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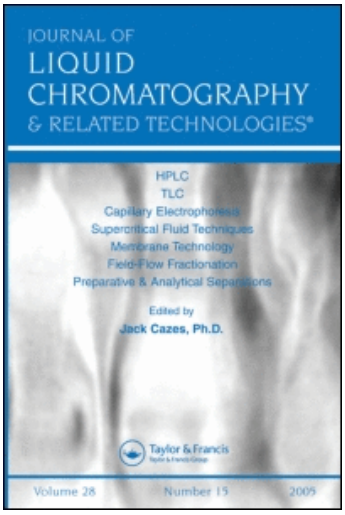

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Gradient Elution for Triterpene Separation from *Cecropia lyratiloba* Miquel by HSCCC

Rodrigo R. Oliveira[a]; Gilda G. Leitão[a]; Michelle C. C. Moraes[a]; Maria Auxiliadora C. Kaplan[a]; Daise Lopes[b]; Jorge P. P. Carauta[c]

[a] Núcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil [b] Agroindústria de Alimentos, EMBRAPA, Rio de Janeiro, Brasil [c] Serviço de Ecologia Aplicada, FEEMA, Rio de Janeiro, Brasil

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Núcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil

Daise Lopes
Agroindústria de Alimentos, EMBRAPA, Rio de Janeiro, Brasil

Jorge P. P. Carauta
Serviço de Ecologia Aplicada FEEMA, Rio de Janeiro, Brasil

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Abstract: The triterpenoid pool from the dichloromethane fraction obtained from the methanolic extract of roots of *Cecropia lyratiloba* Miquel was submitted to CCC using a gradient elution consisting of Hex/EtOAc/MeOH/H_2O—1/2/X/1 (X = 0.5 (A); 0.75 (B); 1.0 (C); 1.5 (D); 2.0 (E)) in five steps. The lower aqueous phase was used as mobile phase, 2 mL/min at 850 rpm. This procedure led to the isolation of tormentic acid and a mixture of tormentic and euscaphic acids. In order to improve the triterpene separation the fractions Fr 31–49 were submitted to a new CCC run using a fine adjustment of the methanol concentration in the gradient elution system. This separation procedure led to the isolation of euscaphic acid, 3-acetyl tormentic acid and a mixture of tormentic and isoarjunolic acids.

Keywords: *Cecropia lyratiloba*, Cecropiaceae, Terpenoids, Gradient elution, Countercurrent chromatography

Address correspondence to Gilda Leitão, Núcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil. E-mail: ggleitao!@nppn.ufrj.br

INTRODUCTION

Triterpenoids are secondary metabolites derived from squalene cyclization through epoxide formation. These compounds can be found in free form, as well as glycosides belonging to several botanical families.[1]

Concerning bioactivity, this class of compounds is known to exhibit a wide spectrum of pharmacological activities such as anti-microbial, hypocholesterolemic, anti-atherosclerotic, anti-inflammatory, anti-HIV, anti-hepatotoxic, and in the treatment of tumors and leukemia.[2–5]

Triterpene separation may be difficult using traditional chromatographic techniques.[6] Countercurrent chromatography (CCC) is a separation technique that uses a liquid stationary phase without solid support matrix. Therefore, it eliminates the irreversible loss of sample onto the solid support matrix used in the conventional adsorption chromatographic column.[7] Little attention has been given to the use of gradients in CCC, in contrast to the usual isocratic elution.[8–10] However, gradient elution in CCC could be the choice for the purification of mixtures containing compounds with a large range of polarities and partition coefficients.[11] This alternative has been successfully applied to the CCC technique for the separation of triterpenes in the enriched dichloromethane fraction from roots of *Cecropia lyratiloba* (Figure 1), described in this paper.

