Separation of the toxic zierin from *Zollernia ilicifolia b*y high speed countercurrent chromatography

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RESUMO: Separação da toxica (z)-zierinade de *Zollernia ilicifolia* por cromatografia em corrente de alta velocidade. Ensaios farmacológicos preliminares do extrato metanólico 70% das folhas da espécie medicinal brasileira *Zollernia ilicifolia* Vog. (Fabaceae) exibiu efeitos analgésicos e antiulcerogênicos. Análises realizadas previamente mostraram que esse extrato contém, além de flavonóides glicosilados, saponinas e glicosídeo cianogênico. Flavonóides e saponinas são substâncias descritas na literatura com atividade antiulcerogênica. Neste trabalho, desenvolvemos uma metodologia para separar o glicosídeo cianogênico dos outros componentes para obter quantidade suficiente de material para realização de ensaios farmacológicos. O glicosídeo cianogênico zierina (2*S*-□-D-glicopiranosiloxi-(3-hidroxifenil) acetonitrila) foi separado dos outros componentes por cromatografia contracorrente a alta velocidade. O sistema de solvente utilizado foi uma mistura clorofórmio-metanol-n-propanol-água (5:6:1:4), v/v/v/v). Esta técnica levou à separação da zierina dos outros componentes de *Zollernia ilicifolia.*

Palavras-chave: *Zollernia ilicifolia*, zierina, cromatografia contracorrente a alta velocidade

ABSTRACT: Preliminary pharmacological assays of the 70% methanol extract from the leaves of the Brazilian medicinal plant *Zollernia ilicifolia* Vog. (Fabaceae) showed analgesic and antiulcerogenic effects. Previous analyses have shown that this extract contains, besides flavonoid glycosides and saponins, a toxic cyanogenic glycoside. Flavonoids and saponins are compounds reported in literature with antiulcerogenic activity. In this work, we developed a methodology to separate the cyanogenic glycoside from these compounds in order to obtain enough amount of material to perform pharmacological assays. The cyanogenic glycoside zierin (2*S*)-□-D-glucopyranosyloxy-(3-hydroxy-phenyl)-acetonitrile was separated from the other components by high speed countercurrent chromatography (HSCCC). The solvent system used was composed of chloroform-methanol-n-propanol-water (5:6:1:4, v/v/v/v). This technique led to the separation of zierin from the possible active compounds of *Zollernia ilicifolia*.

Key words: *Zollernia ilicifolia*, zierin, high-speed counter-current chromatography

INTRODUCTION

Zollernia ilicifolia Vog. (Fabaceae) is used as an herbal remedy in traditional medicine with the same uses of the true “espinheira-santa” (*Maytenus ilicifolia*, Celastraceae). This species is native from the Brazilian Tropical Atlantic Forest region, Brazil (Di Stasi et al., 2002). In an investigation, a new flavonol glycoside kaempferol-3-*O*-□-L-rhamnopyranosyl(1□ 2)-*O*-[□-L-rhamnopyranosyl (1□ 6)]-*O*-□-D-galactopyranoside-7-*O*-□-L-rhamnopyranoside and a cyanogenic glycoside zierin (2*S*)-□-D-glucopyranosyloxy-(3-hydroxy-phenyl)-acetronitrile were identified in the infusion of leaves from *Z. ilicifolia* (Coelho et al., 2003). Zierin was isolated for the first time from *Zieris laevigata* (Rutaceae) (Gmelin et al., 1973). Studies evidenced the analgesic and antiulcerogenic effects of the 70% methanolic extract of *Z. ilicifolia* on lesions induced by indomethacin/bethanechol in mice. Pharmacological

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tests have also shown that animals treated with *Z. ilicifolia* extract exhibited increase of irritability and respiratory rate, loss of corneal reflex and decrease of motor activity (Gonzalez et al., 2001).

Chemical screening by TLC and HPLC of the 70% methanolic extract showed the presence of flavonoids and saponins but also of a cyanogenic glycoside. It is well known that cyanogenic glycosides are very toxic components from plants and they can cause death (Vetter, 2000). In fact, we observed a significative percentage of deaths of the mice submitted to pharmacological testings (Gonzalez et al., 2001).

Since literature reports that flavonoids and saponins are compounds with antiulcerogenic activity, it is important to develop a methodology to rapidly separate the cyanogenic glycoside from these compounds in order to obtain enough amount of material to perform pharmacological assays (Matsuda et al., 1998; La Casa et al., 2000).

