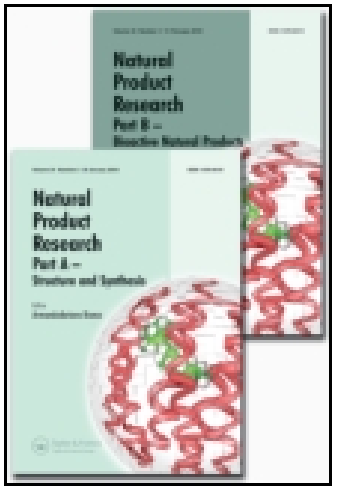

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Cyclooxygenase inhibitory properties of *nor*-neolignans from *Styrax pohlii*

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Chemical investigation of the *n*-hexane and EtOAc fractions of the ethanolic extract from *Styrax pohlii* (Styracaceae) aerial parts resulted in the isolation of the benzofuran *nor*-neolignan derivatives egonol (**1**), homoegonol (**2**), homoegonol gentiobioside (**3**), homoegonol glucoside (**4**) and egonol gentiobioside (**5**). This is the first report of compounds **1–5** in *S. pohlii*. Compounds **1–5**, the acetyl derivatives **1 a** and **2 a**, the ethanolic extract (**EE**), the *n*-hexane fraction (**HF**) and EtOAc fraction (**EF**) were tested for their inhibitory activities against COX-1 and COX-2. The results showed that **EE**, **HF**, **EF** and compounds **1–5** and **1 a–2 a** shown weak to moderate inhibition of COX-1 and COX-2. Among the assayed *nor*-neolignans, **4** gave a COX-1 inhibition of 35.7% at 30 μM. Compound **5** displayed a COX-2 inhibition of 19.7% at 30 μM.

Keywords: *Styrax pohlii*; Styracaceae; cyclooxygenase – 1/2 inhibitory activity

1. Introduction

Prostaglandins (PGs) are mediators of pain, fever and other symptoms associated with inflammation. Their biosynthesis is rate-limited by the initial conversion of arachidonic acid to PG G_2, and by conversion of the latter to PG H_2, which involves a single enzyme, namely cyclooxygenase (COX). Nonsteroidal anti-inflammatory drugs exert their therapeutic effects by inhibiting PG synthesis in COX (Smith, DeWitt, & Garavito, 2000). It is well established that COXs exist as three isoforms that catalyse the same reaction but differ in terms of expression regulation. More specifically, COX-1 is constitutively expressed in most tissues and is thought to be responsible for regulating normal physiological functions, while COX-2 is induced in macrophages, fibroblasts and several other cell types by pro-inflammatory stimuli. COX-3 is a splice variant of COX-1 (Chandrasekharan et al., 2002; Smith et al., 2000; Warner & Mitchell, 2002).

Styrax pohlii A. DC. (Styracaceae), known in Brazil as 'benjoeiro', 'estoraqueiro', 'árvore-de-bálsamo', 'pindaíba', 'pindaubuna' or 'pindauvuna', is a tree that grows in the States of São Paulo, Minas Gerais, Goiás, and Mato Grosso do Sul and is commonly employed in folk medicine to relieve fever (Lorenzi, 1998; Rodrigues & de Carvalho, 2008). The resinous material that is usually secreted, when the barks and trunks of this plant are

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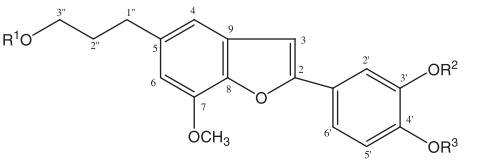
cut by sharp objects is often utilised in traditional medicine to substitute the benzoin resin, which is known for its anti-inflammatory properties (Lorenzi, 1998; Pauletti, Teles, Silva, Araújo, & Bolzani, 2006). Previous studies on this genus have resulted in the isolation of lignan derivatives of 3,7-dioxabicyclo[3.3.0]octane, butanolide and tetrahydrofuran; neolignan derivatives of dihydrobenzofuran, *nor*-neolignan derivatives of benzofuran, phenylpropanoids, phenolic acids, pentacyclic saponins and triterpenes (Pauletti et al., 2006). In addition, biological investigations have shown that the *nor*-neolignans from *Styrax*, particularly egonol (**1**) and homoegonol (**2**) and their derivatives, display moderate antimicrobial and cytotoxic actions (Hirano, Gotoh, & Oka, 1994; Öztürk, Akgül, & Anil, 2008; Pauletti, Araújo, Young, Giesbrecht, & Bolzani, 2000; Teles et al., 2005). Moreover, egonol has been shown to inhibit the hemolytic activity of the complement system, which could be beneficial in the therapy of inflammatory diseases (Min et al., 2004).