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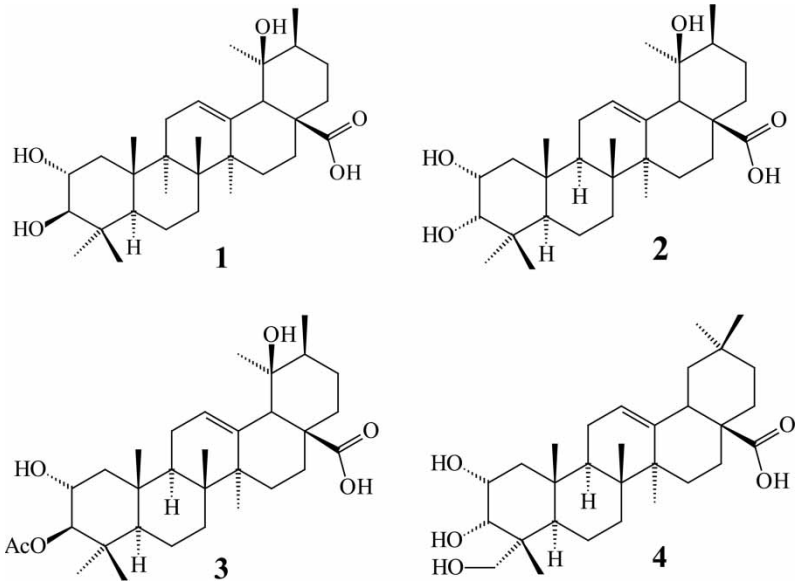

Figure 1. Structures of the triterpenes isolated from *C. lyratiloba.* **1**-Tormentic acid, **2**-Euscaphic acid, **3**-3-acetyl-tormentic acid **4**-Isoarjunolic acid.

EXPERIMENTAL

Apparatus

High speed countercurrent chromatography (HSCCC) was performed in a P.C. Inc Multilayer Coil Separator-Extractor CCC apparatus, equipped with interchangeable triple coil of 1.68 mm, 240 mL PTFE tubes. The HSCCC system was equipped with a solvent pump SD-200, Dynamax; a manual injection valve with a 5 mL loop; and a fraction collector FC-1, Dynamax.

Preparation of Triterpene Mixture from *Cecropia lyratiloba*

Roots of *Cecropia lyratiloba* Miquel (1.3 kg) were dried, crushed, and extracted with methanol at room temperature. The methanol extract was dissolved into a mixture of MeOH/H_2O 50/50 v/v and partitioned successively with hexane, dichloromethane, ethyl acetate, and butanol. The dichloromethane fraction (8.18 g) was fractionated by silica gel column chromatography using chloroform and ethyl acetate in increased polarity gradient. According to TLC profile, the fraction eluted with $CHCl_3$/EtOAc 50% was characterized as a triterpene mixture.

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Selection of the Solvent System

The empirical screening of the solvent system was made by the direct measurement of the distribution coefficients by the shake-flask method.[12] A small amount of the triterpene mixture was first dissolved in a test tube with the solvent system hexane–ethyl acetate–methanol–water (1:2:1:2, v/v/v/v). The test tube was shaken and the compounds were partitioned between the two phases. An aliquot of each phase was spotted on a TLC plate, which was eluted with the organic phase of the solvent system chloroform–ethyl acetate–methanol–water (7:3:5:7). The result was visualized after spraying with $CeSO_4$/heat. The gradient system was adjusted by slightly changing the methanol content in the aqueous polar lower phase of the solvent system hexane–ethyl acetate–methanol–water (1:2:1:2). Five hexane–ethyl acetate–methanol–water compositions, referred as A to E, were prepared. They can be represented by (1:2:X:2), with the amount of methanol, X, being: **A**—X = 0.5, **B**—X = 0.75, **C**—X = 1.0, **D**—X = 1.5, **E**—X = 2.0. The triterpene mixture was added to five test tubes containing the five A to E biphasic systems. TLC plates were prepared for all 10 liquid phases to have an idea of solute partitioning.

The injected sample solution was prepared by dissolving 500 mg of the triterpene extract sample in 5 mL of both phases of the solvent system **A**.

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HSCCC Separation Procedure

Separation of the triterpenes was carried out in two steps, using two different gradient systems (Tables 1 and 2). In the first separation the coil was entirely filled with the upper phase of solvent system **A**. Then, the apparatus was rotated at 890 rpm, while the lower phase of the same solvent system was pumped into the column at a flow-rate of 2.5 mL/min in the head to tail direction resulting in 81.7% retention of stationary phase ($V_S = 196$ mL). After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the 10 mL triterpene mixture was injected through the injection valve. The mobile phase eluting from the chromatographic column was collected in 10 mL fractions. After 34 fractions (340 mL or 136 min) were collected, the rotation was stopped and the mobile phase was changed. The lower phase of system **B** was used as the new mobile phase, pumped at 2.5 mL/min in the head to tail direction, and the same collection procedure was applied for 24 fractions (240 mL or 96 min). The procedure was done again with the lower phase of solvent system **C**. Smaller 5 mL fractions were collected at 2.5 mL/min. The lower phase of solvent system **C** produced 48 fractions (240 mL or 96 min.). The same procedure was sequentially applied with the lower phase of the two other solvent systems (**D** and **E**). After the elution of 240 mL of lower phase of system **E**, the rotation was stopped and the coil content (upper and lower phases) was collected. Fractions were pooled together according to their TLC profile.