Among the faster methods for the separation of secondary metabolites from plants, high speed countercurrent chromatography (HSCCC) is a special liquid-liquid partition chromatography that uses no solid support matrix, eliminates tailing of solute peaks and minimizes the loss of material due to decomposition (Conway, 1990). The method has been successfully applied to the analysis and separation of various natural products (Hostettmann et al., 1984); (Marston et al., 1990); (Marston et al., 1994); (Hostettmann et al., 2001). However, this method had not been applied to the separation of cyanogenic glycosides.

MATERIAL AND METHOD

Reagent

All organic solvents used for HSCCC were of p.a. grade from Merck. The solvents used for HPLC were of analytical grade from Mallinckrodt Baker S.A., Brazil. Water was nanopure quality.

Preparation of crude sample

Leaves of *Zollernia ilicifolia* (Fabaceae) (485.0 g) were collected at Intervales State Park, Saibadela, Sete Barras, Vale do Ribeira, State of São Paulo, Brazil. *Zollernia ilicifolia* was authenticated by Dr. Ademir Reis, Herbario Barbosa Rodrigues, Itajaí, State of Santa Catarina, Brazil, where voucher specimens were deposited. *Z. ilicifolia* is popularly known in this region as “espinheira-santa”. The plant material was dried in an oven at 45°C during 1 week and powdered. The resulting material (110.0 g) was macerated (four times) at room temperature with 70% methanol 1 L during 48 hours. After filtration and evaporation of the solvents under reduced pressure we obtained about 14.0 g of extract from the dried powder (12,7% yield).

Apparatus

The present study was performed with a P.C. Inc., Potomac, USA. It was equipped with a multilayer with two coils of 1.68 mm i.d polytetrafluoroethylene (PTFE) tubing of approximately 80 and 240 mL with a total capacity of 320 mL. The □ value varied from 0.50 at the internal terminal to 0.85 at the external terminal and the revolution radius was 10 cm (□ = *r/R*, where *r* is the distance from the coil to the holder, and *R*, the revolution radius or the distance between the holder axis and the central shaft). The speed (varying between 0 and 1200 rpm) was adjusted with a controller to a speed of 850 rpm. The flow-rate was controlled with a Waters 4000 constant-flow pump. The sample was injected with a P.C. Inc. Injection Module with a 16 mL sample injection loop.

Preparation of two-phase solvent system and sample solution

Two-phase solvent system composed of chloroform-methanol-n-propanol-water (5:6:1:4, v/v/v/v) was thoroughly equilibrated overnight in a separatory funnel at room temperature and the two phases separated shortly before use.

The sample solution was prepared by dissolving 1.0 g of 70% methanolic extract in 16 mL of a mixture consisting of 8 mL lower phase + 8 mL upper phase of the solvent system chloroform-methanol-n-propanol-water (5:6:1:4, v/v/v/v).

HSCCC separation procedure

For the separation, the column was first entirely filled with the stationary phase (upper phase). Then the apparatus was rotated forward at 850 rpm, while the mobile phase (lower phase) was pumped into the column in a head to tail (H□ T) direction at a flow-rate of 2.0 mL min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 16 mL of the sample solution containing 1.0 g of the crude extract was injected through the injection valve. We collected 60 fractions of 5 mL each with a Redifrac automated fraction collector (Pharmacia, Sweden), in approximately 2.5 h. After the rotation was stopped, washoff was collected in 50 mL fractions.

Analyses of the compounds by TLC and HPLC

The crude sample and the collected fractions were analyzed using silica gel 60G TLC plates on glass (Merck, 20 cm x 20 cm x 0.2 mm) developed with a solvent mixture composed of n-butanol-acetic acid-water (65:15:25, v/v/v) or with chloroform-methanol-n-propanol-water (5:6:1:4, v/v/v/v, lower phase). The spots on the TLC plates were observed under ultraviolet lamp (254 nm) and by spraying with NP/PEG reagent and anisaldehyde/H_2SO_4 followed by heating at 110°C for 5 min (Wagner et al., 1984).

HPLC analysis was performed with a Varian ProStar 330 chromatograph managed by a Varian workstation equipped with a Varian ProStar 220 diode array detector (DAD) operating from 200 to 600 nm and a Rheodyne injector with a 1 ml loop for analytical analyses. The column used was a Phenomenex C_{18} 250 x 4.6 mm i.d., at 25°C. Gradient elution was performed starting from MeOH 20% until MeOH 100%, during 30 min, as mobile phase at a flow rate of 1.0 mL min^{-1}. Monitoring wavelength were 205, 254 and 273 nm.

