5.2 and 100 mg/kg: 31.4 ± 6.8) ($p < 0.001$) (Figure 1a). This inhibition was due to polymorphonuclear inhibition (% inhibition: 400 mg/kg: 56.1 ± 5.1; 200 mg/kg: 57.3 ± 5.1 and 100 mg/kg: 32.0 ± 6.7) ($p < 0.01$) (Figure 1b). As expected, the positive control group that was pre-treated with dexamethasone 0.5 mg/kg also reduced leukocyte migration (% inhibition: 62.1 ± 2.7) ($p < 0.001$) (Figure 1), due to the decrease in polymorphonuclear migration (% inhibition: 63.4 ± 2.7) ($p < 0.001$) (Figure 1b).

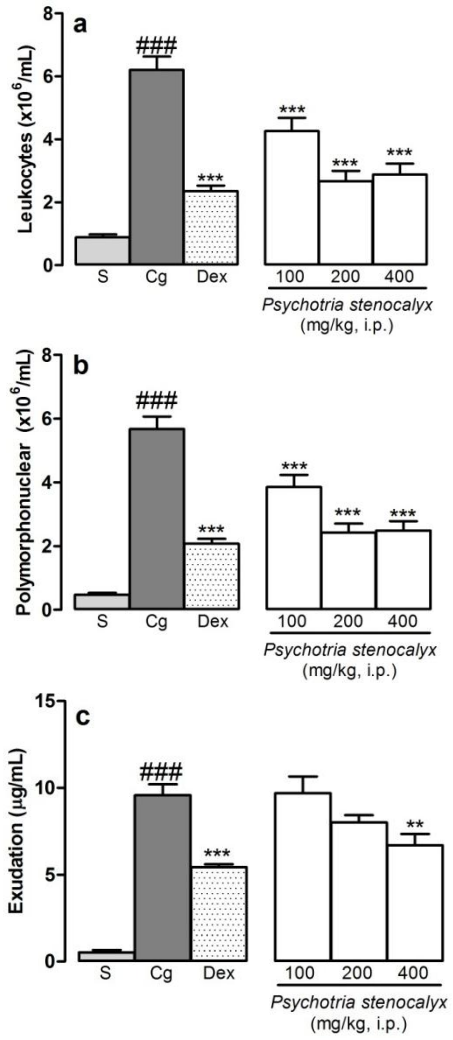

Fig. 1: *Psychotria stenocalyx* leaf extract (100-400 mg/kg)effect on (a) leukocyte migration, (b) polymorphonuclear migration, and (c) exudate concentration when administered intraperitoneally 0.5 h prior to carrageenan induced pleurisy in mice. S: blank control group, animals pre-treated with intraperitoneal (i.p.) saline 0.5 h before pleurisy induction with saline; Cg: negative control, animals treated with i.p. saline 0.5 h before pleurisy induction with carrageenan; Dex: positive control, animals pre-treated with i.p. dexamethasone 0.5 mg/kg before pleurisy induction with carrageenan; *Psychotria stenocalyx*: experimental groups, animals pre-treated with i.p. *Psychotria stenocalyx* extract at doses ranging from 100 to 400 mg/kg before pleurisy induction with carrageenan. The results are expressed as mean ± SEM. N = 6 animals/group. ** $p< 0.01$ compared to Cg group, *** $p< 0.001$ compared to Cg group, ### $p< 0.001$ compared to S group.

Exudate formation was significantly inhibited by *P. stenocalyx* extract only at the 400 mg/kg dose (% inhibition: 30.1 ± 6.7) ($p < 0.01$) (Figure 1c). This result corroborates Moraes *et al.* 2011, who showed that the species *P. suterella P. stachyoides*, and *P. capitata* may have inhibitory activity upon nitric oxide formation *in vitro*, and this soluble gas is closely related to exudate formation. As expected, the group that was pre-treated with dexamethasone 0.5 mg/kg also showed reduced exudate formation (% inhibition: 43.1 ± 1.6) ($p < 0.001$) (Figure 1c).

Based on these results, we consider 400 mg/kg to be the optimum dose, as this dose was able to inhibit cell migration and exudate formation triggered by the phlogistic agent in this pleurisy model.