As a part of our ongoing biological and chemical studies on *Styrax* (Pauletti et al., 2000; Pauletti, Araújo, Young, & Bolzani, 2002; Teles et al., 2005), as well as our investigations on the anti-inflammatory action of natural and semi-synthetic compounds (Coimbra et al., 2004; Neto et al., 2005; Vasconcelos et al., 2006), we now report on the isolation and structural identification of chemical constituents from the *n*-hexane and EtOAc fractions of *S. pohlii* aerial parts, as well as their COX inhibitory activities.

2. Results and discussion

The spectral data of all the isolated compounds (Figure 1) are in agreement with previously published data and allowed for identification of egonol (**1**), homoegonol (**2**), homoegonol glucoside (**4**) and egonol gentiobioside (**5**) (Hopkins, Ewing, & Chisholm, 1967; Pauletti et al., 2000; Schreiber & Stevenson, 1976; Takanashi & Takizawa, 2002). Homoegonol gentiobioside (**3**) had been previously isolated from *S. officinalis* seeds (Anil, 1980), although no NMR data were provided. To the best of our knowledge, this is the first report of compounds **1–5** in *S. pohlii*.

Earlier studies with lignans, neolignans and *nor*-neolignans have revealed that some display significant anti-inflammatory activity (Ban et al., 2002; Blunder et al., 2010; Coy, Cuca, & Sefkow, 2009; Su et al., 2004; Tzeng & Liu, 2004). This has encouraged us to continue our biological and chemical investigations of lignans, neolignans and *nor*-neolignans from *Styrax* species that display different structural features. To establish the potential of crude extracts, fractions and isolated compounds as anti-inflammatory agents, an *in vitro* screening for their ability to inhibit COX isozymes was accomplished.



	R^1	R^2	R^3
1	H	-CH_2-	
1a	CH_3CO	-CH_2-	
2	H	CH_3	CH_3
2a	CH_3CO	CH_3	CH_3
3	6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl	CH_3	CH_3
4	β-D-glucopyranosyl	CH_3	CH_3
5	6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl	-CH_2-	

Figure 1. Chemical structures of the isolated compounds.

Regarding the COX assay (Table 1), incubation of COX-1 and COX-2 with the ethanolic extract (**EE**, 50 μg mL^{-1}) inhibited PG production by 4.8 ± 7.7% and 84.1 ± 3.9%, respectively. Considering these values, the crude extract exhibited a promising selectivity toward COX-2.

Diminished inhibitory activity was detected during assessment of the anti-inflammatory action of the *n*-hexane fraction (**HF**, 50 μg mL^{-1}) and EtOAc fraction (**EF**, 50 μg mL^{-1}) with respect to COX-1 and COX-2. Indeed, **HF** and **EF** were completely unable to inhibit PG production by COX-1. Compared to the crude extract (**EE**) at the same concentration, a small inhibitory activity was observed for COX-2 in the case of **HF** and **EF** (19.3 ± 4.8% and 4.1 ± 3.1%, respectively).

Compounds **1–5** and **1 a–2 a** were also evaluated for their inhibitory activity toward COX-1 and COX-2 (Table 1). Each compound was tested at final concentrations of 30, 20, 10 and 5 μM. Egonol (**1**) and homoegonol (**2**) at 30 μM gave COX-1 inhibition of 19.1 ± 4.3% and 10.6 ± 3.5%, respectively, and COX-2 inhibition of 4.5 ± 3.8% and 7.1 ± 4.9%, respectively. On the other hand, acetylation of **1** and **2** did not improve COX-1 inhibition by **1 a** or **2 a**. In fact, **1 a** and **2 a** were unable to inhibit COX-1 at any extension, but these derivatives enhanced COX-2 inhibition as compared to **1** and **2** at 30 μM by 11.1 ± 1.6% and 16.0 ± 0.2%, respectively.