Fr 31–49 (253 mg) was found to contain a triterpene mixture. This fraction was submitted to a second CCC run, using a similar gradient system slightly optimized around the **D** composition [hexane–ethyl acetate–methanol–water (1:2:1.5:2)]. The aqueous phase polarity was adjusted in small steps. The amount of methanol, X, was gradually increased in four steps as follows: **F** with X = 1.25, **G** (=**D**) with X = 1.5, **H**—X = 1.75, and **I** (=**E**)—X=2.0, (Table 2). Initially, the coil was entirely filled with the upper phase of solvent system **F**. Then, with the coil rotating at 890 rpm, the lower phase of the same solvent system **F** was pumped into the coil at a flow-rate of 2.5 mL/min (head to tail) resulting in

Table 1. Solvent systems used in the first CCC gradient

Solvent system	Hexane (mL)	Ethyl acetate (mL)	Methanol (mL)	Water (mL)
A	1	2	0.5	2
B	1	2	0.75	2
C	1	2	1	2
D	1	2	1.5	2
E	1	2	2	2

Table 2. Solvent systems used in the second CCC gradient

Solvent system	Hexane (mL)	Ethyl acetate (mL)	Methanol (mL)	Water (mL)
F	1	2	1.25	2
G(=D)	1	2	1.5	2
H	1	2	1.75	2
I (=E)	1	2	2	2

81.25% retention of stationary phase ($V_S = 195$ mL). The enriched triterpene mixture (10 mL) was injected through the injection valve. Fractions of 10 mL were collected. After collection of 51 fractions (510 mL or 204 min), the rotation was stopped and mobile phase was changed to the lower phase of system **G**. Sixty one fractions of 5 mL (305 mL or 120 min.) were collected before again changing the mobile phase to the lower phase of solvent systems **H** (54 fractions or 270 mL or 108 min) and **I** (58 fractions or 290 mL or 116 min). After collecting Fr 224, the machine content was extruded by pushing it with compressed nitrogen.

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RESULTS AND DISCUSSION

Silica gel column chromatography of the dichloromethane fraction from *Cecropia lyratiloba* roots afforded a fraction consisting of a triterpene mixture. Preliminary attempts to separate these triterpene mixtures by isocratic HSCCC with the solvent system hexane–ethyl acetate–methanol–water (1:2:1:2 v/v/v/v) showed that this solvent system alone was not effective enough to promote separation of the constituents. The use of a gradient system, in which the strength of the mobile phase is increased by increasing the amount of MeOH in the solvent system, was necessary in order to explore the small differences in the triterpene partition coefficients. The gradual change in methanol concentration on the solvent system hexane–ethyl acetate–methanol–water (1:2:1:2), proved to be appropriate to improve separation, leading to a better distribution of the triterpenes in both phases of the solvent system (Table 1).

The HSCCC experiment was carried out using the aqueous phase of system **A** as mobile phase, followed by the aqueous phase of systems **B**, **C**, **D**, and **E** till completion of total elution. This procedure promoted the separation of nine distinct spots on the TLC — one in step **B**, four in step **D**, besides another four compounds in the last step **E** (Figure 2). This separation procedure resulted in the isolation of tormentic acid (step **D**, Fr 20–22, 29 mg) and a mixture of tormentic and euscaphic acids (step **D**, Fr 26–28,