Structural identification of the compound

Nuclear magnetic resonance (NMR) spectra in DMSO d_6 were obtained using a Varian INOVA 500 spectrometer, operating at 500 MHz for ^1H and 125 MHz for ^{13}C and 2D-NMR (^1H-^1H COSY, HMQC, TOCSY, HMBC).

ES-MS was performed on a Fisons VG Platform spectrometer in positive (70 V) mode. The sample was dissolved in MeOH and injected directly.

IR spectrum was performed in a FT-IR-Nicolet Impact IMACT-400, KBr.

Optical rotation was performed in a Polamat A Carl Zeiss Jena.

Compound **1**. $C_{14}H_{17}NO_7$, UV □ max (MeOH): 218, 277 nm. IR (KBr): 3264 cm^{-1} (OH), 2476 cm^{-1} (C□N). ES-MS *m/z* (rel int) (70 V, positive ion): 311 $[M+H]^+$ (100), 149 $[M-162+H]^+$ (30). ^1H NMR (DMSO d_6): □5.90 (s, H-2), 6.92 (dd, *J*= 1.5 and 1.5 Hz, H-4), 6.83 (ddd, *J*= 8.0, 1.5 and 1.5 Hz, H-6), 7.24 (dd, *J*= 8.0 and 8.0 Hz, H-7), 6.96 (ddd, *J*= 8.0, 1.5 and 1.5 Hz, H-8), 4.18 (d, *J*= 8.5 Hz, H-1'), 3.07 (dd, *J*= 9.0 and 9.0 Hz, H-2'), 3.12 (dd, *J*= 9.0 and 9.0, H-3'), 3.20 (dd, H-4'), 3.25 (ddd, H-5'), 3.69 (dd, *J*= 11.9 and 2.0 Hz, H-6'a), 3.48 (dd, *J*= 5.5 and 11.8, H-6'b). ^{13}C NMR (DMSO d_6): □118.8 (C-1), 66.5 (C-2), 134.9 (C-3), 114.2 (C-4), 157.8 (C-5), 116.6 (C-6), 130.0 (C-7), 118.0 (C-8), 100.9 (C-1'), 73.2 (C-2'), 77.2 (C-3'), 69.9 (C-4'), 76.6 (C-5'), 61.1 (C-6'). $[□]_D$= -29.5.

RESULT AND DISCUSSION

TLC analyses of the 70% methanolic extract obtained from *Z. ilicifolia* with compounds isolated by Coelho (2003) showed a greenish zone after detection with anisaldehyde/H_2SO_4 suggesting the presence of cyanogenic glycoside. Spots corresponding to flavonoids and saponins were also detected in this extract.

Figure 2 shows the HPLC-DAD analysis of the crude 70% methanolic extract of *Zollernia ilicifolia*. The UV spectra of the peak with retention time 9.8 min presented bands at 218 and 277 nm typical of a cyanogenic glycoside (Seigler, 1975). The UV spectra of the peaks with retention times 16.7 min and 17.6 min presented bands at 254 nm and 273 nm, and 264 nm and 346 nm typical of flavonoids (Markham, 1982), while those between 3.0 and 7.0 min presented bands at 210 nm, suggesting the presence of saponins (Oleszek, 2002). Identification of the peak with retention time 9.8 min as being zierin **1** arose from the co-injection of the extract enriched with the previously isolated compound (Coelho et al., 2003).

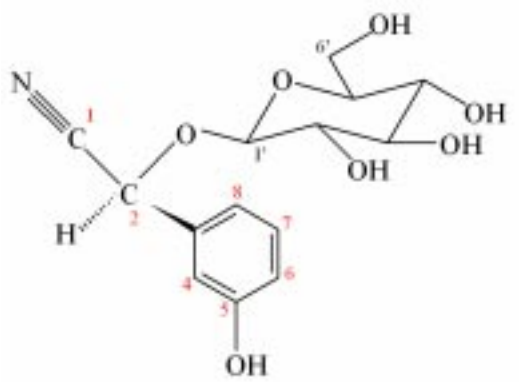

FIGURE 1. Structure of the isolated cyanogenic glycoside **1**, (z)-zierin.