The glycosylated compounds homoegonol gentiobioside (**3**), homoegonol glucoside (**4**) and egonol gentiobioside (**5**) at 30 μM yielded COX-1 inhibition of 1.3 ± 0.6%, 35.7 ± 6.7%, and 23.3 ± 9.1%, respectively. Furthermore, COX-2 inhibition was 6.5 ± 0.1%, 11.9 ± 0.2% and 19.7 ± 3.2%, respectively. Thus, a very small inhibitory activity was observed for COX-1 and COX-2 in the case of compound **3**. Additionally, among the assayed *nor*-neolignans, **4**, furnished the best value of COX-1 inhibition at 30 μM. As for **5**, it provided the greatest percentage of COX-2 inhibition at this same concentration.

The compounds SC-560 and DuP697 were used to standardise the assay for COX-1 (tested concentration = 20 nM, 48.1 ± 9.0%) and COX-2 (tested concentration = 40 nM, 49.3 ± 3.9%), respectively. The IC_{50} for Ibuprofen, used as positive control, were determined to be 2.6 and 1.53 μM for COX-1 and COX-2, respectively.

Table 1. COX-1 and COX-2 inhibitory effects of the extracts, fractions and compounds from *S. pohlii*.

		COX inhibition (percentage)[c]	
Extract/compound	Concentration	COX-1	COX-2
EE	50[a]	4.8 ± 7.7	84.1 ± 3.9
HF	50[a]	n.a.	19.3 ± 4.8
EF	50[a]	n.a.	4.1 ± 3.1
1	30[b]	19.1 ± 4.3	4.5 ± 3.8
2	30[b]	10.6 ± 3.5	7.1 ± 4.9
3	30[b]	1.3 ± 0.6	6.5 ± 0.1
4	30[b]	35.7 ± 6.7	11.9 ± 0.2
5	30[b]	23.3 ± 9.1	19.7 ± 3.2
1 a	30[b]	n.a.[d]	11.1 ± 1.6
2 a	30[b]	n.a.	16.0 ± 0.2

Notes: [a]Conc., μg mL^{-1}.
[b]Conc., μM.
[c]The data represent the mean ± S.E.M.
[d]Not active.

Compounds **1** and **2** are structurally quite similar, both have a benzofuran moiety and differ mainly in the presence of a methylenedioxy substituent in **1** *versus* two methoxyl groups in **2**. An attempt to improve the weak activity showed by **1** and **2** was made by the production of their acetyl derivatives. Although, **1 a** and **2 a** lost COX-1 inhibitory activity.

Regarding the glycosylated *nor*-neolignans, that enhance the hydrosolubility of the egonol and homoegonol derivatives, the number of glucosyl groups in the homoegonol moiety suggests that the presence of one glucosyl group, as in the case of compound **4**, may improve the activity of this derivative, since **4** was more active than **3** and **2**. Furthermore, it can be observed that compound **5** is quite similar to egonol (**1**), differing only in the presence of two glucose unites, exhibited a slight enhanced COX-2 inhibitory activity.

Considering their previous reported biological activities, egonol (**1**) and homoegonol (**2**), which occur widely in *Styrax* (Styracaceae), have attracted researchers' attention mainly because of their antibacterial, antifungal and moderate cytotoxic properties (Pauletti et al., 2000; Teles et al., 2005). Additionally, egonol has been shown to be able to inhibit the hemolytic activity of the complement system with IC_{50} values of 33 μM, while homoegonol was inactive. The modulation of the complementary activity might be beneficial in the therapy of inflammatory diseases (Min et al., 2004). Other *nor*-neolignans and neolignans having a benzofuran moiety, such as 9′-nor-7,8-dehydro-isolicarin B, ocophyllals A and ocophyllals B, have been described to exhibit COX-2 selectivity, affording the best results among other *nor*-neolignans and neolignans cores, with IC_{50} lying in the range of 3.32–16.8 μM (Coy et al., 2009).

3. Experimental

3.1. *General*

^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 *or MeOD*-d_4 on a Bruker AVANCE DRX 500 spectrometer, using TMS as internal standard. Both analytical and preparative HPLC separation analyses were carried out on a Shimadzu LC-6AD system equipped with a degasser DGU-20A5, a UV–Vis detector SPD-20 A series, a communication bus module CBM-20 A, and a Rheodyne manual injector. Separations of the compounds were carried out on a SHIMADZU Shim-pack ODS (particle diameter 5 μm, 250 mm × 4.60 mm and 250 mm × 20 mm) columns equipped with a pre-column of the same material. The MeOH used in the experiments was HPLC grade, J.T. Baker. Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system, Millipore. Silica gel 60 (230–400 mesh, Sigma-Aldrich) and Sephadex LH-20 (Sigma-Aldrich) were employed for column chromatography, as well as silica on TLC Alu foils with fluorescent indicator 254 nm (Sigma-Aldrich). Prep-TLC was conducted on silica gel type G (Sigma-Aldrich). Silica gel 90 reverse-phase ODS (Fluka, 230–400 mesh) was utilised for sample preparation prior to injection into the HPLC system.

