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Isolation of lignans glycosides from *Alibertia sessilis* (Vell.) K. Schum. (Rubiaceae) by preparative high-performance liquid chromatography

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Abstract: Enantiomeric aglycone lignans contained in a mixture were separated from a fraction of the extract of the stems of *Alibertia sessilis* (Vell.) K. Schum. (Rubiaceae) by preparative high-performance liquid chromatography. An efficient and fast separation can be achieved with methanol-water (30:70, v/v). Their structures were identified as (+)-lyoniresinol 3α-*O*-β-glucopyranoside and (-)-lyoniresinol 3α-*O*-β-glucopyranoside, being reported for the first time in Rubiaceae.

Keywords: Rubiaceae; *Alibertia sessilis*; enantiomeric aglycone lignans; HPLC.

Introduction

Rubiaceae is widely distributed in Brazilian main ecosystems (Amazon, Cerrado and Atlantic Forest). This family is well known due to economic and therapeutic importance of these species, specially *Coffea arabica* and *Cinchona ledgeriana* [1-2]. Reported chemical constituents of Rubiaceae revealed a great diversity of secondary metabolites such as iridoids, alkaloids, anthraquinones, flavonoids, phenolics derivatives, triterpenes and diterpenes [1].

Few chemical studies of *Alibertia* have been reported resulting in the isolation of several triterpenes [2-4], iridoids [5], flavonoids [6] and caffeic acid esters [2].

Alibertia sessilis (Vell.) K. Schum., popularly known as "marmelada-de-cachorro", is distributed in the states of Ceará, Mato Grosso, Goiás and Minas Gerais. The plant possesses fruits with succulent pulp, black when ripe, which are consumed by birds in general [7].

From the stems of *A. sessilis* we report the isolation of a mixture of lignan glycosides. The separation of (+)-lyoniresinol 3α-*O*-β-glucopyranoside (**1**) and (-)-lyoniresinol 3α-*O*-β-glucopyranoside (**2**) by conventional methods such as silica gel and RP18 low pressure column chromatography was unsuccessful. However, it was quite easy and fast to purify each compound by preparative high-performance liquid chromatography (HPLC). Reversed-phase HPLC is commonly used for the separation of compounds present in complex mixtures of plant extracts [8-9].

Nuclear magnetic resonance (NMR) analysis was used to identify the compounds. Compound **1** exhibit antioxidant [10] and antitumor-promoting activities [11]. These compounds, whose chemical structures are given in Figure 1, are being reported for the first time in Rubiaceae.

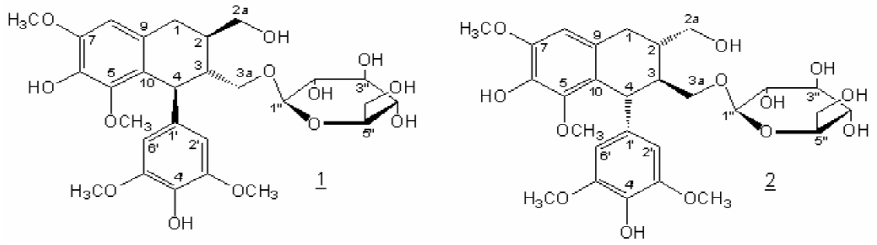

Figure 1. Lignans glucosides isolated from the stems of *Alibertia sessilis*.

Experimental details

Plant material

Alibertia sessilis (Vell.) K. Schum. (Rubiaceae) was collected in the Estação Ecológica Experimental de Mogi-Guaçu, São Paulo State, Brazil in November 2003. A voucher specimen was deposited at the Herbário do Instituto Botânico da Secretaria do Meio Ambiente de São Paulo (nº SP 370.914).

Apparatus

The high-performance liquid chromatography (HPLC) (Shimadzu, Japan) equipment used was a CLASS-VP Ver.5.41 system comprised a Shimadzu SPD10Avp UV detector, a Shimadzu LC-8ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, a Degasser Shimadzu DGU-14A and a CLASS-VP Ver.5.41 workstation (Shimadzu, Japan). HPLC-grade methanol was purchased from J.T. Baker (Baker-Mallinckrodt, Phillipsburg, NJ, USA). HPLC-grade water (18 MΩ cm) was obtained using a Millipore Milli-Q purification system (Millipore Co., Bedford, MA, USA).