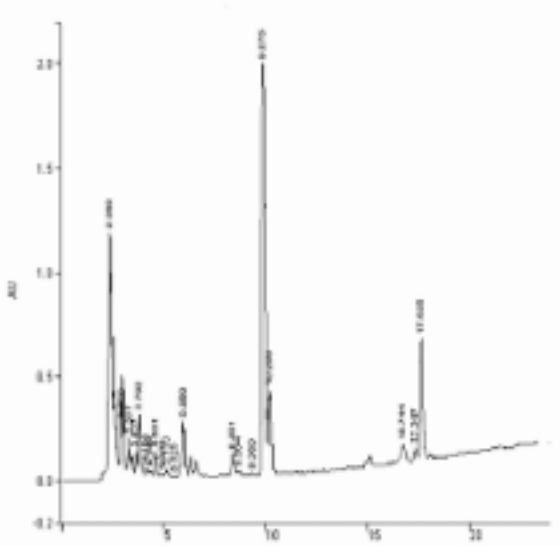

FIGURE 2. Chromatogram of the 70% methanolic extract from *Z. ilicifolia* leaves by HPLC analysis. Conditions: column: reversed-phase Phenomenex Luna, C_{18} column (250 x 4.60 mm I.D., 5 □m); mobile phase: linear gradient of MeOH/water (20-100%) over 30 min, flow rate: 1.0 mL min^{-1}, detection at 254 nm.

For the HSCCC separation, experiments were performed to determine the optimal two phase solvent system. They were based on the results of the distribution of sample between a small amount of the two phases and by the results of TLC tests (Conway, 1990). The mixture of chloroform-methanol-n-propanol-water (5:6:1:4, v/v/v/v) gave the best result, with **1** almost equally distributed between the two phases (partition coefficient value K of approximately

1). Upon elution of the crude extract on silica gel TLC plates using the lower phase of the two phase solvent system the retention factors (Rf) of the secondary metabolites present in the extract of *Z. ilicifolia* were 0.4 (saponins), 0.5 (flavonoids) and 0.7 (cyanogenic glycoside). The relatively low proportion of chloroform in the solvent mixture and the Rf values of flavonoids and saponins indicate the high polarity of the compounds extracted from *Zollernia ilicifolia* suggesting the presence of three or four sugar units of flavonoids and saponins. Thus, the lower phase was chosen as mobile phase for the CCC separation of 70% methanol extract.

Table 1 shows the collected fractions. Screening by TLC showed that the cyanogenic glycoside **1** eluted in fractions 10-18, whereas a mixture containing flavonoid glycosides and saponins eluted in fractions 22-48. Therefore, the separation of **1** from the crude extract was accomplished between 22.5-45 min of chromatographic run and the mixture of flavonoids and saponins was obtained between 55-120 min. TLC analyses of the washoff fractions showed no significative retention of flavonoids and saponins in the stationary phase. As the organic lower phase was used as mobile phase, zierin **1** eluted before the mixture of highly polar flavonoids and saponins.

TABLE 1. Fractions obtained from the 70% methanolic extract from *Z. ilicifolia* leaves by HSCCC. (%)

Substances	Fractions	Isolated amount (mg)	Purity (%)
1 (z)-zierin	10-18	80	95
Mixture of flavonoids and saponins	22-48	600	-

The HSCCC separation described above yielded 80 mg of zierin **1** from 1 g of the crude 70% methanol extract, with 95% purity as determined by HPLC analysis.

The use of HSCCC proved to be effective in the fast isolation of zierin. Clean-up procedure was not necessary in this experiment. Zierin **1** was previously isolated by gel permeation (GPC) followed by purification by RP-HPLC (Coelho et al., 2003). GPC separation is designed to separate mixtures predominantly on the basis of their molecular size and also by adsorption. Since **1** is a small molecule compared to flavonoids and saponins GPC is relatively time-consuming and causes peak broadening. Thus the results of our study showed that HSCCC was a useful tool to isolate the toxic component from the crude 70% methanol extract of *Zollernia ilicifolia* in a one-step separation, faster than GPC separation. Again, compared to GPC (Coelho et al., 2003) the procedure developed here can be used to obtain large amounts of the possible bioactive fraction (mixture of flavonoids and saponins) free from the toxic component. The amount of the sample can also be increased several times using a larger coil.

CONCLUSION

The results of our study clearly demonstrate the advantages of HSCCC for the isolation of zierin **1** from the leaves of *Z. ilicifolia* in only one step without need of time-consuming clean-up. The fast separation and minimum solvent consumption offers a very efficient method for the separation and purification of natural products.

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