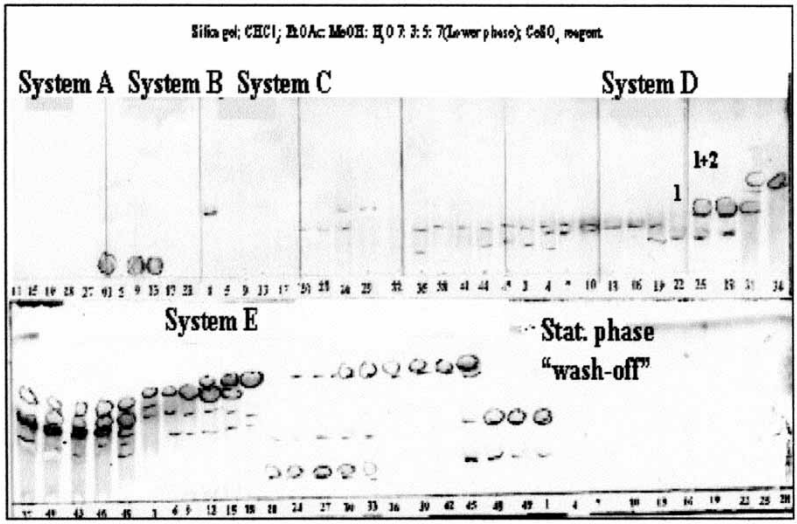

Figure 2. TLC results from the fractionation of the triterpene sample obtained from the dichloromethane extract from *Cecropia lyratiloba.* CCC conditions: Hex:EtOAc:MeOH:H_2O 1/2/X/1 [X = 0.5 (**A**); X = 0.75 (**B**); X = 1.0 (**C**); X = 1.5 (**D**); X = 2.0 (**E**)]. Flow rate: 2.5 mL/min. (tormentic acid, **1**; mixture of tormentic and euscaphic acids, **1 + 2**). The TLC was eluted with the organic phase of the solvent system chloroform–ethyl acetate–methanol–water (7:3:5:7). Spray reagent: $CeSO_4$/heat.

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26 mg). These compounds were identified by comparison of ^{13}C-NMR data with those from the literature.[13–15] The other pure compounds could not be identified due to very low isolated amounts.

In order to improve the separation of the triterpenes found in fractions Fr 31–49 (step **D**) a second run was done. The methanol ratio in the original hexane–ethyl acetate–methanol–water system **D** was optimized (Table 2). The separation procedure was the same as described above.

The optimization of methanol concentration in the second gradient system showed an excellent result: two compounds were obtained at step **G** (Fig. 3). This separation procedure resulted in the isolation of a mixture of tormentic and isoarjunolic acids (Fr 37–45, 50 mg), euscaphic acid (Fr 55–61, 20 mg), and 3–acetyl–tormentic acid (Fr 67–78, 20 mg), identified by comparison of ^{13}C-NMR data with those from the literature.[13–15]

CONCLUSIONS

The elaborated gradient systems were successful in the isolation and purification of triterpenes from *Cecropia lyratiloba.* The isolated

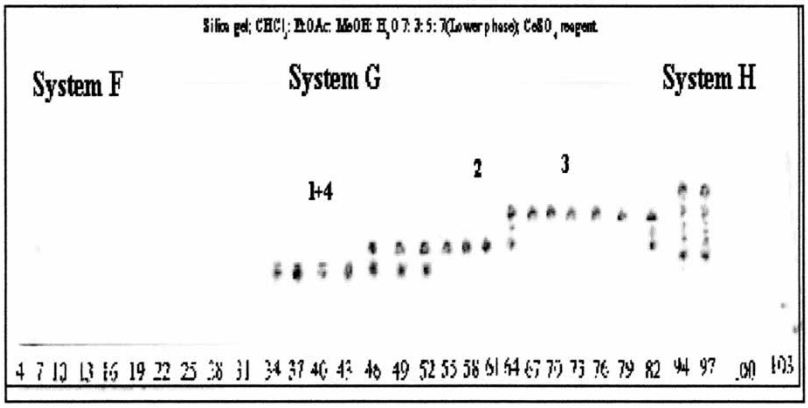

Figure 3. TLC results from the fractionation of fraction Fr 31–39. CCC conditions: Hex:EtOAc:MeOH:H_2O 1/2/X/1 [X = 1.25 (**F**); X = 1.5 (**G**); X = 1.75 (**H**); X = 2.0 (**I**)]. Flow rate: 2.5 mL/min. (isoarjunolic and tormentic acids, **1 + 4**; euscaphic acid, **2** and 3-acetyl-tormentic acid, **3**). The TLC was eluted with the organic phase of the solvent system chloroform–ethyl acetate–methanol–water (7:3:5:7). Spray reagent: $CeSO_4$/heat.

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compounds have small structural differences such as hydroxyl position and configurational isomerism. The use of countercurrent chromatography for triterpenes separation confirms that this technique is very powerful in the isolation of this class of compounds.

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