Extraction, fractionation and preparation of sample

Dried and powered stems (50.0 g) of *A. sessilis* were successively extracted with ethanol at room temperature. The solvent was removed under reduced pressure yielding 3.7 g of crude extract. This extract was dissolved in methanol-water (80:20, v/v) and partitioned with hexane, ethyl acetate and *n*-butanol to yield 0.5 g of hexane extract, 1.4 g of ethyl acetate extract and 1.3 g of *n*-butanol extract after evaporated to dryness under reduced pressure.

The ethyl acetate extract was submitted to column chromatography using Sephadex LH-20. The column was eluted with methanol, to give 15 fractions reunited after thin layer chromatography (TLC) analysis. Fractions 2-3 (146.4 mg) were subjected to chromatography on a RP18 column using a water-methanol gradient system affording iridoid geniposidic acid (11.8 mg), a derivative fenolic glycoside (6.7 mg) not yet identified and the mixture of lignans glycosides (36.6 mg).

Separation procedure

The separation of lignans was performed using a Phenomenex Luna RP18 (2) column (250 x 21.20 mm I.D. x 10 µm, Torrance, CA, USA) in a Rheodyne 7125 sample injector with a 2.0 mL sample loop (Rheodyne, Cotati, CA, USA). The mobile phase composed of methanol-water (30:70, v/v, isocratic system) was eluted using a flow-rate of 10.0 mL min^{-1}, the injection volume was 1.0 mL. Pure lignans were examined by HPLC on an analytical column. The analysis was performed with a Phenomenex Luna RP18 (2) column (250 x 4.6 mm I.D. x 5 µm, Torrance, CA, USA) in a Rheodyne 7125 sample injector with a 20 µL sample loop (Rheodyne, Cotati, CA, USA). The mobile phase composed of methanol-water (30:70, v/v, isocratic system) was eluted at a flow-rate of 1.0 mL min^{-1}, the injection volume was 20 µL. For both preparative and analytical HPLC, the detector was set as 238 nm.

Structural identification of the compounds

The NMR spectra in CD_3OD of all compounds were obtained using a Varian, INOVA 500 spectrometer (Varian, Palo Alto, CA, USA), operating at 500 MHz for ^1H and 125 MHz for ^{13}C, two-dimensional technique such as *g*COSY (gradient chemical shift correlation spectroscopy), *g*HMQC (gradient heteronuclear multiple quantum coherence) and *g*HMBC (gradient heteronuclear

multiple bond connectivity) were used to fully elucidate the structure of compounds. Chemical shifts were given in δ (ppm) using tetramethylsilane (TMS) as internal standard.

(+)-lyoniresinol 3α-*O*-β-glucopyranoside (6.7 mg, **1**): ESI-MS (negative): m/z 581.2303 [M - H]$^-$. ^{1}H NMR (CD_3OD, 500 MHz): δ 1.61 (1H, *m*, H-2), δ 1.99 (1H, *m*, H-3), δ 2.51 (1H, *dd*, *J* = 12 and 15 Hz, H-1) and 2.61 (1H, *dd*, *J* = 5 and 15 Hz, H-1), δ 3.11-3.15 (2H, *m*, H-2" and H-3"), δ 3.18 (1H, *m*, H-4"), δ 3.24-3.27 (1H, *m*, H-5"), δ 3.25 (3H, s, CH_3O-5), δ 3.35 (1H, *dd*, *J* = 4 and 10 Hz, H-3a), δ 3.43 (1H, *dd*, *J* = 6.5 and 11 Hz, H-2a), 3.55 (2H, *m*, H-2a and H-6"), δ 3.64 (6H, s, CH_3O-3' and CH_3O-5'), δ 3.73 (1H, *m*, H-6"), δ 3.76 (3H, s, CH_3O-7), δ 3.78 (1H, *dd*, *J* = 5.5 and 10 Hz, H-3a), δ 4.18 (1H, *d*, *J*= 7.5 Hz, H-1"), δ 4.32 (1H, *d*, J = 5.5 Hz, H-4), δ 6.32 (2H, *s*, H-2' and H-6'), δ 6.48 (1H, *s*, H-8). ^{13}C NMR (CD_3OD, 125 MHz): δ 33.8 (C-1), δ 40.6 (C-2), δ 42.7 (C-4), δ 46.7 (C-3), δ 56.6 (CH_3O-7), δ 56.9 (CH_3O-3' and CH_3O-5'), δ 60.2 (CH_3O-5), δ 62.8 (C-6"), δ 66.2 (C-2a), δ 71.5 (C-3a), δ 71.7 (C-4"), δ 75.2 (C-2"), δ 77.9 (C-3"), δ 78.2 (C-5"), δ 104.8 (C-1"), δ 107.0 (C-2' and C-6'), δ 107.9 (C-8), δ 126.4 (C-10), δ 130.2 (C-9), δ 135.0 (C-4'), δ 138.9 (C-6), δ 139.3 (C-1'), δ 147.6 (C-5), δ 148.6 (C-7), δ 149.0 (C-3' and C-5').