Phytochemical characterization of the alkaloids

In order to isolate and/or identify the compounds from *P. stenocalyx* crude extract, an acid-base extraction was performed to obtain a purified alkaloid fraction. The HPLC chromatogram at 280 nm showed the presence of nine major compounds, of which seven were identified (

Fig). The UV spectra displayed by the peaks for these compounds are consistent with *β*-carboline (**1**, **2** and **4**), tetrahidro-*β*-carboline (**3**) (Passos *et al.*, 2013a) and vallesiachotamine-like nuclei (**5-7**) (Djerassi *et al.*, 1966).

The alkaloids lyaloside **1** and (*E*)-O-(6′)-cinnamoyl-4”-hydroxy-3”,5”-dimethoxy-lyaloside **2** were identified without isolation by their UV and HRMS data compared to the data available in the literature (Valverde *et al.*, 1999; Passos *et al.*, 2013a). The hyphenated technique HPLC-DAD-SPE was used to isolate the peaks 3-7 of the chromatogram, which was characterized by UV, HRMS, and 1D and 2D NMR spectra compared with data available in the literature.

In the ^{1}H NMR spectra, the specific hydrogen chemical shifts of each isolated compound compared with the literature is coherent with the alkaloids strictosamide **3** (Atta-ur-Rahman *et al.*, 1991), pauridianthoside **4** (Levesque *et al.*, 1977), vallesiachotamine lactone **5** (Passos *et al.*, 2013a), *E*-vallesiachotamine **6**, and *Z*-vallesiachotamine **7** (Waterman *et al.*, 1982). The structures of all compounds are shown in

Fig. The compiled data on retention time, mass and UV spectra for all compounds are given in Table 1.

The ^{1}H NMR spectra of all isolated compounds showed, due to the presence of the benzene ring of the indole system, four aromatic hydrogens: two doublets (δ_{H9} and δ_{H12}) ortho-coupled with two double-doublets (δ_{H10} and δ_{H11} – mutually ortho-coupled). All the couplings were confirmed by the 2D COSY spectrum. For the *β*-carboline-like alkaloid (**4**), the ^{1}H NMR spectra showed two doublets (δ_{H5}-δ_{H6}), while for the tetrahidro-*β*-carboline-like (**3**) and vallesiachotaman-like alkaloid (**5**, **6**, and **7**) it showed four signals (δ_{H5a-b}-δ_{H6a-b}) presenting multiplicities coherent with the pattern AA’BB’ coupling system. The glycosyl alkaloids **3** and **4** showed the signal of the anomeric hydrogen as a doublet with coupling constant coherent with a *β*-glucose ($J \approx 8$ Hz). The HMBC spectrum showed correlations between C21–H1’ and C1’–H21, confirming that the glucoside was linked to the acetal carbon C21.

The isomers *E/Z*-vallesiachotamine, **6** and **7**, were distinguished by the difference between their olefinic H19 signals in the ^{1}H NMR spectra. The *E*-isomer showed a quartet at δ_{H19} 6.79 ppm while for the *Z*-isomer, there was a quartet at δ_{H19} 6.58 ppm. The olefinic hydrogen is relatively more deshielded in the *E*-isomer due to the anisotropy of the carbonyl C21 of the aldehyde group. On the other hand, in the *Z*-isomer, the anisotropy of the aldehyde leads to a more deshielded 18-methyl group. Thus, a doublet at δ_{H18} 2.10 ppm was observed for the *E*-isomer and at δ_{H18} 2.20 ppm for the *Z*-isomer (Waterman *et al.*, 1982). The 1:1 ratio of these *E*/*Z* isomers was calculated by the integration of these correspondent peaks in the HPLC chromatogram at λ_{max} 290 nm. For the vallesiachotamine lactone **5**, the NMR data were quite similar to compounds **6** and **7**, except for the signals of the *α*-*β*-unsaturated *γ*-lactone ($\delta_{CH18-21}$), linked to the rest of molecule by the carbons C15-C20.