3.2. *Plant material*

Aerial parts of *Styrax pohlii* A. DC. were collected in Luis Antonio city, (21°33′–21°37′ S and 47°45′–47°57′ W, in October 2008), and identified by Prof. V.M.M. Gimenez and Prof. M. Groppo. A voucher specimen (SPFR12168) was deposited in the Herbarium of the Department of Biology, Laboratory of Plant Systematics, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, University of São Paulo, Brazil (Herbarium SPFR).

3.3. *Extraction and isolation*

The air-dried, powdered stems and leaves (2.4 kg) of *S. pohlii* were extracted with EtOH. After filtration, the solvents were removed under reduced pressure, to yield 87 g of

the extract. The **EE** (40 g) was then dissolved in MeOH/H_2O (2 : 8 v/v) and successively partitioned with *n*-hexane, EtOAc and *n*-BuOH. After solvent removal using a rotary evaporator, each partition phase yielded 2.8, 8.9, 7.6 and 8.2 g, respectively. The **HF** (1.4 g) was chromatographed over silica using a gradient of *n*-hexane/EtOAc as eluent, which afforded 111 fractions. Compounds **1** (27 mg) and **2** (22 mg) were isolated from fractions 92–100 and 69–80, respectively, after a prep-TLC silica using CH_2Cl_2/MeOH (98 : 2 v/v). The EtOAc residue (3.2 g) was submitted to a Sephadex LH-20 chromatographic column employing MeOH as eluent, which furnished seven fractions. Fractions 8–10 (336 mg) were dissolved in MeOH/H_2O (68 : 32 v/v), chromatographed over a reverse phase ODS cartridge, and subsequently submitted to preparative RP-HPLC purification using MeOH/H_2O/AcOH (68 : 31.9 : 0.1 v/v/v), UV detection at 254 nm and 9 mL min^{-1} flow rate, which yielded six fractions. Fractions 2, 3 and 5 gave compounds **3** (18 mg, Rt 15.9), **4** (10 mg, Rt 18.8) and **5** (7 mg, Rt 26.2), respectively.

Homoegonol gentiobioside (**3**). ^{1}H NMR (500 MHz, δ, methanol-d_4): 1.98 (m, 2 H, H-2″), 2,82 (t, $J = 7.4$, 2 H, H-1″), 3.19 (d, $J = 10.5$, 1 H, H-6‴), 3.21 (m, 1 H, H-2‴ and H-2⁗), 3.28 (m, 1 H, H-5⁗), 3.36 (m, 3 H, H-3‴, H-4‴ and H-3⁗), 3.44 (m, 1 H, H-5‴), 3.48 (m, 1 H, H-4⁗), 3.59 (m, 1 H, H-3‴), 3.75 (dd, $J = 5.4$ and 11.4, 1 H, H-6‴), 3.86 (dd, $J = 5.4$ and 11.4, 1 H, H-6⁗), 3.90 (s, 3 H, 3′-OCH_3), 3.94 (m, 1 H, H-3″), 3.95 (s, 4′-OCH_3), 4.04 (s, 3 H, 7-OCH_3), 4.10 (dd, $J = 1.3$ and 11.4, 1 H, H-6″), 4.28 (d, $J = 7.8$, 1 H, H-1‴), 4.34 (d, $J = 7.5$, 1 H, H-1⁗), 6.79 (s, 1 H, H-6), 7.05 (s, 1 H, H-3), 7.07 (s, 1 H, H-4), 7.08 (d, $J = 8.4$, 1 H, H-5′), 7.46 (d, $J = 1.7$, 1 H, H-2′), 7.49 (dd, $J = 1.7$ and 8.4, 1 H, H-6′). ^{13}C NMR (125 MHz, δ, methanol-d_4): 32.2 (C-2″), 32.5(C-1″), 55.6 (3′-OCH_3), 55.7 (4′-OCH_3), 55.8 (7-OCH_3), 66.0 (C-6⁗), 68.8 (C-6‴), 69.0 (C-3″), 70.2 (C-4⁗), 70.5 (C-4‴), 74.0 (C-2⁗), 74.2 (C-2‴), 76.1 (C-5‴), 76.9 (C-5⁗), 76.9 (C-3‴), 77.1 (C-3⁗), 100.7 (C-3), 103.7 (C-1‴), 104.7 (C-1⁗), 107.9 (C-6), 108.7 (C-2′), 112.7 (C-4), 112.7 (C-5′), 118.1 (C-6′), 124.0 (C-1′), 131.6 (C-9), 138.4 (C-5), 142.7 (C-8), 145.2 (C-7), 149.9 (C-4′), 150.2 (C-3′), 156.4 (C-2).