(-)-lyoniresinol 3α-*O*-β-glucopyranoside (5.8 mg, **2**): ESI-MS (negative): m/z 581.2303 [M - H]$^-$. ^{1}H NMR (CD_3OD, 500 MHz): δ 1.58 (1H, *m*, H-2), δ 2.03 (1H, *m*, H-3), δ 2.56 (2H, *m*, H-1), δ 3.03-3.11 (4H, *m*, H-2", H-3", H-4" and H-5"), δ 3.23 (3H, s, CH_3O-5), δ 3.50 (2H, *m*, H-2a), δ 3.51 (1H, *m*, H-3a), δ 3.58 (1H, *m*, H-6"), δ 3.65 (6H, s, CH_3O-3' and CH_3O-5'), δ 3.72 (1H, *m*, H-3a), δ 3.74 (1H, *m*, H-6"), δ 3.75 (3H, s, CH_3O-7), δ 4.03 (1H, *d*, *J*= 7.5 Hz, H-1"), δ 4.13 (1H, *d*, *J* = 6.5 Hz, H-4), δ 6.31 (2H, *s*, H-2' and H-6'), δ 6.48 (1H, *s*, H-8). ^{13}C NMR (CD_3OD, 125 MHz): δ 33.8 (C-1), δ 41.6 (C-2), δ 43.2 (C-4), δ 46.6 (C-3), δ 56.6 (CH_3O-7), δ 56.9 (CH_3O-3' and CH_3O-5'), δ 60.1 (CH_3O-5), δ 62.7 (C-6"), δ 66.2 (C-2a), δ 71.6 (C-4"), δ 72.0 (C-3a), δ 75.1 (C-2"), δ 77.9 (C-3"), δ 78.2 (C-5"), δ 104.2 (C-1"), δ 107.1 (C-2' and C-6'), δ 107.8 (C-8), δ 126.2 (C-10), δ 130.2 (C-9), δ 135.0 (C-4'), δ 138.9 (C-6), δ 139.4 (C-1'), δ 147.5 (C-5), δ 148.7 (C-7), δ 149.0 (C-3' and C-5').

Results and Discussion

The crude extract from stems of *A. sessilis* was partitioned using hexane, ethyl acetate and *n*-butanol. The ethyl acetate extract afforded geniposidic acid, a derivative fenolic glycoside and a third compound. The ^{1}H- and ^{13}C-NMR data for this last showed overlapping signals, suggesting that third compound could be a diastereomeric mixture of lignans containing enantiomeric aglycone parts that were not separated. Conventional methods such as silica gel and RP18 low pressure column chromatography were not successful to separate this lignans.

So, this partially purified sample was analysed by HPLC and a series of experiments was performed to determine the optimal solvent system for the HPLC separation. The following systems were tested: methanol-water (35:65, 30:70, 25:75, v/v), and the separation time were: 18 min, 25 min and 42 min, respectively, for an analytical separation run. But, despite of first system has faster separation time, the peaks concerning to lignans didn't have good separation, and the third system had a long separation time. A good and fast separation can be achieved with the system methanol-water (30:70, v/v).

Another evidence that the mixture was composed of a enantiomeric aglycone parts is the identical UV spectras with absorption maximum at 215 and 270 nm and one spot when analysed by TLC.