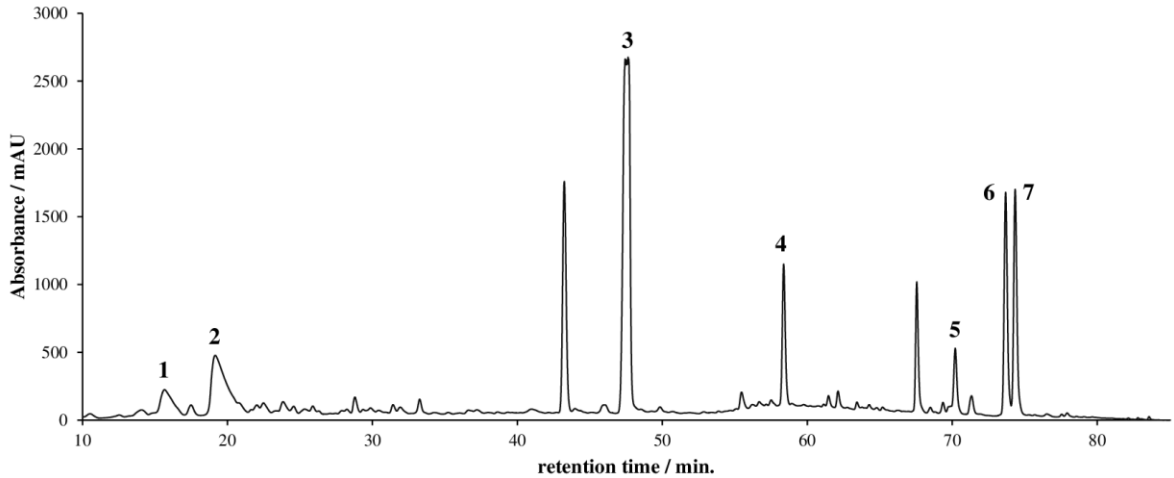

Fig. 2: HPLC chromatogram at 280 nm. The peaks numbered 1-7 (corresponding to compounds **1**-**7**, respectively) were analysed in HPLC-DAD-ESI-TOF-MS mode [Phenomenex C18 Luna column, 150 x 4.6 mm, 3µm, 40 ºC, elution rate 0.5 mL/min, elution gradient 0 min, 15% B; 60 min, 65% B; 65-75 min, 100% B; 76-85 min, 15% B. Injection volume 10 µL, and UV traces monitored at 254, 280, and 330 nm].

Table 1: Description of the main peaks observed in the HPLC-DAD-ESI-TOF-MS analyses performed for the alkaloid fraction of *P. stenocalyx* leaf extract.

Peak nº	Retention time (min.)	UV spectrum λ_{max}(nm)	Molecular ion $[M + H]^+$	Molecular formula (calc.)	compound
1	15.9	247, 303, 370	527.2025	$C_{27}H_{31}N_2O_9^+$	**1**
2	19.5	239, 308, 338	733.2582	$C_{38}H_{41}N_2O_{13}^+$	**2**
3	47.4	224, 280	499.2067	$C_{26}H_{31}N_2O_8^+$	**3**
4	58.4	221, 286, 385	541.1831	$C_{27}H_{29}N_2O_{10}^+$	**4**
5	70.3	219, 290	365.1500	$C_{21}H_{21}N_2O_4^+$	**5**
6	73.7	225, 291	351.1709	$C_{21}H_{23}N_2O_3^+$	**6**
7	74.3	225, 291	351.1714	$C_{21}H_{23}N_2O_3^+$	**7**