3.4. *Acetylation of egonol (1) and homoegonol (2)*

Four milligrams of **1** and **2** were treated with acetic anhydride (3 mL) and pyridine (3 mL) overnight. The reaction was quenched with cold H_2O (30 mL) and extracted with CH_2Cl_2 (2 mL × 15 mL), HCl 10% (2 mL × 15 mL) and H_2O (3 mL × 15 mL). Anhydrous sodium carbonate was added to the organic phase, which was filtered and concentrated under reduced pressure, to give acetate derivatives **1 a** and **2 a**.

3.5. In vitro *COX-1 and COX-2 inhibitory activity*

The extracts, fractions, isolated and acetylated compounds were tested *in vitro* for their COX-1 and COX-2 inhibitory activity using a COX inhibitor screening assay kit (catalogue no. 560131, Cayman Chemicals, MI, USA), according to the manufacturer's instructions. COX-1 and COX-2 catalyse the conversion of arachidonic acid to PGH_2; $PGF_{2\alpha}$ is produced from PGH_2 by reduction of the latter with stannous chloride and this product is quantified via an enzyme immunoassay [Acetylcholinesterase, AChE]. In our experiments, the samples were dissolved in DMSO and diluted to the desired concentration using the reaction buffer. The reaction mixtures (1.15 mL) containing 950 µL 0.1 M Tris-HCl (pH 8.0) as well as 5 mM EDTA, 2 mM phenol, 10 µL heme and 10 µL COX-1 (ovine) or COX-2 (human recombinant) were pre-equilibrated to 37°C in a water bath. Next, 20 µL of the sample with the desired concentration of the test solution (final concentrations of 30, 20, 10 and 5 µM for the pure compounds and 50 µg mL^{-1} for the crude extract and fractions) were added to the tube containing the inhibitor

(Gautam, Srivastava, Jachak, & Saklani, 2010). 20 μL of the buffer were added to the 100% initial activity tubes. The incubation period was 10 min. The reaction was initiated by addition of 10 μL arachidonic acid at a final concentration of 100 μM to all the test tubes. After 2 min, the reactions were finalised by addition of HCl 1 M (50 μL), followed by 100 μL stannous chloride. Then, $PGF_{2\alpha}$ was quantified by means of the ELISA method. For this purpose, the contents of the reaction tubes were diluted and transferred to a 96-well plate coated with a mouse anti-rabbit IgG, which was followed by addition of the PG screening AChE tracer and the PG screening antiserum. Plates were incubated in an orbital shaker for 18 h, at room temperature. Next, the reaction mixtures were removed, and the wells were washed five times with buffer containing 0.05% Tween 20. Ellman's reagent (200 μL) was then added to each well, and the plate was incubated in an orbital shaker for 60 min, at room temperature, until the control wells yielded an OD lying between 0.3–0.8 at 405 nm. A standard curve with PG was generated from the same plate, which was used to quantify the PG levels produced in the presence of the samples. Results are expressed as the percentage of inhibition relative to a control (100% initial activity, solvent-treated samples). All determinations were carried out in duplicate. The compounds SC-560 (Cayman Chemicals) and DuP697 (Cayman Chemicals) were used to standardise the assay for COX-1 (tested concentration = 20 nM, 48.1 ± 9.0%) and COX-2 (tested concentration = 40 nM, 49.3 ± 3.9%), respectively. Ibuprofen (Sigma-Aldrich) was employed as positive control. The concentration of drug giving 50% of inhibition (IC_{50}) was calculated by non-linear regression analysis of percentage of inhibition *versus* concentration.

4. Conclusions

In summary, chemical investigations of *n*-hexane and EtOAc fractions of the **EE** from *S. pohlii* resulted in the isolation and identification of compounds **1–5**. Additionally, biological results indicated that **EE**, **HF**, **EF** and compounds **1–5** and **1 a–2 a** are able to weakly or moderately inhibit COX-1 and COX-2.

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