The result obtained from the partially purified sample of *Alibertia sessilis* by preparative HPLC is showed in Fig. 2.

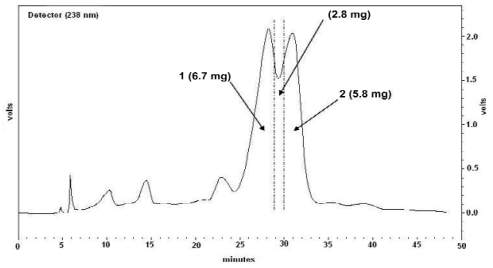

Figure 2. Preparative HPLC chromatogram of the partially purified sample of *Alibertia sessilis*. Column: Phenomenex Luna RP18 (2) column (250 x 21.20 mm I.D. x 10 µm); mobile phase: methanol-water (30:70, v/v); flow-rate: 10.0 mL min^{-1}; monitoring at 238 nm. Peak 1: (+)-lyoniresinol 3α-*O*-β- glucopyranoside. Peak 2: (-)-lyoniresinol 3α-*O*-β- glucopyranoside.

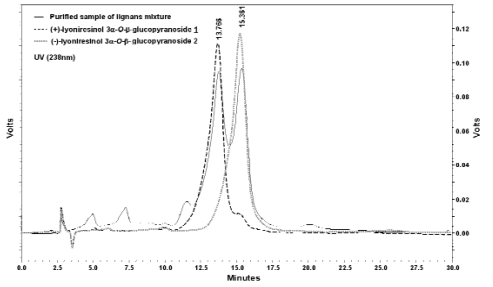

Figure 3. Analytical HPLC chromatogram of the partially purified sample of *Alibertia sessilis* and the compounds isolated. Column: Phenomenex Luna RP18 (2) column (250 x 4.6 mm I.D. x 5 µm); mobile phase: methanol-water (30:70, v/v); flow-rate: 1.0 mL min^{-1}; monitoring at 238 nm. Peak at t_R = 13.766 min: (+)-lyoniresinol 3α-*O*-β-glucopyranoside **1**. Peak at t_R = 15.361 min: (-)-lyoniresinol 3α-*O*-β-glucopyranoside **2**.

After this separation, each fraction was collected, weighed and analysed by ^{1}H- and ^{13}C-NMR and HPLC (fig. 3). The relative configurations at C-2, C-3 and C-4 were estabilished by comparison with literature data [12]. The coupling constants (J = 7.5 Hz) of the anomeric proton signal of the glucosyl moiety demonstrated that sugar has b-anomeric configuration [13]. The peak 1 fraction was identified as (+)-lyoniresinol 3α-*O*-β-glucopyranoside (**1**) and the peak 2 fraction as (-)-lyoniresinol 3α-*O*-β-glucopyranoside (**2**).

Conclusions

Reversed-phase HPLC was successful in the preparative separation of a mixture containing enantiomeric aglycone parts, it was easy and fast to purify each compound of the mixture. So, this study contributed significantly to improve the knowledge about secondary metabolites of more one species of Brazilian Cerrado, when these compounds are being reported for the first time in Rubiaceae.

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V. C. da Silva, G. H. Silva, V. da S. Bolzani, M. N. Lopes. Isolamento de lignanas glicosiladas de *Alibertia sessilis* (Vell.) K. Schum. (Rubiaceae) por cromatografia líquida de alta eficiência preparativa.

Resumo: Agliconas enantioméricas de lignanas, contidas numa mistura, foram separadas de uma fração do extrato dos galhos de *Alibertia sessilis* (Vell.) K. Schum. (Rubiaceae) por cromatografia líquida de alta eficiência preparativa. Uma separação eficiente e rápida pode ser conseguida com a fase móvel metanol-água (30:70, v/v). As estruturas foram identificadas como (+)-lioniresinol 3α-*O*-β-glicopiranosídeo e (-)-lioniresinol 3α–*O*-β-glicopiranosídeo, sendo descritas pela primeira vez em Rubiaceae.

Palavras-chave: Rubiaceae; *Alibertia sessilis*; agliconas enantioméricas de lignanas; HPLC.

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