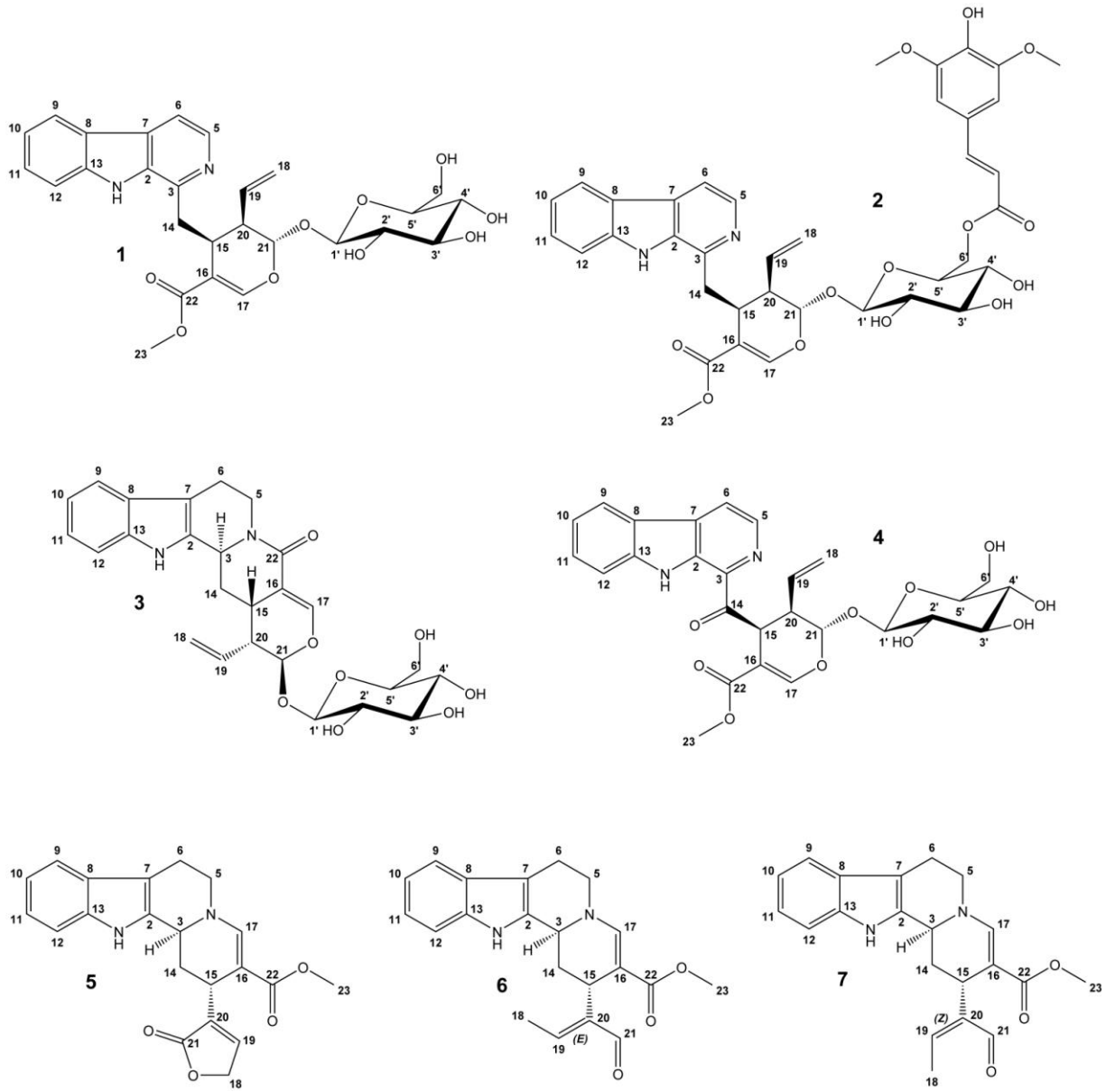

Fig. 3: Monoterpene indole alkaloids (compounds **1**-**7**) identified in the alkaloid fraction of *P. stenocalyx* extract of leaves.

CONCLUSIONS

P. stenocalyx extract of leaves showed preliminary anti-inflammatory activity at the 400 mg/kg dose, by inhibiting leukocyte influx and exudate formation to the pleural cavity after carrageenan administration. From the alkaloid fraction of *P. stenocalyx* extract, the seven known monoterpene indole alkaloids were identified: lyaloside **1**, (E)-O-(6′)-cinnamoyl-4”-hydroxy-3”,5”-dimethoxy-lyaloside **2**, strictosamide **3**, pauridianthoside **4**, vallesiachotamine lactone **5**, E-vallesiachotamine **6** and Z-vallesiachotamine **7**. Although these alkaloids are already known, this is the first biological and phytochemical study with the species *P. stenocalyx*.

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Conflict of interests: there are no conflict of interests.

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